Integrated bioprocessing for the sustainable manufacture of poly (methyl methacrylate) precursors.

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Copyright Declaration

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1,2-PG	1,2-Propylene glycol
1,3-PG	1,3-Propylene glycol
3-HIBA	3-Hydroxyisobutyric acid
3HP	3-Hydroxypropionate
AAT	Alcohol acyltransferase
ACH	Acetone cyanohydrin
Acx4	Acyl-coenzyme A oxidase
ATP	Adenosine triphosphate
AU	Absorbance units
BCKD	Branched chain ketoacid dehydrogenase
BIB	Butylisobutyrate
BMA	Butyl methacrylate
CGG	*C. glutamicum* glucose growth medium

CoA	Coenzyme A
DAD	Diode array detector
DCW	Dry cell weight
DO	Dissolved oxygen
ECH	Enoyl–CoA hydratase
EG	Ethylene glycol
EMCP	Ethylmalonyl-CoA pathway
FAD	Flavin adenine dinucleotide
GC-MS	Gas chromatography–mass spectrometry
GRAS	Generally recognised as safe
h	Hour
HCH	3- Hydroxyisobutyryl-CoA hydrolase
HPLC	High performance liquid chromatography
IBA	Isobutyric acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside

KIV	α-Ketoisovalerate
kton	Kiloton
LB	Lysogeny broth
LC-MS	Liquid chromatography–mass spectrometry
MAA	Methacrylic acid
MAAL	3-Methylaspartate ammonia lyase
MMA	Methyl methacrylate
MOPS	3-(N-morpholino)propanesulfonic acid
NAD^+	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
OD	Optical density
OPA	Ortho-phthalaldehyde
P3HB4HB	Poly-3-hydroxybutyrate-co-4-hydroxybutyrate
PE	Polyethylene

PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PHB	Poly (3-hydroxybutyrate)
PHBHHx	Poly-3-hydroxybutyrate-co-3-hydroxyhexanoate
PHVB	Poly-3-hydroxybutyrate-co-3-hydroxyvalerate
PLA	Poly (lactic acid)
PMMA	Poly (methyl methacrylate)
PP	Polypropylene
PPG	Polypropylene glycol
PS	Polystyrene
PTA	Purified terephthalic acid
PTFE	Polytetrafluoroethylene
PVC	Polyvinylchloride
qPCR	Quantitative polymerase chain reaction
RID	Refractive index detector

RPM	Revolutions per minute
tMAAL	3-Methylaspartate ammonia lyase from *Clostridium tetanomorphum*
TPP	Thiamine pyrophosphate
USDoE	US Department of Energy
VVM	Volume per volume per minute
YSI	Yellow Springs Instrument

Abstract

Industrial focus is shifting from the utilisation of petrochemicals and is moving towards the utilisation of biomass for the production of platform chemicals. The finite lifespan of petrochemicals, the dramatic fluctuation in the price of oil and environmental concerns are all significant drivers for this change. Polymers such as poly (methyl methacrylate), a mass market polymer, can be sustainably produced using platform chemicals derived from biomass. This study investigates the production of two platform chemicals, mesaconate and butyl-methacrylate, from biomass which would allow the sustainable production of poly (methyl methacrylate). Mesaconate was initially targeted with butyl methacrylate later identified as a potential target with envisaged reduced downstream processing required for integration onto poly (methyl methacrylate) production.

Initially a mesaconate production pathway was incorporated into *Corynebacterium glutamicum* selected due to the inherent ability of *C. glutamicum* to produce large quantities of glutamate. Mesaconate production was demonstrated through bioprocesses containing the constructed *Escherichia coli* strains with improvement in the analysis method providing accurate quantification. Adaption of the bioprocess in particular through alteration of the dissolved oxygen improved the mesaconate titre further.

Secondly a novel pathway for the production of a poly (methyl methacrylate) precursor butyl methacrylate was constructed within *E. coli*. Strains containing this pathway demonstrated production of butyl methacrylate within biotransformations. Adaptation of the biotransformation method and strain development produced an increase in the butyl methacrylate titre. Further engineering allowed the elimination of by-products from the bioprocess and improved the tolerance of the host to the toxicity of butyl methacrylate. Production of butyl methacrylate directly from glucose was attempted although this was not successful.

Chapter 1

Introduction & Literature Review

1.1. Platform chemicals

Platform chemicals are building block chemicals which can be converted into a wide range of chemicals or materials. As such they are fundamental to the production of many items we use today, not only in industry but also domestically. As multi use "building blocks" platform chemicals provide a starting point to produce items with applications spanning a large variety of sectors (Table 1). The majority of these chemicals are produced using fossil fuels such as oil, coal and gas which are non-renewable resources. It is estimated that these petrochemical resources will begin to deplete at some point within the next 30 years (Michaelides, 2017) largely due to the continuing growth of the global energy and chemical sectors (Sheldon, 2018; Sravan *et al.*, 2019).

Table 1: Examples how platform chemicals are utilised over a variety of sectors. Adapted from Werpy and Peterson (2004).

Sector	**Uses For Platform Chemicals**
Industrial	Corrosion inhibitors, gas purification, lubricants, seals, hoses, emission abatement
Transportation	Fuel, moulded plastics, fabrics, anti-corrosion chemicals, anti-freeze
Textiles	Carpets, fibres, fabrics, foam, upholstery, Lycra, Spandex
Safe Food Supply	Food packaging, preservatives, fertiliser, pesticides, beverage bottles, appliances, vitamins
Environment	Water chemicals, flocculants, chelators, cleaners, detergents
Communication	Moulded plastics, computer casings, liquid crystal displays, pens, pencils, inks, dyes, paper products
Housing	Paints, resins, siding, insulation, cements, coatings, varnishes, flame retardants, adhesives, carpeting
Recreation	Footgear, protective equipment, camera and film, bicycle parts & tires, wet suits, tapes-CD's-DVD's, golf equipment, camping gear, boats
Health and Hygiene	Plastic eyeglasses, cosmetics, detergents, pharmaceuticals, suntan lotion, medical-dental products, disinfectants, aspirin

Coupled with the continual depletion of petrochemicals, the economics of traditional methods of platform chemical production can be heavily influenced by the cost of substrates. The value of the substrates required for synthetic processes are tied directly to the value of crude oil. The value of oil can fluctuate due to a multitude of factors such as variation in the production and consumption levels, geopolitical reasons and the state of the global economy to name a few (Demirbas, Omar Al-Sasi and Nizami, 2017). This instability can either be of benefit or detriment to the economy of a platform chemical process, but with the price of Brent crude oil in Europe fluctuating between $40 and $120 in the last 10 years, the difference can be significant (Byrne, Lorusso and Xu, 2018). The fluctuation in the price of petrochemical precursors for platform chemical production is a major disadvantage which can be alleviated through the development of a biological process.

Platform chemical production in a biorefinery can provide numerous benefits over traditional petrochemical based production. A biorefinery is a facility which utilises renewable biomass as the precursor for the production of biofuels, chemicals, plastics and polymers. Often multiple products are produced if possible to maximise value streams from the carbon source (Erickson, Nelson and Winters, 2012). The biomass used within a biorefinery can often be of extremely low value, such as organic waste, which for a process can greatly improve the economics over the use of more expensive petrochemical precursors (Coma *et al.*, 2017). In addition to low value carbon sources biorefineries for platform chemical production can often have lower environmental pollution and greenhouse gas emissions over their petrochemical equivalents (Becker *et al.*, 2015). The stability and environmental advantages provided by biorefineries is an attractive proposition for industrial producers of platform chemicals with 30% of platform chemicals expected to be produced from biomass by 2025 (Kamm, 2007).

Although biorefineries appear to be the future for platform chemical production, the development of a viable process can be a significant challenge. Many platform chemicals cannot currently be produced biologically at titres and production rates which allow the product to compete economically with chemical production. Therefore substantial research is required into identification of the optimum biological pathways and hosts to reach the production levels required. An early challenge can be to identify an appropriate biorefinery product which will provide the maximum value to the bioprocess.

A report published by the US Department of Energy (USDoE) in 2004 analysed 300 potential platform chemicals which could be produced from biomass to determine which provided the highest potential value to industry (Werpy and Peterson, 2004). This extensive list of 300 chemicals was first narrowed down to 30 through analysis of a variety of factors including potential applications, market size, current price and estimated cost of setting up a bioprocess. The 30 top candidates were then further refined to a list of 15 chemicals which were estimated to provide the highest value as platform chemicals derived from biomass (Table 2).

Table 2: The top 15 platform chemical candidates identified in 2004 by the US department of energy and their potential applications within industry. Adapted from Werpy and Peterson, (2004).

Platform Chemical	**Potential Applications**
Succinic, fumaric and malic acid	Solvents, Polyesters
2,5 Furan dicarboxylic acid	Polyesters
3 Hydroxy propionic acid	Acrylic Acid
Aspartic acid	Chelating agents, Sweeteners
Glucaric acid	Solvents, Adipic acid
Glutamic acid	Polyesters, Polyamides
Itaconic acid	Poly(itaconic acid), Methyl methacrylate
Levulinic acid	Fuel oxygenates, Solvents
3-Hydroxybutyrolactone	Pharmaceuticals, Solvents
Glycerol	Propylene glycol, Acrylic acid
Sorbitol	Polyethylene isosorbide terephthalates
Xylitol/arabinitol	Sweeteners, Polyester resins

The publication of this report served as guidance for industry and academic researchers as to help drive innovation in the bioproduction of these chemicals. As the list was never intended to be a definitive ranking, but rather a guide to highlight the chemicals with the highest industrial potential at the time, this list was revisited and re-evaluated again by the US department of energy in 2010 (Bozell and Petersen, 2010). Upon re-evaluation it was

observed that a number of chemicals included in the original list did not attract the expected quantity of research indicating a lack of feasibility or interest industrially. This, along with changes in the potential of other chemicals resulted in adaptation of the original list. The chemicals which dropped off the list as a result were several organic acids (fumaric, malic, aspartic, glucaric, glutamic and itaconic acid) along with 3-hydroxybutyrolactone. Ethanol was added to the list as technological advances have allowed it to shift from a commodity chemical used primarily for biofuel to a platform chemical which can be used for production of other chemicals. Lactic acid was also added to the list with advances in the current production pathways opening new avenues for utilisation. The revised list is shown in Table 3 with potential applications of each chemical.

Table 3: The top 10 platform chemical candidates identified in 2010 by the US department of energy and their potential applications within industry. Adapted from Bozell and Petersen, (2010).

Platform Chemical	**Potential Applications**
Ethanol	Polyethylene, polyvinylchloride
Furans	Polyesters
3 Hydroxy propionic acid	Acrylic acid
Lactic acid	Polylactic acid, lactate esters, solvents
Succinic acid	Succinate esters, nylons, polyesters
Levulinic acid	Fuel oxygenates, Solvents, novel polymers
Glycerol	Propylene glycol, Acrylic acid
Sorbitol	Polyethylene isosorbide terephthalates
Xylitol	Sweeteners, Polyester resins
Biohydrocarbons	Isoprene production

From analysis of the revised list (Table 3) it can be seen that all of the platform chemicals highlighted have applications as precursors for polymer production. Ethanol, the furans, succinic acid, sorbitol, xylitol, 3 hydroxy propionic acid, glycerol and the biohydrocarbons can be incorporated into the production of existing mass-produced polymers such as

polyethylene, polyacrylic acid and polyesters while lactic acid and levulinic acid can be used for production of novel polymers such as polylactic acid. The ability for a platform chemical to be incorporated into the production of a commercial polymer allows it to fulfil several of the criteria identified by the USDoE in the form of a potential high-volume product, an avenue for substitution into a commercial pathway and multiple valuable end products. This highlights the importance of research into the bio-production of platform chemicals which have commercial polymer applications and in particular mass market applications in the production of plastics.

1.2. The significance of the plastics market

Plastics have become a staple of modern life with a plastic or polymer present within a large portion of the items commonly found within a household. The first fully synthetic polymer, Bakelite, was patented in 1907. This mouldable resin produced from phenol and formaldehyde is credited with sparking the "plastics age" (Crespy, Bozonnet and Meier, 2008) with global mass market production of plastics in action by the 1950's. Plastics showed revolutionary potential as a material due to their physical properties. Plastics in various forms as a material showed a unique set of properties being lightweight with a high tensile strength and durability, inexpensive, mouldable to any shape required, high resistant to corrosion and insulation to heat and electricity (Thompson *et al.*, 2009). As a result of this unique combination of properties the growth in plastic production has significantly outpaced other materials in the last 65 years with the global annual production of plastic polymers increasing from 2 million tonnes to 380 million tonnes (Geyer, Jambeck and Law, 2017). This rapid growth in the industrial production of plastics has resulted in this sector becoming the largest application of crude oil (Siracusa *et al.*, 2008; Kishna *et al.*, 2017). This therefore represents a large and expanding market into which biologically produced platform chemicals can not only be integrated but also help to improve and optimise.

Originally prized for their favourable physical properties and cost-effective production from petrochemicals plastics have since become a significant environmental issue. None of the commonly used plastics are biodegradable therefore without effective recycling plastics

become a near permanent source of waste in the environment (Siracusa *et al.*, 2008). It is estimated that since 1950 over 7800 million tons of synthetic resins and fibres have been produced with only 2500 millions tons estimated to currently be in use (Geyer, Jambeck and Law, 2017). As a result, this amounts to 6300 million tons of plastic waste of which it is estimated only 12% is incinerated and 9% is recycled. As a result, plastic waste is a significant pollutant on land and within the ocean. Packaging products make up 39.9% of the plastics market and therefore is one of the reasons plastic is a significant pollutant as by nature products of this type are largely disposable (Narancic and O'Connor, 2017).

Plastic waste can be particularly devastating to marine ecosystems. Due to the high resistance to degradation presented by polymers upon release into the ocean they remain present until removal. This can cause significant damage to the marine ecosystem through entanglement and ingestion (Gregory, 2009; Koelmans *et al.*, 2014). Polymer nets are one of the main causes of entanglement which is an issue that caused relatively limited damage previously due to the unwanted biodegradable properties of rope. Ingestion of plastics can harm marine species through damage to the stomach, obstructing the gut and through passive leeching of hazardous toxins into the food chain (Villarrubia-Gómez, Cornell and Fabres, 2017). Further environmental cost from plastics arises in the form of greenhouse gas emissions related to the current production methods. The five most common plastic polymers produce 70 million tons of CO_2 emissions in the US alone (Posen *et al.*, 2017). As CO_2 emissions have been shown to be directly correlated with global warming plastic production currently is a driver for climate change (Yang, Zou and Zhou, 2017).

Although continued production of plastics by the current petrochemical methods prove to be detrimental to the environment plastics are now essential to industry. The sheer size of the synthetic polymer market prevents production processes from slowing therefore there is significant interest in the production of biopolymers and sustainable routes to the current mass market plastics. Biologically produced platform chemicals are key to both of these routes to environmentally sustainable plastic.

1.2.1. Novel biodegradable polymer production

The extreme resistance to biodegradation exhibited by the traditional plastics was originally one of the novel physical properties which allowed plastics and polymers to be utilised in a plethora of applications. As time has progressed the extreme market demand for plastics and polymers has resulted in a large amount of plastic waste and as a result environmental damage. Currently plastics and polymers which are not biodegradable are used for applications such as packaging and one-time use items e.g. plastic cutlery. These products do not require resistance to biodegradation and therefore traditional plastics such as polyethylene (PE), polystyrene (PS) and polyvinylchloride (PVC) are not the ideal for this function. As a result research has been carried out into the production of biodegradable polymers which can be used as replacements for disposable plastic products (Siracusa *et al.*, 2008; Song *et al.*, 2009).

1.2.1.1. Poly (Lactic acid)

Currently a biodegradable bioplastic under full commercial production is polylactic acid (PLA) which has a global production capacity of 200 kton/year although, due to its unique physical properties, polylactic acid is expected to eventually replace petrochemical derived plastics (Di Lorenzo and Androsch, 2018). As a plastic, PLA has similar physical properties to polystyrene (PS) and polyethylene terephthalate (PET) but has the advantage of being biodegradable which makes it an attractive biopolymer for replacing traditional plastics. PLA is has a high melting point ($175^{O}C$), has high transparency, is biocompatible and is biodegradable (Nagasawa *et al.*, 2005). Due to these properties PLA is suitable for use in food packaging, bottle production and medical applications. As PLA is biodegradable all applications are based around products and uses which do not require long term stability or require breakdown after a certain length of time. It is this property which makes PLA unique.

Biodegradation of PLA has been analysed in detail as the plastic degrading at the correct time is key to mass utilisation of the polymer. PLA is converted to water soluble oligomers through hydrolytic degradation and is a process which can be influenced by multiple factors such as

the temperature, pH, the molecular weight and crystallinity of the polymer (Di Lorenzo and Androsch, 2018). It has been shown that, in compostable conditions, a PLA bottle is fully degraded within 30 days, opening up new avenues for disposal of plastic products (Kale *et al.*, 2007).

Commercial production of PLA is currently only carried out by a small number of companies including Purac-Toyobo, Futerro and Natureworks LLC. In order to produce PLA lactic acid is first self-esterified to produce lactide which is followed by polymerisation to produce PLA (Choi *et al.*, 2015). The current commercial production process for Ingeo PLA by NatureWorks LLC has greenhouse gas emissions of 1.3 kg CO_2 eq /kg of PLA which by comparison, is much lower than the CO_2 produced by traditional plastic manufacture (3.2 kg CO_2 eq /kg for PET and 3.4 kg CO_2 eq /kg for PS) (Kolstad *et al.*, 2012). This significant difference in CO_2 emissions is directly related to the source of the monomer, lactic acid.

Lactic acid can either be produced synthetically or through bacterial fermentation. Microbial production of lactic acid has a distinct advantage over synthetic production as microbial production allows enantiomerically pure L or D-lactic acid to be produced. Chemical synthesis of lactic acid from petrochemicals produces a racemic mix of both enantiomers which requires purification before lactic acid can be utilised for PLA production (Ghaffar *et al.*, 2014). Enantiomeric purity is essential for PLA production as impurity as small as 1 mol% can greatly effect the crystallinity of the polymer (Matsumoto and Taguchi, 2013). Along with the purity microbial production of lactic acid can also use low cost substrates and as a process uses a low temperature and has a low energy cost (John, Nampoothiri and Pandey, 2007). These factors provide biological production of lactic acid with an advantage not only economically but also environmentally (Madhavan Nampoothiri, Nair and John, 2010). As such lactic acid is currently produced by fermentation for PLA commercially, the predicted future scale of PLA production resulted in the identification of lactic acid by the USDoE as an important biologically produced platform chemical (Bozell and Petersen, 2010).

Lactic acid is an organic acid originally discovered in sour milk in 1780. It has since become an important food additive due to its status as generally recognised as safe (GRAS). Food related applications account for 85% of lactic acid demand with widespread use as a preservative and acidulant (John, Nampoothiri and Pandey, 2007). The market for lactic acid was 260 ktons in 2012 and was expected to rise to 600 ktons by 2020 due to the growing demand for PLA (Di

Lorenzo and Androsch, 2018). Lactic acid is now almost exclusively produced biologically through fermentation which results in production of either the L or D isomer. Synthetic production was unable to compete with biological production as the resulting racemic mixture of both L and D isomer required further downstream processing before it could be effectively used (Wee, Kim and Ryu, 2006).

Production of the L isomer has been demonstrated in numerous microorganism species including *Rhizopus, Lactobacillus, Enterococcus and Bacillus*. The dominant microorganisms for the production of L-lactic acid are the fungi *Rhizopus oryzae* and the lactic acid bacteria species *Lactobacillus paracasei* (Di Lorenzo and Androsch, 2018). High yields have been produced in immobilised fed batch biotransformations of *Rhizopus oryzae* using glucose as the carbon source where 231 g/L of L-lactic acid was observed (Yamane and Tanaka, 2013). *Lactobacillus paracasei* was shown to produce 198 g/L of L-lactic acid when using fresh sweet potato as the carbon source in a simultaneous saccharification and fermentation process (Nguyen *et al.*, 2013). Although the yields of L-lactic acid are high D-lactic acid is also required for production of PLA polymers with altered physical properties.

Recently production of D-lactic acid has been demonstrated in strains of *C. glutamicum*, *E. coli* and *B. subtilis.* It was demonstrated that *C. glutamicum* grown in oxygen deprived conditions overexpressing the D-Lactate dehydrogenase from *Lactobacillus delbrueckii* with deletion of the endogenous L-Lactate dehydrogenase could produce 120 g/L of D- lactic acid with an optical purity of 99.9% (Okino *et al.*, 2008). Further optimisation of this strain through deletion of the phosphoenolpyruvate carboxylase and overexpression of *glk, gapA, pfk, tpi* and *fba* reduced succinic acid production while maintaining glucose utilisation and resulted in a yield of 195 g/L of D- lactic acid (Tsuge *et al.*, 2015). *E. coli* naturally produces the D isomer of lactic acid and through deletion of multiple genes to reduce the production of acetate, ethanol, pyruvate and succinate produced 125 g/L of D- lactic acid (Zhou *et al.*, 2011). In *B. subtilis* a strain containing *idhA* from *L. delbrueckii* was able to produce 87 g/L of D-lactic acid within a minimal media opening up an additional option for industrial production (Awasthi *et al.*, 2018).

Although both isomers can be produced using expensive carbon sources such as glucose in order for mass production of PLA to occur is it predicted that the selling price of lactic acid will need to drop by half and the production cost should be below $0.8/ kg (Juturu and Wu,

2016). In order to achieve this low cost production further research needs to be carried out to achieve similar high yields from inexpensive carbon sources such as lignocellulose (Cubas-Cano *et al.*, 2018), food waste (Pleissner *et al.*, 2017), starch (Okano *et al.*, 2018) and glycerol (Bruno *et al.*, 2018). Achieving this low cost production of the platform chemical lactic acid will allow PLA to be fully utilised on a similar scale to comparable plastics such as PET and PS.

1.2.1.2. Polyhydroxyalkanoate polymers

In addition to polylactic acid, polyhydroxyalkanoates (PHAs) provide another source of biodegradable polymers produced directly from biomass which eliminates petrochemicals from the process. PHA's are a family of polyesters which are produced intracellularly which can have a large degree of customisation depending on which monomers the microorganism is engineered to produce. This can open up a variety of applications and physical properties to the produced polyesters (Gao *et al.*, 2011). PHA's are produced by over 250 species of bacteria (Jiang and Zhang, 2017) and are accumulated intracellularly upon limitation of key nutrients but availability of a carbon source. These stores are intended to be used as energy sources upon availability of the limiting nutrient and as such are readily biodegradable in microbial environments (Somerville, Poirier and Nawrath, 1995).

PHA's have a wide variety of uses again similar to PLA these uses are driven by the novel biodegradability of the produced polymers. Applications for PHA's has emerged in the packaging industry, along with uses in medicine and pharmaceuticals due to the biocompatibility of microbial polymers (Andreeßen and Steinbüchel, 2010). The PHA's with the greatest study to date are poly(3-hydroxybutyrate) (PHB) and its copolymer with 3-hydroxyvalerate poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHVB). PHB has physical properties similar to Polypropylene (PP), it is a highly crystalline thermoplastic with a similar tensile strength (40Mpa). However PHB is extremely brittle in comparison to PP therefore the copolymer PHVB has been produced which assists in limiting this brittle property (Jiang and Zhang, 2017). These polymers are highly degradable and have been known to fully degrade within seawater in 350 weeks (Lee, 2000), due to the environmental concerns of plastic within

marine environments which has been outlined previously (Section 1.2) this property makes commercial replacement of traditional plastics with PHA's an attractive proposition.

PHB and 3 copolymers (poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV), poly-3-hydroxybutyrate-co-4-hydroxybutyrate (P3HB4HB), and poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHx) are the only PHA's which are commercially produced on a large scale by 25 companies worldwide. These polymers are produced in batch fermentations of microorganisms such as *E. coli, Bhurkolderia sp* and *Ralstonia eutropha* but, as many downstream processing steps are required to extract the polymers from the cellular biomass, the high cost of production limits the mass market adaption of these polymers (Chen, 2010). The carbon feedstock is thought to contribute up to 40% of the production cost therefore research is currently directed to low value carbon sources (White, Laird and Hughes, 2017). It has been shown recently that fish waste (Mohapatra *et al.*, 2017), oxalate waste from the alumina industry (White, Laird and Hughes, 2017), xylose (Huo *et al.*, 2017) and seaweed (R. *et al.*, 2017) could be potential low value feedstocks for the production of PHA's.

While research is continuing to lower production costs in renewable feedstocks, research is also being carried out into alternative PHA polymers which may provide additional value. A PHA which is may provide additional value to poly(3-hydroxypropionate) (poly (3HP)). Poly (3HP) combines the physical properties of both PHB and PLA and as such has a higher stability than PLA but is more readily enzymatically cleaved than PHB (Andreeßen and Steinbüchel, 2010). This polymer has an excellent tensile strength and has been promising potential for use in the packaging industry and can also be used as a plasticiser in blends (Tingirikari, Ahmed and Yata, 2016). Bio-production of this polymer has been shown through conversion of 3-hydroxypropionate, malonyl- coenzymeA and propionaldehyde dehydrogenase by recombinant strains although yields remain low (Andreeßen, Taylor and Steinbüchel, 2014).

Along with its physical properties, which may be attractive commercially, poly (3HP) can be hydrolysed to produce its monomer 3-hydroxypropionate (3HP). 3HP was identified by the USDoE as a platform chemical with the potential to be used in a wide variety of applications such as the production of the industrial precursor acrylic acid along with antimicrobial applications in the food industry (Bozell and Petersen, 2010). This chemical was identified as a product which would provide high value if derived from biomass and therefore this may

provide additional value streams to poly (3HP) production processes if a lower demand for the polymer exists.

It is clear that there is a demand for biodegradable polymers and biological production of the platform chemicals required for their production. As innovation continues into controlling biodegradation opening the way for a wide variety of new applications the market for biodegradable biopolymers is sure to grow making them an important class of plastics for the future.

1.2.2. Bioproduction of existing mass market polymers

While biodegradable bioplastics represent a promising growing market with superior environmental attributes the mass production of the largest petrochemical derived plastics and polymers will remain due to the familiarity of these materials and the mass market applications which are already established. In order to replace the common plastics used today biodegradable plastics require either comparable attributes or a much lower cost (Ross, Ross and Tighe, 2017). Along with this some applications of plastics have functions for which degradation would be undesirable. As such replacement with biodegradable polymers is not possible currently but production of the common polymers from biomass is. Due to the necessity for a low and stable production cost of these mass market polymers, bioproduction is gaining increased popularity as the price of oil continues to fluctuate. A biomass derived process from a sustainable source should allow production to continue with minimal variability in price due to having a carbon source with a stable price and supply. As such research and innovation on the common polymers is now focused on elimination of petrochemical precursors.

1.2.2.1. Polyethylene terephthalate

A prominent example of industry led exploration, into incorporation of biomass derived substrates into the production of a common polymer, is the future bio based production of

polyethylene terephthalate (PET). Originally discovered in 1941, PET was quickly patented in 1946 (Ke and Yongping, 2005). This polymer was shown to be lightweight, strong, had low permeability of CO_2, no negative health effects associated with contact and had good optical clarity. Due to these properties PET has been used for a large variety of applications. Commonly used for the production of plastic drinks bottles PET fibres can also be used when blended with cellulosic fibres in the production of clothes, curtains, upholstery, threads, tire cord filaments and industrial fibres (Saleh, Bayoumi and Shatta, 2012). The excellent barrier properties and electrical insulation of PET allows it to be used in tapes, x-ray films, as mouldings for electronic devices and general electrical applications where conduction is not desired (Farah *et al.*, 2015).

As a result of these applications it is estimated that 700 million tons of PET has been produced since 1950 which accounts for 8% of all plastics ever produced. PET production accounts for 70% of all polyester, polyamide and acrylic fibres (Geyer, Jambeck and Law, 2017). In 2016 it was estimated that 100 million tons of PET was produced with production growing by 4% per annum. Commercially PET is produced using two substrates, ethylene glycol (EG) and purified terephthalic acid (PTA) both of which are derived from petrochemicals feedstocks (Pang *et al.*, 2016). These substrates are subjected to esterification and polycondensation under a high temperature and pressure resulting in the formation of PET (Xiao *et al.*, 2015).

EG for PET production originates from petrochemicals as ethylene which is oxidised to ethylene oxide then hydrated to form EG. PTA begins as a benzene, toluene, xylene mix from which the xylene is separated through crystallisation before oxidation and purification to form PTA (Pang *et al.*, 2016). This chemical pathway for the production of PET from petrochemicals is shown in Figure 1. The combination of fossil fuels dwindling along with massive fluctuations in the price of petrochemical precursors has driven industry to investigate how they can produce both of these substrates from biomass as demonstrated by Coca-Cola who announced the production of PET bottle produced entirely from biomass which is expected to reach the market in 2020 (Hottle, Bilec and Landis, 2017).

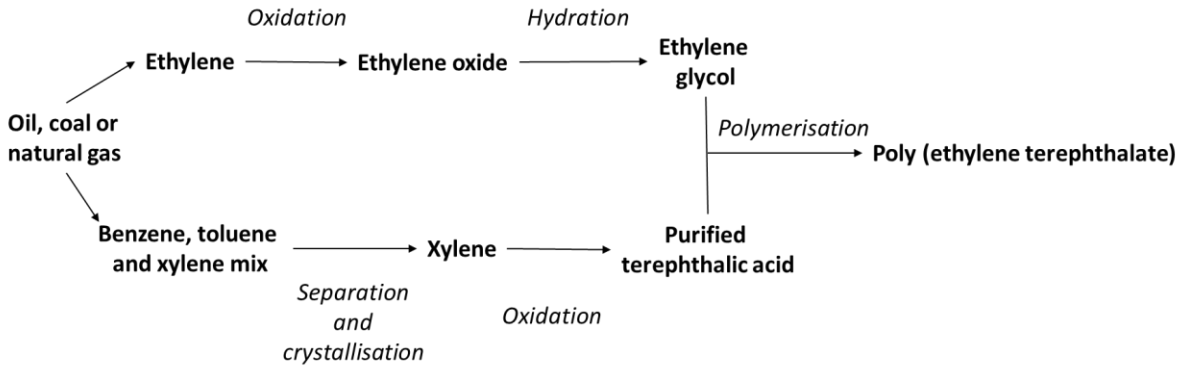


Figure 1: The petrochemical derived production process for Poly ethylene terephthalate (PET).

Two routes through which EG can be produced from biomass eliminating petrochemicals is through the utilisation of bio-produced ethanol or glycerol. Both ethanol and glycerol were identified by the USDoE in the top 10 platform chemicals with the highest potential in industrial applications (Bozell and Petersen, 2010). Ethanol has long been produced biologically through fermentation and is a prominent biofuel with over 25 billion gallons produced annually worldwide. For ethanol production as a biofuel the yeast *Saccharomyces cerevisiae* and a bacterial species *Zymomonas mobilis* are the common microorganisms used for fermentation , using sugarcane and corn used as the carbon source for the majority of ethanol production (Zabed *et al.*, 2017). Ethanol has recently become of interest as a platform chemical and not a fuel due to the growing demand for biopolymer production.

Ethylene, currently produced by the steam cracking of naphtha and ethane, is used for the production of polyethylene (PE), PET and can be alkylated to produce ethylbenzene for polystyrene (PS) production (Mohsenzadeh, Zamani and Taherzadeh, 2017). These 3 polymers account for almost 50% of all plastics produced and therefore the market for biologically produced ethylene is extremely large (Geyer, Jambeck and Law, 2017). Ethanol can be efficiently dehydrated to ethylene, and was the original source of ethylene before the rise of the petrochemical industry and cheap oil which made petrochemical cracking the superior option for production (Bozell and Petersen, 2010). Ethylene has been identified as a naturally produced hormone in plants (McManus, 2012) and as a metabolomic component of cyanobacteria (Kuchmina *et al.*, 2017) although neither route has been optimised for large scale production of ethylene within a bioreactor. With bio-ethanol production well

established at a large scale it is currently the superior route for ethylene production. Bio-ethylene produced from bio-ethanol is not currently used for PET production due to the cost. Bio-ethylene derived from sugar cane bio-ethanol which is the lowest price bio-ethanol costs $1200 / ton and bio-ethylene from lignocellulosic bio-ethanol costs $1900 / ton. For comparison petrochemical ethylene has an average cost of $1100 / ton (Pang *et al.*, 2016) and therefore demonstrates that further work is required into optimisation of the bio-ethanol process before it can be utilised for PET production.

Glycerol can also be utilised to produce EG. Glycerol was again identified by the USDoE as a bio –produced platform chemical with potential for many applications. Glycerol was included due as large volume of inexpensive glycerol is available due to increased biodiesel production (Bozell and Petersen, 2010). In 2015 2 million tons of glycerol was produced as a by-product of the biodiesel industry. Glycerol is used industrially as a feedstock for the production of dihydroxyacetone, 1,2-propylene glycol (1,2-PG), 1,3- propylene glycol (1,3-PG), acrolein and hydrogen. During the production of 1,2-PG, through hydrogenolysis, EG can be produced as a by-product depending on the catalyst used, with Raney nickel catalysts showing the highest selectivity (Yin *et al.*, 2009). Although EG can be produced in this reaction glycerol has two disadvantages for industrial EG production when compared to ethanol. The current volume of glycerol produced from biodiesel is not high enough to sustain mass production of PET and propylene glycol is a higher value product from hydrogenolysis therefore adaptation of hydrogenolysis for higher EG production is of economic disadvantage (Pang *et al.*, 2016).

PTA, the second substrate required for the production of PET can be produced from biomass by three routes all of which have only been demonstrated on a small scale (Pang *et al.*, 2016). Limonene which is present in the peel of citrus fruit can be used to produce PTA. The dehydrogenation of limonene yields p-cymene which can then be oxidised to PTA (Mathers, 2012). Although this is a viable source of biomass derived PTA, a sufficient quantity of limonene is unlikely for mass production of PET. Along with this limonene currently has applications within foods and perfumes further limiting supply (Ciriminna *et al.*, 2014).

A second method for bio-production of PTA is through the chemical conversion of furfural. The furans were included in the USDoE top 10 platform chemicals due to substantial improvement in their production (Bozell and Petersen, 2010) opening up additional applications. Furfural is an aldehyde, produced through the acid hydrolysis and dehydration

of xylose, with applications in the production of plastics, pharmaceuticals and agrochemicals (Mamman *et al.*, 2008). The production of furfural is a commercial process with annual production over 300 ktons (Nhien *et al.*, 2017). In order to produce PTA furfural is oxidised to fumaric acid and maleic acid followed by dehydration to maleic anhydride. Maleic acid and furfural derived furan is then subject to a Diels-Alder reaction yielding a Diels-Alder adduct which is dehydrated to produce phthalic anhydride. Phthalic anhydride is then hydrolysed to dipotassium phthalate before a transfer reaction and acidification produces PTA (Tachibana, Kimura and Kasuya, 2015). Although a cheap feedstock is utilised in xylose the number of reactionary steps may be detrimental to commercial production of PTA using this method.

The final method outlined in the literature for production of PTA from biomass is through the utilisation of isoprene and acrylic acid. Isoprene is a hydrocarbon commonly used to produce elastomers for products such as rubber bands, golf balls and shoes. With only 5% used for chemical production. Bio-isoprene is produced through sugar fermentation of bacteria with species of *Bacillus* demonstrating high yields (Sivy, Shirk and Fall, 2002; Morais *et al.*, 2015). Acrylic acid is a monomer and chemical intermediate with applications in the production of superabsorbent plastics, polymers and rubbers. Biobased acrylic acid can be derived from various sources including lactic acid, glycerol, glutamic acid and glucose (Beerthuis, Rothenberg and Shiju, 2015). PTA can be produced from these two substrates through a Deils-Alder reaction to yield 4-methylcyclohex-3-enecarboxylic acid followed by dehydro-aromatisation to yield 4-methylbenzoic acid. This is then oxidised using sulphuric acid to produce PTA. Although this process has a high yield of PTA (78%) bio-isoprene and acrylic acid are high value chemicals and therefore the current process would not be economical for PET production (Pang *et al.*, 2016).

In summary both substrates for PET production, EG and PTA, can be bio-produced but currently no method exists which in its current state is suitable for mass production of bio-PET. The majority of methods are hindered by the low value of PET which therefore requires a low production cost. As companies such as PepsiCo and The Coca-Cola Company have committed to the production of bio-PET industrial incorporation of biological routes to EG and PTA is inevitable with unpublished processes addressing this economic divide. This drive for the production of bio-PET along with biodegradable biopolymers demonstrates that the industrial production of polymers is quickly moving away from the use of petrochemicals.

1.3. Methyl-methacrylate and poly (methyl-methacrylate)

An organic compound which has significance industrially is the ester methyl methacrylate (MMA). At room temperature MMA is a colourless volatile liquid, has a fruity odour which is common to esters and exhibits toxicity through damage to mitochondria upon exposure (de la Casa-Resino, Pérez-López and Soler-Rodríguez, 2014). First produced commercially in Germany in 1933 (Olah, Prakash and von R. Schleyer, 1997) the demand for MMA was thought to be greater than 2.5 million tons per annum in 2018 (Degnan, 2018), a figure which is likely to rise in future years as new applications for MMA arise. The demand for MMA is driven by the variety of applications which can be provided by this compound and the applications of the polymer, poly methyl-methacrylate (PMMA).

As a monomer MMA can be used for the production of polymer dispersions in paints (Nagai, 2001) along with the production of various copolymers such as poly(acrylic acid-co-methyl methacrylate) used as a chemotherapy drug delivery platform (Yan and Gemeinhart, 2005), poly(styrene-co-methyl methacrylate) used in polymer-clay nanocomposite production (Xu *et al.*, 2003) and poly(N-isopropylacrylamide-co-methyl methacrylate) used for the production of thermosensitive hydrogels (Zhang and Zhuo, 2002). Although MMA is involved in the production of a large variety of copolymers the application which the majority of MMA produced is utilised for is the production of its polymer PMMA.

PMMA is a lightweight, optically clear, durable polymer which is also biocompatible opening avenues for use in biomedical applications (Norakankorn *et al.*, 2007; Fan *et al.*, 2015). Its production rose to prominence in World War II when it was used for aircraft glazing. Since that point PMMA has continued to be produced on a large scale across the world due to the favourable physical properties displayed by the polymer (Mosley, 2017). Commercially, PMMA sheets are branded under names such as Perspex (Lucite International), Acrylite (Evonik) and Plexiglas (Arkema) depending on which company they are produced by.

In addition to its primary application, as a replacement for glass, applications for PMMA are emerging in a plethora of fields. In biomedicine PMMA bone cement has been used for knee and hip replacements since 1953 with the use of the cement increasing 300% since 2004 in England and Wales (Ayre *et al.*, 2016). Due to the cost, clarity and durability PMMA has also

been shown to be a suitable alternative to glass for the production of plastic optical components (He *et al.*, 2016). It can also be used in microfluidic research as a low cost alternative to glass or silica (Haiducu *et al.*, 2008) and has been used to create battery components when electrospun into nanocomposite fibres with graphene (Khan *et al.*, 2014). It has also been found to have applications in the creation of dye sensitised solar cells due to the strength and clarity of the polymer (Ali, Karim and Buang, 2015). All of these current and potential applications contribute to the rising demand for this polymer and, as such, the methods by which it is produced commercially continues to evolve.

1.3.1. MMA Production

As time has progressed, three significant processes have been developed for the commercial production of the PMMA monomer MMA. These are the acetone cyanohydrin (ACH) process, the C4 oxidation process and the Alpha process developed by Lucite International. The eldest of these processes is the ACH process which continues to be used to date due to its efficiency.

Conventional petrochemical based production of MMA utilises the acetone cyanohydrin (ACH) process which was first commercialised in 1937 (Nagai, 2001). The first step in the process is the condensation of acetone and hydrogen cyanide to produce ACH. This is then hydrolysed, in the presence of sulphuric acid, to produce methacrylic acid (MAA) and ammonium sulphate by-products. The methacrylic acid is then esterified in the presence of methanol to produce MMA (Gogate, Spivey and Zoeller, 1997). After esterification downstream processing steps are employed to separate the mixture of MMA, unused methanol, water, ammonium bisulphate and sulphuric acid. Effective downstream processing allows the MMA to be recovered while recycling unused sulphuric acid and methanol back into the process (Mosley, 2017). An outline of this process is shown in Figure 2.

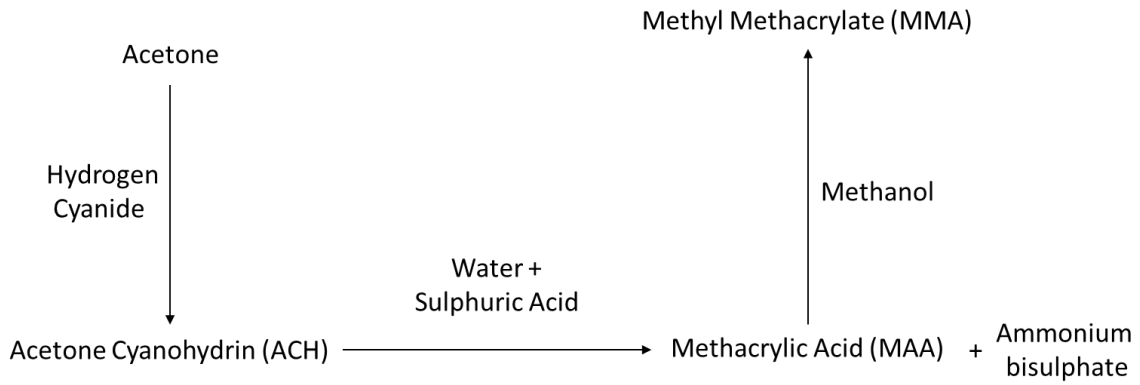


Figure 2: An outline of the ACH process commonly used for the production of MMA.

Due to the decreased availability of hydrogen cyanide, particularly in Japan, an alternative route of MMA production was developed utilising C4 intermediates as the precursors for production which effectively eliminated the need for hydrogen cyanide (Nagai, 2001; Mosley, 2017). The C4 oxidation process for the production of MMA is a two stage oxidation process employing isobutylene or *tert*-butanol as the precursor for production. The first step in the production pathway is the oxidation of each precursor to methacrolein using air. The second stage in the oxidation process then oxidises methacrolein to methacrylic acid. The final step in the process which is also seen in the ACH process is the esterification of methacrylic acid with methanol producing MMA. An outline of this process is shown in Figure 3. Initially a rate limiting step in this process was the performance of the oxidation catalysts which when slower was economically prohibitive and prevented mainstream adoption of this process (Nagai, 2001).

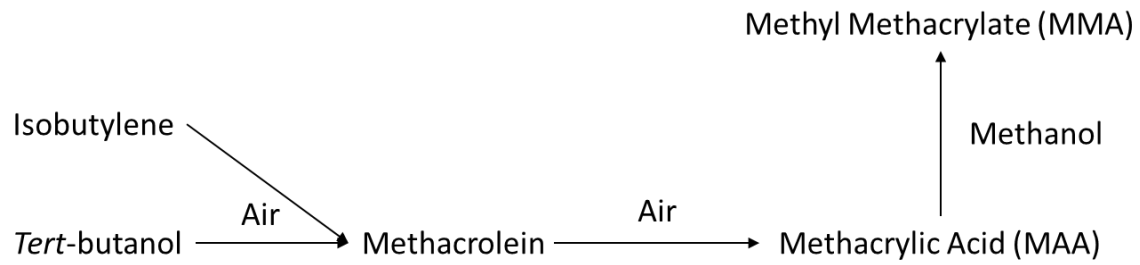


Figure 3: An outline of the C4 oxidation process used for MMA production. The process consists of two oxidation steps using air and an esterification reaction using methanol.

Both of the described MMA production processes had undesirable attributes in the form of high energy costs and precursors which can vary greatly in availability (Mosley, 2017). Therefore Lucite International developed the Alpha process which involves no toxic reactants or waste products and utilises readily available precursors (Raeissi, Florusse and Peters, 2013; Zhu *et al.*, 2015). The Alpha process uses ethylene, carbon monoxide, methanol and formaldehyde as precursors and produces MMA through two reactions. Initially methyl propionate is synthesised from ethylene, carbon monoxide and methanol through carbonylation. This is then followed by gas phase condensation of methyl propionate with formaldehyde to produce MMA (Nagai and Ui, 2004) The outline of this process is shown in Figure 4.

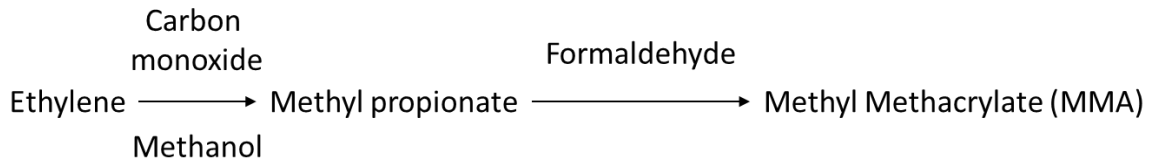


Figure 4: The Alpha process for the production of MMA from ethylene. The process involves the carbonylation of ethylene followed by the condensation of methyl propionate to form MMA.

1.3.2. Advantages of MMA bioproduction

All of the MMA production processes outlined previously have disadvantages associated with them contributing to the drive to develop a bioprocess for the production of MMA. The ACH process which is the most widely used MMA production process has several distinct disadvantages associated with it which makes a sustainable alternative an attractive proposition. The global demand for acetone, the substrate for this process, which is produced from fossil fuel reserves is increasing while fossil fuel reserves continue to deplete which may affect the process through increased cost or limited availability (Hoffmeister *et al.*, 2016). In conjunction with an unsustainable substrate, the process uses large volumes of toxic and hazardous materials such as hydrogen cyanide and produces ammonium bisulphate waste, both are issues which raise the cost of the process through the implementation of safe handling, disposal and remediation.

The C4 oxidation process and the Alpha process produce few waste products particularly the Alpha process but again both processes depend upon a supply of petrochemical substrates in the form of isobutylene or *tert*- butanol for the C4 process and ethylene for the alpha process. As such both processes rely on a low price of Brent crude oil which has an unpredictable value in Europe in the last 10 years with the price varying between $40 - $120 a barrel (Byrne, Lorusso and Xu, 2018). In conjunction with this availability of these substrates can also be unpredictable. Isobutylene in particular is used as a gasoline additive in western countries which limits its availability explaining why the C4 oxidation process is less common in western countries (Nagai, 2001).

Due to these disadvantages to the common MMA production processes research is shifting towards the biological production of MMA precursors. A potential precursor which has attracted early research is itaconic acid. Itaconic acid is an organic acid which was identified by the US department of energy (USDOE) in 2004 as an important building block chemical with a potential application in the production of MMA (Werpy and Peterson, 2004). It was subsequently removed from a paper revising this list in 2010 because the market for itaconic acid did not expand as predicted (Bozell and Petersen, 2010).

Itaconic acid is currently commercially produced through the submerged fermentation of *Aspergillus* species, particularly *Aspergillus terreus,* with chemical synthesis from petrochemicals unable to compete due to the cost effectiveness of the fungal fermentation process (Willke and Vorlop, 2001). The current production capacity of itaconic acid is thought to be 80,000 tons/ annum, however, due to limited commercial applications of itaconic acid supply outstrips demand (Bafana and Pandey, 2018). A significant hurdle in the use of itaconic acid as a platform chemical for the production of polyacrylic acid and PMMA is the cost which is currently $2 per kilo (Saha, 2017; Bafana and Pandey, 2018). It was originally suggested by the USDOE that in order for itaconic acid to be utilised commercially as a platform chemical the cost would need to decrease to $0.55 per kilo (Werpy and Peterson, 2004) although use specifically as a sustainable replacement for polyacrylate may be possible at $1.5 per kilo (Bafana and Pandey, 2018).

Lucite International have filed a patent covering the production of MMA from itaconic acid, citraconic acid or mesaconic acid, the carboxylation of which would produce methacrylic acid which can then be esterified (Johnson *et al.*, 2015). Using this process they aim to produce

50% of MMA from itaconic acid which would significantly increase the global demand for itaconic acid (Chenyu Du, 2014). A similar process is also in development by Evonik who also produce MMA on a commercial scale (Choi *et al.*, 2015). This investment into the production process for MMA using itaconic acid again demonstrates the current shift in attitude of commercial entities from the status quo of petrochemical derived precursors towards sustainable bio produced alternatives.

1.4. Strategic aim of this body of work

As outlined in section 1.3 innovation in the production of MMA is shifting from the development of new processes which utilise precursors derived from petrochemicals to the incorporation of bio produced precursors. The incorporation of bio produced precursors such as mesaconic acid into the MMA production process has several advantages. A successful process would have sustainability beyond the lifespan of petrochemicals, a precursor with a stable market value and upon development itaconic acid may be produced from a waste feedstock improving the economics of the process beyond that possible from a petrochemical precursor. The publishing of a patent outlining MMA production from itaconic acid, citraconic acid and mesaconic acid (Johnson *et al.*, 2015) highlights that industrial perspective has shifted towards sustainability.

Although bio-production of itaconic acid is well established the current process is unable to produce itaconic acid at a low enough value for cost effective utilisation as a platform chemical (Saha, 2017; Bafana and Pandey, 2018). It was therefore possible that another organic acid may prove economically superior for incorporation into a MMA production pathway. Along with itaconic acid, mesaconic acid had also been identified as a potential bio produced precursor for the production of MMA. Very little published research is available for the bio production of mesaconate from sugars having only been demonstrated within engineered strains of *E. coli* (Wang and Zhang, 2015; Bai *et al.*, 2016). As glutamate was a significant component in the mesaconate production pathway it was hypothesised that mesaconate production may be improved within *C. glutamicum* a microorganism known industrially as the superior host for glutamate production (Lothar Eggeling and Bott, 2005).

Also presented within this body of work is the novel production of butyl methacrylate within *E. coli* utilising initially α-ketoisovalerate (KIV) and butanol as the substrates followed by experiments aimed at completing the pathway by producing the KIV from glucose. Requiring only transesterification to produce MMA, butyl methacrylate upon successful bio-production at a sufficient rate has the potential to provide a superior pathway to MMA in comparison to organic acid precursors which require multiple steps for conversion to MMA.

The strategic aim of this research was therefore to investigate the production of mesaconate in *Corynebacterium glutamicum* and butyl methacrylate in *Escherichia coli* to produce a novel sustainable process for the production of MMA. To the knowledge of the author, mesaconate has never been produced within *C. glutamicum* and butyl methacrylate has never been produced within a microbial host demonstrating the novelty of this research. As this project was carried out in conjunction with Ingenza Ltd it therefore has significant commercial and industrial interest.

Chapter 2

Materials

and

Methods

2.1. Methods for Chapter 3

2.1.1. Bioprocess Details

2.1.1.1. Media Composition

For the bioprocesses using *Corynebacterium glutamicum* three separate media had been produced by Jeffrey, (2016) which were shown to optimise growth and glutamate production. The *C. glutamicum* glucose growth medium (CGG) and biotin depletion media were used for inoculum growth and the Batch media was used within the bioreactor. The composition of the three media are shown below.

The CGG media components are outlined in Table 4.

Table 4: The components and relative concentrations included in the CGG media.

Component	**Concentration (/L)**
Soya Peptone	10 g
Yeast Extract	5 g
Sodium Chloride	5 g
Glucose	5 g
Kanamycin	25 mg
Spectinomycin	200 mg

The Biotin depletion media components are outlined in Table 5.

Table 5: The components and relative concentrations included in the biotin depletion media.

Component	**Concentration (/L)**
Ammonium Sulphate	20 g
Urea	5 g
Calcium chloride	0.01 g
3-(N-morpholino)propanesulfonic acid (MOPS)	42 g
Magnesium Sulphate Heptahydrate	0.25 g
Potassium Phosphate Dibasic	1 g
Monopotassium Phosphate	1 g
Glucose	40 g
Protocatechuate 3,4-dioxygenase	33.3 mg
Biotin	5 µg
Kanamycin	25 mg
Spectinomycin	200 mg
Iron (II) Sulphate Heptahydrate	10 mg
Zinc Sulphate Heptahydrate	1 mg
Manganese (II) Sulphate Monohydrate	10 mg
Copper (II) Sulphate	0.2 mg
Nickel (II) Chloride Hexahydrate	20 µg

The Batch media components are outlined in Table 6.

Table 6: The components and relative concentrations included in the Batch media.

Component	**Concentration (/L)**
Ammonium Sulphate	20 g
Urea	5 g
Calcium chloride	0.01 g
Magnesium Sulphate Heptahydrate	0.25 g
Potassium Phosphate Dibasic	1 g
Monopotassium Phosphate	1 g
Glucose	44 g
Polypropylene Glycol	0.2 ml
Iron (II) Sulphate Heptahydrate	10 mg
Zinc Sulphate Heptahydrate	1 mg
Manganese (II) Sulphate Monohydrate	10 mg
Copper (II) Sulphate	0.2 mg
Nickel (II) Chloride Hexahydrate	20 µg
Protocatechuate 3,4-dioxygenase	33.3 mg
Biotin	1 µg
Kanamycin	25 mg
Spectinomycin	200 mg
Coenzyme B12	379 mg
Cobalt Chloride Hexahydrate	0.36 mg

For the bioprocess using gluconic acid as the carbon source glucose was replaced by the same concentration of gluconic acid in all three of the media.

2.1.1.2. Inoculum growth procedure

All strains were provided as glycerol stocks and were stored at -80^{O}C and defrosted prior to producing the inoculum. For 1 L scale experiments 50 µl of cell bank is added to 50 ml of CGG media in a 500 ml baffled shake flask and grown at 30^{o}C and 250 RPM until an OD600 of 10-15 is reached. The volume of cells needed to produce an OD600 of 0.1 in 100 ml of biotin limitation media was calculated. This volume is then centrifuged at 5000 RPM and the supernatant is discarded. The cells are then washed in 0.9% NaCl (w/v) before being re-centrifuged, the cells are then added to 100 ml of biotin depletion media in a 1 L baffled shake flask. The biotin depletion flask was then grown at 30^{o}C and 250 RPM until an OD600 of 10 is reached. The cells are then centrifuged at 5000 RPM and used to inoculate the batch media within the reactor. This resulted in a starting OD600 of 1 within the bioreactor.

For 4 L scale experiments the second stage is changed to 2x 2 L baffled shake flasks containing 200 ml of biotin depletion media. All other steps are the same.

2.1.1.3. Bioprocess conditions

The bioprocesses were carried out at both a 4 L and 1 L scale. The 4 L batch bioprocesses were carried out in a Bioflo 110 bioreactor (New Brunswick Scientific, Enfield, CT, USA) with a maximum working volume of 10 L. The bioreactor consisted of a borosilicate glass vessel with a stainless steel head plate including ports for pH and DO probes, a condenser, a temperature probe and feed ports. Mixing was provided through two six-blade Rushton turbines on the agitation shaft along with stainless steel baffles. Aeration was provided through the sparger which was positioned below the Rushton turbines to provide an equal distribution of air bubbles. The air flow rate was 1VVM.

The temperature of the vessel was controlled through an external heating jacket and internal cooling fingers controlled by a solenoid. The temperature was maintained at 30^{o}C and monitored by the control unit. pH was maintained at 7 through controlled addition of 2M sulphuric acid and 25% (v/v) ammonium hydroxide / 2M NaOH through peristaltic pumps. The pH is monitored on the control unit and through a pH probe (Mettler Toledo Ltd., Leicester,

UK). The dissolved oxygen is monitored through a dO_2 probe (Broadley-James Ltd., Bedford, UK) and maintained at 30% through an agitation cascade. Foam was controlled through the addition of small quantities of polypropylene glycol (PPG). The off gas measurements were made using a Servomex 4100 gas purity analyser (Servomex, Netherlands).

All 1 L bioprocesses were carried out in a modular Dasgip system (Eppendorf, Hamburg, Germany) consisting of a borosilicate glass vessel with a stainless steel head plate including ports for pH and DO probes, a condenser, a temperature probe and feed ports. Mixing was provided through two six-blade Rushton turbines on the agitation shaft. Aeration was provided through the sparger which was positioned below the Rushton turbines to provide an equal distribution of air bubbles. The air flow rate was 1VVM. The modules used in the operation of the system were a CWD 4 temperature control unit, a MP4 pump system for additional feed lines, a MP8 pump system for acid and base control, a PH4 (PO4) unit for processing dissolved oxygen and pH measurements, a MX4/4 gas control unit, a TC4 (SC4) unit to control temperature and stirrer speed and a GA4 gas analyser unit. pH was maintained at 7 through controlled addition of 2M sulphuric acid and 25% (v/v) ammonium hydroxide / 2M NaOH through peristaltic pumps. The pH is monitored on the control unit and through a pH probe (Mettler Toledo Ltd., Leicester, UK). The dissolved oxygen is monitored through a dO_2 probe (Broadley-James Ltd., Bedford, UK) and maintained at 30% through an agitation cascade. Foam was controlled through the addition of a drop of polypropylene glycol (PPG) upon seeing foaming, if more antifoam was required PPG was added slowly drop by drop. The system was controlled using DASGIP control software.

In all bioprocesses when induction was required isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM within the bioreactor at the time point of induction.

2.1.2. Shake flask method for IPTG toxicity testing

IL5.0.1(G2).2(F16-F17) (strain outlined in section 3.5) was grown as outlined in section 2.1.1.2 before addition to the shake flasks. The experiment was carried out at a 50 ml scale within 500 ml baffled shake flasks. The cells were diluted so as to have a starting OD600 of 1 within the batch media at the start of the shake flask experiment. 0.125 ml of IPTG was added, which

resulted in a final concentration of 1 mM, to the appropriate shake flask at either 0, 10 or 20 hours with 1 control flask having no IPTG addition. Addition was aseptic within a laminar flow hood. The flasks were incubated at 30^{O}C and 200 RPM for the duration of the experiment within a rotating incubator. Samples were analysed as outlined in section 2.1.3 to determine the optical density at 600 nm.

2.1.3. Optical Density Measurements

The optical density of samples was measured at an absorbance of 600nm using a Biomate 5 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA.). Samples were diluted with distilled water to ensure measurements were below 1 Absorbance Unit to comply with the Beer Lambert Law.

2.1.4. Dry Cell Weight (DCW)

The dry cell weight of fermentation samples was determined using a microcentrifuge tube weighing method. For each sample 1 ml was dispensed into three 1.5ml microcentrifuge tubes which had been pre-dried for at least 24 hours in a 100^{o}C oven and weighed before the sample was added. The sample was then pelleted by spinning at 13000 RPM for 5 minutes. After centrifugation the supernatant was removed and stored at -20^{o}C, the pellet was then washed in distilled water and centrifuged again. The water was then removed from the microcentrifuge tubes before drying in a 100^{o}C oven for 24 hours followed by 24 hours in a desiccator. The tubes were then weighted again and the DCW determined by gravimetric difference.

2.1.5. High Performance Liquid Chromatography (HPLC)

Analysis of glucose, amino acids and mesaconate was carried out on a modular HPLC System (All parts were acquired from Gilson (Middleton, USA) unless otherwise stated.). The HPLC system is shown in Figure 5. The system consists of a Jones chromatography Model 7971

Column Heater (Colorado, USA), a Viscotek, Model VE3580 RI Detector (Malvern Instruments Ltd, Worcestershire, UK) and a Diode array model 170. The system was controlled from a single point through Gilson Unipoint Version 5.11 Software (Middleton, USA). All samples added to 0.8 ml HPLC sample vials was filtered through 13 mm, 0.22 µm, PTFE Syringe Filters (Gilson Scientific, Middleton, USA) and each sample was injected in triplicate.

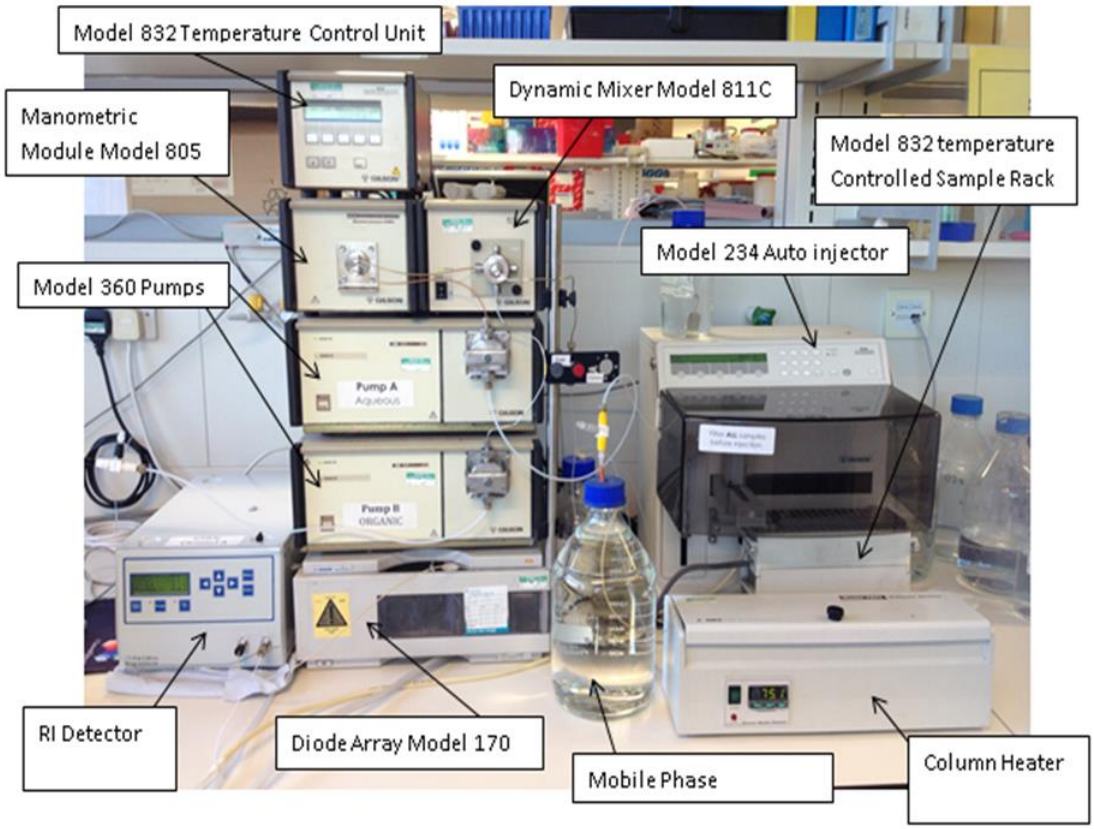


Figure 5: The modular HPLC system including part labels.

2.1.5.1. Glucose

For glucose analysis an isocratic method is used with a Phenomenex, Rezex ROA- Organic Acid H^+ (8%) column (Cheshire, UK) 150/7.8 mm in size, 8 µm particle size. The column temperature was set to 75°C. The mobile phase used was 2.5 mM sulphuric acid with a flow rate of 0.6 ml/min. The RI detector was used for the identification of glucose. All samples were analysed by technical triplication with a standard curve of samples produced for every

analytical run. The R^2 value of the standard curve for authentic external analyte calibrants was checked each run to ensure it was >95%.

2.1.5.2. Amino Acids

To quantify amino acids within the samples a derivatisation method was used to create a chromophore containing molecule allowing improved sensitivity of detection by the DAD detector. This was achieved by the derivatisation method involving the reaction between the amino acid, ortho-phthalaldehyde (OPA) and 2- mercaptoethanol. This method is well documented in the literature (Hanczkó and Molnár-Perl, 2003; Gardner *et al.*, 2013). The column used was a reverse phase C_{18} chromatography column 150/4.6 mm in size with a 5 µm particle size (Grace Davidson Discovery Sciences, Illinois, USA). The column temperature was 40°C throughout the process and analytes were examined at 340 nm. All samples were analysed by technical triplication with a standard curve of samples produced for every analytical run. The R^2 value of the standard curve for authentic external analyte calibrants was checked each run to ensure it was >95%. The gradient used for the process is shown in Table 7.

Table 7: The gradient method used for the amino acid quantification method using HPLC.

Time (Mins)	**0.2% Formic acid + H_2O (%)**	**Methanol (%)**
0-2	80	20
2-4	48	52
4-5.5	48	52
5.5-10	10	90
10-18	10	90
18-20	80	20

2.1.5.3. Mesaconate- Original Method

For mesaconate analysis an isocratic method was used with a Phenomenex, Rezex ROA-Organic Acid H^+ (8%) column (Cheshire, UK) 150/7.8 mm in size, 8 µm particle size. The column temperature was set to 75^oC. The mobile phase used was 5 mM sulphuric acid with a flow rate of 1 ml/min. The UV detector was used for the identification of mesaconate at 210 nm. All samples were analysed by technical triplication with a standard curve of samples produced for every analytical run. The R^2 value of the standard curve for authentic external analyte calibrants was checked each run to ensure it was >95%.

2.1.5.4. Mesaconate- Improved Method

For mesaconate analysis an isocratic method was used with a Phenomenex, Rezex ROA-Organic Acid H^+ (8%) column (Cheshire, UK) 150/7.8 mm in size, 8 µm particle size. The column temperature was set to 75^oC. The mobile phase used was 5 mM sulphuric acid with a flow rate of 0.6 ml/min. The UV detector was used for the identification of mesaconate at 240 nm. All samples were analysed by technical triplication with a standard curve of samples produced for every analytical run. The R^2 value of the standard curve for authentic external analyte calibrants was checked each run to ensure it was >95%.

2.1.6. Yellow Springs Instrument (YSI) Analysis

For the fermentations described in sections 3.12, 3.17 and 3.19 the glucose, glutamine and glutamate concentrations within the samples was determined using a YSI 2950 Select Biochemistry Analyser (YSI incorporated, OH, USA). The system operates through electrochemical probes attached to membranes with imbedded oxidases depending on the substrate being analysed. When the substrate being measured meets the membrane hydrogen peroxide is released which is detected by the probe leading to the creation of a signal current with which the analyser can display the concentration within the sample.

Sample supernatant was diluted to ensure the concentration of the substrate was below the operational limit of <5 g/L. In order to be analysed the sample the supernatant was transferred into a 1.5ml microcentrifuge tube and each sample was measured in triplicate.

2.1.7. Fraction Collection

Fraction collection was carried out using the method outlined in section 2.1.5.3 with the mobile phase replaced by distilled water. Fractions were collected using the Gilson modular HPLC system (Section 2.1.5) with attachment of a fraction collector. A 1 g/L mesaconate standard was run to determine the retention time before the sample was injected. Three 2 minute fractions were collected in universal vials from the injection of the sample at 13-15, 15-17 and 17-19 minutes. This 6 minute window covered the complete mesaconate peak. This was repeated for multiple replicate sample injections to collect a sufficient volume of fraction.

2.1.8. NMR Sample Preparation

For NMR analysis sample was evaporated using a rotary evaporator (Rotavapor model R3, Buchi Ltd, Oldham, UK) before being resuspended in 1 ml of deuterium oxide and added to NMR tubes. A 1 g/L mesaconate standard was also produced diluted in deuterium oxide. The sample and standard were then sent for proton NMR analysis.

2.1.9. LC-MS Sample Preparation and Analysis

For LC-MS analysis fractions collected using the method outlined in section 2.1.7 were evaporated using a rotary evaporator (Rotavapor model R3, Buchi Ltd, Oldham, UK) before being resuspended in 1 ml of methanol and added to HPLC vials. LC-MS analysis was then performed by Dr Christina Viegelmann (University of Strathclyde) using an Exactive mass spectrometer with an electrospray ionization source attached to an Accela 600 HPLC pump with Accela autosampler and UV/Vis detector (Thermo Scientific, Bremen, Germany). Mass spectrometry was carried out over a mass range of 100–2000 m/z in positive and negative

ionization modes with spray voltage of 4.5 kV and capillary temperature at 270 °C. A flow rate of 300 µL/min was used and the column used was an ACE5 C18 column (5 µm × 75 mm × 3 mm) (Hichrom Limited, Reading, UK). The mobile phases used were water + 0.1% formic acid (v/v) and Acetonitrile + 0.1% formic acid (v/v). LC-MS data was acquired using Xcalibur version 2.2 (Thermo Scientific, Bremen, Germany).

2.2. Methods for Chapter 4

2.2.1. Inoculum production methods

2.2.1.1. Batch biotransformation inoculum production (Constitutively expressed plasmids)

Miller lysogeny Broth (LB) (Sigma Aldrich, Missouri, USA) was used for the growth of the biotransformation inoculum. This LB medium was used for all experiments throughout chapter 4 and was prepared as outlined in the manufacturer's instructions (25 g/L in deionised water). For any strain, 100 µl from a cell bank vial was added to 300 ml of LB medium in a 1L Erlenmeyer flask. For strains containing a pMMA plasmid, 300 µl of the 0.2 g/L stock (1000x Stock) of ampicillin was added to the LB resulting in a 200 µg/L concentration of ampicillin. For strains containing the pKIV plasmid 300 µl of the 0.025 g/L stock (1000x Stock) of chloramphenicol was added to the LB resulting in a 25 µg/L concentration of chloramphenicol. If both plasmids were present both antibiotics were added as outlined above. The inoculum flasks were incubated at 30^{o}C and 200 RPM in a rotating incubator (IKA KS4000i control, Staufen im Breisgau, Germany). The inoculum was grown overnight for approximately 18 hours. The OD600 was checked at 18 hours by aseptically removing 1 ml of culture using a graduated pipette. The 1 ml sample was then diluted 1/10 in 3 cuvettes to provide a triplicate. The triplicate cuvettes were then read in a spectrophotometer (DR3900, Hach Lange Ltd, UK) at 600nm and the OD was recorded. For successful biotransformations the OD600 was required to be above 3. If the OD600 was below 3 the inoculum was returned to the incubator and the OD600 was repeatedly checked until the culture OD600 measured above 3.

Once the desired OD600 was reached the inoculum was transferred into 500 ml sterile centrifuge tubes and centrifuged (Avanti J-E Series, Beckman Coulter, California, USA) at 5000 RPM for 20 minutes at 4°C. After centrifugation the supernatant was removed and the pellet was re-suspended in 0.1M sodium phosphate buffer (pH 7) to produce a buffer/cell solution with an OD600 of 50. This solution was then ready to be used in the batch biotransformations where it will be diluted ½ by KIV stock giving a final OD600 of 25 in 0.05 M sodium phosphate buffer (pH 7).

2.2.1.2. Batch Biotransformation inoculum production (With an inducible plasmid)

For batch biotransformations using strains containing an IPTG induced plasmid the inoculum process was the same as section 2.2.1.1 until after the first OD600 reading. At this point, after an approximate 18 hour growth, the inoculum was diluted in the same medium to an OD600 of 0.3. The OD600 was monitored until an OD600 of 0.6- 0.8 was reached. At this point IPTG was added to a final concentration of 1 mM the inoculum was then incubated at 30°C and 200 RPM until an OD600 above 3 was reached. After this point the same protocol was followed as in section 2.2.1.1.

2.2.1.3. Fed-batch biotransformation inoculum production (Constitutively induced plasmids)

For fed-batch biotransformations with no induction the same protocol was followed as in section 2.2.1.1. When re-suspending the cell pellet 0.05 M sodium phosphate buffer (pH 7) was used and the cell/buffer solution was re-suspended to an OD600 of 25 not 50 for these experiments as there was no ½ dilution step with a KIV stock in this method.

2.2.2. Biotransformation methods

2.2.2.1. Batch Biotransformations

All batch biotransformations were carried out in 250 ml Schott bottles unless otherwise stated using a working volume of 30 ml. All Schott bottles were sterilised before use. To start a batch biotransformation 15 ml of the cell/buffer mixture with an OD600 of 50, from the inoculum preparation step (Section 2.2.1.1), was added to the Schott bottle along with 15 ml of 80 mM KIV stock and 165µl of butanol (60 mM final concentration). This resulted in a biotransformation vessel containing a final volume of 30 ml, a cell OD600 of 25 in 0.05 M sodium phosphate buffer (pH 7), 40 mM of KIV and 60 mM of butanol. The biotransformation vessel was then incubated at 30^{o}C and 200 RPM in a rotating incubator (IKA KS4000i control, Staufen im Breisgau, Germany). The vessel was sampled periodically for HPLC analysis. The sample preparation for HPLC analysis is outlined in sections 2.2.3.6 and 2.2.3.7. For the biotransformation with increased oxygen the Schott bottle was sparged with oxygen for 2 minutes before sealing.

2.2.2.2. Sparged batch biotransformations

The inoculum for the sparged batch biotransformations was produced using the same method outlined in section 2.2.1.1. The sparged batch biotransformations were carried out in three different vessels 100 ml baffled Erlenmeyer flasks, 100 ml Erlenmeyer flasks and 100 ml serum bottles. All three vessel types were glass and were sterile before use, the working volume for each biotransformation was 20 ml. To start the biotransformations 10 ml of the cell and 0.1M sodium phosphate buffer (pH 7) mixture with an OD600 of 50 prepared in the inoculum step (Section 2.2.1.1) was added to the vessel with 10 ml of 80 mM KIV stock and 110 µl butanol (to give a final concentration of 60 mM).

After the aseptic addition of the substrates and the inoculum the vessels were sealed using rubber Suba-seals (Sigma Aldrich, Missouri, USA). The vessels were then placed in a rotating incubator (IKA KS4000i control, Staufen im Breisgau, Germany) at 30^{o}C and 180 RPM and the sparge system was attached. The vessels were sparged using needles which pierced the Suba-

seals, the air sparge was provided by an air pump set to 1 VVM. The air was filtered before entering the vessel through 0.2 µm PTFE filters, there was a second needle in each vessel to provide an outlet for the air to prevent a build up of pressure. A diagram of the sparged biotransformation setup is shown in Figure 6.

Samples (0.5 ml) were taken through the Suba-seal using a sterile needle and syringe periodically and were then processed for HPLC analysis as outlined in sections 2.2.3.6 and 2.2.3.7.

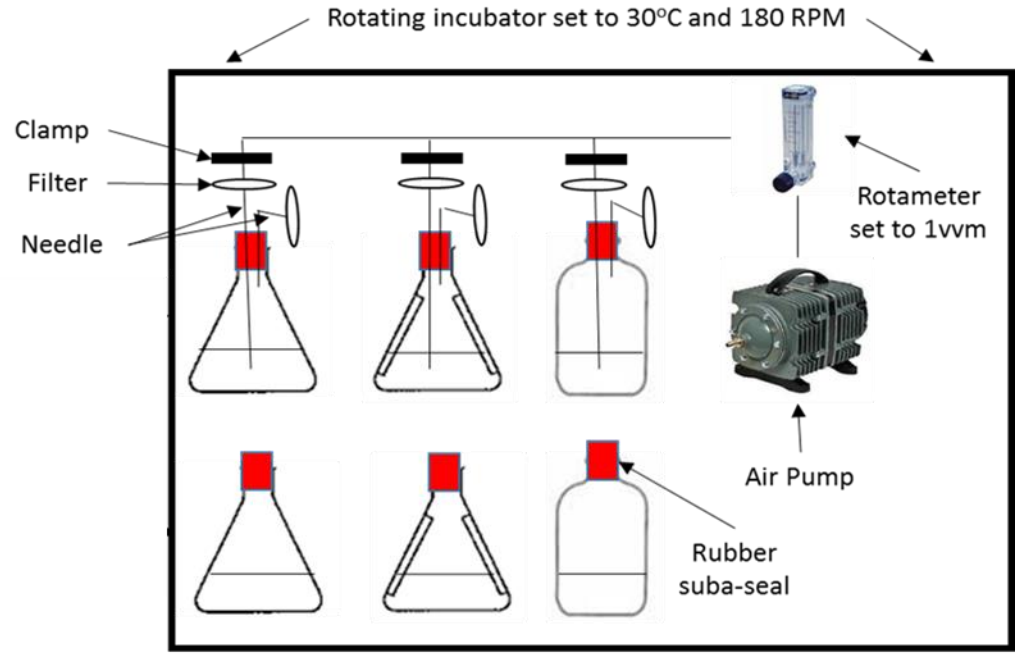


Figure 6: The outline of the sparged biotransformation setup.

2.2.2.3. Fed-batch biotransformations

All fed batch biotransformations were carried out in 250 ml sterile Schott bottles unless otherwise stated, using a working volume of 30 ml. To start a fed batch biotransformation 29 ml of the cell/ buffer suspension with an OD600 of 25 from the inoculum growth protocol (Section 2.2.1.1) was added to the Schott bottle. Depending on the experiment a KIV and/or butanol stock was prepared at a concentration at which the addition of 1 mL of stock would result in the desired KIV and/or butanol concentration upon addition. The stocks prepared for specific sections are shown below in Table 8.

Table 8: The outline of the KIV and butanol stocks prepared for fed batch biotransformations.

Section	Stocks Prepaired	Resulting Feed Concentration
4.6	150 mM butanol	5 mM/ ml
	150 mM KIV	5 mM/ ml
	150 mM KIV and 150 mM butanol	5 mM/ ml of both
	30 mM KIV and 30 mM butanol	1 mM/ ml of both
4.6.1	150 mM KIV and 150 mM butanol	5 mM/ ml of both
4.6.2	300 mM KIV and 300 mM butanol	10 mM/ ml of both
4.6.3	150 mM KIV and 150 mM butanol	5 mM/ ml of both
	300 mM KIV and 300 mM butanol	10 mM/ ml of both
	300 mM KIV and 150 mM butanol	10 mM/ ml of KIV and 5 mM/ ml of butanol
	150 mM KIV and 300 mM butanol	5 mM/ ml of KIV and 10 mM/ ml of butanol

The vessel containing the 30 ml biotransformation volume was then incubated at 30^{o}C and 200 RPM. 1 ml samples were taken from the fed-batch biotransformation at each time point before the addition of the 1 ml of KIV and butanol stock, this ensured that the volume within the biotransformation remained at 30 ml. The HPLC sample preparation is outlined in sections 2.2.3.6 and 2.2.3.7.

2.2.2.4. Co-factor Biotransformations

Cofactor biotransformations were carried out within 250 mL Schott bottles with a 30 ml working volume. For biotransformations in which cofactors were added the inoculum was prepared as outlined in section 2.2.1.1 for batch biotransformations. A mixed coenzyme stock was produced containing the following concentrations – coenzyme A (CoA) = 30 mM (24.275 g/L), nicotinamide adenine dinucleotide (NAD^+) = 30 mM (19.925 g/L) and thiamine pyrophosphate (TPP) = 6 mM (2.5 g/L). This stock was produced by dilution of the required quantity of all cofactors with sterile water. This stock was then filter sterilised for use in the biotransformation.

The concentration of the coenzyme stock therefore required the addition of 1 ml to result in a 30 ml biotransformation with final coenzyme concentrations of 1 mM of CoA, 1 mM of NAD^+ and 0.02 mM of TPP. The biotransformations were set up by adding 15 ml of cell/ buffer inoculum with an OD 600 of 50, 1 ml of the coenzyme stock solution and 14 ml of a KIV stock solution which resulted in a final KIV concentration of 40 mM. 165 µl of butanol was then added to have a final concentration of 60 mM. The Schott bottles were then incubated at 30^{O}C and 200 RPM within a rotating incubator (IKA KS4000i control, Staufen im Breisgau, Germany). Samples were taken periodically and prepared as outlined in sections 2.2.3.6 and 2.2.3.7 for HPLC analysis.

2.2.3. Analytical methods

2.2.3.1. GC-MS Method

GCMS analysis was carried out using an Agilent 7694 headspace sampler and an Agilent 5973 inert mass selective detector (Agilent, Santa Clara, USA). Helium was used as the carrier gas. The column used was a ZB-WAX + column (Phenomenex, Torrance, USA). The method had a max temperature of 260^{o}C and a flow rate of 1 ml/ min. The temperature gradient was 60^{o}C at 5 minutes, 200^{o}C at 20 minutes and 260^{o}C at 30 minutes. The headspace sample vials were heated to 90^{o}C prior to sampling. The analysis and equipment operation was carried out by

Jonathan Selfridge (Ingenza Ltd). Biotransformations which were analysed by GC-MS were carried out in sealed GC-MS vials.

2.2.3.2. HPLC method for α-ketoisovalerate (KIV), isobutyric acid (IBA), glucose and butanol analysis

Analysis of α-ketoisovalerate (KIV), isobutyric acid (IBA), glucose and butanol was carried out on the same HPLC method. The method was isocratic and was carried out on a modular Agilent 1200 HPLC (Agilent, Santa Clara, USA). The column used was a REZEX ROA Organic Acid H^+ (8%) 300 mm x 7.8 mm (Phenomenex, Torrance, USA) which was heated to 65^oC in the internal column heater. The mobile phase used was 0.005 Mol/L sulphuric acid produced using 95-97% (v/v) sulphuric acid and HPLC grade water. The flow rate of the mobile phase in the method was 1 mL/min. The injection volume for each sample was 20 µl and all samples were injected in triplicate. For every analyte at least 5 standards were produced the concentrations of which varied depending on the expected levels of each analyte in the current experiment. A refractive index detector and diode array detector were both used for analysis, the diode array detector used a wavelength of 210nm and the reference was 450 nm.

2.2.3.3. HPLC method for butyl methacrylate analysis (Gradient method)

Analysis of butyl methacrylate (BMA) was carried out using a gradient method on an Agilent 1100 HPLC (Agilent, Santa Clara, USA). The column used was a GraceSmart RP18 C_{18} 150 mm x 4.6 mm (Fisher Scientific, New Hampshire, USA) which was heated to 40^oC in the internal column heater. The method gradient and solvents are shown below in Table 9.

The combined flow rate of the solvents was 1 mL/min with an injection volume of 20 µL per injection. Each sample was injected in triplicate for an accurate analysis along with at least 5 standards which were produced, the concentrations of which varied depending on the expected levels of each analyte in the current experiment. The diode array detector was used to analyse BMA, the wavelength used was 215 nm and the reference wavelength was 360 nm.

Table 9: The outline of the gradient method for BMA analysis.

Time (Minutes)	Water + 0.1% Formic Acid (%)	Acetonitrile + 0.1% Formic Acid (%)
0 - 1	50	50
1 - 5.10	20	80
5.10 - 9	10	90
9 – 9.25	50	50
9.25 - 13	50	50

2.2.3.4. HPLC method for butyl methacrylate analysis (Isocratic method)

Analysis of BMA was carried out using an isocratic method on an Agilent 1100 HPLC (Agilent, Santa Clara, USA). The column used was a GraceSmart RP18 C_{18} 150 mm x 4.6 mm (Fisher Scientific, New Hampshire, USA) which was heated to 40^{o}C in the internal column heater. The mobile phase was 50%/50% distilled water and methanol (v/v) with 1 ml of trifluoroacetic acid added per litre. The flow rate used was 1 mL/min with an injection volume of 20 µL per injection. Each sample was injected in triplicate for an accurate analysis along with at least 5 standards which were produced, the concentrations of which varied depending on the expected levels of each analyte in the current experiment. The diode array detector was used to analyse BMA, the wavelength used was 210 nm and the reference wavelength was 360 nm.

2.2.3.5. Cell banking method

All cell banks were created as follows unless otherwise stated. To prepare the inoculum to be cell banked either a single colony from a plate or a master vial was used to inoculate 30 ml of LB containing the appropriate antibiotic concentration in a 500 ml Erlenmeyer flask. The

inoculum was incubated at 37°C and 250 RPM in a rotating incubator (IKA KS4000i control, Staufen im Breisgau, Germany). The inoculum was grown until and OD600 of 5 was measured on a spectrophotometer (DR3900, Hach Lange Ltd, UK). The inoculum was then transferred aseptically into a sterile centrifuge tube and centrifuged at 3900 RPM and 4°C for 10 minutes in a centrifuge (Avanti J-E Series, Beckman Coulter, California, USA). The supernatant was then removed and the cells were resuspended in LB medium and 50% glycerol (v/v) to the same volume that was originally transferred into the centrifuge tube. The LB and 50% glycerol (v/v) were mixed to create a final concentration of glycerol at 10% (v/v). The resuspended solution was then aliquoted into freezer vials each containing 1 ml. These vials were then stored in the -80°C freezer.

In order to confirm that a new cell bank was not contaminated a cell bank vial was streaked onto each of the following agar plates: LB, LB + Antibiotic (s), Nutrient agar, Yeast extract peptone dextrose, CGG, CGG + Nalidixic acid, Potato dextrose agar, MacConkey agar and Mannitol salt agar. Each plate was streaked in duplicate with a plate of each type incubates in a 30°C and a 37°C incubator for 24- 48 hours. The plates were then checked for contamination and if no contamination was present the cell bank was certified for use.

2.2.3.6. BMA sample preparation for HPLC analysis

For BMA analysis by HPLC a 0.5 ml sample was aseptically removed from the biotransformation vessel using a Gilson P1000 pipette and transferred into a 1.5 ml microcentrifuge tube. A 1 ml volume of HPLC grade acetonitrile was then added to the 1.5ml microcentrifuge tube which was then shaken briefly by hand. The microcentrifuge tube was then shaken for 20 minutes on an orbital shaker (IKA labortechnik KS125, Staufen im Breisgau, Germany) at 800 RPM. The microcentrifuge tube was then centrifuged (5415C centrifuge, Eppendorf, Hamburg, Germany) at 14000 RPM for 3 minutes before 1 ml was syringe filtered through a 0.2µm PTFE filter into an HPLC vial. The HPLC vial was then sealed using a metal crimp cap and placed in a -20°C refrigerator for storage before analysis.

2.2.3.7. KIV, IBA, and butanol sample preparation for HPLC analysis

For KIV, IBA and butanol analysis by HPLC a 0.5 ml sample was removed from the biotransformation vessel aseptically using a Gilson P1000 pipette and transferred into a 1.5ml microcentrifuge tube. Samples required different dilutions depending on the substrate or product concentrations, this was to ensure samples fell within the range of the HPLC standards. Depending on the dilution required a volume of the sample was transferred to another 1.5ml microcentrifuge tube and the required volume of deionised water was added to dilute the sample. The diluted sample was then centrifuged (Sigma 1-14 Microfuge, Sigma Aldrich, Missouri, USA) for 3 minutes at 14000 RPM. The centrifuged sample was then syringe filtered through a 0.2µm PTFE filter into an HPLC vial. The HPLC vial was then capped and stored in the -20^{o}C refrigerator prior to analysis by HPLC.

2.2.3.8. Cell viability assay

For cell viability plating a 1 ml sample was taken aseptically from the biotransformation vessel into a 1.5ml microcentrifuge tube . The sample was then serially diluted in 1/10 increments. Sterile 1.5ml microcentrifuge tubes each containing 900µl of sterile water were labelled. 100µl from the sample was pipetted into the first 1.5ml microcentrifuge tube which was mixed by shaking. 100µl was then transferred from the mixed dilution into the next 1.5ml microcentrifuge tube, this was repeated until all dilutions were complete. 100µl from each dilution was pipetted onto LB + 1 mM antibiotic (depending on strain) agar plates. The sample was spread around the plate before incubation for 24-48 hours in a 30^{o}C incubator until countable colonies formed. The colonies were then counted to provide cell viability numbers.

2.2.4. Media compositions

2.2.4.1. Lund media composition

Lund media is a medium which has previously been used by Ingenza to scale bioprocesses successfully to up to 50,000 L. The components of this media and the concentrations used are shown below in Table 10. Any adaptations to this base media are outlined within the text if

applicable. If required antibiotic stock solutions were added to the completed media to give the appropriate concentration of antibiotic.

Table 10: The components and concentration of components within Lund medium.

Component	**Concentration (/L)**
Glucose	10 g
Ammonium sulphate	2 g
Dipotassium phosphate	14.6 g
Sodium phosphate monobasic dihydrate	3.6 g
Ammonium citrate dibasic	0.5 g
Magnesium sulphate heptahydrate	0.493 g
Calcium chloride dihydrate	1 mg
Iron (III) chloride	20 mg
Zinc sulphate heptahydrate	0.36 mg
Copper (II) sulphate pentahydrate	0.32 mg
Manganese (II) sulphate monohydrate	0.3 mg
Cobalt (II) chloride hexahydrate	0.36 mg
Ethylenediaminetetraacetic acid disodium salt dihydrate	44.6 mg

Chapter 3

Mesaconate production using *Corynebacterium glutamicum*

3.1. Introduction

Mesaconate is a branched unsaturated dicarboxylic acid and has industrial significance as a platform chemical due to potential applications across a variety of industry sectors. It has previously been used for the production of fire retardant materials (DiGiulio and Bauer, 1981) a quality also shared by the diallyl ester of mesaconate (Currier, Gerald and Ellis, 1972). It can also be incorporated into acrylamide hydrogels which have applications spanning the biomedical, agricultural, food, building and communication sectors (Üzüm, Kundakci and Karadağ, 2009). The incorporation of mesaconate into such materials has been shown to improve the functionality of the gel in relation to biomedical and drug delivery related applications in particular (Üzüm and Karadağ, 2005). The application of mesaconate with the greatest commercial significance is as a precursor for the production of methyl methacrylate (MMA) (Johnson *et al.*, 2013).

The current demand for MMA is around 2.5 million tons per annum (Degnan, 2018) and is driven by the numerous applications for the polymer poly- (methylmethacrylate) (PMMA) as outlined in section 1.3. All of these current and potential applications make finding a renewable source of MMA and therefore its precursor mesaconate an important endeavour which in turn reduces our reliance on finite petrochemicals.

3.2. Mesaconate Production

Mesaconate was originally produced in 1880 through the decarboxylation of citric acid (Apelblat, 2014). Currently industrial production of unsaturated dicarboxylic acids such as mesaconate occurs through oxidation of petrochemically derived C_4 hydrocarbons (Cornils and Lappe, 2000). This process of production is commonly used at a large scale for the production of industrially important organic compounds such as fumaric acid and maleic anhydride (Lohbeck *et al.*, 2000). Two significant disadvantages are associated with this process. The oxidation process for the production of mesaconate employs nitric acid resulting in the formation of nitrous oxide which can be damaging to the environment (Bai *et al.*, 2016)

while also relying on a steady supply of petrochemicals the price of which can fluctuate greatly (Byrne, Lorusso and Xu, 2018).

The biological production of mesaconate could provide a sustainable production route for MMA and PMMA independent from current petrochemical based processes. Mesaconate is known to be produced in *Clostridium tetanomorphum* and *Clostridium cochlearium* as an intermediate in the catabolic pathway of glutamate (Wachsman, 1956; Zelder *et al.*, 1994). While the presence of a mesaconase was also demonstrated to be present within *Burkholderia xenovorans* (Kronen, Sasikaran and Berg, 2015). The glutamate degradation pathway (Figure 7) is driven by two key enzymes, to reach mesaconate, namely glutamate mutase and 3-methylaspartate ammonia lyase (MAAL) (Buckel and Barker, 1974). This pathway allows the metabolism of glutamate resulting in the production of acetate and pyruvate used for incorporation into central metabolism.

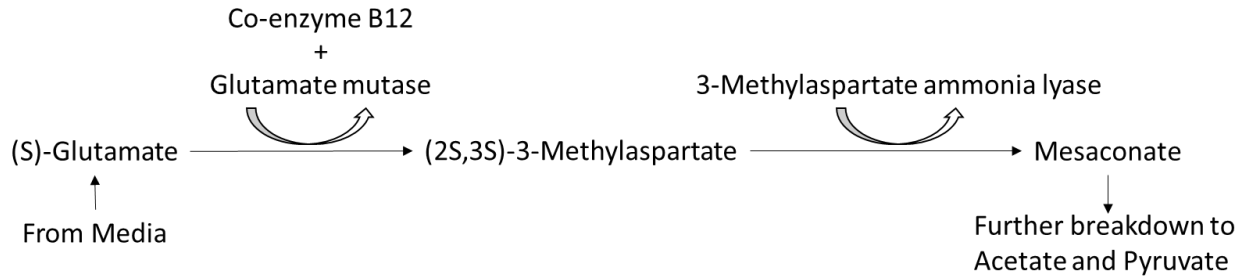


Figure 7: The glutamate degradation pathway highlighting the steps involved in mesaconate production.

3.2.1. Glutamate Mutase

Glutamate mutases are isomerases which catalyse the conversion of (S)-glutamate to (2S,3S)-3-methylaspartate (Figure 8) and the reverse reaction. Structurally glutamate mutase have two components parts, heterodimeric protein subunits linked to catalysis and a monomeric subunit for the binding of co-enzyme B12 (Reitzer *et al.*, 1999). The enzyme glutamate mutase is a co-enzyme B12 dependant enzyme. The presence of coenzyme B12 binds the two subunits to form active glutamate mutase (Heinzelmann *et al.*, 2003). Co-enzyme B12 dependant enzymes can be inactivated by oxygen exposure, co-enzyme analogues and

substrates which leads to an inactive cofactor remaining bound to the glutamate mutase inhibiting the activity of the enzyme. It has been reported that reactivation of the enzyme is possible through adenosine triphosphate (ATP) introduction into the system via reactivating factors (Toraya, 2003).

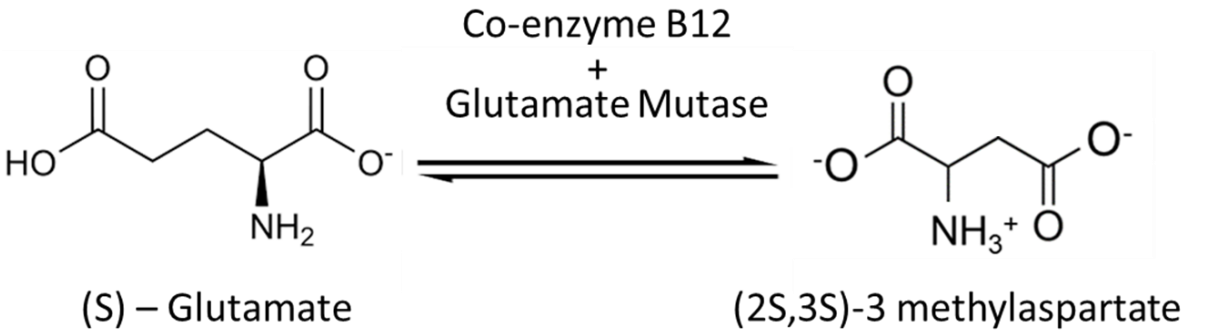


Figure 8: The reversible reaction catalysed by glutamate mutase.

A third component of the glutamate mutase gene cluster of *Clostridium tetanomorphum* has also been identified (*mutL*) which until recently was thought to encode a non-essential reactivation factor. This was confirmed by Wang & Zhang (2015) who demonstrated that this gene is indeed a reactivation factor which improves the inactivation turnover number of the mutase within *E. coli*, this result is further explored in section 3.3. Glutamate mutases have been publicised as components in the glutamate fermentation pathways of *Clostridium tetanomorphum* (Marsh and Holloway, 1992)*, Clostridium cochlearium* (Hoffmann *et al.*, 1999), members of the *Enterobacteria* such as *Citrobacter* spp., *Klebsiella planticola*, *Morganella morganii* (Kato and Asano, 1997) and in *Actinoplanes fruliensis* (Heinzelmann *et al.*, 2003).

3.2.2. Methylaspartate ammonia lyase (MAAL)

Methylaspartate ammonia lyase (MAAL) is a homodimeric enzyme which catalyses the reversible removal of ammonium from (2S,3S)-3methylaspartate to form mesaconate and ammonium (Figure 9). MAAL was first identified in *Clostridium tetanomorphum* as part of the

glutamate degradation pathway (Levy *et al.*, 2002). It has also been found in other facultative and obligate anaerobes such as *Citrobacter* spp., *Klebsiella planticola* and *Morganella morganii* (Kato and Asano, 1997). In addition to being part of the glutamate degradation pathway for glutamate fermentation MAAL has alternative potential uses in the creation of enantiomerically pure L-Aspartic acid derivatives (de Villiers *et al.*, 2012) and as a base enzyme for the creation of asymmetric amino acids not found in nature (Raj *et al.*, 2012). As it stands MAAL is an essential component in the biosynthetic pathway from glutamate to mesaconate for the generation of acetate and pyruvate and is the only enzyme which facilitates the formation of mesaconate in a natural biological pathway.

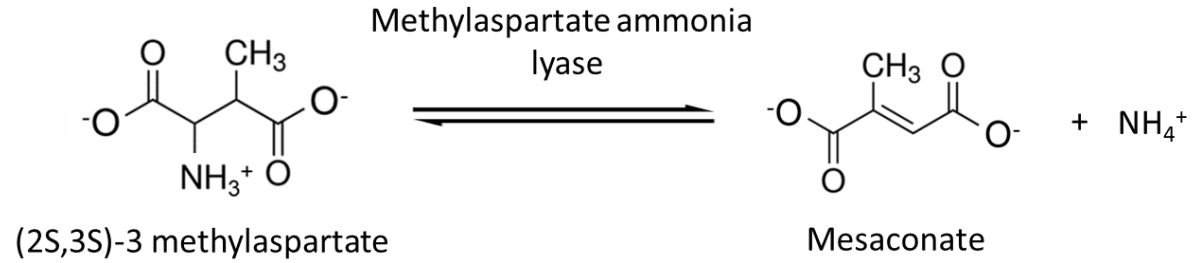


Figure 9: The reversible reaction catalysed by methylaspartate ammonia lyase (MAAL).

3.3. Bioproduction of mesaconate directly from sugars

To date the only literature known to the author outlining the production of mesaconate ,from sugars, through a bioprocess is in two papers by Wang & Zhang (2015) and Bai *et al.*, (2016) in which *E. coli* is the preferred expression system. The first paper outlines a systematic approach to engineering the co-enzyme B12 dependant glutamate mutase pathway into the *E. coli* strain BW25113 which was purchased from the Coli Genetic Stock Center. This systematic approach allows progressive improvements in mesaconate yield to be achieved and helps to outline the key components of this pathway. Glutamate was selected to be the carbon source for the pathway to mesaconate as high production rate and yield systems of glutamate are well documented and established. The first strain included glutamate mutase (*mutS/mutE*) and MAAL both of which were cloned from *C. tetanomorphum*. This new strain

(EM1) was incubated in a shake flask with a glutamate feed resulting in 1.17 g/L of mesaconate in the flask broth.

As the glutamate mutase-Coenzyme B12 complex has been shown to inactivate quickly, as previously discussed, the next step outlined was the incorporation of MutL, a reactivatase from *C. tetanomorphum*, into strain EM2. Previous to this paper the function of the *mutL* gene present on the glutamate mutase operon in *C. tetanomorphum* was unknown (Zelder *et al.*, 1994), Wang & Zhang (2015) elucidate the function of this gene through an in vitro enzymatic assay demonstrating that it can reactivate O_2^- induced deactivated glutamate mutase but also non-functional B12 induced deactivate glutamate mutase. Fermentation of this strain in a shake flask with a glutamate feed resulted in 2.65 g/L of mesaconate being produced. This experiment highlights the importance of MutL for the operation of glutamate mutase with a 162.4% increase in mesaconate production being demonstrated between the two strains EM1 and EM2. MutL removes the inactive co-enzyme B12 from the glutamate mutase freeing the glutamate mutase for further use. The next step which the authors of this paper took was to regenerate this inactive co-enzyme B12 in an attempt to create a constant available supply of active co-enzyme within the media.

Coenzyme B12 is known to be able to be produced from cobalamine through the adenosylcobalamine salvage pathway (Lawrence and Roth, 1996). The authors selected four genes from this pathway: *btuB* (encoding an outer membrane porin), *btuR* (cobalamin adenosyltransferase), *fldA* (flavodoxin) and *fpr* ($NADP^+$: ferredoxin reductase) and created strains based on EM2 which had each gene individually (*fldA* and *fpr* kept together) and all of the genes combined. Each individual gene improved the mesaconate titre with the strain EM8 including all four genes achieving a titre of 5.91 g/L an improvement of 74.3% on strain EM2. This experiment confirms that co-enzyme B12 regeneration can have a beneficial effect on the mesaconate production pathway.

In an effort to improve the yield of mesaconate the authors replaced the MutE subunit of glutamate mutase to GlmE from *Clostridium cochlearium* which has been reported to have a greater oxygen resistance (Leutbecher *et al.*, 1992). This strain (EM9) achieved a titre of 7.81 g/L showing a 39.7 % increase in mesaconate production indicating that the stability of the glutamate mutase can have a significant effect on the pathway. This strain, EM9, was then used to test weather direct production of mesaconate from glucose was possible without

glutamate feeding. The same shake flask bioprocess was attempted with the only alteration being the removal of the glutamate feed from the media. A titre of 6.96 g/L was achieved at a productivity of 0.1 g/L/h with a yield of 0.21 mol/mol of glucose which is 21% of the theoretical maximum. Increasing the concentration of co-enzyme B12 was also shown to have no significant effect on mesaconate production. This paper demonstrates the feasibility of introducing the glutamate degredation pathway into a microorganism for the production of mesaconate and also highlights how little about the enzymes and limiting factors involved in the pathway is known which the authors make significant progress in expanding.

The second publication followed on from the research in the first paper and focussed on the biological production of mesaconate in *E. coli* demonstrates how lignocellulosic sugars can be utilised (Bai *et al.*, 2016). The sugars which were the focus of the paper was L-arabinose and D-xylose. In order to incorporate these sugars into the pathway previously outlined in *E. coli* both sugars were first converted to 2,5-Dioxopentanoate using an operon from *Burkholderia xenovorans LB400* for D- xylose and *Burkholderia multivorans* for L- arabinose. 2,5-Dioxopentanoate was then converted to 2- ketoglutarate by a 2-ketoglutarate semialdehyde dehydrogenase. Mesaconate production could then occur using the pathway and enzymes demonstrated in the previous paper for mesaconate production from 2- ketoglutarate and coenzyme B12 regeneration. Using these pathways a mesaconate yield of 3.47 g/L in 48 hours was observed from D- xylose and a yield of 3.08 g/L in 48 hours was observed from L-arabinose.

Overexpression and variation of pentose transporters within the strains further enhanced the yields to 6.59 g/L from D-xylose and 5.37 g/L from L-arabinose. Screening for improved nonphosphrylative metabolism operons allowed the yield to be improved further which along with metabolic engineering of the strains, eliminating succinyl-CoA through deletion of *sucA* and overexpression of the glutamate dehydrogenase *gdhA,* produced the highest respective titres of 12.53 g/L from D-xylose and 13.25 g/L from L- arabinose. When both the pathways for production of 2,5-Dioxopentanoate from D-xylose and L-arabinose were combined within one strain along with the rest of the mesaconate production pathway a mesaconate yield of 14.75 g/L was observed after 48 hours. This strain had a mesaconate production rate of 0.3 g/L/h and is the highest mesaconate titre and production rate published to date outlining bioproduction from a process utilising a sugar at the carbon source.

3.3.1. Bioproduction of mesaconate from methanol

The only other biological method of mesaconate production outlined in published literature allows for methanol to be utilised as the primary carbon source in the process. Methanol provides several advantages over sugars as a carbon source for the production of mesaconate. In comparison to sugars methanol has a lower cost with very little price fluctuation (Bertau *et al.*, 2014), an attractive property when designing a bioprocess for large scale production of a platform chemical. Along with this economic advantage methanol can be produced from both petrochemicals and plant waste if required making it readily available while also ethical for use as a large scale carbon source as it is not involved in food production (Sonntag *et al.*, 2015).

Mesaconate production from methanol was first demonstrated by Sonntag *et al.,* (2014) through utilisation of the ethylmalonyl-CoA pathway (EMCP) in *Methylobacterium extorquens* AM1. The EMCP was recently fully elucidated (Erb *et al.*, 2007; Erb, Fuchs and Alber, 2009) and is a pathway which facilitates the assimilation of acetate in bacterial species which are isocitrate lyase–negative and can therefore not assimilate acetate through the glyoxylate shunt. Using the ECMP species such as *Methylobacterium extorquens* AM1 and *Rhodobacter sphaeroides* can produce (3S)-malate and succinyl-CoA to drive the TCA cycle from three molecules of acetate, CO_2 and bicarbonate. The pathway consists of 14 enzymatic steps all of which are outlined in Figure 10 for *Rhodobacter sphaeroides*.

Using this pathway in *Methylobacterium extorquens* AM1 it was hypothesised by (Sonntag *et al*., 2014) that the inclusion of a thioesterase with the appropriate specificity would allow the cleavage of mesaconyl-CoA resulting in the production of mesaconate. After the screening of various thioesterases it was shown that *Methylobacterium extorquens* AM1 containing the thioesterase *yciA* from *E. coli* grown in a minimal media including methanol produced 0.07 g/L of mesaconate. In this paper it was also shown that this mesaconate titre reduced over time suggesting that mesaconate was not only being produced but was later utilised, a quality that was not desirable for producing larger titres of mesaconate.

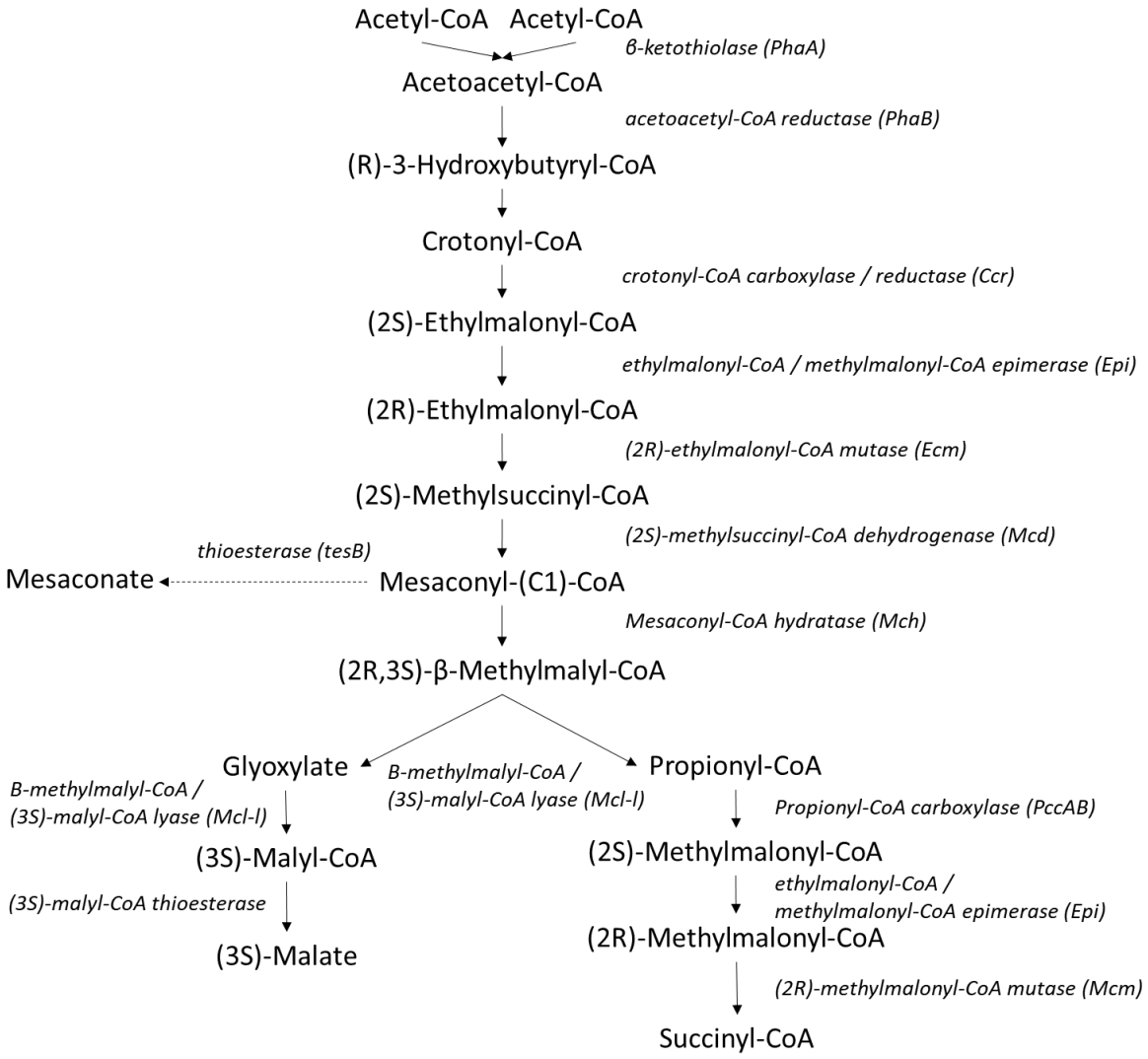


Figure 10: The ethylmalonyl-CoA pathway outlining the enzymes used by Rhodobacter sphaeroides and the proposed route to mesaconate. Adapted from Erb, Fuchs and Alber (2009).

Expanding on this work, it was shown in a future publication by the same authors that by decreasing the sodium availability in the media by 30-fold prevented the utilisation of produced mesaconate. After the elimination of this undesirable effect flux improvement through the deletion of a regulator for polyhydroxybutyrate production ,*phaR,* facilitated a mesaconate titre of 0.443 g/L to be produced (Sonntag *et al.*, 2015). Along with the mesaconate titre of 0.443 g/L the limited specificity of *yciA* allowed 0.222 g/L of (2S)-methylsuccinate to be produced from methylsuccinyl-CoA. As methylsuccinyl-CoA occurs

before mesaconyl-CoA in the EMCP this by-product ((2S)-methylsuccinate) likely reduced the possible mesaconate titre.

The bioprocesses using *E. coli* and *Methylobacterium extorquens* outlined two approaches to mesaconate production allowing both sugars and methanol to be used as the carbon source. Although the pathway in *E. coli* resulted in a higher yield the presence of publications outlining two separate pathways for mesaconate production provide different possibilities for a large scale mesaconate bioprocess. An advantage of two separate processes is that it allows a large variety of host microorganisms to be viable for a mesaconate production bioprocess. Both processes were carried out at a small scale 5 ml for *E. coli* and 50 ml for *M. extorquens*. As mesaconate currently is not a high value chemical an effective process for bioproduction would require a very large scale to be economically viable. As such the host microorganism is an important consideration.

3.4. Corynebacterium glutamicum as a host for mesaconate production

This chapter will present results which outline the use of *Corynebacterium glutamicum* as a novel expression system for the enzymes of the glutamate degradation pathway. This pathway should allow the production of mesaconate as was observed when the same pathway was included in *E. coli* (Wang and Zhang, 2015). An outline of the proposed pathway is shown below in Figure 11.

The production of mesaconate through this pathway relies on the availability of the precursor glutamate. As such *Corynebacterium glutamicum* appeared to be a logical choice of expression system for this pathway. *Corynebacterium glutamicum* is a Gram-positive, facultatively anaerobic bacterium which was originally discovered due to its ability to effectively and efficiently produce glutamate (Kinoshita, Udaka and Shimono, 1964; Boulahya *et al.*, 2010). *Corynebacterium glutamicum* has since become the industrial workhorse for the production of glutamate with market demand reaching more than 2.5 million tonnes per annum (Hirasawa and Shimizu, 2016). Strains of *C. glutamicum* are known to be able to produce titres of over 80 g/L of glutamate (Sanchez and Demain, 2008) although it is likely that unpublished industrial processes are able to produce much higher titres. The natural

ability of *C. glutamicum* to overproduce glutamate provides a notable advantage for the selection of this expression system to produce a mesaconate over others such as *E. coli* which cannot naturally produce high titres of glutamate.

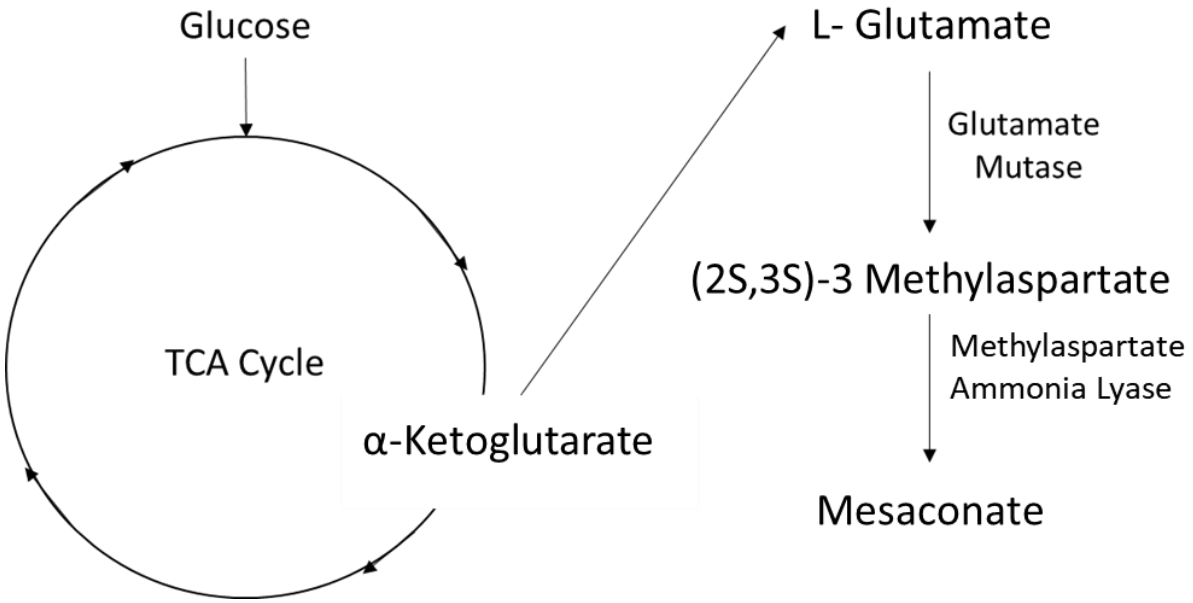


Figure 11: The proposed mesaconate production pathway in C. glutamicum designed by Ingenza Ltd.

E. coli can be engineered to produce higher titres of glutamate than normal through strategies such as overexpression of glutamate dehydrogenase to increase the flux of α- ketoglutarate to glutamate or through deletion of the α- ketoglutarate dehydrogenase (sucA) that catalyses the production of succinyl-CoA from α-ketoglutarate (Bai *et al.*, 2016). Despite this the levels of glutamate produced from engineering of *E. coli* cannot compare to *C. glutamicum* as evidenced by *C. glutamicum* remaining the industry workhorse for this particular amino acid (Ma *et al.*, 2017). As with all industrial bioprocesses the efficiency of mesaconate production from glutamate would improve over time as further insight into the optimum operation conditions for the enzymes becomes available and engineering of the enzymes can be investigated. As such it is possible that *E. coli* at some point may no longer be a viable option as an expression system for this pathway due to inability to compete with the glutamate production capacity of *C. glutamicum*.

Another advantage of using *C. glutamicum* as an expression system for the production of mesaconate is that it has GRAS (Generally regarded as safe) status opening up additional uses for the product due to this certification (Wieschalka *et al.*, 2013). Along with this *C. glutamicum*, as a well-established workhorse for amino acid production (Fisher *et al.*, 2014), has a familiarity within industry. Although *E. coli* is also used industrially at a large scale for amino acid production (Ma *et al.*, 2017) *C. glutamicum*'s use for glutamate production specifically provides information which may be invaluable in the creation of a biorefinery for large scale production of mesaconate from glutamate. This understanding of *C. glutamicum* bioprocess conditions required to maximise glutamate production and the downstream processing required when producing glutamate in *C. glutamicum* (Cheng *et al.*, 2012) may give it an advantage over *E. coli* for the ideal expression system for this pathway.

The overproduction of glutamate by *C. glutamicum* makes it a logical alternative to *E. coli* which as previously discussed has been used as an expression system for the production of mesaconate (Wang and Zhang, 2015). Through the addition of similar enzymes in the form of glutamate mutase and methylaspartate ammonia lyase it is thought that an increased titre of mesaconate may be possible from *C. glutamicum* due to the efficiency of glutamate production within this host.

3.5. Strain construction

All of the *Corynebacterium glutamicum* strains which were used throughout this chapter were produced in house by Ingenza Ltd, Roslin, UK and are outlined in Table 11. The same MAAL was used in all strains originating from *C. tetanomorphum* which was the same gene used in the literature outlining mesaconate production in *E. coli* (Wang and Zhang, 2015). A variety of glutamate mutases were utilised throughout the chapter with the reasoning for the different selections outlined in the experimental results and discussion.

The strains were designated a title based on the in-house cataloguing system at Ingenza, IL5.0.0.0 represented the unaltered host strain *C. glutamicum* ATCC 13869 which was deposited in the ATCC by Ajinomoto. Within each strain name the 5 represented *C. glutamicum*, a 1 represented plasmid 1 with the in house labelling for the genes contained

within brackets after the plasmid number. For example in IL5.0.1(G2).2(F16-F17) the 1(G2) section of the name indicated that the strain contained plasmid 1 containing the tMAAL gene. Plasmid 1 in all strains bar the empty vector strain (IL5.0.1.2) contained the MAAL gene and contained a kanamycin resistance marker for selection. Plasmid 2 in all strains bar the empty vector strain (IL5.0.1.2) contained the glutamate mutase genes and contained a spectinomycin resistance marker for selection. All plasmids were under the control of a *tac* IPTG inducible promotor with 1 mM of IPTG added to experiments for induction. The *tac* promotor subjects the plasmid to an all or none induction response and as the synthetic analogue of lactose can be used for induction (IPTG) this is not metabolised and expression will continue indefinitely after induction (Khlebnikov and Keasling, 2002).

Table 11: The strains of C. glutamicum used within this chapter. All strains were produced at Ingenza Ltd.

Strain Name	***C. glutamicum* Host Strain**	**Glutamate Mutase Genes (Origin)**	**MAAL Genes (Origin)**
IL5.0.0.0	ATCC 13869	None	None
IL5.0.1.2	ATCC 13869	None	None
IL5.0.1(G2).2(F16-F17)	ATCC 13869	*glmA, glmB* (*Actinoplanes friuliensis*)	*tMAAL (Clostridium tetanomorphum)*
IL5.0.1(G2).2(F1-F2)	ATCC 13869	*glmS, glmE* (*Desulfosporosinus meridiei*)	*tMAAL (Clostridium tetanomorphum)*
IL5.0.1(G2).2(F1-F2-F3)	ATCC 13869	*glmS, glmE, glmL* (*Desulfosporosinus meridiei*)	*tMAAL (Clostridium tetanomorphum)*
IL5.0.1(G2).2(F7-F8-F9)	ATCC 13869	*mutS mutE, mutL* (*Clostridium tetanomorphum*)L	*tMAAL (Clostridium tetanomorphum)*

3.6. Previous work on mesaconate production by C. glutamicum

The work presented in this chapter, on the production of mesaconate by *Corynebacterium glutamicum*, is a continuation of work carried out by Jeffrey, (2016). It was shown in this work that the inclusion of the mesaconate pathway in the strain IL5.0.2(G2).1(F1-F2) was unable to

produce measurable mesaconate concentrations. Quantitative polymerase chain reaction (qPCR) analysis showed that a low transcription of both genes was observed.

The inclusion of a reactivatase for the glutamate mutase improved the expression of the glutamate mutase and the MAAL. Although expression of the enzymes was observed no mesaconate was able to be measured by HPLC. LC-MS analysis indicated that low levels of mesaconate, although unmeasurable by HPLC, may have been produced in the bioprocess.

A strain containing a more oxygen tolerant glutamate mutase was produced IL5.0.1(F16.F17.G2).0. This strain contained the glutamate mutase and methylaspartate ammonia lyase on the same plasmid under the control of a constitutive promoter. Samples from the bioprocess were analysed by qPCR showing that the mRNA target gene transcripts were being expressed albeit at a low copy number. No mesaconate was observed during the bioprocess although as a high level of foaming was observed the strain was not thought to be suitable for further bioprocess work.

3.7. Aims

The aim of the research presented in this chapter was to demonstrate the novel production of mesaconate using *Corynebacterium glutamicum* as the host expression system for the enzymes of the glutamate degradation pathway. The initial aim was to demonstrate successful production of mesaconate by *C. glutamicum* which had not been demonstrated to date within literature. As it was expected that the strains of *C. glutamicum* would produce a large quantity of glutamate the aim after successful demonstration of mesaconate production was to improve the mesaconate production rate and titre to fully utilise the abundance of available substrate.

The overarching aim was to provide evidence that efficient mesaconate production was possible within *C. glutamicum*, which may allow a mesaconate bioprocess to feed into the production chain for poly- methyl methacrylate (PMMA). As no literature was available outlining mesaconate production within a controlled bioreactor a further aim of the experiments was to provide insight into process considerations which may have relevance upon scaling of the process to an industrial scale.

3.8. Batch bioprocesses using an inducible glutamate mutase with increased oxygen tolerance

It had been shown previously by Jeffrey (2016) that a constitutively induced strain containing a methyl-aspartate ammonium lyase gene (*tMAAL*) from *Clostridium tetanomorphum* and the two genes for a glutamate mutase (*glmA/glmB*) with increased oxygen tolerance from *Actinoplanes fruiliensis* on a single plasmid did not produce a measurable concentration of mesaconate. This was unexpected as qPCR demonstrated that both genes were transcribed in the bioprocess. As a large quantity of foaming was observed in the bioprocess this strain was not suitable for further experiments. The strain was therefore altered to separate the glutamate mutase and MAAL on to separate plasmids under the control of an IPTG inducible promoter. This strain format had been shown previously to have detectable mesaconate, when LC-MS analysis was used, while also having limited foam production. It was therefore hypothesised that mesaconate production may be improved in the new inducible strain IL5.0.1(G2).2(F16-F17).

In order to determine if the glutamate mutase with improved oxygen tolerance could facilitate the production of mesaconate within an inducible strain of *C. glutamicum,* two 4 L bioprocesses were carried out using IL5.0.1(G2).2(F16-F17), one with and one without induction using IPTG. For mesaconate to be produced high initial concentrations of glutamate are required. It is well documented that in order to get high titres of glutamate a method to induce overproduction must be chosen for the bioprocess (Shimizu and Hirasawa, 2006). Biotin limitation has been determined by previous research to be the best method of induction for the overproduction of glutamate providing a higher glutamate titre or a greater degree of stability in the bioprocess than temperature induction, tween 40 addition or ethambutol addition (Jeffrey, 2016). Therefore biotin limitation will be the induction method of choice for the overproduction of glutamate in all of the strains analysed throughout this chapter. The concentration of biotin which was used in the batch media to trigger glutamate overproduction was 1 µg/L as it has been shown that concentrations of 3 µg/L and above do not trigger glutamate overproduction (Shiio, Otsuka and Takahashi, 1962).

Both bioprocesses were carried out on a 4L scale as outlined in method section 2.1.1.3 and the inoculum was produced as outlined in method section 2.1.1.2. Samples were collected for

analysis of dry cell weight (Section 2.1.4) along with HPLC analysis to determine glucose, glutamate and mesaconate concentrations (Section 2.1.5). Each bioprocess was stopped when the O_2 and CO_2 concentrations measured in the off gas returned to the values recorded at 0 hours as this indicated the end of respiration within the bioprocess. The results of the mesaconate analysis of both bioprocesses is shown in Figure 12.

Only the mesaconate analysis of both bioprocess is shown as it was immediately noticed in the HPLC chromatograms that the mesaconate peak in the samples varied slightly in retention time and peak shape from the mesaconate standards analysed. The shift in retention time and peak shape may have been due to the samples originating from a bioprocess broth which can contain a large variety of compounds. Although this hypothesis was possible, similar distortion was not observed during HPLC analysis for glutamate and glucose. The second anomaly detected in the mesaconate analysis was that both the induced and uninduced bioprocesses produced a similar concentration of mesaconate. The induced bioprocess produced 1.33 g/L of mesaconate at 48 hours while the bioprocess without induction produced 1.27 g/L of mesaconate by 44 hours. Without IPTG induction expression of the plasmids should not occur and as a result it would be expected that no mesaconate would be produced.

It was possible that a leaky promoter may allow the production of mesaconate prior to induction although a lower concentration would be expected if this was the case. Before further analysis of both bioprocesses could be carried out it was important to first confirm if the peak observed in both bioprocesses was mesaconate.

Strain Genotypes

IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

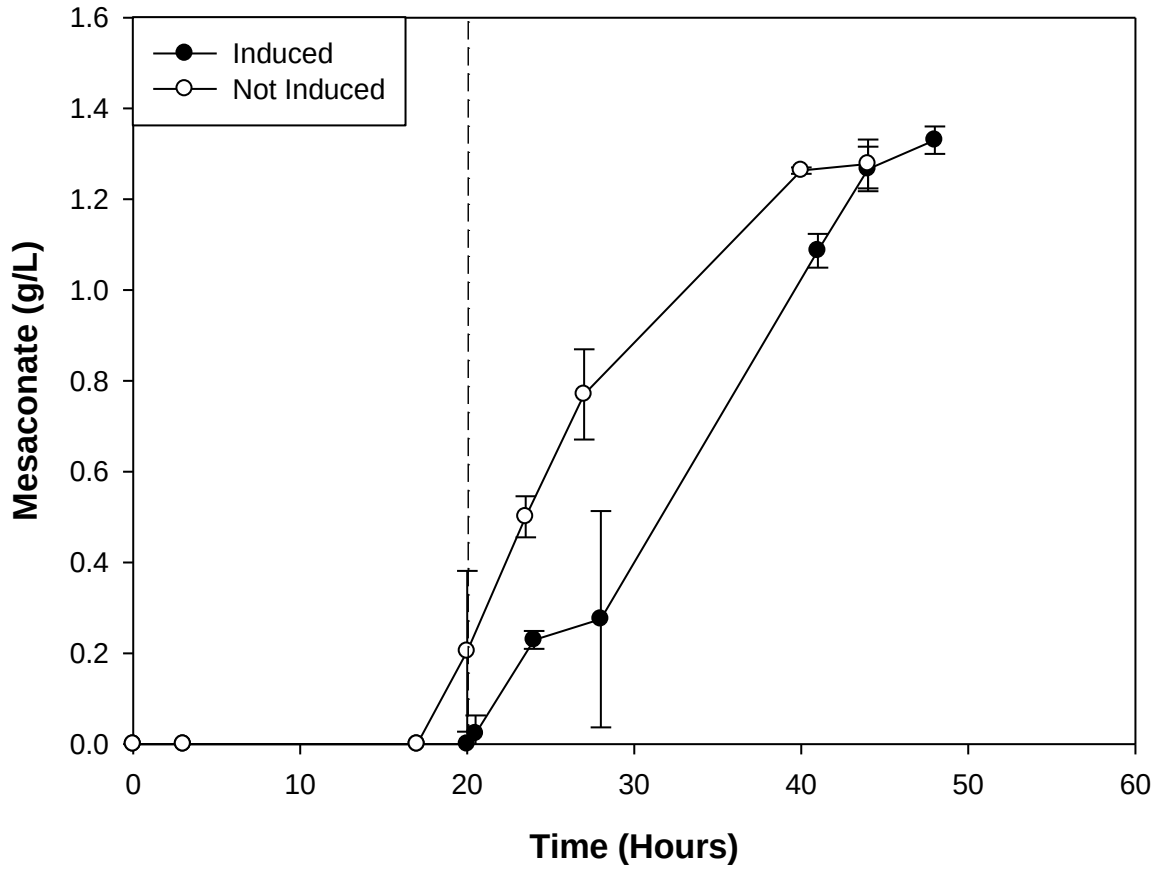


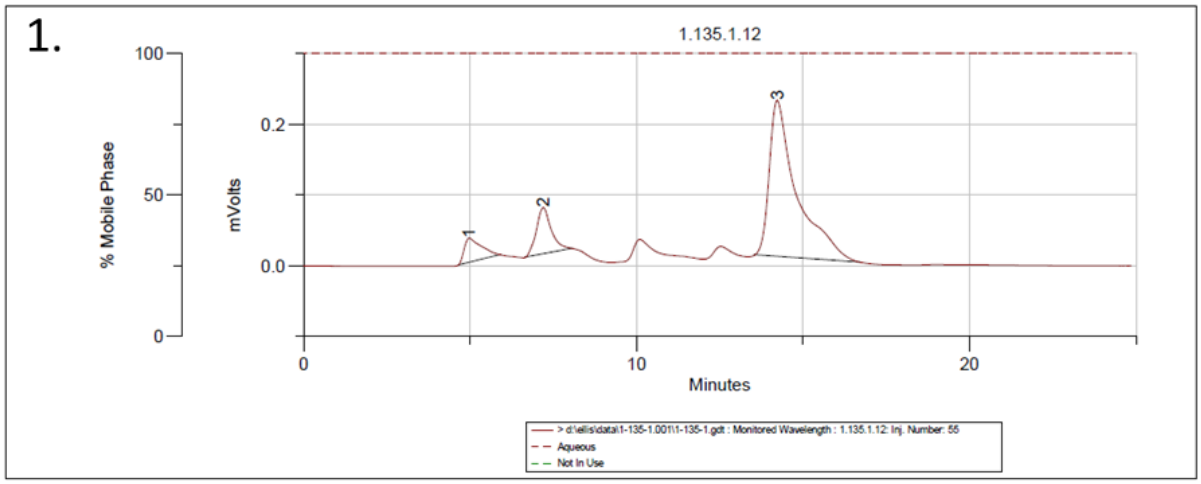
Figure 12: The mesaconate concentration measured from the induced and uninduced bioprocess of IL5.0.1(G2).2(F16-F17). The IPTG induction time is shown by the dashed line (---). Each sample was measured in triplicate with the error bars representing the standard deviation of the samples.

3.8.1. Mesaconate production by the wild type control strain

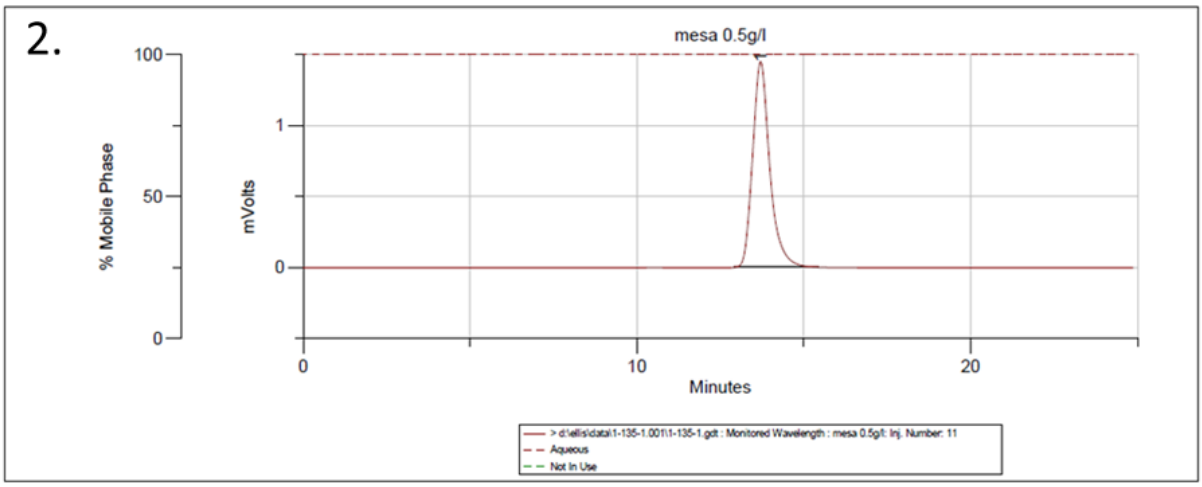
In order to confirm if the possible mesaconate peak observed in the previous bioprocesses was indeed mesaconate a bioprocess was carried out using the wild type control strain of *C. glutamicum* IL5.0.0.0. This strain did not contain the enzymes required for mesaconate production therefore if the same peak appeared during HPLC analysis of the control strain it could be concluded that the peak was not mesaconate.

A 1L bioprocess was set up using the same method as the previous experiment with samples taken for HPLC analysis to determine the mesaconate concentration present. The HPLC analysis of the samples collected from the bioprocess showed the presence of the same suspected mesaconate peak at a retention time similar to that of the mesaconate standards. As the chromatograms of the blank injections showed no peaks it could be confirmed that the presence of the mesaconate peak was not due mechanical error but the responsible compound was indeed contained within the samples. A chromatogram of a mesaconate standard and a sample from the bioprocess of IL5.0.0.0 is shown in Figure 13.

As it is not possible for mesaconate to be produced in this bioprocess this peak was confirmed not to be mesaconate but another product which on the initial HPLC method had a similar retention time to mesaconate and may be preventing mesaconate production from being observed. As the unknown product was formed in a bioprocess of the control strain it was hypothesised that it would be present in all bioprocesses carried out under the same conditions, as such development of the HPLC method was required to separate this peak from mesaconate which may be produced in bioprocesses where mesaconate production is possible.



	Inj. Number	Peak Name	R. Time	Area	Sample Descrip.	Purity	Spectral Match 1	Spectral Match 2	
1	55.00	1	4.97	20598132.00	1.135.1.12	957.32	<No Library>	<No Library>	
2	55.00	2	7.19	34280208.00	1.135.1.12	969.68	<No Library>	<No Library>	
3	55.00	3	14.23	211133520.00	1.135.1.12	979.82	<No Library>	<No Library>	



	Inj. Number	Peak Name	R. Time	Area	Sample Descrip.	Purity	Spectral Match 1	Spectral Match 2	
1	11.00	1	13.70	863933632.00	mesa 0.5g/l	999.96	<No Library>	<No Library>	

Figure 13: The chromatograms from the organic acid HPLC method. Chromatogram 1 is the 75 hour sample from the bioprocess of IL5.0.0.0 (Retention time = 14.23 minutes). Chromatogram 2 is the 0.5 g/L mesaconate standard (Retention time = 13.70 minutes). Both chromatograms are from the UV detector.

3.9. HPLC method development for accurate mesaconate quantification.

3.9.1. Confirming the presence of mesaconate in the bioprocess

The HPLC method used initially for the identification of organic acids is outlined in section 2.1.5.3. This method had been used successfully for the quantification of a variety of organic acids including mesaconate with good peak symmetry and low limits of detection being observed. It has been shown that when *Corynebacterium glutamicum* strains were run under the conditions required for mesaconate production that an unknown product was produced resulting in a competing peak forming around the retention time of mesaconate in the current organic acid detection method.

This hypothesis was formed as it could be seen that in the wild type strain a peak was observed at the same timepoint as mesaconate (Section 3.8.1). As this strain could not produce mesaconate a separate compound must be produced in the bioprocess which can block mesaconate detection by the current method. In order to alter the HPLC method for accurate detection of mesaconate work was first carried out to determine what the unknown product was.

From the bioprocess using IL5.0.1(G2).2(F16-F17) which should produce mesaconate (Section 3.8) 20 ml samples were taken at each timepoint. The final sample (48 Hours) from that bioprocess was taken to be used for NMR and LC-MS analysis. The aim of this analysis was twofold, to see if it would be possible to identify the unknown product and to confirm that mesaconate was indeed produced.

As the bioprocess media contained a large variety of compounds and the mesaconate concentration within the sample may have been small therefore the sample was separated using an HPLC fraction collector. As the injected sample passed through the HPLC column the compounds within the sample are separated based on the HPLC retention within the column. The fraction collector allows specific portions of the injected sample to be collected within separate vials after passing through the refractive index detector. On the column using distilled water as the mobile phase mesaconate passed through the refractive index detector at around 14 minutes after injection of the sample. Three 2 minute fractions were collected

in separate vials, 13-15 minutes which was fraction 1, 15-17 minutes which was fraction 2 and 17-19 minutes which was fraction 3. Three fractions were collected to ensure that the mesaconate and unknown compound was not missed in the first fraction. This was repeated multiple times as the maximum injection size into the HPLC column was 20 µL. This process resulted in the original 48 hour sample from the bioprocess of IL5.0.1(G2).2(F16-F17) being split based off of retention time into 3 separate fractions. As the HPLC process used distilled water as a mobile phase the fractions were now diluted with water. The fractions were therefore evaporated to eliminate the water before NMR and LC-MS analysis could take place.

For NMR and LC-MS analysis the evaporated fractions were resuspended in 1 ml of deuterium oxide and methanol respectively before being added to an NMR tube and a HPLC vial. A 1 g/L mesaconate standard and a 1 g/L citraconate standard were also prepared in methanol for LC-MS analysis and a 1 g/L mesaconate standard was prepared in deuterium oxide for NMR analysis. The sample preparation process for NMR and LC-MS analysis is outlined in sections 2.1.8 and 2.1.9. It was hypothesised that the unknown peak may have been citraconate as it is structurally similar to mesaconate and may therefore have an HPLC peak at a similar retention time. The samples were then analysed by proton NMR and LC-MS.

The proton NMR spectra is shown in Figure 14. It can be seen from the NMR spectra that the media components were causing significant noise which masked one of the peaks seen in the standard. From the NMR spectra it could not be concluded if mesaconate was present in the sample. As fraction 1 was the concentration of 5 ml of original sample into 1 ml it was expected that if the 1.33 g/L concentration of mesaconate detected by the HPLC method was accurate that a greater signal than the 1 g/L standard would be detected. As mesaconate could not be observed in the NMR sample it was possible that a much lower concentration of mesaconate was in the sample than expected. This supports the hypothesis that the peak detected by HPLC was not mesaconate.

The result of the LC-MS analysis is shown in Figure 15. It can be seen from the top half of the figure where the retention time of peaks within the sample is shown that the mesaconate standard and the 3 fractions had peaks with a similar retention time. As the citraconate peak did not have a similar retention time to the fractions it could be concluded that the unknown peak which would be contained within the fractions was not citraconate.

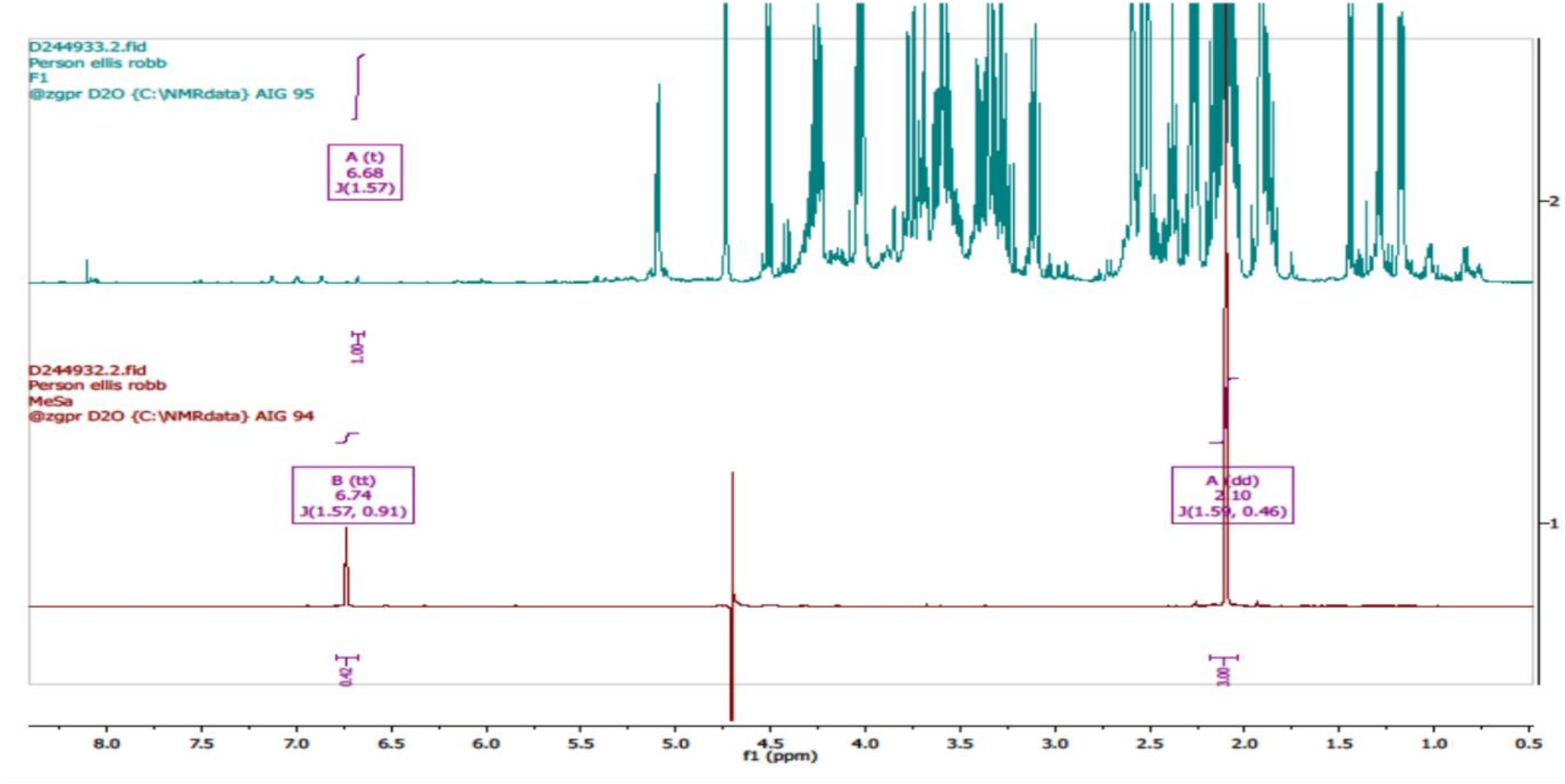


Figure 14:The proton NMR spectra of the 1 g/L mesaconate standard (Bottom) and the sample fraction (Top).

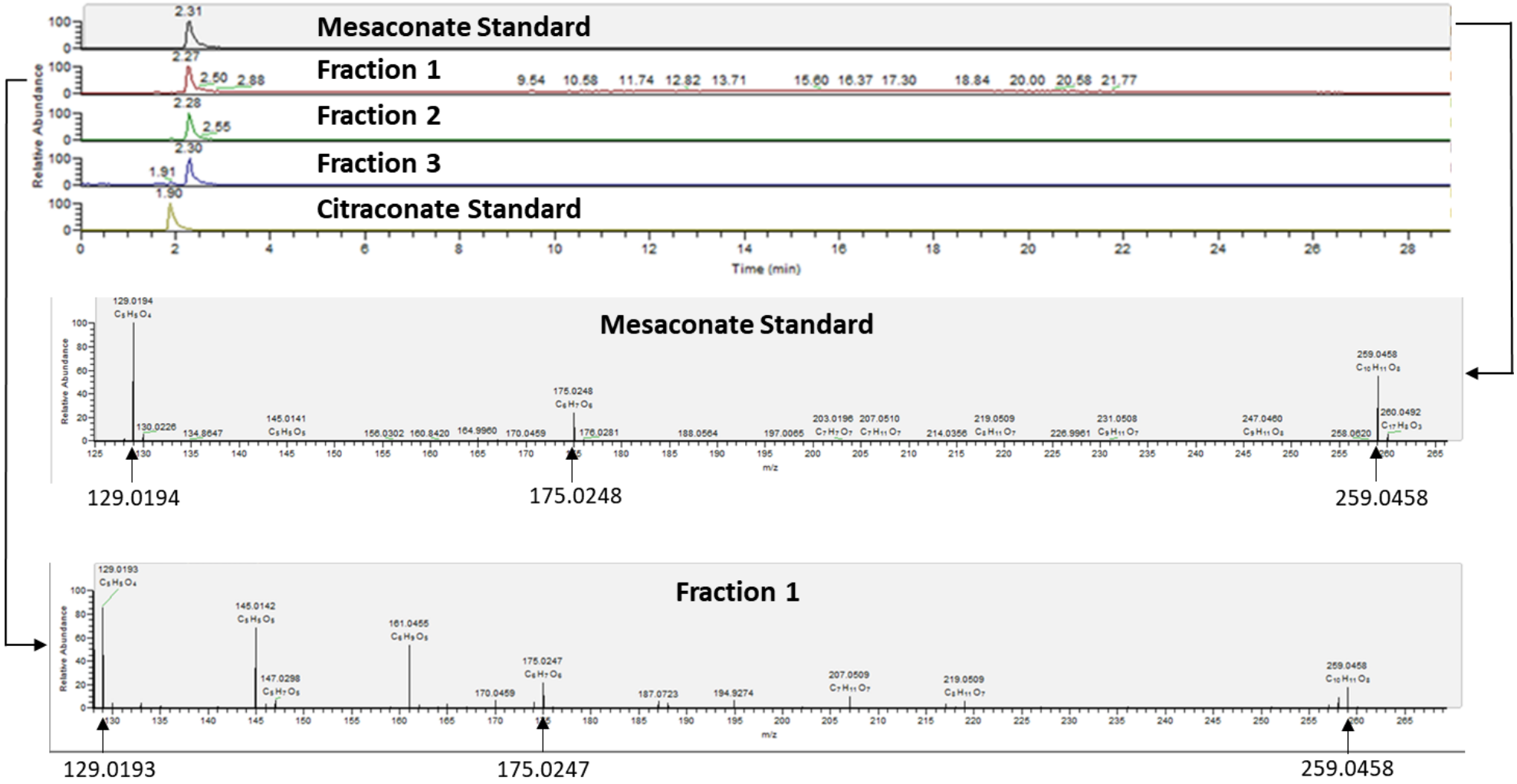


Figure 15: The LC-MS analysis of the 1 g/L mesaconate standard, the three collected fractions (1,2 and 3) and a 1g/L citraconate sample. The top portion of the figure shows the retention times of the samples in the following order. Mesaconate standard, F1, F2, F3 and citraconate standard. The bottom portion of the figure shows the mass analysis in the following order. Mesaconate standard and fraction 1.

When comparing the mass spectroscopy result at retention time 2.31 for the mesaconate standard and retention time 2.27 for fraction 1 it can be seen that fraction 1 contains all three peaks shown in the mesaconate standard. As the mass of each peak is accurate to 3 decimal places it can be concluded that fraction 1 does contain mesaconate. The mesaconate in the sample was not able to be quantified as the sample concentration had been altered during fraction collection, evaporation and resuspension. As mesaconate had been shown to be contained in the 48 hour sample from IL5.0.1(G2).2(F16-F17) work could be carried out on improving the HPLC for elimination of the unknown peak.

3.9.2. Adapting the HPLC method

It had been demonstrated by the LC-MS analysis of the 48 hour sample from the bioprocess of IL5.0.1(G2).2(F16-F17) that mesaconate was present in the sample but the NMR analysis suggested that the concentration was much lower than what was initially measured. Therefore it was hypothesised that a mesaconate peak was present but was masked by the unknown peak, giving rise to an overestimation in the bioprocess derived mesaconate being measured. In order for mesaconate to be accurately detected within the bioprocess samples the HPLC method required alteration to eliminate or shift the peak produced by the unknown product.

In the organic acid HPLC method samples were analysed by both UV and refractive index detectors (RID). Mesaconate has both a UV and RID trace with the UV trace preferred for quantification as there is lower baseline noise especially when analysing the bioprocess media which can contain a large variety of compounds. The lower baseline noise in the UV detector allows a lower limit of quantification which may be necessary if the mesaconate is much lower as suggested.

As the current HPLC method produced a good chromatography for mesaconate standards it was preferred to adapt this method rather than switching organic acid detection to a completely different method. The original method outlined in section 2.1.5.3 used a flow rate of 1 ml/minute and used a wavelength of 210 nm for the detection of mesaconate using the UV detector. It was possible that mesaconate and the unknown compound had different UV

absorbance profiles and could be separated by altering the wavelength of detection. In order to test this 5 samples were run on the HPLC with the area of the mesaconate peak measured at 190, 200, 210, 220 and 230 nm. The 5 samples were a 1 g/L mesaconate standard, a 0.26 g/L mesaconate standard, the 48 hour sample from the induced bioprocess of IL5.0.1(G2).2(F16-F17) and the 40 hour sample from the uninduced bioprocess of IL5.0.1(G2).2(F16-F17) with and without spiking. The spiked sample contained 1 g/L of mesaconate. The resulting peak areas detected are shown in Figure 16.

From Figure 16 it can be seen that the two mesaconate standards increase in peak area until 220 nm and then the peak area drops at 230 nm. This showed that the lambda max of mesaconate is between 210 and 230 nm. The 48 hour sample from IL5.0.1(G2).2(F16-F17) with induction shows that the peak area decreases as the wavelength increases. As this sample does not show an increase in peak area at 220 nm which was seen in the standards this suggested that the peak was not mesaconate. The same trend was observed in the 40 hour sample from the uninduced bioprocess of IL5.0.1(G2).2(F16-F17) with the peak area dropping to 0 at 230 nm. As the unknown/mesaconate peak was observed in this sample, while mesaconate was not envisioned to be produced in this bioprocess due to the lack of induction, it may be possible that increasing the wavelength above 230nm could eliminate the unknown peak from the sample.

If this hypothesis was correct the sample spiked with 1 g/L of mesaconate and the 1 g/L mesaconate standard should have the same peak area at 230 nm as the mystery peak would be eliminated. It can be seen that this was the case with the spiked sample only having a peak area 1% higher than the 1 g/L standard at 230 nm. This confirms that at this wavelength mesaconate is the only peak present. As mesaconate still has absorbance at 240nm this wavelength was selected to ensure the unknown peak was no longer present. Along with changing the wavelength of UV detection in the method the flow rate was changed to 0.6 ml/min which should separate the mesaconate and unknown peak to ensure the unknown peak was not still present behind the mesaconate peak which may cause inaccuracy at low mesaconate concentrations. The new HPLC mesaconate detection method was outlined in section 2.1.5.4.

Strain Genotypes
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

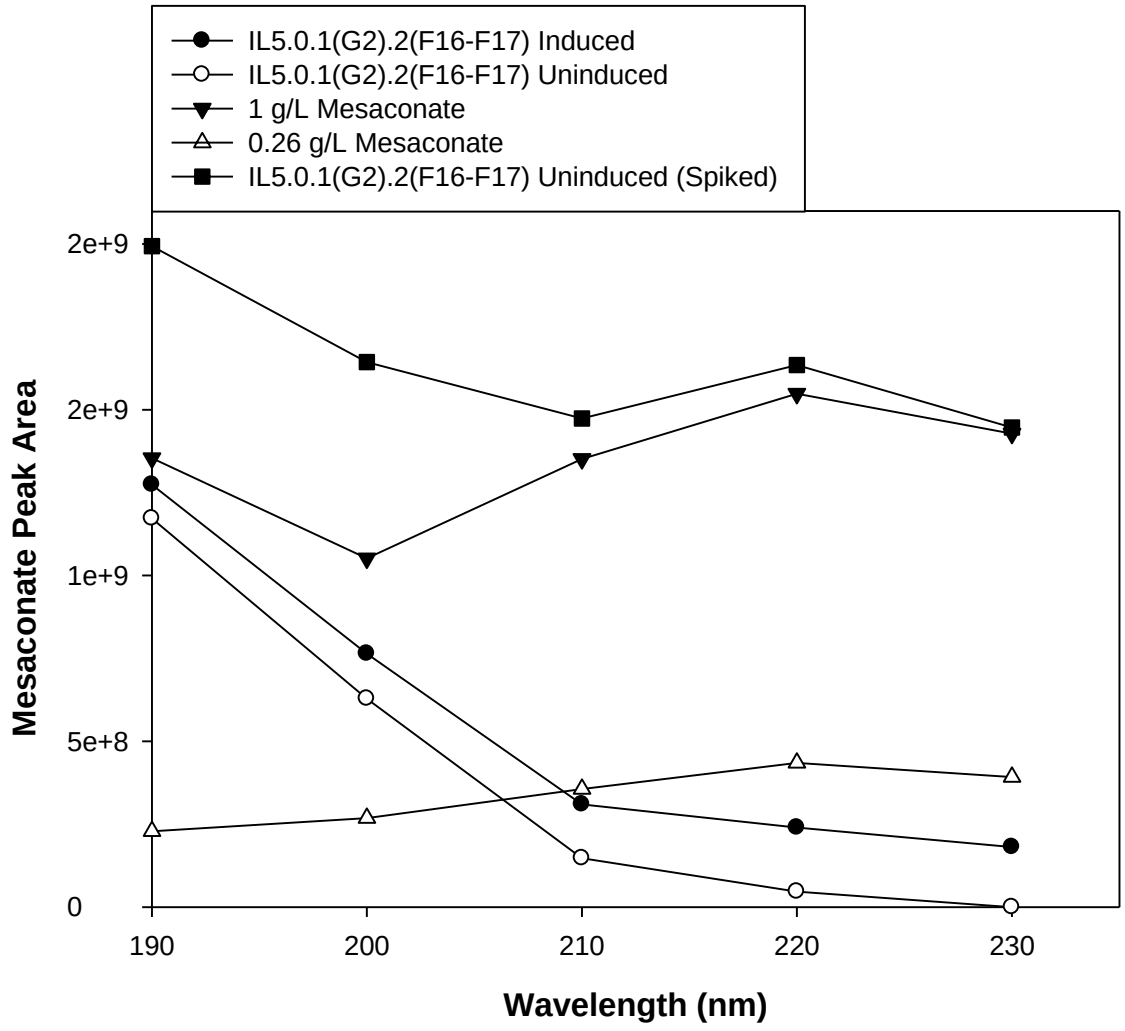


Figure 16: A comparison of the mesaconate peak area at different wavelengths. Each sample was analysed using the current organic acid HPLC method. The IL5.0.1(G2).2(F16-F17) induced sample was the 48 hour sample, the IL5.0.1(G2).2(F16-F17) uninduced sample was the 40 hour sample and the IL5.0.1(G2).2(F16-F17) spiked sample was the 40 hour sample spiked with 1 g/L of mesaconate.

3.9.3. Testing the improved HPLC method

To determine the accuracy of the new HPLC method the bioprocesses shown in section 3.8 were reanalysed at 240 nm to again determine the new mesaconate concentrations. The new mesaconate concentration for the three bioprocesses are shown in Figure 17. It can be seen from this figure that no mesaconate was detected in the bioprocess using IL5.0.0.0 and the uninduced bioprocess of IL5.0.1(G2).2(F16-F17). This result was expected as IL5.0.0.0 cannot produce mesaconate due to the absence of glutamate mutase and MAAL and the uninduced bioprocess should not have expression of these enzymes. The maximum mesaconate titre now produced by IL5.0.1(G2).2(F16-F17) was 0.08 g/L which was much lower than the concentration previously observed in section 3.8 (1.33 g/L). This result is supported by the NMR analysis where mesaconate could not be detected which would be expected at a concentration of 0.08 g/L but not at 1.33 g/L which was previously measured.

An improvement in peak shape can also be seen when comparing a mesaconate standard with the 48 hour IL5.0.1(G2).2(F16-F17) bioprocess sample to the old HPLC method and the improved HPLC method. The chromatograms from the new HPLC method has an improved symmetry when compared to the standard, with a closer retention time. This suggests that mesaconate is now being analysed as opposed to the unknown peak. As the unknown peak has been eliminated from the analysis method it was not thought to be essential that the identity of the peak was determined.

In conclusion the mesaconate concentrations in the three bioprocesses now appear to have mesaconate profiles in keeping with what is possible for each strain. The wild type strain IL5.0.0.0 produced no mesaconate and the bioprocess of IL5.0.1(G2).2(F16-F17) without induction also produced no mesaconate which was expected. The mesaconate concentration which was produced by IL5.0.1(G2).2(F16-F17) with induction now had a maximum concentration of 0.08 g/L which supports the hypothesis from the NMR analysis that a much lower concentration of mesaconate was present. The accuracy of the method was also supported by the comparison of peaks to the standard. The new method had a much greater peak symmetry resulting in a closer retention time. As this HPLC method for mesaconate detection had been shown to be accurate this method was therefore used to quantify all future mesaconate concentrations.

Strain Genotypes
IL5.0.0.0 – *C. glutamicum* ATCC 13869
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

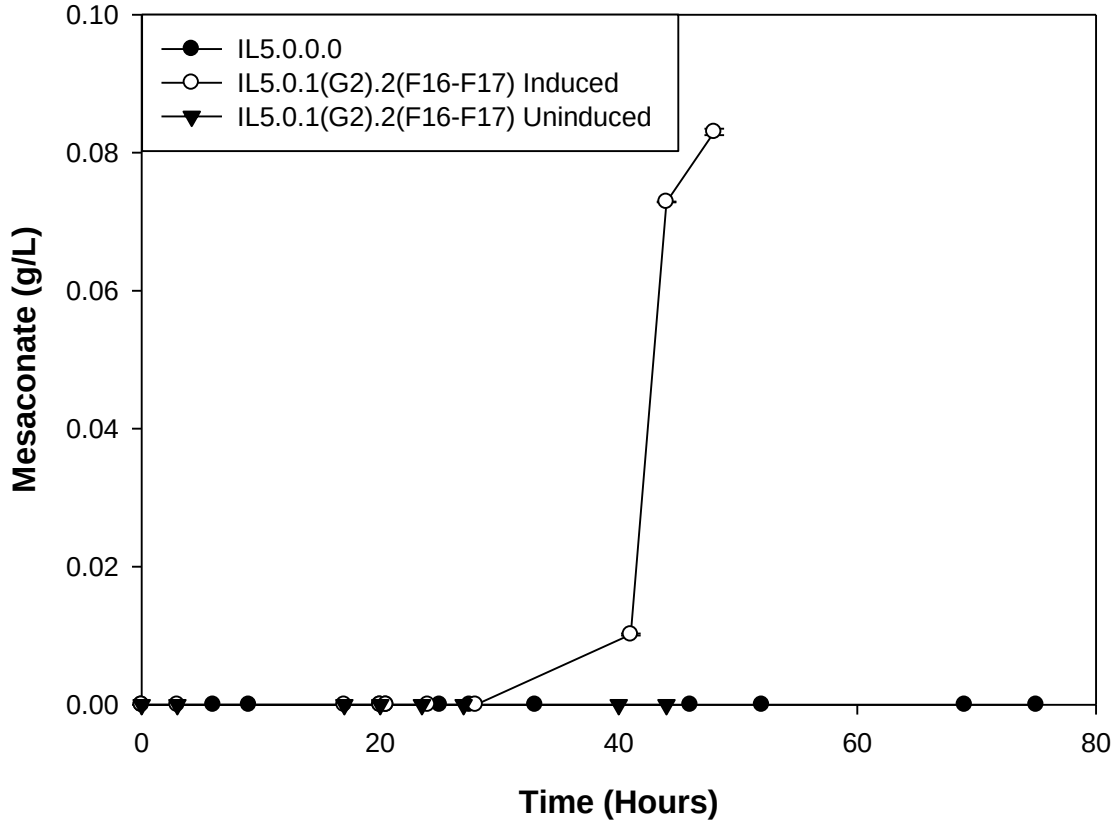


Figure 17: The mesaconate concentrations measured using the improved HPLC method. Each sample was measured in triplicate with the error bars representing the standard deviation of the samples.

3.10. Batch bioprocess using the wild type control strain

As an accurate HPLC method for the analysis of mesaconate had been produced, the wild type strain bioprocess could now be analysed in full. Thus allowing an accurate analysis of the mesaconate production pathway. An important first step in the engineering and validation of a mesaconate production pathway in *C. glutamicum* was to analyse the wild type control strain of *C. glutamicum* ATCC 13869 (IL5.0.0.0) to determine the ability of this strain to produce glutamate under biotin limitation.

The bioprocess for the control strain IL5.0.0.0 was carried out at a 1L scale as outlined in section 2.1.1.3 and the inoculum was prepared as outlined in section 2.1.1.2. The results of the bioprocess for IL5.0.0.0 are shown in Figure 18.

It can clearly be seen that the production of glutamate stops when glucose becomes depleted within the media at 33 hours suggesting that carbon availability is a limiting factor for the production of glutamate. This is expected as glucose is the only carbon source available within the bioreactor. It is therefore possible that, if required, a fed-batch bioprocess may be able to increase the titre of glutamate further. The glucose utilisation rate within the bioreactor was 1.1 g/L/h with a glutamate production rate of 0.56 g/L/h until glucose depletion. The highest dry cell weight (DCW) observed is 5.07 g/L at 25 hours. Growth appears to stop before the complete utilisation of glucose suggesting that the slowing of growth is not related to a lack of carbon availability within the media. The low dry cell weight observed within the bioprocess is expected as biotin has been limited within the media. Biotin is known to be an essential growth factor which cannot be synthesised by *C. glutamicum* (Shiio, Otsuka and Takahashi, 1962) therefore the availability of biotin will have a direct effect on the growth achieved during a bioprocess. A growth rate of 0.15 g/L/h was achieved in the bioprocess between 0 and 25 hours.

The glutamate concentration of 18.98 g/L (4.06 g/g of DCW) produced by IL5.0.0.0 is higher than the concentration observed in the literature (~12 g/L) when Tween 40 was used as the overproduction stimulator (Shirai *et al.*, 2007) and directly comparable to a glutamate titre of 4.09 g/g of DCW observed under biotin limiting conditions by Asakura *et al.*, (2007). No mesaconate was detected during HPLC analysis of samples using the improved mesaconate detection method. This was expected as the wild type strain cannot produce mesaconate due

to lack of a glutamate mutase and methyl aspartate ammonium lyase. As *C. glutamicum* ATCC 13869 was shown to produce a substantial quantity of glutamate under biotin limiting conditions it was selected for the engineering of a mesaconate production pathway.

Strain Genotypes

IL5.0.0.0 – *C. glutamicum* ATCC 13869

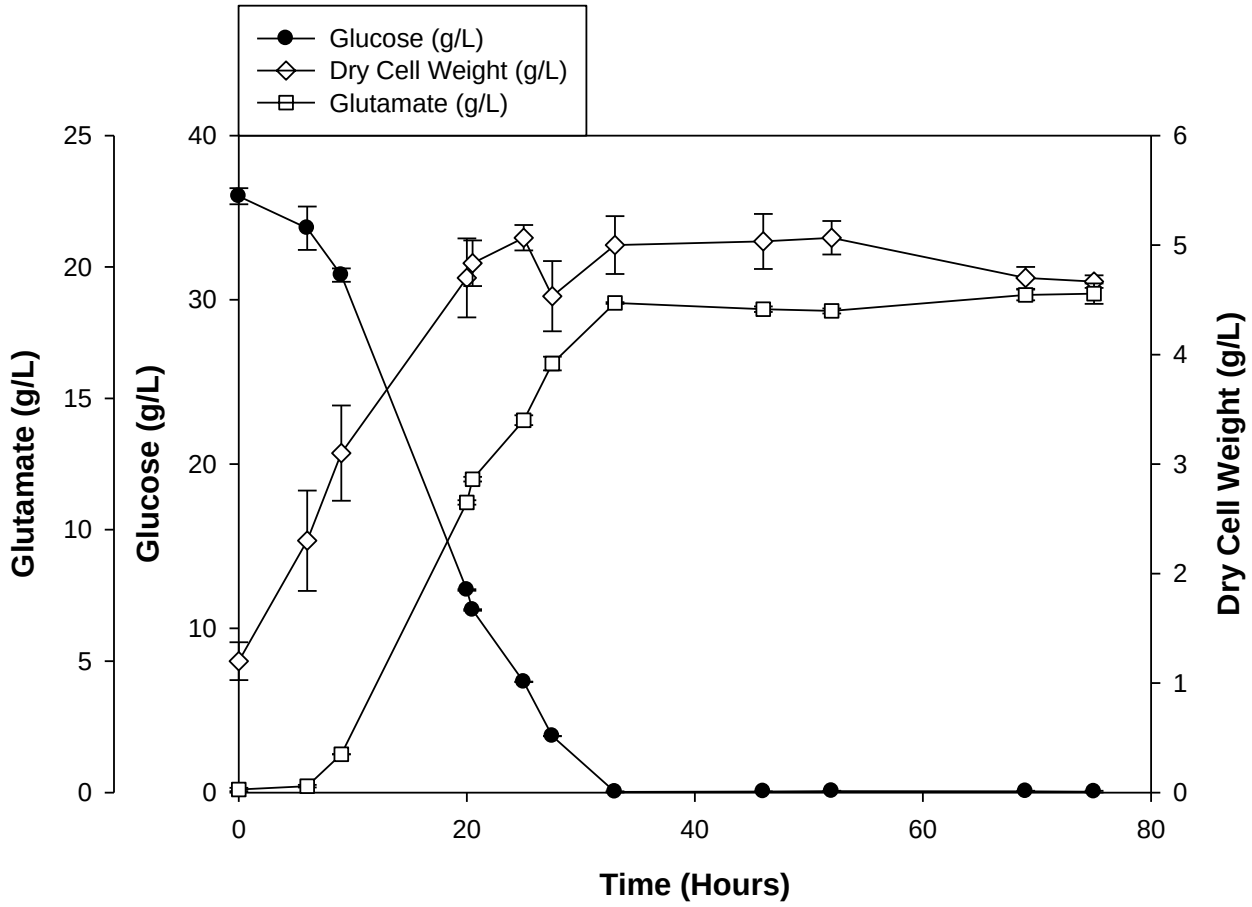


Figure 18: The analysis of the wild type control strain C. glutamicum IL5.0.0.0. The bioprocess was carried out in a 1L Dasgip vessel. The media used was the "batch media" including 1 µg/L of biotin, no kanamycin or spectinomycin was included in the media or inoculum. The inoculum when added gave a starting OD600 of 1. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

3.11. Batch bioprocess using the empty vector strain

In order to have a true control strain for comparison to the production strains which were created, a strain containing two empty vectors was produced to see if the addition of the vectors had an effect on the bioprocess. This strain was entitled IL5.0.1.2. Two vectors were added as two enzymes were added to the production strain a glutamate mutase and a methylaspartate ammonia lyase. Both vectors were under the control of a *tac* promotor.

The *tac* promotor is an inducible promotor which allows for tight regulation of gene expression, this high level of control makes it a commonly employed promotor when engineering biological systems within bacteria. The *tac* promotor system is a hybrid of the *trp* and *lac* promotors using the *lac* repressor molecule to prevent transcription (de Boer, Comstock and Vasser, 1983). The genes under the control of this *lac* repressor molecule are therefore transcribed upon the addition of isopropyl β-D-thiogalactoside (IPTG) to the media which blocks the transcription of the *lac* repressor molecule.

This strain with empty vectors was to act as a control for the production strain which will have vectors containing the required enzymes, as such IPTG was added to the bioprocess to replicate the conditions of the production strain. The vector which encodes for glutamate mutase in the production strain contains *spc*R encoding spectinomycin resistance and the vector which encodes methylaspartate ammonia lyase in the production strain contains *kan*R which encodes kanamycin resistance. Therefore both kanamycin and spectinomycin were required in the medium for IL5.0.1.2 the concentrations of which are outlined in section 2.1.1.1.

The bioprocess for strain IL5.0.1.2 was carried out at a 1L scale and the bioprocess conditions are outlined in section 2.1.1.3 the inoculum for the bioprocess was grown as outlined in section 2.1.1.2. The result of the bioprocess is shown in Figure 19. The bioprocess was run for 70.75 hours at which point the experiment was stopped as the CO_2 and O_2 readings had returned to the values recorded at 0 hours indicating that respiration was no longer taking place.

Strain Genotypes

IL5.0.1.2 – *C. glutamicum* ATCC 13869 (Kan^R: , Spc^R:)

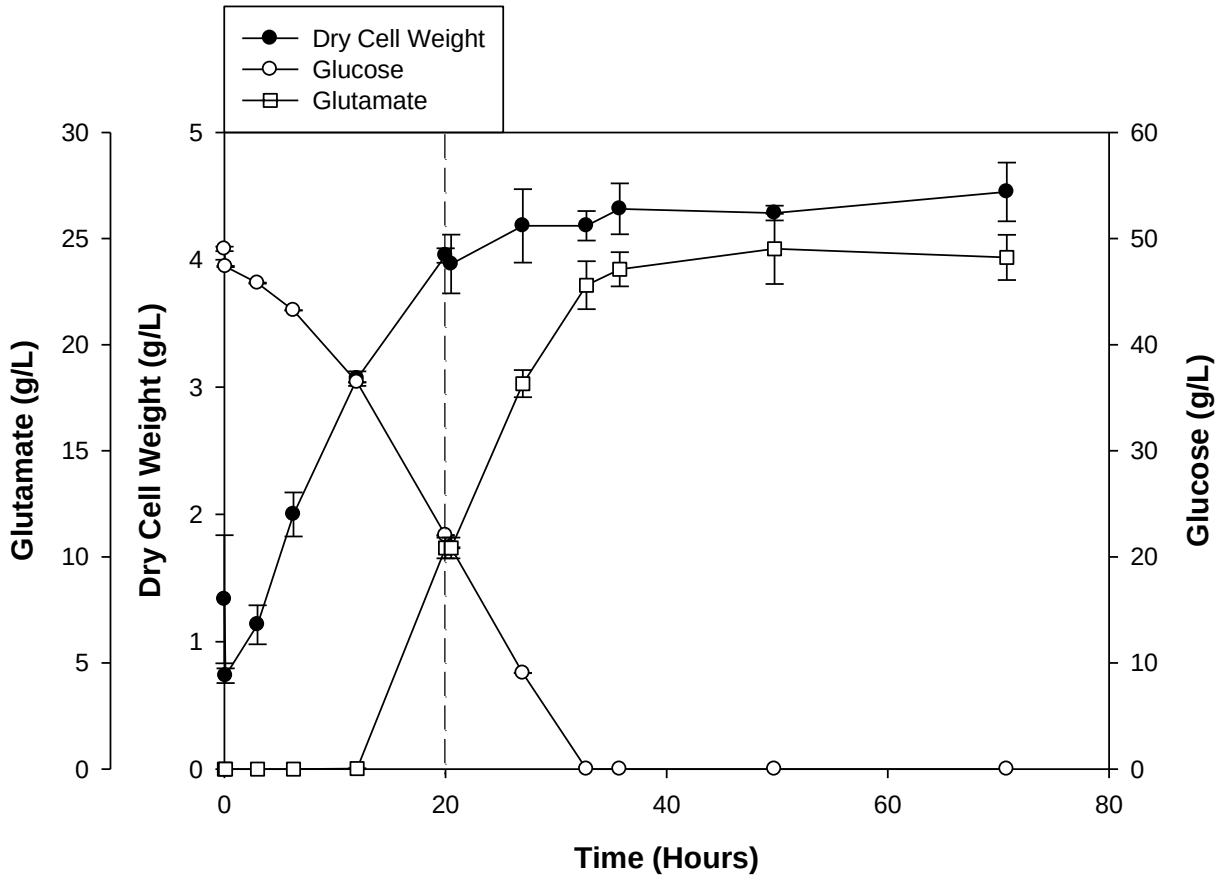


Figure 19: The analysis of the empty vector strain C. glutamicum IL5.0.1.2. The bioprocess was carried out in a 1L Dasgip vessel. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave a starting OD600 of 1. The vectors were induced at 20 hours indicated by the dashed line (---) through the addition of 1 mM of IPTG. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

As with all bioprocesses it is important to monitor the effect the addition a substrate has on the process, IPTG addition at 20 hours does not appear to have an effect on glutamate production with a production rate of 1.3 g/L/h observed before the addition and a production rate of 1.2 g/L/h observed following the addition. The overall production rate of glutamate was 0.70 g/L/h from the start of the bioprocess until the glucose is depleted at 32.75 hours this is a 25% increase on the control strain IL5.0.0.0. The maximum titre of glutamate observed during the bioprocess was 24.51 g/L at 49.75 hours which is 29% higher than that observed from IL5.0.0.0 (18.98 g/L). This can be explained by looking at the available carbon in the two bioprocesses.

The control bioprocess IL5.0.0.0 had 36.31 g/L of glucose available at 0 h and IL5.0.1.2 had 47.36 g/L at 0 h. This difference may be due to experimental error during the preparation and autoclaving of the media. The fact that a higher titre of glucose is related to a higher titre of glutamate is confirmed when comparing the yield of glutamate in relation to glucose used. IL5.0.0.0 had a glutamate yield of 0.52g/g of glucose and IL5.0.1.2 had a yield of 0.52g/g of glucose therefore it appears that the higher yield in IL5.0.1.2 is directly related to the greater glucose availability.

The glucose utilisation rate was 1.45 g/L/h which is 32% higher than IL5.0.0.0 (1.1 g/L/h). When looking at this in combination with the lower overall titre of DCW from IL5.0.1.2 of 4.53 g/L (10.6% lower than IL5.0.0.0), a titre of glutamate from DCW of 5.61 g/g (38% higher than IL5.0.0.0) and the overall production rate of glutamate mentioned previously which was 25% greater than IL5.0.0.0. It could be concluded that strain IL5.0.1.2 is the more efficient strain for the production of glutamate. Why this is the case is not clear, although it does indicate that the addition of the plasmids did not negatively affect glutamate production. As the production of glutamate remains stable after the addition of the empty vectors the next step was to create a production strain incorporating the enzymes necessary for mesaconate production.

3.12. Induced batch bioprocess using the mesaconate production strain

In order to produce mesaconate in *C. glutamicum* two enzymes were added to the empty vector strain IL5.0.1.2., these enzymes were a glutamate mutase and a methyl aspartate ammonium lyase (Figure 20).

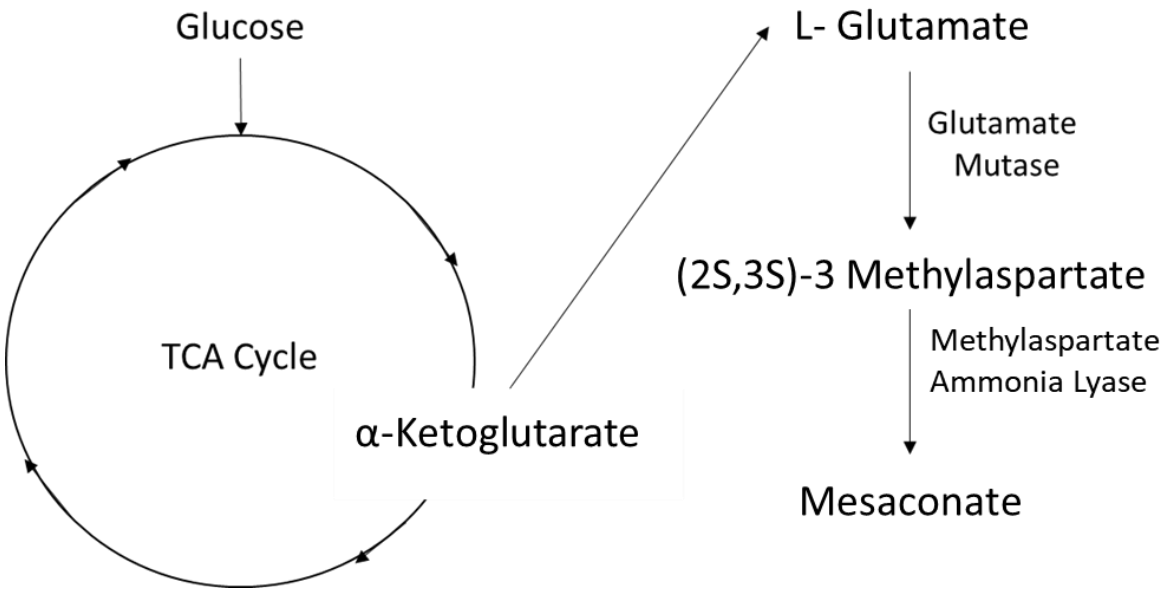


Figure 20: The proposed mesaconate production pathway in C. glutamicum.

These genes were incorporated into the empty vectors to create IL5.0.1(G2).2(F16-F17). This strain was then run under the same conditions as the empty vector strain. It was hypothesised that the addition of these enzymes to the system would enable mesaconate production. It had been previously shown that this strain may have produced up to 1.33 g/L of mesaconate when analysed using the old HPLC method, although upon further inspection this was thought to be an interfering peak and not mesaconate. This bioprocess was analysed using the new method which eliminates an interfering peak. The bioprocess for strain IL5.0.1(G2).2(F16-F17) was carried out at a 4L scale in a Bioflo 110 reactor vessel using the conditions outlined in section 2.1.1.3. The inoculum was prepared as outlined in section 2.1.1.2. The result of the bioprocess is shown in Figure 21.

Strain Genotypes

IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

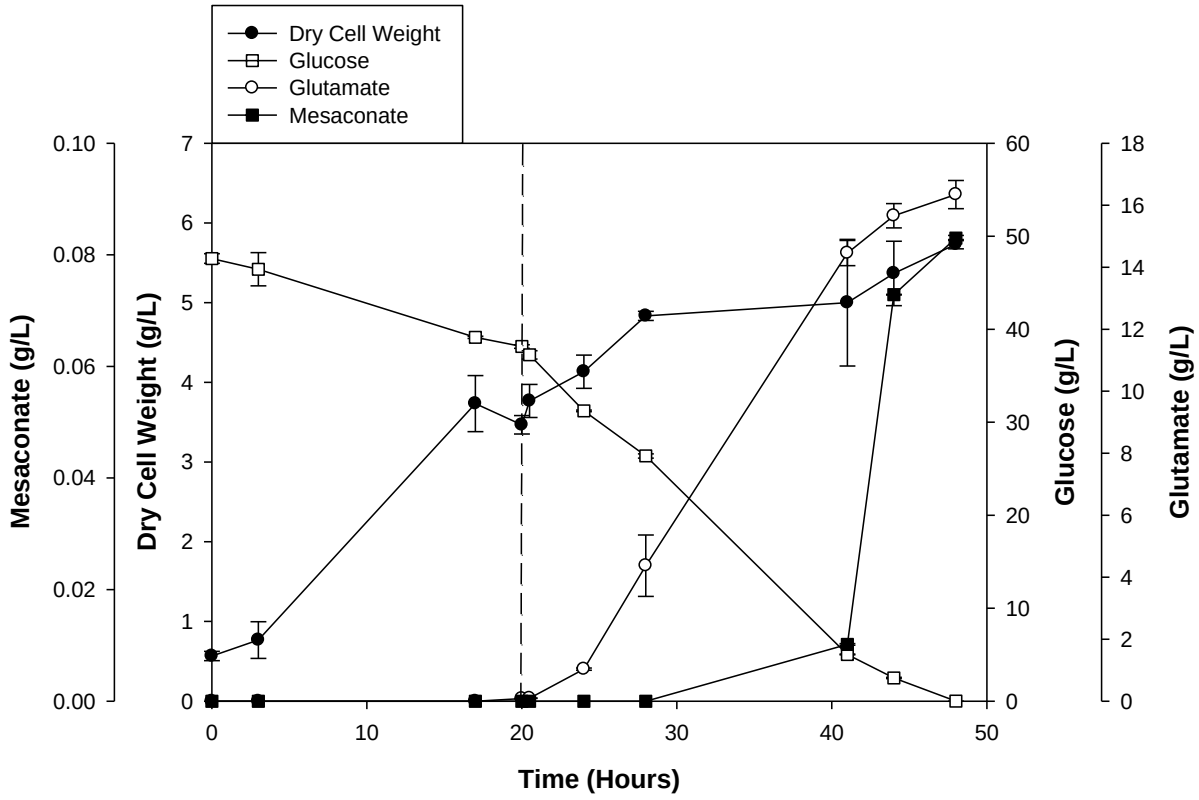


Figure 21: The analysis of the production strain C. glutamicum IL5.0.1(G2).2(F16-F17). The bioprocess was carried out in a 4L Bioflo vessel with a 10L working volume. The media used was the "batch media" including kanamycin and spectinomycin and 1µg/L of biotin. The inoculum when added gave a starting OD600 of 1. The vectors were induced at 20 hours indicated by the dashed line (---) through the addition of 1 mM of IPTG. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

The bioprocess was run for 44 hours at which point the experiment was stopped as the CO_2 and O_2 readings had returned to the values recorded at 0 hours indicating that respiration was no longer taking place.

Through the addition of glutamate mutase and methyl-aspartate ammonium lyase (MAAL) a mesaconate titre of 0.083 g/L was successfully produced in the production strain *C. glutamicum* IL5.0.1(G2).2(F16-F17). This was lower than the apparent titre of 1.33 g/L which was observed upon analysis with the old HPLC method. This result confirms that the previous HPLC method was not measuring mesaconate but another compound, this is confirmed by the fact that NMR analysis of the final sample from this bioprocess showed that less than 1 g/L of mesaconate was present (Section 3.9.1). To the authors knowledge this was the first reported bio- production of mesaconate from glucose in an expression system other than *E. coli* which was demonstrated by Wang & Zhang (2015). The titre of mesaconate produced was lower than expected when looking at the titres achieved in *E. coli*. The *E. coli* strain EM1 containing a glutamate mutase and a MAAL from *C. tetanomorphum ATCC15920* produced a mesaconate titre of 1.17 g/L when grown in shake flasks with a glutamate feed.

As glutamate is naturally produced by *C. glutamicum* IL5.0.1(G2).2(F16-F17) there was no need to feed glutamate although a similar mesaconate titre would be expected as the production pathways were very similar in design. One possible explanation for the lower titre of mesaconate observed is that the oxygen concentration within the bioreactor was too high. Shake flasks have poor control of the oxygen availability within whereas the bioprocess for IL5.0.1(G2).2(F16-F17) had the dissolved oxygen concentration tightly controlled at 30% throughout the experiment by increasing the stirrer speed. For both of the experiments the MAAL is from *Clostridium tetanomorphum* wherein it is used in the anaerobic glutamate fermentation pathway (Goda *et al.*, 1992; Raj and Poelarends, 2013). It was therefore possible that the oxygen concentration had an inhibitory effect on the MAAL and the mesaconate titre. This hypothesis was also backed up by the fact that mesaconate started to be produced as the measured CO_2 in the bioprocess was declining indicating reduced respiration was a factor.

In this instance, this hypothesis does not appear to be accurate. The glutamate mutase appears to be the bottleneck in the production pathway for two reasons. No (2S,3S)-3 methylaspartate appears during HPLC analysis of the samples (Data not shown) and mesaconate is present within the samples. This suggests that all of the available (2S,3S)-3

methylaspartate is converted to mesaconate and the presence of mesaconate means that the MAAL is both expressed and active. It is therefore not believed that the MAAL is responsible for the low titre of mesaconate.

A significant difference between the demonstrated pathway and the pathway demonstrated in *E. coli* is the origin of the glutamate mutase. The glutamate mutase used by Wang & Zhang was acquired from *Clostridium tetanomorphum ATCC15920* whereas the glutamate mutase demonstrated in IL5.0.1(G2).2(F16-F17) was acquired from *Actinoplanes fruiliensis*. Glutamate mutase is known to be produced in *C. tetanomorphum* as a component of the anaerobic glutamate fermentation pathway converting L-glutamate to (2S,3S)-3 methylaspartate (Holloway and Marsh, 1994). This *C. tetanomorphum* mutase has been shown to be inactivated by O_2 as the inactive form of the co-factor (Co-enzyme B12) attaches to the enzyme binding subunit (Toraya, 2003). This inactivation was confirmed by Wang & Zhang and through the addition of a reactivatase (mutL) the mesaconate titre was increased by 162%.

Glutamate mutase is a known component of the friulimicin production pathway in *A. fruiliensis* (Heinzelmann *et al.*, 2003) and was selected for the production strain IL5.0.1(G2).2(F16-F17). The perceived advantage of this selection was that the production of friulimicin is an aerobic process (Aretz *et al.*, 2000) therefore the mutase may not be as sensitive to O_2 inactivation. From the analysis of the mesaconate production it can be seen that the perceived stability did not translate to a higher titre of mesaconate. As previously described it appears that the glutamate mutase is the bottleneck for mesaconate production, it is possible that the glutamate mutase is simply being expressed in low concentrations. This could be investigated by repeating the experiment and carrying out RNA sequencing on the samples to determine the expression. As mesaconate was produced in the bioprocess glutamate mutase was being expressed although the intensity of the expression is unknown. It has been reported that the *A. fruiliensis* glutamate mutase can also isomerise α-ketoglutarate to produce aspartic acid which is unique to this mutase (Heinzelmann *et al.*, 2003). This does not appear to have had an effect on the mesaconate production in this bioprocess as no aspartic acid was detected when analysing the samples.

A DCW titre of 5.7 g/L was achieved with a growth rate of 0.15 g/L/h achieved between 0 and 28 hours which was the main growth phase of the bioprocess. When compared to the growth

rate of the empty vector strain (0.13 g/L/h) and the wild type control strain (0.15 g/L/h) it can be seen that the addition and expression of the vectors does not have an effect on the growth of the bacterium.

The maximum glutamate titre achieved was 16.34 g/L which is lower than the wild type control (18.98 g/L) and the empty vector strain (24.51 g/L) which suggested that IL5.0.1(G2).2(F16-F17) may not be as efficient at producing glutamate as the control strains. It was also possible that the lower titre may have been due to a difference in the carbon to dry cell weight ratio. In order to confirm this the rate of glucose utilisation and the yield of glutamate to dry cell weight was calculated.

The bioprocess had a glucose utilisation rate of 0.99 g/L/h which is lower than both of the control strains, the empty vector strain had a rate of 1.45 g/L/h (46% higher) and the wild type control had a rate of 1.1 g/L/h (11% higher), suggesting that the production strain will be less efficient at producing glutamate as the growth rate has already been shown to be comparative. This is confirmed by looking at the glutamate production in relation to DCW. The wild type strain had a yield of 4.06 g/g of DCW, the empty vector strain had a yield of 5.61 g/g of DCW and the production strain had a yield of 3.16 g/g of DCW. The lower glutamate titre was therefore due to a slower production rate by the cells and not the availability of carbon.

It is unclear why the mesaconate titre was much lower in the production strain in comparison to the *E. coli* strain from the literature although it appears that the glutamate mutase is the bottleneck in the process. In order to determine the effect the IPTG induction had on the bioprocess the experiment was repeated without the addition of IPTG.

3.13. Un-induced batch bioprocess using the mesaconate production strain

The bioprocess for strain IL5.0.1(G2).2(F16-F17) with no IPTG induction was carried out at a 4L scale in a Bioflo 110 reactor vessel using the conditions outlined in section 2.1.1.3 the only alteration to the outlined method was that no IPTG was added to the bioprocess. The inoculum was prepared as outlined in section 2.1.1.2. The result of the bioprocess is shown in Figure 22.

No mesaconate was produced in this bioprocess which demonstrates that the *tac* promotor and the IPTG induction method is successful in regulating efficiently the expression of the vectors. It also demonstrates that the mesaconate production seen in the induced bioprocess was indeed a product of the inserted vectors. This confirms that the new HPLC analysis method is effective and that the 1.27 g/L of mesaconate previously observed was an interfering compound and not mesaconate.

A DCW titre of 5.33 g/L was achieved during the bioprocess a similar titre to the induced experiment which had a titre of 5.73 g/L (7.5% higher) with a growth rate of 0.20 g/L/h measured between 0 and 20 hours which is the growth phase of the experiment. In comparison to the induced bioprocess a 33% faster rate of growth is observed with the growth plateauing at 20 hours. The glucose in the reactor was fully utilised at 40 hours and was utilised at a rate of 1.35 g/L/h. This was a faster rate than that which was observed during the induced bioprocess (0.99 g/L/h). The combination of a faster glucose utilisation rate along with a lower titre of DCW suggests that the carbon will have been used to produce a higher titre of glutamate.

A maximum titre of 19.94 g/L of glutamate was achieved with a production rate of 0.54 g/L/h between 3 hours when production started and 40 hours which was the point at which the glucose within the reactor became limiting. Although the maximum titre of glutamate is 22% higher than the induction strain (16.34 g/L) the production rate is only 2% higher suggesting that the productivity of the cells is not the major factor in the different glutamate titres.

Strain Genotypes
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

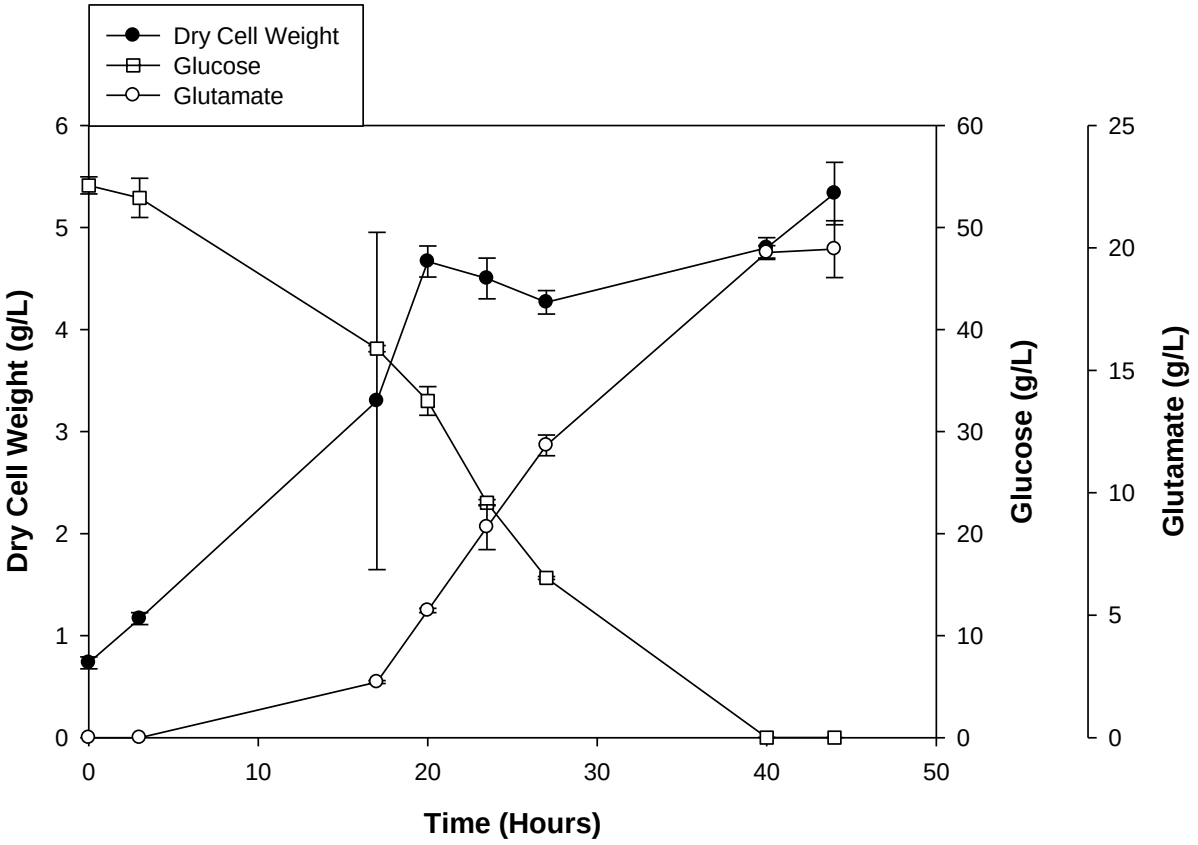


Figure 22: The analysis of the production strain C. glutamicum IL5.0.1(G2).2(F16-F17). The bioprocess was carried out in a 4L Bioflo vessel with a 10L working volume. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave a starting OD600 of 1. No mesaconate was detected in the samples. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

When looking at the titre of glutamate in relation to DCW the induced bioprocess has a titre of 3.16 g/g of DCW and the bioprocess without induction has a titre of 3.74 g/ g of DCW. This shows that the cells in the bioprocess without induction were 18% more productive which was a factor in the higher maximum titre. The major factor in relation to the maximum titre of glutamate was the available carbon during the bioprocess. Both bioprocesses had similar titres of glutamate in relation to glucose of 0.34 g/g of glucose from the induced bioprocess and 0.37 g/g of glucose in the bioprocess without induction. This means that the available glucose is what will have had the greatest effect on the maximum titre and this is reflected in a starting concentration of 47.6 g/L in the induced bioprocess and 54.1 g/L in the bioprocess without induction. This higher glucose concentration explains the higher glutamate titre observed in the bioprocess without induction.

As the mesaconate titre in the induced bioprocess was so low it was expected that the bioprocess without induction would have similar characteristics. Had the titre of mesaconate been higher there would have been differences in the glutamate titre in relation to glucose as a greater proportion of the glutamate would have been converted to mesaconate.

3.14. The effect of biotin excess on the mesaconate production strain

Although mesaconate production was observed in IL5.0.1(G2).2(F16-F17) the yield was lower than that observed by Wang and Zhang (2015) using *E. coli*. This may have been due to the use of an alternative glutamate mutase but may also be due to the effect of biotin limitation as this was not required for *E. coli* to produce glutamate.

In order to determine if mesaconate could be produced when biotin was not limited a 1L bioprocess was set up using IL5.0.1(G2).2(F16-F17) in batch media with additional biotin. The handbook of *Corynebacterium glutamicum* (L Eggeling and Bott, 2005) defines biotin excess as being above 200 µg/L. In all previous bioprocesses the biotin present at the start was 1 µg/L which allows growth and glutamate overproduction. For this experiment 200 µg/L of biotin was added to the media which should prevent glutamate overproduction as the biotin concentration would not drop below 30 µg/L.

The inoculum was set up as outlined in section 2.1.1.2 and the bioprocess was set up as outlined in section 2.1.1.3 with the only differences being the higher biotin concentration of 200 µg/L. Samples were taken periodically for HPLC analysis of glucose, mesaconate and glutamate along with DCW measurements. The result of the analysis is shown in Figure 23.

It can be seen from Figure 23 that a small titre of glutamate was produced in this bioprocess with 0.34 g/L present at 28 hours. This glutamate titre was much less than that observed in the same bioprocess with biotin limitation (Section 3.12) where 16.3 g/L of glutamate was produced. This lower titre was expected as the bioprocess did not have biotin limiting conditions. Although glutamate was produced no mesaconate production was observed, as a low concentration of mesaconate was observed when high concentrations of glutamate was produced (Section 3.12) this was not unexpected. This result suggested that the presence of biotin did not improve the efficiency of mesaconate production from glutamate.

A maximum dry cell weight of 13.9 g/L was produced by 24 hours with a growth rate of 0.58 g/L/h. This titre was 237% higher than the DCW observed by 24 hours in the bioprocess with biotin limitation (4.13 g/L). The growth rate was also 241% higher than that observed in the previous bioprocess (0.17 g/L/h). This increase in growth was due to the concentration of

Strain Genotypes

IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

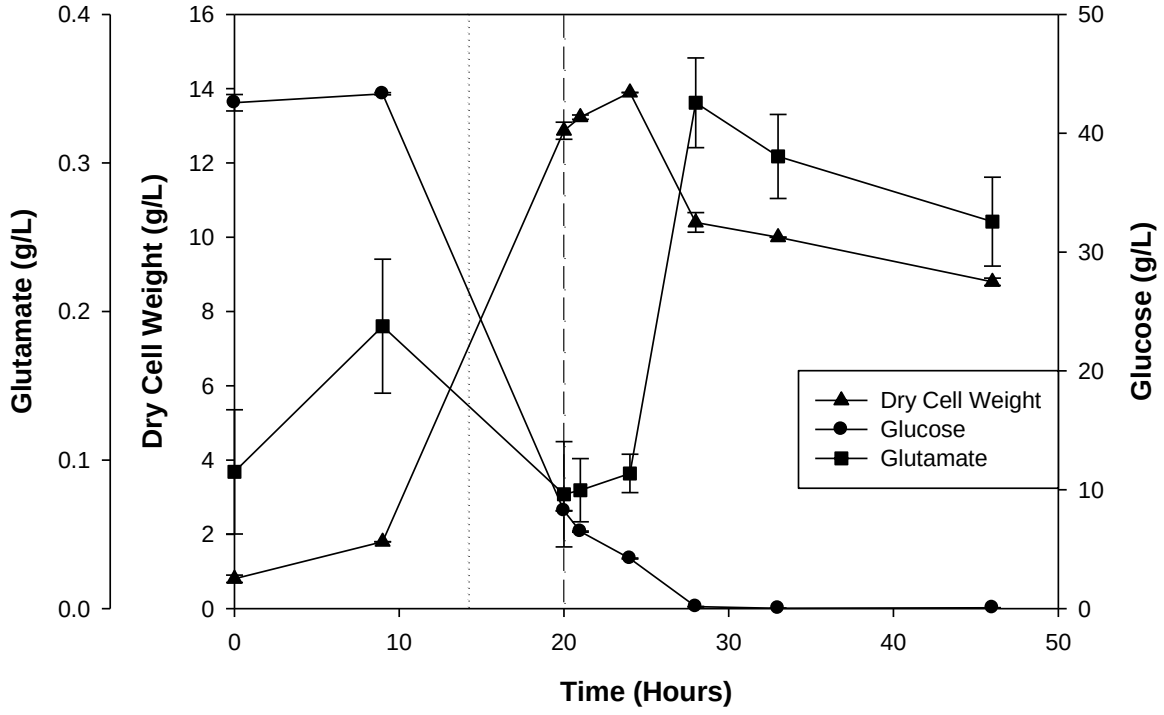


Figure 23: The analysis of the production strain C. glutamicum IL5.0.1(G2).2(F16-F17) with an excess of biotin. The bioprocess was carried out in a 1L Dasgip vessel with a working volume of 1.5L. The media used was the "batch media" including kanamycin and spectinomycin and 200 µg/L of biotin. The inoculum when added gave a starting OD600 of 1. No mesaconate was detected in the samples. The dotted line represents the point at which excessive foaming occurred (·····). The dashed line represents the time at which IPTG was added (----). All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

biotin in the bioprocess. Biotin is an essential component for the activity of pyruvate carboxylase. As *C. glutamicum* is a biotin auxotroph the addition of biotin increases pyruvate carboxylase activity which increases growth (Sato *et al.*, 2008). The increased growth rate resulted in excessive foaming in the bioprocess causing glutamate to be lost through the off-gas filter. This foaming was the cause of the dip in glutamate observed in the samples.

It was concluded that although the presence of a higher concentration of biotin increased the dry cell weight and growth rate, it did not result in the production of mesaconate. It was therefore also concluded that as glutamate was present and was not converted to mesaconate that a larger concentration of glutamate was required in the biotin excess bioprocess. As such the next step was to incorporate a feed of glutamate.

3.15. The effect of biotin excess and a glutamate feed on the mesaconate production strain.

It was concluded in the bioprocess with 200 µg/L of biotin (Section 3.14) that a glutamate feed may be required for mesaconate production when an excess of biotin was present. The increased biotin concentration facilitated growth but this did not result in conversion of the available glutamate to mesaconate. In the paper which outlined the production of mesaconate by *E. coli* (Wang and Zhang, 2015) an external glutamate feed was employed which allowed mesaconate production. It was therefore hypothesised that if biotin limitation was a factor that was detrimental to mesaconate production a *C. glutamicum* bioprocess with excess biotin and a glutamate feed may demonstrate a higher mesaconate production capacity.

In order to determine if the incorporation of an external glutamate feed into the media would allow the production of mesaconate a bioprocess was set up using IL5.0.1(G2).2(F16-F17) with a 0.2 g/L/h glutamate feed. This concentration of feeding was selected as the bioprocess with mesaconate production had a glutamate production rate of 0.34 g/L/h which resulted in a build-up of glutamate. Therefore the slightly lower rate of 0.2 g/L/h was selected.

It was decided that IPTG would be added at 0 hours. In previous bioprocesses IPTG was added at 20 hours which as this was when glutamate was being overproduced. As there would be no glutamate overproduction but instead a constant feed of glutamate it was thought that the addition of IPTG at 0 hours would maximise mesaconate production through availability of glutamate mutase and MAAL throughout the bioprocess. The concentration of biotin was also lowered from 200 µg/L to 20 µg/L as the excessive growth seen in the biotransformation with 200 µg/L caused foaming which was detrimental to the bioprocess. At 20 µg/L no biotin limitation was expected and therefore no glutamate overproduction.

The bioprocess inoculum was set up as outlined in section 2.1.1.2 with the bioprocess set up as outlined in section 2.1.1.3. The glutamate feed was set up by continuously feeding 2 ml/h of a 100 g/L glutamate stock solution. Samples were taken periodically and analysed for glutamate, mesaconate and glucose by HPLC and separate DCW analysis. The result of the analysis is shown in Figure 24.

Strain Genotypes
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

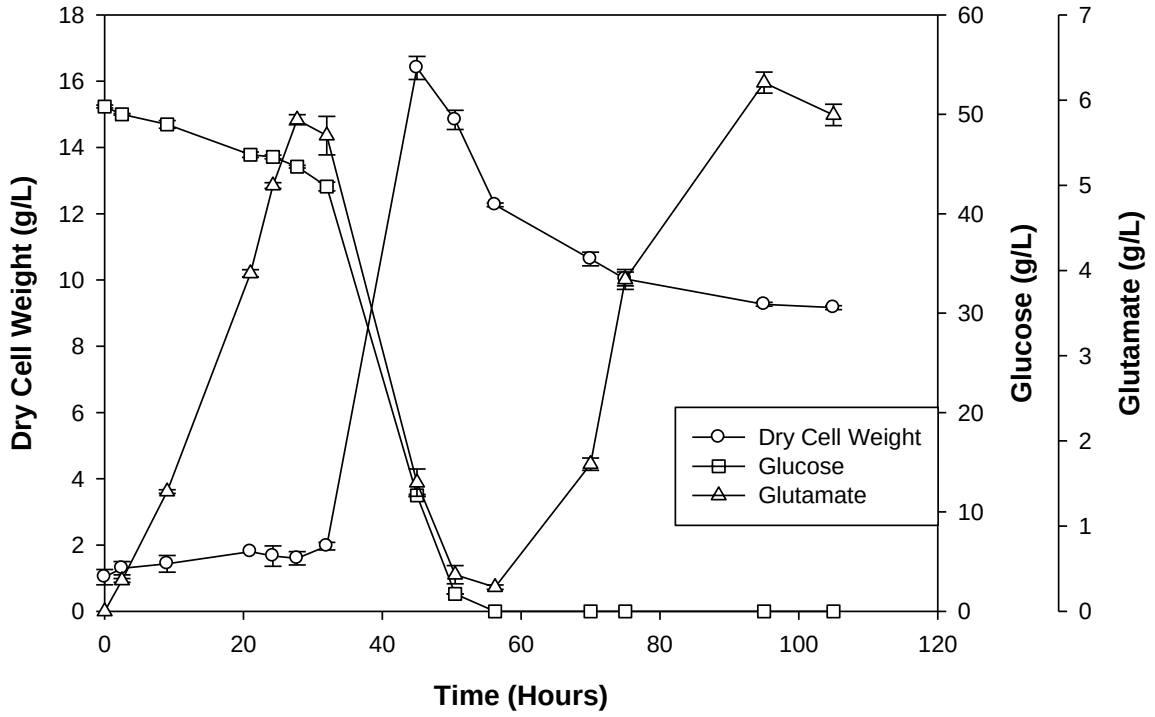


Figure 24: The analysis of the production strain C. glutamicum IL5.0.1(G2).2(F16-F17) with an excess of biotin and a glutamate feed. The bioprocess was carried out in a 1L Dasgip vessel with a working volume of 1.5L. The media used was the "batch media" including kanamycin and spectinomycin and 20 µg/L of biotin. The inoculum when added gave a starting OD600 of 1. No mesaconate was detected in the samples by HPLC. The glutamate feed was 0.2 g/L/h. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

From Figure 24 it can be seen no mesaconate was produced in the bioprocess when biotin was not limited and glutamate was present at a higher concentration. This result was not expected as it had previously been shown in *E. coli* (Wang and Zhang, 2015) that mesaconate could be produced with an external glutamate feed. It can be seen that glutamate was utilised during growth therefore it would be expected to be utilised for mesaconate production.

The early addition of IPTG to the bioprocess at 0 hours was shown to have a detrimental effect on growth with limited growth observed until after the 32 hour sample with the maximum dry cell weight observed in the 45 hour sample of 16.4 g/L. This maximum dry cell weight was higher than that observed in the bioprocess with biotin in excess (13.9 g/L) demonstrating that growth was still possible after the lag phase. IPTG has previously been shown to have a detrimental effect on the growth rate of *C. glutamicum* (Equbal *et al.*, 2013) depending on when IPTG is added to the bioprocess. It was possible that the toxicity of IPTG early in the bioprocess may have prevented the production of mesaconate later after growth when glutamate and sufficient biomass was available.

The glucose within the bioprocess was initially utilised slowly until growth began with glucose becoming depleted at 56 hours. This was a utilisation rate of 0.91 g/L/h was slower than that observed in the previous bioprocess without the glutamate feed which showed a utilisation rate of 1.52 g/L/h. This lower rate of glucose use was due to the lag in growth observed from the early induction of IPTG. Mesaconate had only been observed previously when glucose was present within the bioprocess (section 3.12). Therefore it is possible that no mesaconate was observed as glucose had been completely utilised for biomass production.

In this bioprocess and the previous bioprocess there was a timepoint where glucose and glutamate were present after induction and no mesaconate was observed. Therefore it was concluded that biotin limitation was not responsible for the low mesaconate titre observed in bioprocess where it was produced. It may be possible that glucose availability is important for mesaconate production, therefore the next step was to run a bioprocess containing glucose throughout.

3.16. Fed batch bioprocess of the mesaconate production strain

The available carbon for growth and production has a significant effect on bioprocesses. Fed batch bioprocesses often demonstrating higher titres of product than batch bioprocesses due to an increase in the concentration of active biomass within the reactor and increases in the length of the bioprocess due to a greater carbon availability (Luli and Strohl, 1990). Fed batch and continuous processes are the standard processes employed industrially for the production of glutamate from *C. glutamicum* shifting from the traditional batch process (Hermann, 2003). Therefore it was hypothesised that by providing a continuous feed of glucose to the bioprocess it may be possible to increase the titre of mesaconate within the bioreactor.

The bioprocess for strain IL5.0.1(G2).2(F16-F17) with IPTG induction and a continuous feed of glucose was carried out at a 1L scale in a Dasgip bioreactor using the conditions outlined in section 2.1.1.3 the only alteration to the outlined method was that a continuous feed of glucose was added to the bioprocess. The glucose feed (250 g/L solution) was started at 20 hours at which point the base was also changed to 2M NaOH and IPTG induction occurred. The speed of the glucose feed was adjusted to maintain an approximate glucose concentration of less than 20 g/L within the reactor. The glucose concentration was monitored at-line, as samples were collected, through the use of reagent test strips. The inoculum was prepared as outlined in section 2.1.1.2. The result of the bioprocess is shown in Figure 25.

A maximum mesaconate titre of 0.0325 g/L was achieved during the fed batch bioprocess which was 60.8% lower than the titre achieved in the batch process (0.083 g/L). A possible explanation for the lower mesaconate titre is that the blocked filter caused an increase in pressure with the reactor limiting production. The air out filter became blocked between 35 hours and 49 hours due to foaming within the bioreactor, this will have causes pressure to build up until 49 hours at which point the filter was replaced with another sterile filter. This process did not result in contamination of the bioreactor as samples were streaked on nutrient agar plates and no colonies other than *C. glutamicum* were observed.

Strain Genotypes

IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

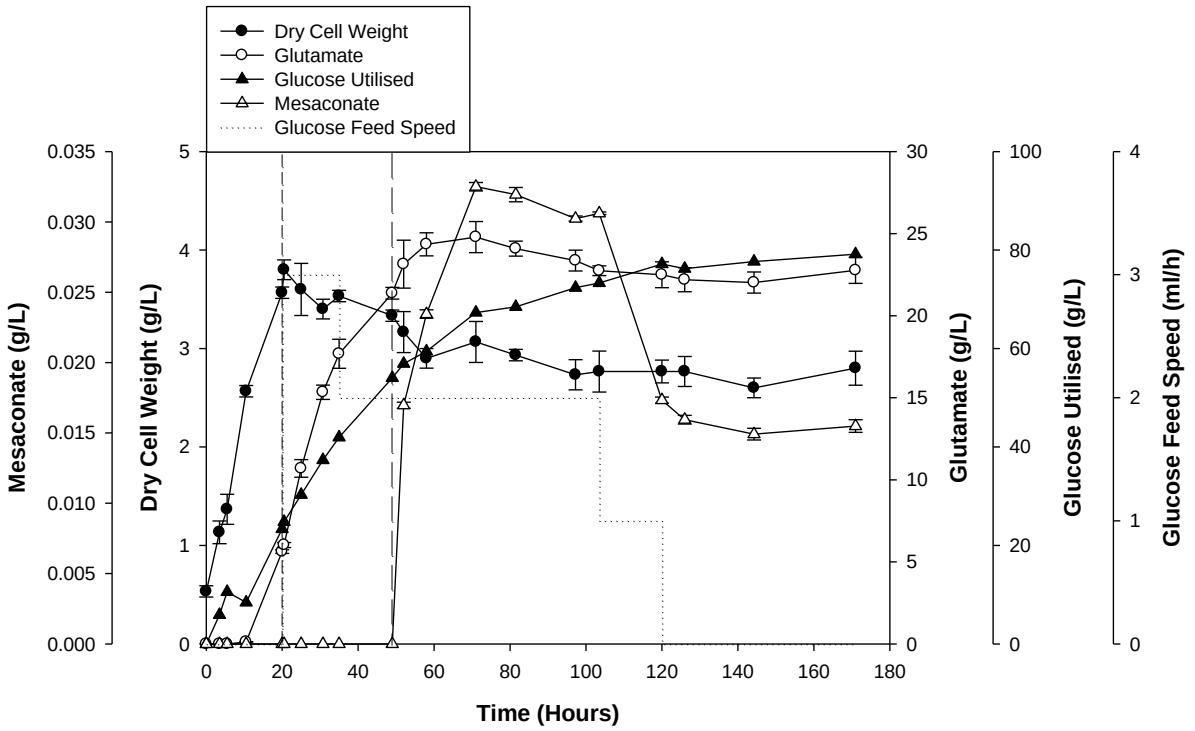


Figure 25: The analysis of the production strain C. glutamicum IL5.0.1(G2).2(F16-F17) with a continuous glucose feed. The bioprocess was carried out in a 1L Dasgip reactor with a 1.5L working volume. The inoculum when added gave an OD600 = 1. Induction occured at 20 hours indicated by the short dashed line (---) through the addition of 1 mM of IPTG. The glucose feed speed is indicated by the dotted plot (.....) on the graph. The off-gas filter was replaced at 49 hours indicated by the line with larger dashes (– – –). All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

Mesaconate production appears immediately after the pressure is released from the reactor suggesting that the pressure may have had an effect on production. It is possible that the higher pressure caused a stress reaction within the *Corynebacterium* cells resulting in either no transcription of the vectors or diversion of the carbon away from the mesaconate pathway. As glutamate continues to be produced before the pressure was relieved the former is more likely than the latter. The mesaconate titre increases until 80 hours at which point the titre begins to decrease, why this occurs is not clear as this is not observed in the batch bioprocess. It is possible that the batch bioprocess was not run for long enough in order to observe the decrease in the mesaconate titre.

A maximum biomass titre of 3.8 g/L was achieved during the bioprocess with a growth rate of 0.16 g/L/h between 0 and 20 hours which can be seen to be the growth period of the bioprocess. This maximum titre is 34% lower than the titre of the batch bioprocess with IPTG induction (5.73 g/L) although the growth rate is comparable with the fed-batch process having a 7% higher growth rate than the batch process. Growth stops abruptly at 20 hours which may be due to the addition of IPTG and the switch of the base from 25% NH_4OH (v/v) to 2M NaOH which also occurs at 20 hours. IPTG has been shown in previous batch bioprocesses to not have a substantial effect on the growth of the strains (Section 3.11 and 3.12) therefore it is not likely to have caused the stop in growth. The base was swapped as the large volume of NH_4OH being added to the reactor to control the pH change due to glutamate production may have become inhibitory to the bioprocess especially as the fed batch bioprocess had a higher projected glutamate yield. It has been shown in the literature that ammonium can have a negative effect on methylaspartate ammonia lyase from *C. tetanomorphum* (Botting and Gani, 1992) therefore it was possible that a high volume of NH_4OH added during a long fed batch process may have had an effect on mesaconate production. The effect of changing the base is explored in section 3.19 but is unlikely to have halted the growth phase with nitrogen for growth available through the media and previous base addition of NH_4OH. It is thought that the termination of the growth phase was not affected by the additions at 20 hours but instead was due to the transition into the production phase falling naturally on 20 hours as has been previously demonstrated in section 3.12.

Despite a lower titre of DCW being observed in this bioprocess the maximum titre of glutamate was 24.79 g/L which is 52% higher than the induced batch bioprocess. This suggests

that either the production rate of glutamate was faster in the fed batch bioprocess or that the production phase lasted for longer in the fed batch bioprocess. The production rate of glutamate was 0.46 g/L/h which was 13% slower than the production rate of the induced batch bioprocess. The fed batch bioprocess must therefore have a longer production phase due to the increased availability of carbon due to the glucose feed. This was expected as fed batch processes are commonly employed in industry for this reason (Hermann, 2003). The production phase of glutamate lasted 52.5 hours which was 24.5 hours longer than the production phase observed in the induced batch bioprocess. The increase in the length of the production phase of glutamate did not have any effect on the mesaconate titre.

It could be concluded from this bioprocess that increasing the glucose present increased the glutamate titre but did not increase the mesaconate produced. Therefore glucose limitation appears not to be responsible for the low mesaconate titre observed. Growth stopped on the addition of IPTG and the switching of the base used in the bioprocess, as IPTG had been added at 20 hours with no detrimental effect observed it was possible that using NaOH as the base could negatively affect growth.

3.17. The effect of an alternative carbon source on mesaconate production

In order to determine if the presence of glucose within the bioreactor had an effect on mesaconate production, a bioprocess was carried out using an alternative carbon source. Gluconic acid was selected as the alternative carbon source as *C. glutamicum* has been shown in literature to be able to grow effectively on this carbon source reaching comparable dry cell weight titres to growth on glucose (Vallino and Stephanopoulos, 1994). It has also been shown that growth on gluconic acid can have a beneficial effect on amino acid production with higher lysine titres observed on gluconic acid (Bianchi *et al.*, 2001). Therefore it was hypothesised that changing the carbon source may improve glutamate production and therefore mesaconate production.

To confirm that *C. glutamicum IL5.0.1(G2).2(F16-F17)* could grow on gluconic acid a shake flask experiment was set up to measure growth before moving into a bioreactor to determine mesaconate production. Three shake flasks were set up containing 50 ml of CGG media outlined in section 2.1.1.1. One flask contained glucose (5 g/L) as outlined in the method and the other two shake flasks contained gluconic acid (5 g/L) instead of glucose. Two flasks were required for gluconic acid as both a gluconic acid solution and the sodium salt of gluconic acid were tested as it was not known if the form of gluconic acid would affect *C. glutamicum IL5.0.1(G2).2(F16-F17)* growth. The flasks were inoculated with 50 µl of cell bank and placed in a rotating incubator at 30^{o}C and 250 RPM. Five samples were taken from each flask with the OD measured at 600nm for each sample to measure the growth of the cells within the flask. The result of the OD600 measurements are shown in Figure 26.

It can be seen from Figure 26 that growth occurred in the flask containing glucose and the sodium salt of gluconic acid with final measurements of 17.87 AU (Absorbance units) and 13.07 AU measured respectively at 2 hours. Glucose was shown to be the superior carbon source for growth having a 37% increase in AU at 22 hours. Although the sodium salt of gluconic acid facilitated less growth than glucose the growth was sufficient for a 1 L bioprocess to be set up to test mesaconate production. Why the gluconic acid solution did not produce growth in a shake flask was not investigated further.

Strain Genotypes
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

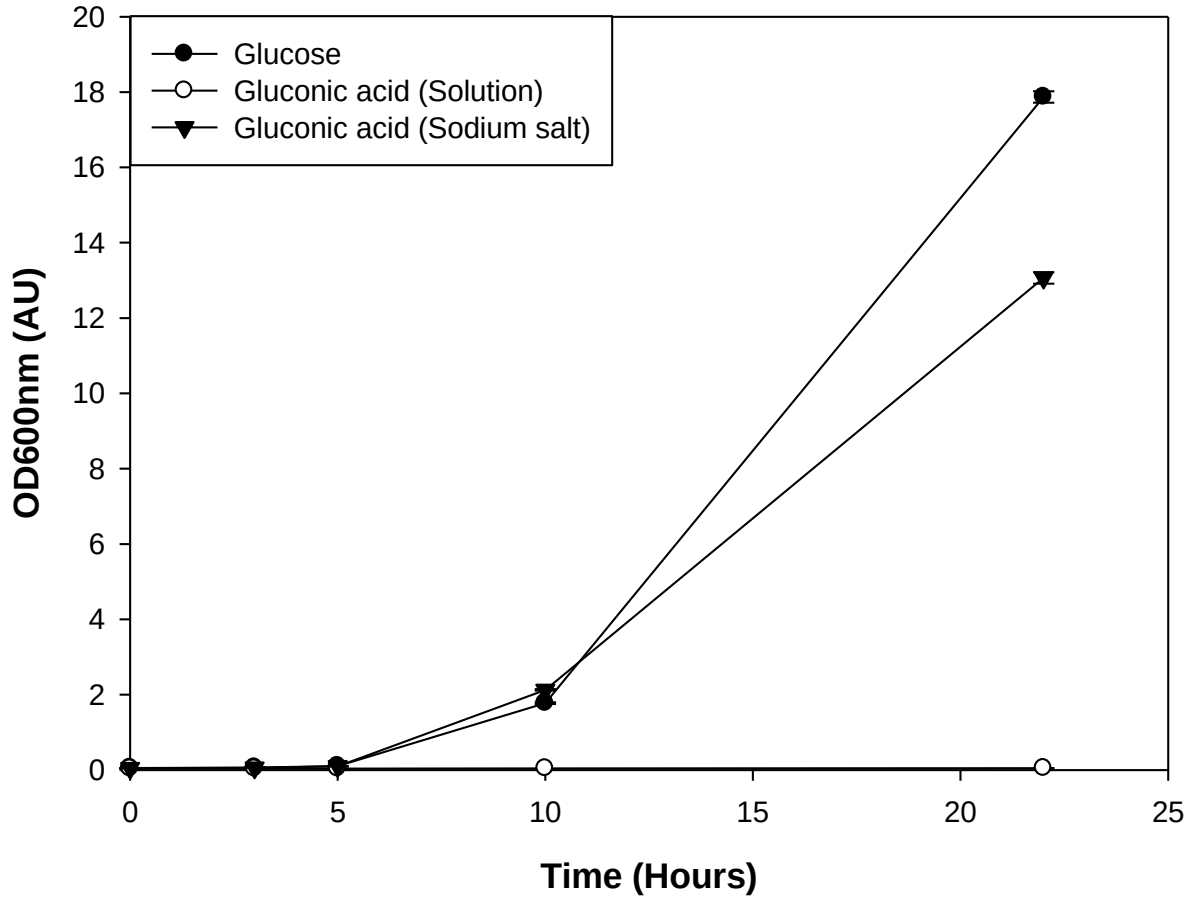


Figure 26: The optical density measurements from three shake flasks of IL5.0.1(G2).2(F16-F17) grown in CGG media containing three different carbon sources, glucose, gluconic acid solution and the sodium salt of gluconic acid. The samples were measured in triplicate with the error bars representing the standard deviation of the samples.

As gluconic acid has been shown to allow the growth a bioprocess for strain IL5.0.1(G2).2(F16-F17) using gluconic acid as the sole carbon source was carried out at a 1L scale in a using the conditions outlined in section 2.1.1.3. For this bioprocess the glucose within all media were replaced with gluconic acid at the same concentration. Due to the timing of the inoculum growth IPTG induction was carried out at 12.5 hours instead of 20 hours. The inoculum was prepared as outlined in section 2.1.1.2. The result of the bioprocess is shown in Figure 27.

A dry cell weight tire of 4.43 g/L was achieved with a growth rate of 0.14 g/L/h between 0 and 25.5 hours. In comparison to the glucose batch bioprocess (Section 3.12) this maximum titre of DCW was 23% lower (5.73 g/L) and the growth rate was 7% lower (0.15 g/L/h). This result demonstrates that gluconic acid is a suitable carbon source for the growth of *C. glutamicum* with a comparable growth rate to the glucose batch process. The dry cell weight titre was lower than the glucose batch process but was comparable to the fed batch process in section 3.16 which was 4.17 g/L. Induction with IPTG at 12.5 hours did not appear to negatively effect growth which was seen previously when IPTG was added at 0 hours (Section 3.15).

The maximum glutamate titre of 10.34 g/L was achieved during the bioprocess with a production rate of 0.48 g/L/h and a yield from DCW of 2.33 g/g of DCW. It can be seen that in comparison to the batch glucose process, which had a maximum titre of 16.34 g/L (58% higher), a production rate of 0.53 g/L/h (10% higher) and a yield on DCW of 3.16 g/g (36% higher) of DCW, that gluconic acid is not an optimal carbon source for achieving high yields of glutamate. The gluconic acid was completely utilised by 36 hours at which point glutamate production stopped. The glutamate yield from gluconic acid was 0.21 g/g which was less than that observed in the glucose bioprocess which was 0.34 g/g. As the DCW was also lower in this bioprocess it was possible that a by-product was produced diverting carbon from glutamate as there should have been more carbon available due to the lower growth.

No mesaconate production was observed in this bioprocess which suggests that glucose within the reactor was not inhibitory to mesaconate production and may be necessary to promote carbon flow towards mesaconate. In conclusion the use of an alternative carbon source did not improve mesaconate production. The addition of IPTG at an earlier timepoint did not appear to be detrimental to growth, it was therefore possible that induction could be moved to an earlier timepoint.

Strain Genotypes

IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

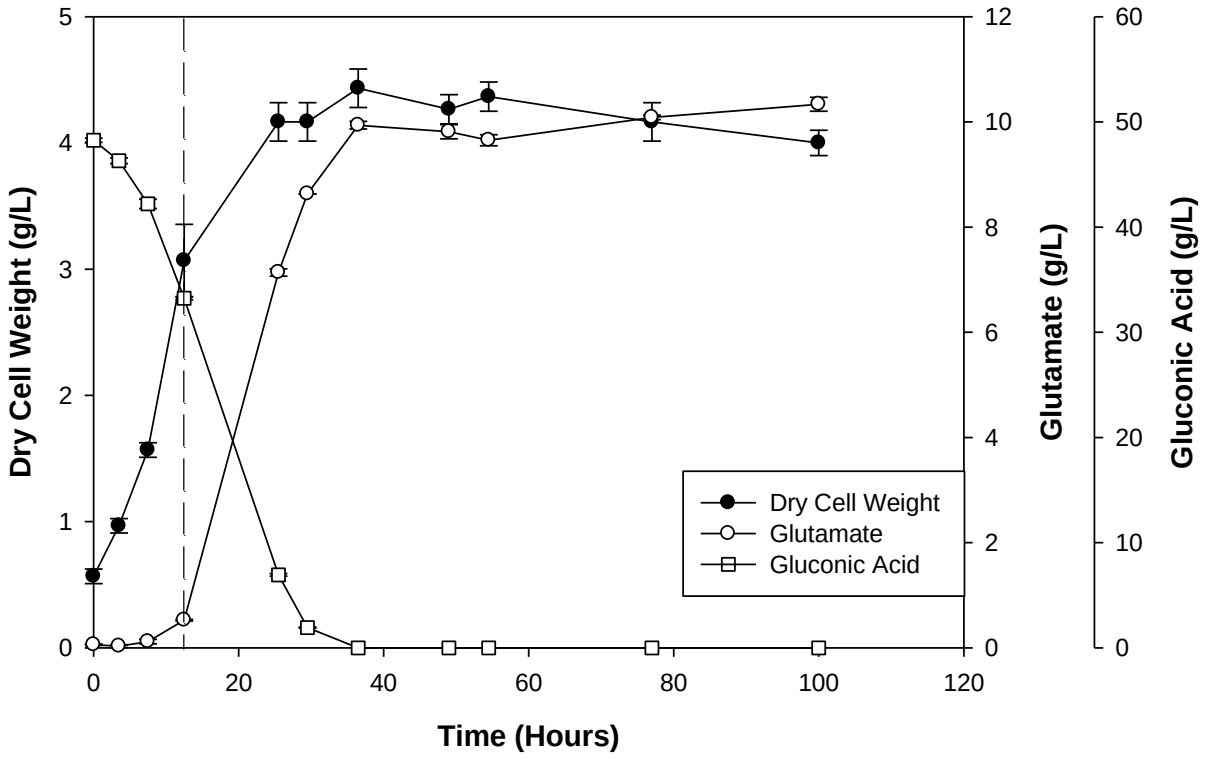


Figure 27: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F16-F17) using gluconic acid as the carbon source. The bioprocess was carried out in a 1L Dasgip vessel. The batch media was altered to include gluconic acid instead of glucose at the same concentration. The inoculum when added gave an OD600 = 1. The vectors were induced at 12.5 hours indicated by the dashed line (---) through the addition of 1 mM of IPTG. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

3.18. The effect of IPTG addition time on the growth of IL5.0.1(G2).2(F16-F17)

In all previous bioprocesses induction of the mesaconate production plasmids was carried out by addition of 1 mM of IPTG at 20 hours the only exceptions were the bioprocess with the glutamate feed (Section 3.15) where IPTG was added at 0 hours and the gluconic acid bioprocess (Section 3.17) where IPTG was added at 12.5 hours. In the bioprocess with 0 hour induction a large lag of 32 hours was observed before growth took place although this was not seen in the other bioprocess where IPTG was added at 12.5 hours.

The 20 hour induction timepoint had been selected to allow sufficient growth of the host cells within the bioprocess before induction. IPTG can be toxic to *C. glutamicum* depending on the concentration used (Sun *et al.*, 2016) which can negatively effect the growth rate of the host strain (Equbal *et al.*, 2013). It was hypothesised that a higher the concentration of cells within the induced bioprocess would allow the production of a larger quantity of enzymes and therefore a greater concentration of mesaconate could be produced. As such the bioprocess was induced at 20 hours to prevent growth limitation but this was often after glutamate production had begun.

As mesaconate production is directly linked to the production of glutamate, as the precursor, an earlier induction time would allow the glutamate mutase and MAAL to be produced and available before glutamate production begins. If an earlier induction time did not have a negative effect on growth this should have a positive effect on mesaconate production and may reduce the lag observed between induction and mesaconate production (Figure 21,Figure 25). In order to determine the effect of induction time on growth of *C. glutamicum IL5.0.1(G2).2(F16-F17)* four shake flasks were set up with either no IPTG, or 1 mM of IPTG added at the 0 hour, 10 hour or 20 hour timepoint. The inoculum was prepared as outlined in section 2.1.1.2 and the shake flask contained the same batch media outlined in section 2.1.1.1 to mimic the previous bioprocesses. The shake flask method is outlined in section 2.1.2. Samples were taken periodically for 30 hours with the optical density measured at 600nm, resulting optical density measurements acquired for each of the four shake flasks is shown in Figure 28.

It can be seen from the growth data in Figure 28 that the addition of 1 mM of IPTG at 0 hours had a negative effect on growth mirroring what had previously been observed in section 3.15. The initial OD600 measured at 0 hours for the 0 hour addition flask was 1.01 AU and only increased to 2.33 AU by 30 hours. This was only an increase of 1.32 AU resulting in a growth rate of 0.044 AU/ hour. By comparison the other 3 flasks had much higher growth rates. The control had an increase of 17.39 AU and a growth rate of 0.58 AU/ hour. Addition of IPTG at 20 hours as was the standard procedure resulted in an increase in AU of 18.52 with a growth rate of 0.62 AU/ hour. This was a 7% increase in growth rate over the control. In comparison to the control the addition of IPTG at 10 hours resulted in a total growth of 15.84 and a growth rate of 0.53 AU/ hour which was a 5% decrease.

From these results it was concluded that the addition of IPTG at 10 hours would not have a large effect on the growth rate and would allow the production of the glutamate mutase and MAAL earlier in the bioprocess. Therefore induction was carried out at 10 hours in future bioprocesses. IPTG induction at 0 hours was not an option due to the low growth observed in the shake flask as a result.

Strain Genotypes
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

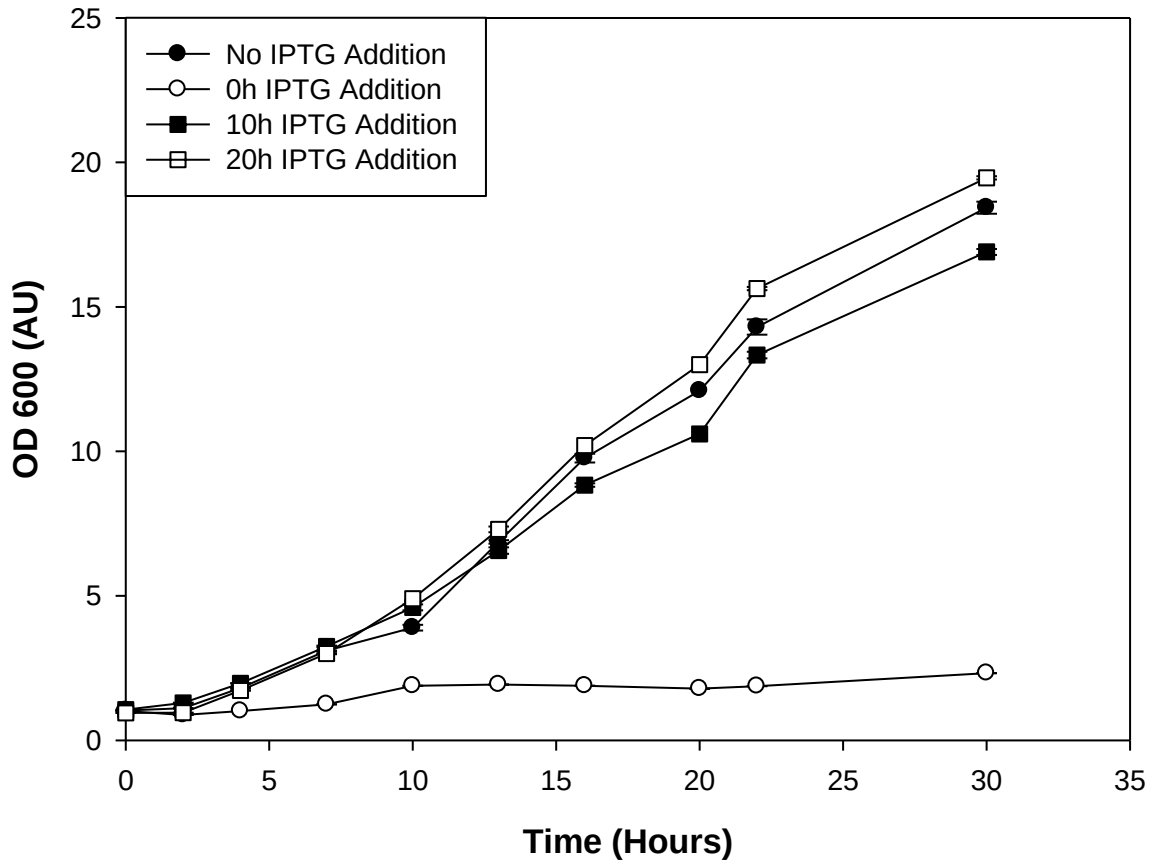


Figure 28: The shake flask experiment to determine the effect of IPTG on growth of the production strain IL5.0.1(G2).2(F16-F17). The flasks contained batch media and the inoculum was prepared as outlined in section 2.1.1.2. 0.125 ml of IPTG stock was added to each flask at a different timepoint 0, 10 or 20 hours. The control flask did not have IPTG added at any point. The error bars represent the standard deviation of the OD600 measurements. Each sample was measured in triplicate.

3.19. The effect of an alternative base on mesaconate production

The DCW measurements observed in the fed-batch experiment (Section 3.16) appears to suggest that changing the base from 25% NH_4OH (v/v) to 2M NaOH had an adverse effect on growth during the bioprocess. In order to determine if changing the base has an effect on growth a bioprocess was carried out using only 2M NaOH as the base for pH control. Under nitrogen limitation glutamate synthetase is upregulated within *Corynebacterium glutamicum* which promotes the conversion of glutamate to glutamine for the production of cell wall components (L Eggeling and Bott, 2005). This is not a desirable condition for the production of mesaconate as it would limit the glutamate available for the glutamate mutase. It has been mentioned previously that ammonium can be inhibitory to MAAL activity (Botting and Gani, 1992) therefore it is possible that using an entirely alternative base may have a positive effect on mesaconate production. It is hypothesised that as no extra nitrogen was being added to the reactor, growth and glutamate production may be limited as nitrogen is essential for both.

In order to determine if using an alternative base would have an effect on mesaconate production a 1L bioprocess was set up using IL5.0.1(G2).2(F16-F17) as outline in section 2.1.1.3. The inoculum was grown as outlined in section 2.1.1.2. As it had been shown that induction using IPTG at 10 hours (section 3.18) did not stop the growth of IL5.0.1(G2).2(F16-F17) this timepoint was selected for induction in the bioprocess. It was hypothesised that an earlier induction time may improve mesaconate production as the production enzymes would be available earlier in the bioprocess. Samples were taken periodically for glucose, glutamate, mesaconate and dry cell weight analysis. The result of the batch bioprocess using the production strain *C. glutamicum IL5.0.1(G2).2(F16-F17)* with the pH control under the influence of an alternative base NaOH is outlined in Figure 29.

A maximum dry cell weight titre of 4.17 g/L was observed in this bioprocess with a growth rate of 0.13 g/L/h between 0 and 24.5 hours which was the growth phase of the experiment. In comparison to the batch production process in section 3.12 this titre was 27% lower (5.73 g/L) and the growth rate was 13% lower (0.15 g/L/h).

Strain Genotypes
IL5.0.1(G2).2(F16-F17) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmA, glmB*)

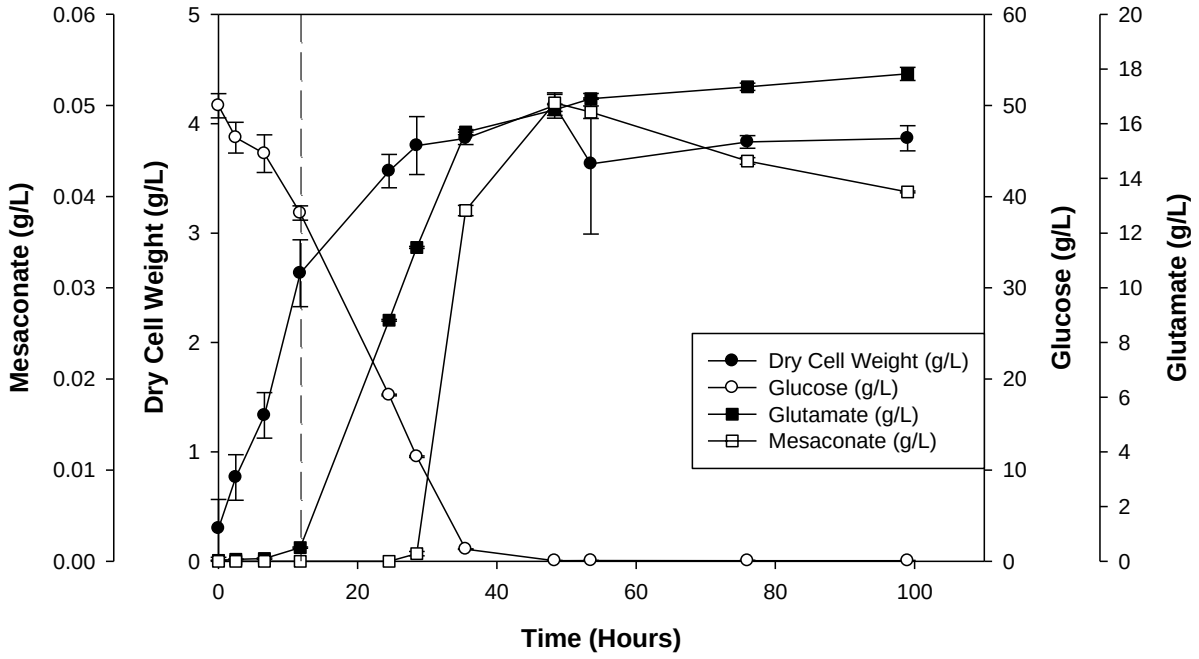


Figure 29: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F16-F17). The bioprocess was carried out in a 1L Dasgip vessel with 2M NaOH as the base replacing 25% NH_4OH (v/v). The inoculum when added gave an OD600 = 1. The vectors were induced at 10 hours indicated by the dashed line (---) through the addition of 1 mM of IPTG. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

These results show that the removal of NH_4OH may have had an adverse effect on the growth. When looking at the bioprocess as a whole it is more likely that the difference in the DCW titre is due to the inherent variation between bioprocesses rather than the base choice as the majority of the growth phase takes place before the glutamate production phase in which base would be added to the reactor.

The maximum glutamate titre was 17.82 g/L with a production rate of 0.51 g/L/h between 6.6 and 35.5 hours. In comparison to the batch production process in section 3.12 the maximum titre was 9% higher (16.34 g/L) and the production rate was 4% lower (0.53 g/L/h). As the titre was higher and the production rate during the production phase was slightly lower it suggests that the cells were more efficient at producing glutamate which can be confirmed by comparing the glutamate production in relation to DCW. The titre of glutamate in relation to DCW was 4.28 g/g which was 35% higher (3.16 g/g) than the batch production strain with NH_4OH as the base. The glutamate production in relation to glucose utilised was 0.36 g/g of glucose. These results show that there is enough nitrogen contained within the media to support glutamate production in the batch process without needing the addition of extra nitrogen through the NH_4OH base. As the cells were shown to be more productive it is possible that that the alternative base may be the best option for the bioprocess if only to eliminate ammonium from the process which may be inhibitory to the MAAL enzyme.

A mesaconate titre of 0.0503 g/L was achieved during the bioprocess which was 39% lower than the titre achieved in the batch process with NH_4OH as a base (0.083 g/L) and 55% higher than the titre achieved in the fed batch process (0.0325 g/L). As with the previous experiments no (2S,3S)-3 methylaspartate was detected in the samples indicating that the glutamate mutase is the bottleneck in the production pathway. It is unclear what the cause of the variation in titres between the three processes is. This experiment again demonstrates gradual decline of the mesaconate titre over time as was shown in the fed batch bioprocess. In all three of the bioprocesses a low titre of mesaconate was observed when a large titre of glutamate was available indicating that the low mesaconate titre may be due to inactivity of the glutamate mutase.

3.20. Batch bioprocess using an alternative glutamate mutase

It was observed that in the bioprocesses where mesaconate was produced (Section 3.12, 3.16 and 3.19) that there was no build-up of β- methylaspartate (Data not shown). It was therefore hypothesised that the glutamate mutase may be inactivated quickly during the bioprocess therefore limiting the mesaconate production ability of the strain. In the previous work on mesaconate production carried out by Jeffrey (2016) the glutamate mutase which was selected for use originated from *Desulfosporosinus meridiei* and was selected as it showed the greatest efficiency in the conversion of glutamate to β- methylaspartate. This glutamate mutase was included in the strain IL5.0.1(G2).2.(F1-F2) which contained the same MAAL from *Clostridium tetanomorphum* used previously in the strain IL5.1(G2).2(F16-F17).

In the previous work using IL5.0.1(G2).2(F1-F2) no mesaconate could be measured by HPLC although the interfering peak was observed at the same timepoint which may have been masking a smaller mesaconate peak. As a new HPLC method was developed in section 3.9 which separates the interfering peak from mesaconate accurate analysis was now possible. This strain containing a glutamate mutase with higher activity was revisited to determine the mesaconate production as it was hypothesised from previous experiments that the glutamate mutase was the bottleneck in the bioprocess.

Two bioprocesses were carried out using IL5.0.1(G2).2(F1-F2), one with IPTG induction and one without IPTG induction. Both bioprocesses were set up as outlined in section 2.1 for a batch bioprocess. IPTG was added at 9 hours in the induced bioprocess with samples taken from each periodically for analysis of dry cell weight, glucose, glutamate and mesaconate. The result of the uninduced bioprocess is shown in Figure 30 and the result of the induced bioprocess is shown in Figure 31.

The bioprocess without induction had excessive foaming between the 25 and 32 hour samples, this experiment was not repeated as the aim was to determine if any mesaconate was produced without induction. This aim was still achievable despite the foaming observed. It can be seen from Figure 30 that this foaming caused the DCW measured to drop which was caused by biomass being sequestered on the sides of the vessel. A maximum DCW of 3.03 g/L was observed before foaming with a growth rate of 0.11 g/L/h. This growth rate was 32%

Strain Genotypes

IL5.0.1(G2).2(F1-F2) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE*)

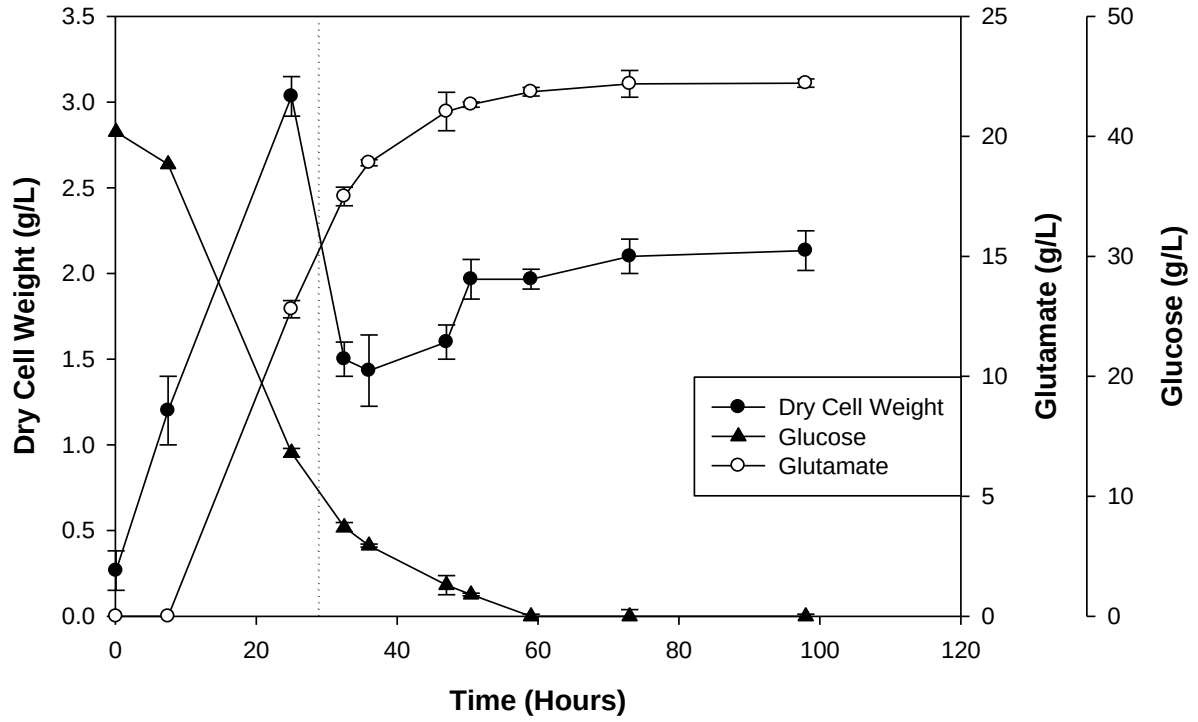


Figure 30: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F1-F2) without induction. The bioprocess was carried out in a 1L dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. Foaming occurred in the bioprocess as indicated by the dashed line (---). All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

Strain Genotypes
IL5.0.1(G2).2(F1-F2) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE*)

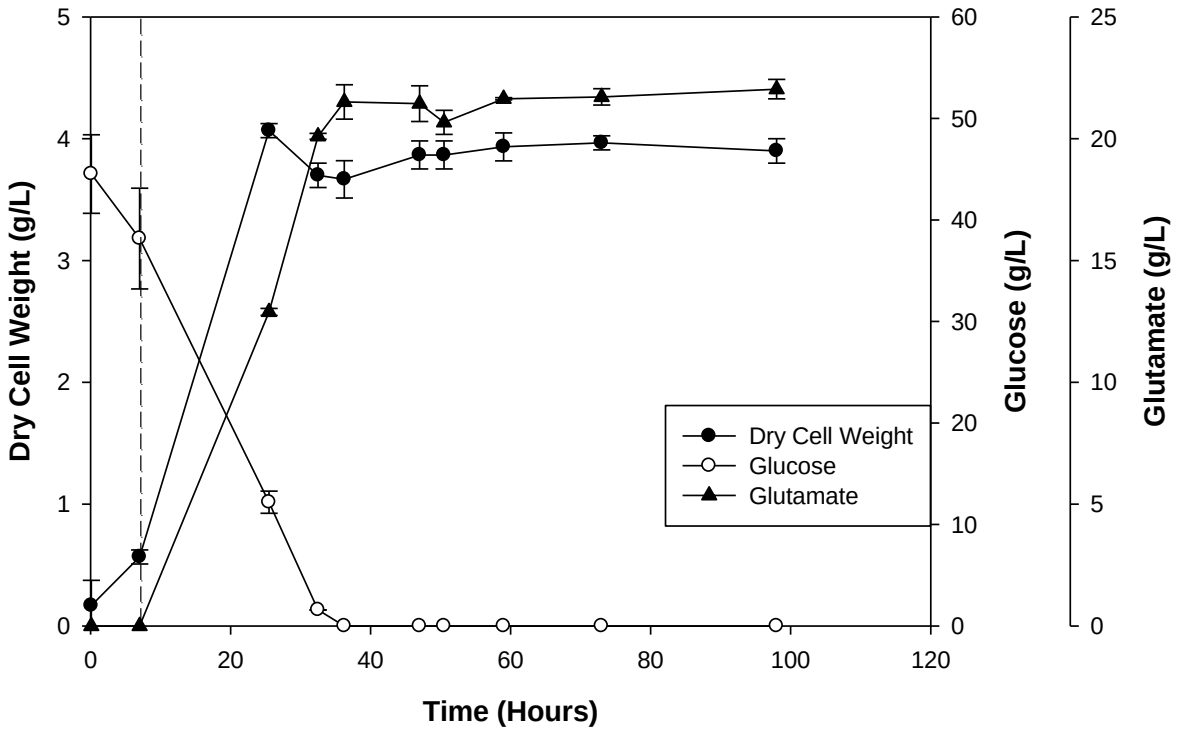


Figure 31: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F1-F2) with induction. The bioprocess was carried out in a 1L Dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. The bioprocess was induced with IPTG at 9 hours as indicated by the dashed line (---). All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

lower than the growth rate observed in IL5.0.1(G2).2(F16-F17) without induction (0.16 g/L/h).

A maximum glutamate titre of 22.2 g/L was observed in the bioprocess with a production rate of 0.37 g/L/h before glucose was depleted at 59 hours. This glutamate titre was higher than that observed from IL5.0.1(G2).2(F16-F17) without induction (19.95 g/L). As glutamate was available it was concluded that mesaconate was not able to be produced by IL5.0.1(G2).2(F1-F2) without IPTG induction.

The bioprocess with IPTG induction at 9 hours produced a maximum DCW titre of 4.06 g/L with a growth rate of 0.15 g/L/h. This growth rate and titre was higher than that observed in the bioprocess without induction which shows that induction with IPTG at 9 hours was not detrimental to growth which was observed when IPTG was added at 0 hours (Section 3.18). A glutamate titre of 21.5 g/L was observed at 36 hours which was the time at which glucose was fully utilised in the bioprocess. Glutamate had a production rate of 0.60 g/L/h in this bioprocess which was a 76% increase over the rate of 0.34 g/L/h observed in the comparable bioprocess using IL5.0.1(G2).2(F16-F17).

No mesaconate was observed in this bioprocess which was unexpected as induction should have allowed mesaconate production using the available glutamate. It was possible that the glutamate mutase was again becoming inactive in the bioprocess as no β- methylaspartate was detected. For glutamate to be produced an aerobic bioprocess is required, it was concluded from this result that as the glutamate mutase had a reduced oxygen tolerance the dissolved oxygen in the bioprocess may be an important factor for mesaconate production. As the glutamate mutase appeared to be inactive further work was required to improve the activity of this enzyme.

3.21. The effect of reactivatase addition of mesaconate production

As no mesaconate or β- methylaspartate was detected in the batch bioprocess using IL5.0.1(G2).2(F1-F2), it was concluded that the glutamate mutase was inactive despite the presence of glutamate. It was hypothesised that this inactivation was due to the oxygen present within the bioprocess as mesaconate production was observed when using the glutamate mutase from *Actinoplanes friuliensis* which has an increased oxygen tolerance.

Previous work carried out by Jeffrey (2016) showed that bioprocesses of IL5.0.1(G2).2(F1-F2) had an extremely low copy number of the glutamate mutase which would explain why no mesaconate was observed. It was shown in this work that the copy number of the two glutamate mutase components *glmE* and *glmS* improved upon the addition of an associated reactivatase *glmL*. At the time no mesaconate was detected due to the limitation of the HPLC method. Therefore the strain containing *glmL* entitled IL5.0.1(G2).2(F1-F2-F3) was revisited to determine if the addition of the reactivatase *glmL* could have a positive effect of mesaconate production.

The *glmL* gene associated with the glutamate mutase enzyme previously had an unknown function but had been identified to have ATP binding sites (Zelder *et al.*, 1994). This gene was not thought to be essential for glutamate mutase function as the *glmE* and *glmS* subunits had been shown to operate in the absence of *glmL*. The work on mesaconate production within *E. coli* (Wang and Zhang, 2015) demonstrated that the addition of the reactivatase into their production strain containing a glutamate mutase and methylaspartate ammonia lyase improved the production of mesaconate from glucose by 162.4% resulting in a mesaconate titre of 2.65 g/L. It was concluded from this work that the reactivatase prevented oxygen related deactivation of the glutamate mutase.

As it was hypothesised that available oxygen was the cause of the low mesaconate titres observed in the bioprocesses using *C. glutamicum* the addition of the reactivatase was expected to improve mesaconate production. There is no reactivatase associated with the glutamate mutase from *Actinoplanes friuliensis* which may be due to the fact that this glutamate mutase has an increased oxygen tolerance therefore this could not be attempted in the previous best production strain IL5.0.1(G2).2(F16-F17).

In order to determine if the addition of the reactivatase gene *glmL* improved the mesaconate production the new strain IL5.0.1(G2).2(F1-F2-F3) containing *glmL* was tested in two bioprocesses. One bioprocess did not have IPTG induction and the other had IPTG induction at 9 hours. The bioprocesses were set up as outlined in section 2.1. Samples were taken periodically for analysis of glucose, glutamate, mesaconate and dry cell weight. The result of the bioprocess without induction is shown in Figure 32 and the result of the bioprocess with induction is shown in Figure 33.

Figure 32 shows the results obtained without induction. IL5.0.1(G2).2(F1-F2-F3) utilised all of the available glucose by 40 hours. At this timepoint a DCW of 4.3 g/L was observed with a growth rate of 0.095 g/L/h. As foaming occurred in the bioprocess of IL5.0.1(G2).2(F1-F2) without induction, which displaced some biomass, the DCW could not be compared. A glutamate titre of 25.65 g/L was measured at 40 hours with a production rate of 0.64 g/L/h, this was higher than the titre of 22.2 g/L observed in IL5.0.1(G2).2(F1-F2) without induction.

Interestingly some mesaconate was observed in this bioprocess in the absence of IPTG induction indicating that the plasmids were leaky, allowing some transcription of both enzymes. 0.014 g/L of mesaconate was produced by 40 hours with production stopping when glucose was depleted and glutamate production stopped. Although this titre was very low it suggested that with induction this strain may produce mesaconate.

With induction (Figure 33) IL5.0.1(G2).2(F1-F2-F3) utilised all of the available glucose at the 46 hour sample. At 46 hours a DCW of 6.63 g/L was produced at a production rate of 0.13 g/L/h. This DCW was higher than the bioprocess without induction showing that the addition of IPTG did not negatively effect growth. A glutamate titre of 21.7 g/L was produced by 46 hours after this timepoint production slowed as there was no longer available glucose. The glutamate production rate was 0.47 g/L/h which was lower than the production rate observed In the bioprocess without induction (0.64 g/L/h), the glutamate production rate may have been lower as mesaconate was produced in this bioprocess as a higher titre.

The highest mesaconate titre thus far was observed in this bioprocess with a maximum titre of 0.58 g/L measured at 72 hours. This mesaconate titre was 599% higher than the previous highest mesaconate titre of 0.083 g/L observed in the batch bioprocess of IL5.0.1(G2).2(F16-F17) (Section 3.12). This bioprocess had a mesaconate production rate of 0.012 g/L/h

Strain Genotypes
IL5.0.1(G2).2(F1-F2-F3) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE, glmL*)

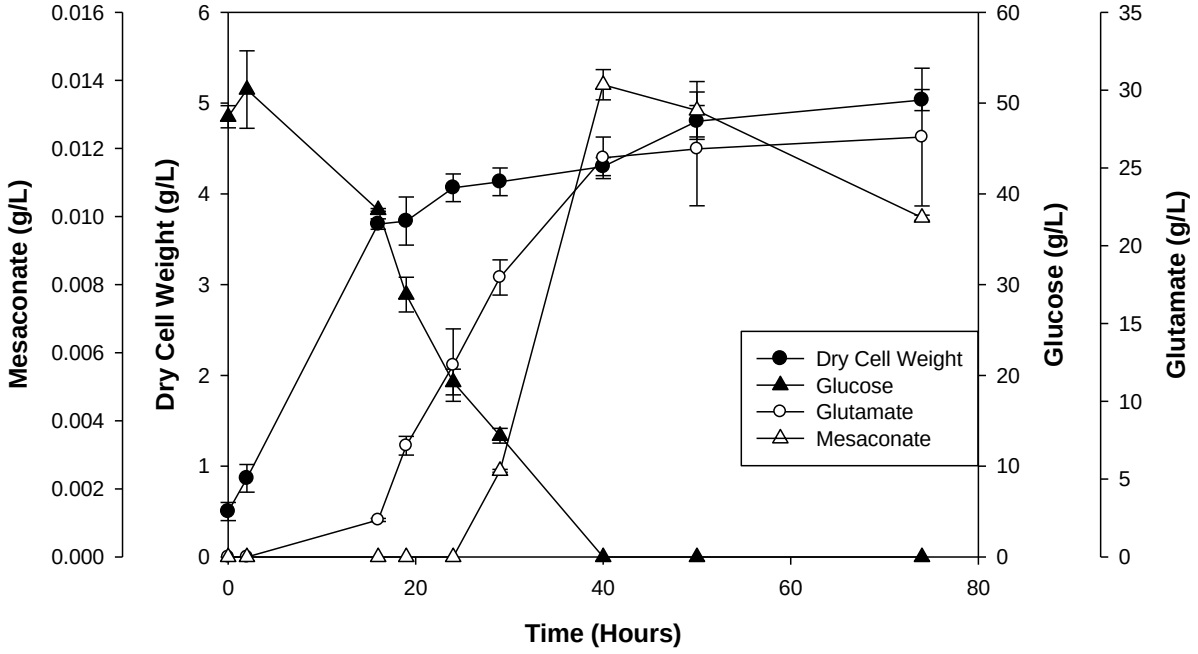


Figure 32: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F1-F2-F3) without induction. The bioprocess was carried out in a 1L Dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

Strain Genotypes
IL5.0.1(G2).2(F1-F2-F3) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE, glmL*)

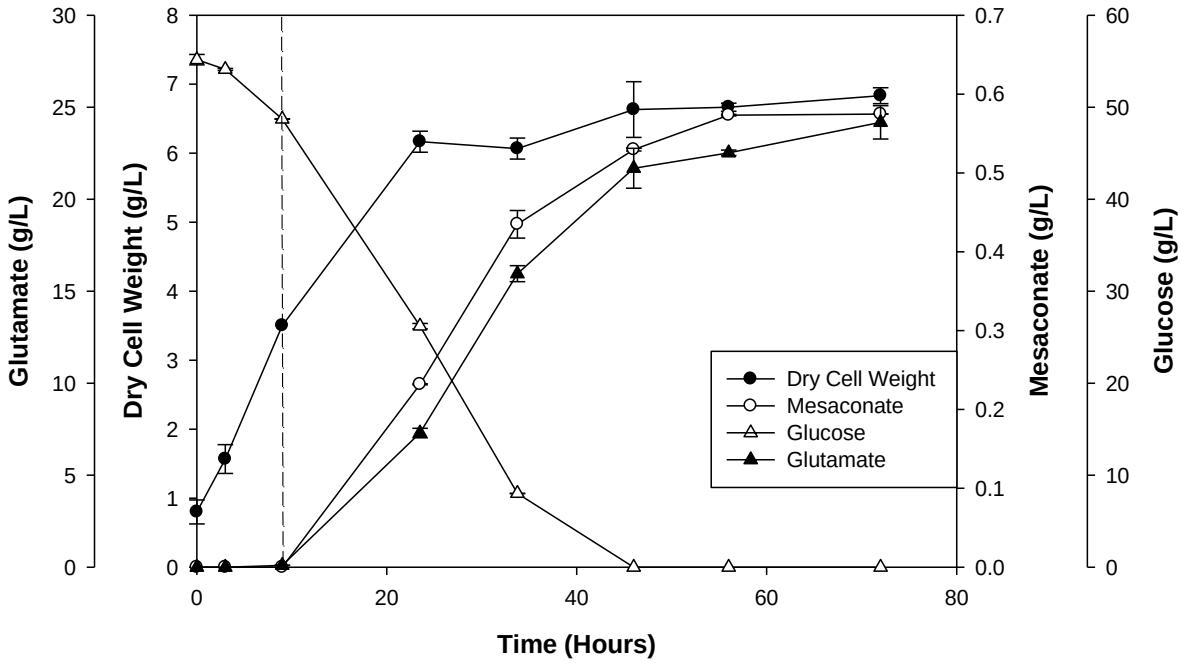


Figure 33: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F1-F2-F3) with IPTG induction at 9 hours. The bioprocess was carried out in a 1L dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

between the 9 and 56 hour timepoints. This mesaconate titre of 0.58 g/L of mesaconate was much lower than the titre observed by Wang and Zhang (2015) where 2.65 g/L was observed. There are multiple reasons which may explain why less mesaconate was observed in this bioprocess than in *E. coli*.

While both experiments used a strain containing the MAAL from *C. tetanomorphum* the glutamate mutase was from a different source, *C. tetanomorphum* for the *E. coli* and *D. meridiei* in IL5.0.1(G2).2(F1-F2-F3). This may effect the titre produced as each glutamate mutase may have a different activity rate for the production of β-methylaspartate. In the *E. coli* experiment a single sample was taken for determination of the mesaconate concentration but the timepoint of the sample is not outlined, therefore the production rates could not be compared.

The bioprocess was very different for both experiments. The *E. coli* experiment was carried out at a 5 ml scale within a shake flask with sodium glutamate supplemented into the fermentation media. By comparison the bioprocess demonstrated here was carried out at a 1L scale within a bioreactor with glutamate produced from the available glucose within the media. It may be possible that as glucose was utilised for glutamate production and growth that this influenced mesaconate production.

Interestingly there appears to be a correlation between glutamate production and mesaconate production, when glucose is depleted glutamate production stops which is expected as glucose is the carbon source for production of glutamate. The highest production rate of mesaconate occurs during the highest production rate of glutamate with both substrates slowing in production rate after the glucose is depleted. It can be concluded from observation that it may be possible that glutamate is unable to be utilised from the external media by *C. glutamicum* and can only be utilised for mesaconate production during production. It may also be possible that the glutamate mutase is unable to be reactivated by the reactivatase in the absence of glucose therefore the activity slows and mesaconate production slows. As the activity of *glmL* requires ATP and the absence of glucose would limit ATP production this is a distinct possibility.

It appears that the presence of oxygen is indeed a limiting factor in the activity of the glutamate mutase. As the *E. coli* experiment was carried out in shake flasks the oxygen

transfer rate into the media would be very low which may explain the increased titre observed. As the *C. glutamicum* bioprocess occurs in a bioreactor with an air sparge the dissolved oxygen concentration was maintained at 30% to maximise glutamate production. This may therefore be reducing the activity of the glutamate mutase and limiting mesaconate production.

In conclusion the addition of the gene encoding the reactivatase subunit, *glmL*, to the glutamate mutase plasmid improved the production of mesaconate by 599%. This result demonstrated that the glutamate mutase was inactivated by oxygen previously which was why low titres were observed. A higher titre of mesaconate may be observed when lowering the dissolved oxygen within the bioprocess as it was shown in *E. coli* in a shake flask experiment, within which a low oxygen transfer rate into the media would be expected, that a higher mesaconate titre was produced.

3.22. IL5.0.1(G2).2(F1-F2-F3) with different dissolved oxygen conditions

The best strain for the production of mesaconate in *C. glutamicum* at this time was IL5.0.1(G2).2(F1-F2-F3) which produced a mesaconate titre of 0.58 g/L (Section 3.21) in a batch bioprocess. As this strain contained a reactivatase *glmL* which was thought to reactivate glutamate mutase which was spontaneously deactivated by O_2- (Wang and Zhang, 2015). The strain IL5.0.1(G2).2(F1-F2) without the reactivatase produced no mesaconate (Section 3.20) demonstrating that the presence of the reactivatase was essential for mesaconate production in the current bioprocess conditions. IL5.0.1(G2).2(F16-F17), the previous best producer of mesaconate with a titre of 0.083 g/L, included a glutamate mutase from *Actinoplanes friuliensis* which was selected for its increased oxygen tolerance. This strain produced mesaconate without a reactivatase, as no reactivatase is associated with this glutamate mutase in *A. friuliensis*, it was hypothesised that the dissolved oxygen within the bioprocess may have an important effect on the activity of glutamate mutase and therefore mesaconate production.

As such two bioprocesses were carried out altering the dissolved oxygen conditions to determine if the mesaconate titre could be increased. One bioprocess had a dissolved oxygen concentration maintained at 10%, down from the 30% concentration which was previously used. The second bioprocess was set up as standard with 30% DO with the air sparge stopped during glutamate production which was hypothesised to produce anaerobic conditions for glutamate mutase activity thereafter. The time at which glutamate was being produced was selected as the air shutoff time as creating anaerobic conditions before glutamate production may have prevented glutamate production altogether. Both bioprocesses were set up as outlined in section 2.1 with the changes in air sparging outlined above. Both bioprocessed were induced at 9 hours by IPTG addition. Samples were taken from both bioprocesses for the analysis of DCW, glucose, glutamate and mesaconate.

The result of the first bioprocess with a 10% dissolved oxygen concentration is shown in Figure 34. In this bioprocess it could be seen that the glucose was fully utilised by 47 hours with a maximum DCW of 5.03 g/L reached at this timepoint which was 26% lower than the bioprocess with a 30% DO concentration (6.83 g/L). A maximum glutamate titre of 23.7 g/L was measured which was 2% lower than in the bioprocess with a 30% DO concentration

Strain Genotypes
IL5.0.1(G2).2(F1-F2-F3) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE, glmL*)

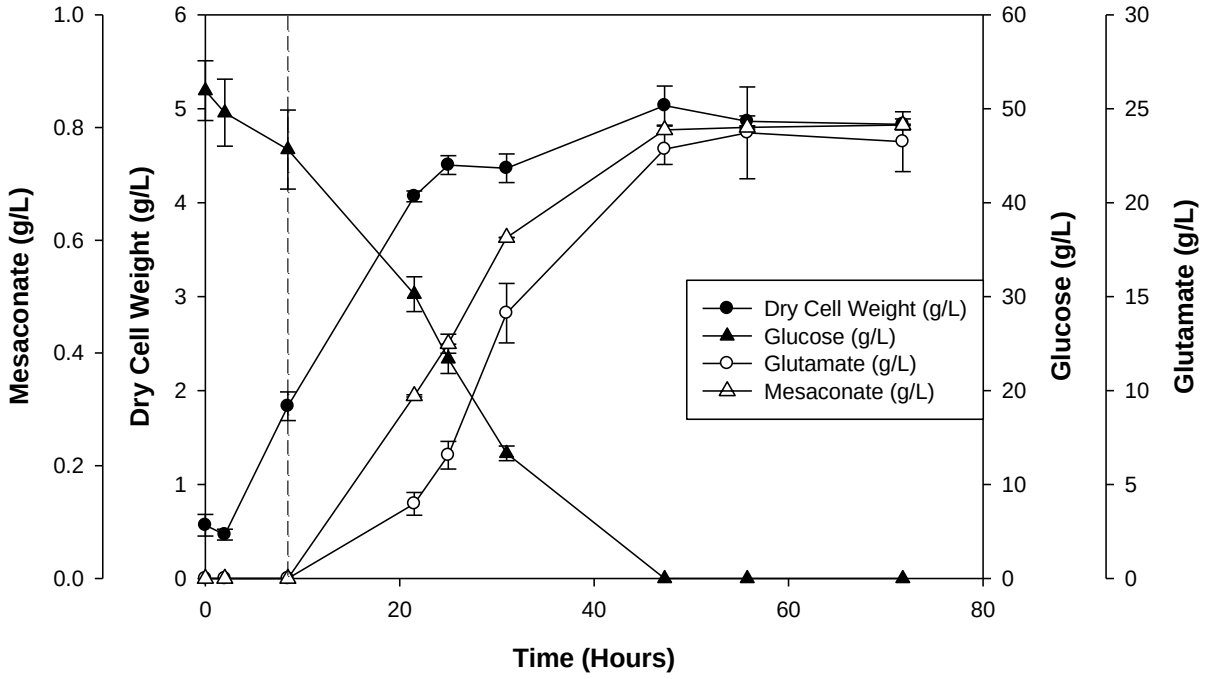


Figure 34: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F1-F2-F3) with IPTG induction at 9 hours. The DO% in the bioprocess was maintained at 10%. The bioprocess was carried out in a 1L Dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

which had a maximum titre of 24.2 g/L (Section 3.21). Interestingly DCW to glutamate ratio was greater when the DO concentration was 10% indicating that the cells had a greater glutamate productivity. This was unexpected as glutamate production requires aerobic conditions therefore a greater DO concentration would be expected to facilitate the production of more glutamate.

The aim of the experiment was to determine if a greater mesaconate titre was observed when the DO concentration was lowered to 10% from 30%. It can be seen from Figure 34 that a maximum mesaconate titre of 0.8 g/L was produced in this bioprocess which was the highest mesaconate titre observed thus far. This mesaconate titre was 38% higher than the titre of 0.58 g/L observed when the DO concentration was maintained at 30%. As a higher mesaconate production rate was also observed of 0.017 g/L/h in comparison to 0.012 g/L/h it could be concluded that reducing the DO concentration within the bioprocess increased the activity of the glutamate mutase. This was likely due to a reduction in spontaneous deactivation by O_2- within the bioprocess. When comparing the g/g ratio of DCW to mesaconate, the bioprocess at 10% produced more mesaconate per gram of DCW (0.16 g/g) than the bioprocess at 30% DO (0.09 g/g). This indicates that it was also possible that the activity of the glutamate mutase was increased at the lower oxygen concentration.

As a higher mesaconate concentration was observed when the DO concentration was reduced it was hypothesised that stopping the airflow during glutamate production would further increase the mesaconate titre as less DO would be present in the bioprocess. The result of the second bioprocess with the airflow shutoff was shown in Figure 35. From this figure it can be seen that the shutoff of airflow at 20 hours stopped both growth and glutamate production thereafter. The maximum DCW produced was 3.7 g/L which was 46% lower than the batch biotransformation with 30% DO (Section 3.21) which had a maximum DCW of 6.8 g/L. The growth rate up until 20 hours was lower 0.15 g/L/h with air disruption than without 0.23 g/L/h suggesting that a similar titre may not have been achieved had the airflow not been disrupted. Although the initial growth rate was lower the growth rate for the hour before air shutoff was 0.2 g/L/h and after was 0.085 g/L/h suggesting that the air shutoff did indeed impact growth.

Strain Genotypes

IL5.0.1(G2).2(F1-F2-F3) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE, glmL*)

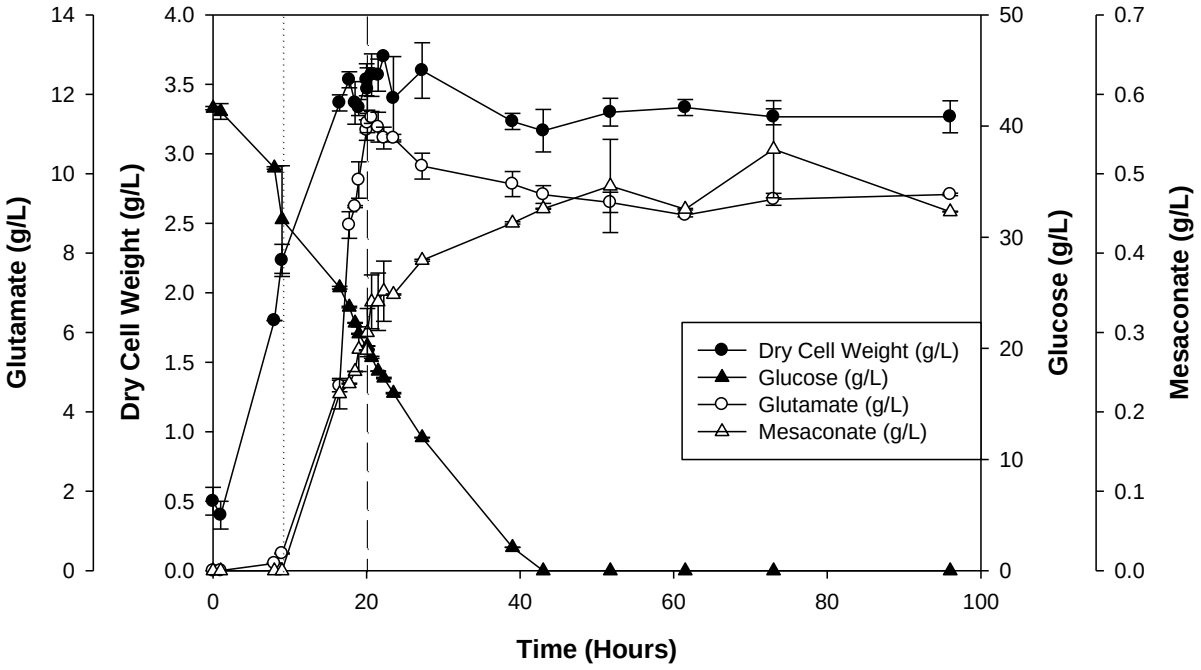


Figure 35: The analysis of the batch bioprocess using the production strain C. glutamicum IL5.0.1(G2).2(F1-F2-F3) with IPTG induction at 9 hours as indicated by the dotted line (····). The DO% in the bioprocess was maintained at 30% with the airflow stopped completely at 20 hours as indicated by the dashed line (---). The bioprocess was carried out in a 1L Dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

A maximum glutamate titre of 11.4 g/L was observed at 20.6 hours after which time no further glutamate was produced. This glutamate titre was 53% lower than the glutamate titre observed in the bioprocess with a constant 30% DO concentration of 24.2 g/L. Again as with the DCW the glutamate production stops upon stopping the air sparge into the bioreactor. This result demonstrates that an air sparge is necessary for glutamate production with anaerobic conditions stopping production entirely. Although glutamate production stopped, 11.4 g/L of glutamate should have been sufficient to prevent glutamate availability from being a limiting factor on mesaconate production.

A maximum mesaconate titre of 0.53 g/L was measured in this bioprocess which was 7% lower than the titre produced when the DO concentration was 30% (0.57 g/L) and 34% lower than the titre produced when the DO concentration was 10% (0.8 g/L). Interestingly the mesaconate production per gram of DCW was the same as the as the bioprocess which had the DO maintained at 10% (0.16 g/g). It was hypothesised that the anaerobic conditions within the bioprocess would increase the mesaconate titre produced, as was seen when the DO concentration was lowered, although this was not the case it appeared that limiting the airflow did improve the specific mesaconate production of the cells. The mesaconate production rate in this bioprocess was higher in the before the airflow was stopped (0.025 g/L/h) than after (0.008 g/L/h) demonstrating that lowering the oxygen concentration was not the only condition required for a high rate of mesaconate production.

It was previously hypothesised that mesaconate could only be produced while glutamate was being produced and extracellular glutamate could not be utilised which had been observed in section 3.21. This was now not thought to be the case as it was shown in Figure 35 that mesaconate production continues after glutamate production stops. The glutamate titre slowly decreases as mesaconate is produced suggesting that extracellular glutamate is being utilised. Mesaconate production appears to stop after glucose is depleted in the bioprocess with the 43 hour, 61 hour and 96 hour titres remaining constant at 0.46 g/L with the higher titres at 52 hours and 73 hours having a large error and therefore may not be reliable. The reason glucose depletion may be that the reactivatase requires ATP for activity which would become limiting in the absence of a carbon source.

As the absence of an air sparge would reduce spontaneous O_2- deactivation of the glutamate mutase another factor must be present which limits glutamate mutase activity. It was possible

that coenzyme B12 was becoming depleted in the bioprocess. It was identified by Wang and Zhang (2015) that upon reactivation of the glutamate mutase – coenzyme B12 complex by a reactivatase inactive coenzyme B12 is removed. It was therefore possible that all of the available coenzyme was inactivated which was the cause of mesaconate production stopping in the absence of glucose and oxygen.

In conclusion the bioprocess with a DO concentration of 10% demonstrated that reduction of the DO concentration improved the mesaconate production rate and titre. This titre of 0.8 g/L was the highest observed to date. This is consistent with the hypothesis that the glutamate mutase was deactivated by oxygen. From the bioprocess with the air supply halted at 20 hours it was concluded that although the mesaconate to DCW ratio was the same as the 10% DO bioprocess glutamate production stopped in anaerobic conditions. ATP may be necessary for mesaconate production as when glucose is depleted mesaconate production stops. Stopping the air supply did not increase the mesaconate titre over the 10% DO bioprocess.

3.23. The effect of changing the glutamate mutase and reactivatase on mesaconate production in C. glutamicum.

It was shown in section 3.22 that reducing the concentration of DO in the bioprocess to 10% improved the mesaconate titre produced to the maximum observed to date of 0.8 g/L. Although this is the highest mesaconate titre which has been demonstrated in *C. glutamicum* it was lower than that observed in *E. coli* (2.65 g/L) also containing a MAAL, glutamate mutase and reactivatase (Wang and Zhang, 2015). Between the two bioprocesses there were various factors which may have influenced the difference in mesaconate titre. The *C. glutamicum* bioprocesses were carried out at a 1L scale within a bioreactor with glutamate being produced directly from glucose. By comparison the *E. coli* bioprocess was carried out in shake flasks at a 5 ml scale with glutamate supplementation into the media. These factors may affect the oxygen availability in the bioprocess and therefore the titre as the shake flask would be expected to have a much lower DO concentration. It was hypothesised that the factor which may have the greatest effect on the mesaconate titre was the glutamate mutase used.

The strain IL5.0.1(G2).2(F1-F2-F3) contained the genes *glmE, glmS* (glutamate mutase) and *glmL* (reactivatase) from *Desulfosporosinus meridiei*. These were selected as *glmE* and *glmS* had been determined by Ingenza Ltd internally to have the greatest activity rate for β-methylaspartate production from glutamate. The mesaconate production demonstrated in *E. coli* utilised a glutamate mutase and reactivatase from *C. tetanomorphum* (*mutE, mutS and mutL*) which was selected due to the glutamate mutase showing the highest k_{cat} value reported (Holloway and Marsh, 1994). It was hypothesised that using this alternative glutamate mutase and reactivatase from *C. tetanomorphum* in the *C. glutamicum* strain may increase the mesaconate titre to be closer to that observed in *E. coli*.

Therefore, in order to determine if changing the glutamate mutase and reactivatase within the *C. glutamicum* host would facilitate the production of a higher mesaconate titre the strain IL5.0.1(G2).2(F7-F8-F9) was produced. This strain contained the genes *mutE, mutS* and *mutL* which encode the glutamate mutase and reactivatase from *C. tetanomorphum.* This strain would allow it to be determined whether the different mutase was the cause of the disparity seen between *E. coli* and *C. glutamicum* mesaconate production. Two bioprocesses

Strain Genotypes

IL5.0.1(G2).2(F1-F2) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE*)

IL5.0.1(G2).2(F1-F2-F3) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *glmS, glmE, glmL*)

IL5.0.1(G2).2(F7-F8-F9) – *C. glutamicum* ATCC 13869 (Kan^R: *tMAAL*, Spc^R: *mutS, mutE, mutL*)

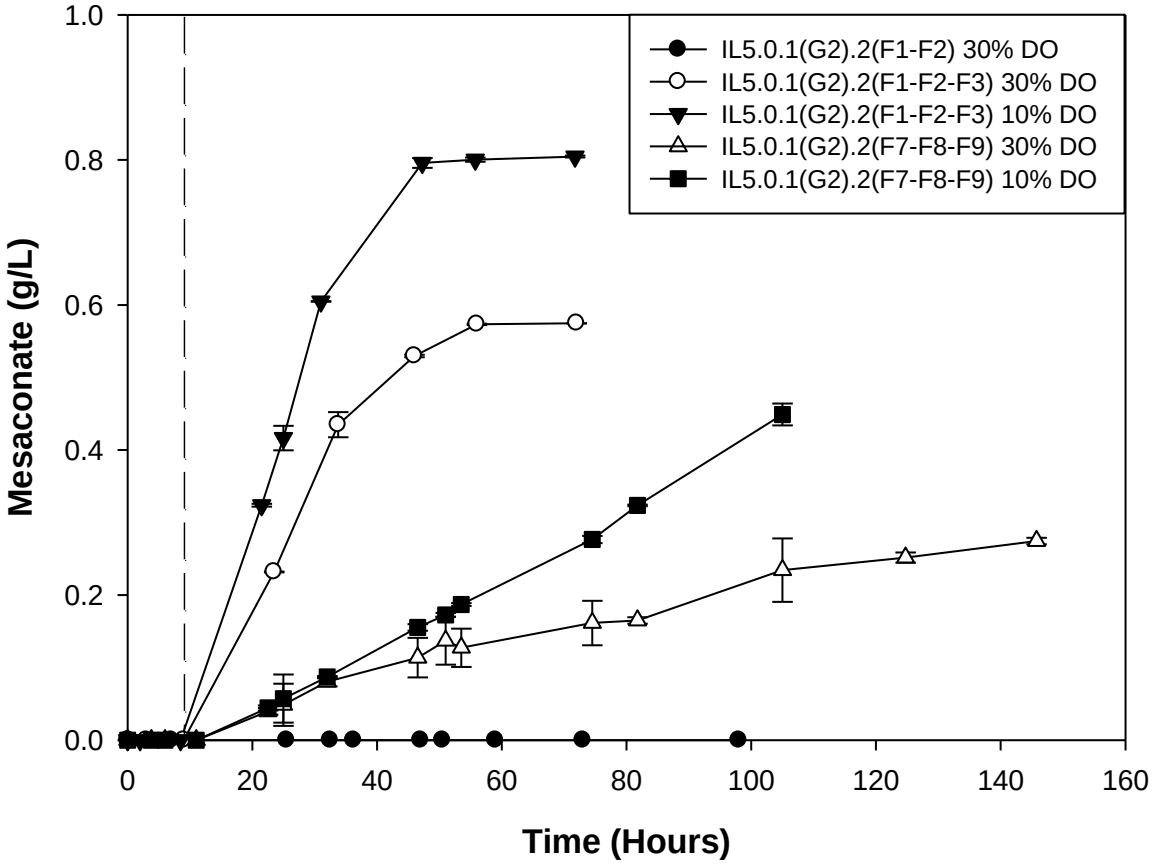


Figure 36: The analysis of the mesaconate production by C. glutamicum IL5.0.1(G2).2(F1-F2), IL5.0.1(G2).2(F1-F2-F3) and IL5.0.1(G2).2(F7-F8-F9) with a DO concentration of 10% and 30%. IPTG induction was at 9 hours as indicated by the dashed line(----). The DO% in each bioprocess was either 30% or 10% which is included in the legend. Each bioprocess was carried out in a 1L Dasgip vessel with 2M NaOH as the base. The media used was the "batch media" including kanamycin and spectinomycin and 1 µg/L of biotin. The inoculum when added gave an OD600 = 1. All measurements were carried out in triplicate. The error bars represent the standard deviation of the measurements.

were set up with the new strain, one at 30% DO and the other at 10% DO. Both bioprocesses were set up as outlined in section 2.1. Samples were taken periodically for mesaconate analysis. The result of the mesaconate analysis is shown in Figure 36 along with the mesaconate production profiles of previous bioprocesses for comparison.

It can be seen from Figure 36 that IL5.0.1(G2).2(F7-F8-F9) did not produce a higher maximum mesaconate titre than either of the bioprocesses using IL5.0.1(G2).2(F1-F2-F3) and also had a lower production rate of mesaconate over the bioprocess.

The maximum mesaconate titres and production rates are summarised in Table 12. It can be seen that similar to the result observed in section 3.22 when the DO% was reduced to 10% a higher titre and production rate of mesaconate was observed. This confirms that reducing the oxygen availability in the bioprocess can improve the activity of the glutamate mutase.

Table 12: Summary of the maximum mesaconate titres and mesaconate production rates shown in Figure 36.

Bioprocess	**Maximum mesaconate titre**	**Mesaconate production rate**
IL5.0.1(G2).2(F1-F2) 30% DO	0 g/L	0 g/L/h
IL5.0.1(G2).2(F1-F2-F3) 30% DO	0.57 g/L	0.012 g/L/h
IL5.0.1(G2).2(F1-F2-F3) 10% DO	0.8 g/L	0.021 g/L/h
IL5.0.1(G2).2(F7-F8-F9) 30% DO	0.27 g/L	0.002 g/L/h
IL5.0.1(G2).2(F7-F8-F9) 10% DO	0.45 g/L	0.005 g/L/h

Although the mesaconate production rate was lower in the bioprocess of IL5.0.1(G2).2(F7-F8-F9) with a 10% DO concentration it could be seen that the mesaconate production rate did not slow over the course of the bioprocess. The bioprocess was stopped at 105 hours as the off gas analysis of the O_2 and CO_2 showed that respiration had stopped which was the time at which all previous bioprocesses were halted. Previously mesaconate production had always stopped before this point. How mesaconate production continued for 105 hours in this strain was not immediately apparent and it was possible that the maximum titre may have reached 2.65 g/L had the bioprocess not been stopped. No production rate was provided for the *E. coli*

bioprocess therefore it could not be determined if this was how the higher titre was achieved. The glutamate mutase appeared to have an increased stability as even in the 30% DO bioprocess the production rate continues until the bioprocess was stopped. It could not be determined from the current data what factor allowed mesaconate production to continue in IL5.0.1(G2).2(F7-F8-F9) but not in IL5.0.1(G2).2(F1-F2-F3) under the same bioprocess conditions. A possible explanation may be that the slower production rate of mesaconate using the *C. tetanomorphum* glutamate mutase inactivated the available coenzyme B12 at a much slower rate allowing production to continue for longer. It had been shown by Wang and Zhang (2015) that the reactivatase mechanism of action was release of inactive coenzyme B12 from the glutamate mutase allowing bonding of active coenzyme B12. The slower activity of IL5.0.1(G2).2(F7-F8-F9) may therefore utilise coenzyme B12 at a slower rate with the halt in mesaconate production in IL5.0.1(G2).2(F1-F2-F3) being due to limitation of active coenzyme B12 in the medium.

It was therefore concluded that the glutamate mutase and reactivatase from *Desulfosporosinus meridiei* included in IL5.0.1(G2).2(F1-F2-F3) was the preferred enzyme for future work on the production of mesaconate in *C. glutamicum*. This was due to the production rate which was 76% higher, although production did not stop in IL5.0.1(G2).2(F7-F8-F9) the production rate was too slow to be an option in a commercial bioprocess. It was hypothesised that coenzyme B12 limitation may be involved in the difference in production time observed. To produce higher titres of mesaconate work was required to determine why mesaconate production stopped in IL5.0.1(G2).2(F1-F2-F3).

3.24. Conclusions and Future Work

3.24.1. Conclusions

Initially the inducible *C. glutamicum* strain IL5.0.1(G2).2(F16-F17) was shown to produce mesaconate in both the induced bioprocess and the bioprocess without induction, with equivalent titres seen in both. It was concluded that this result may not be accurate as the bioprocess without induction should not have produced mesaconate. A bioprocess using the wild type strain IL5.0.0.0 was shown to produce mesaconate which should not be possible therefore it was concluded that the analysis method being used may not be accurate.

From the NMR and LC-MS analysis it was concluded that mesaconate was present within the induced bioprocess of IL5.0.1(G2).2(F16-F17) although at a lower concentration than that measured by HPLC. Using a spectrophotometer it was concluded that at 190 nm, the wavelength used in the HPLC method for analysis, a high absorbance was obtained for all samples. It was therefore concluded that 240 nm would be used to measure mesaconate and eliminate the interfering peak from the analysis method.

The new HPLC method was shown to be accurate with reanalysis of the samples from previous bioprocesses, showing mesaconate production in the induced bioprocess and no mesaconate in the bioprocess with no induction and the wild type control strain. It was concluded that an unknown product had previously been masking mesaconate in the HPLC method.

It was concluded, from full analysis of the wild type strain IL5.0.0.0 and the empty vector strain IL5.0.1.2, that the empty vector strain was superior for glutamate production. From the bioprocess using the production strain IL5.0.1(G2).2(F16-F17) it was concluded that either the glutamate mutase or methyl aspartate ammonium lyase was inactive, as a large titre of glutamate was available but a small titre of mesaconate was produced.

An excess of biotin was concluded to prevent mesaconate production which may have been due to a low glutamate production rate. A higher DCW and growth rate was also observed in this bioprocess which was expected. It was also concluded that adding a glutamate feed did not allow mesaconate production to occur which may have been due to the low availability of glucose late in the bioprocess.

It was concluded from the glucose fed batch bioprocess that increasing the glucose availability increased the glutamate titre but this did not result in a higher mesaconate titre. It was also concluded that changing the base from NH_4OH to NaOH may have been detrimental to growth with growth stopping at this point.

Gluconic acid as an alternative carbon source was shown to facilitate growth in the bioprocess but no mesaconate was observed suggesting that glucose was necessary for mesaconate production. As IPTG induction occurred at 10 hours as opposed to 20 hours with no detriment to growth it was concluded that the addition of IPTG at the earlier time may improve mesaconate production. A growth test with IPTG addition at different times concluded that addition at 10 hours did not impact on growth, whilst addition at 0 hours prevented growth.

A bioprocess using IL5.0.1(G2).2(F16-F17) and the alternative base 2M NaOH showed a higher glutamate titre and dry cell weight but a lower mesaconate titre than previous batch bioprocesses. It was concluded that the additional nitrogen from the previous base NH_4OH was not necessary for growth and glutamate production and as NH_4OH may inhibit the activity of the MAAL 2M NaOH should be used in future bioprocesses.

A *C. glutamicum* strain containing an alternative glutamate mutase IL5.0.1(G2).2(F1-F2) which was known to have a reduced oxygen tolerance showed no mesaconate production. It was concluded form this that the dissolved oxygen within the bioprocess may be responsible for inactivity of the glutamate mutase. The addition of the associated reactivatase for this glutamate mutase in strain IL5.0.1(G2).2(F1-F2-F3) resulted in the highest mesaconate titre thus far confirming that oxygen deactivation of the glutamate mutase was occurring.

Lowering the dissolved oxygen concentration of the bioprocess was shown to increase mesaconate production while stopping the airflow was shown to prevent further mesaconate production. From this it was concluded that the dissolved oxygen was involved in glutamate mutase deactivation but that some oxygen was required for mesaconate production. It was possible that a lack of ATP halted mesaconate production in anaerobic conditions.

IL5.0.1(G2).2(F7-F8-F9), using the same glutamate mutase and reactivatase, demonstrated to be effective for mesaconate production in *E. coli* (Wang and Zhang, 2015), produced mesaconate at a slower rate than IL5.0.1(G2).2(F1-F2-F3) but had prolonged production. It was concluded from this that IL5.0.1(G2).2(F7-F8-F9) was the superior strain for future

mesaconate production due to the higher production rate but IL5.0.1(G2).2(F7-F8-F9) may be able to produce a higher titre over a longer time frame. It was hypothesised that a lack of coenzyme B12 may be responsible for mesaconate production stopping in the bioprocess.

3.24.2. Future Work

- It was shown by Wang & Zhang (2015) that in *E. coli* ,when the reactivatase reactivates the glutamate mutase, inactive coenzyme B12 is released. After implementation of a coenzyme B12 regeneration pathway the mesaconate titre improved. It was therefore hypothesised that coenzyme B12 was a limiting factor for mesaconate production. Implementation of a coenzyme B12 regeneration pathway into the *C. glutamicum* strain IL5.0.1(G2).2(F1-F2-F3) may increase the mesaconate titre further as a result. To test if coenzyme B12 was a limiting factor a bioprocess could also be carried out with addition of further coenzyme B12 to see if mesaconate production was prolonged.

- It was shown, in the bioprocesses using IL5.0.1(G2).2(F1-F2-F3) with different airflow conditions, that reducing the dissolved oxygen concentration to 10% improved mesaconate production but stopping the airflow completely slowed mesaconate production. Therefore it may be possible that the bioprocess could be optimised further through altering the DO to a value between 10% and 0%. Repetition of the bioprocess at 5% DO would allow it to be observed if a DO lower than 10% may improve the mesaconate titre.

- It was seen in the bioprocesses using IL5.0.1(G2).2(F7-F8-F9) that the mesaconate production rate did not slow up until the bioprocesses were stopped. They were stopped as the conditions within the bioreactor indicated that respiration had stopped and therefore no further mesaconate production was expected. With this strain this

was not the case therefore it would be beneficial to run the bioprocess for longer to determine which titres can be reached.

- Identification of the unknown peak may be of importance for future streamlining of the bioprocess. As it was produced over time and was not produced when biotin was in excess it could be hypothesised that this product was linked to glutamate production. In order to do this fraction collection could be carried out to separate the unknown compound from the sample followed by further NMR and LC-MS analysis focused on the unknown product. This identification may allow the removal of this product from the bioprocess if it was found to be detrimental to mesaconate production.

- In order to determine whether the glutamate mutase is being inhibited within the bioprocesses or whether the enzyme is not being transcribed and expressed, it may be necessary to carry out RNA sequencing on samples from a batch bioprocess of IL5.0.1(G2).2(F1-F2-F3). This will allow the expression of the plasmid genes to be quantified leading to a greater understanding of the bottleneck within the mesaconate production pathway.

Chapter 4

Butyl- methacrylate production using *Escherichia coli*

4.1. Introduction

Although using mesaconate for the bioproduction of MMA as outlined in chapter 3 is a viable route substantial disadvantages arise when this route of production is increased in scale. Before chemical conversion of mesaconate to methyl methacrylate (MMA) can occur, mesaconate must first be separated from the fermentation broth. In a large scale bioprocess this can require significant downstream processing which increases the overall cost of the process and reduces the economic viability of the project.

Organic acids as a group provide a particular set of challenges to downstream processing. Often multiple organic acids are present in the broth some of which can be in salt form and a high energy cost is required for the removal of excess water (Zaman *et al.*, 2017). When looking at a complete bioprocess for organic acid production, the downstream processing and concentration of the acid can account for up to 60% of the total cost and is therefore a significant consideration in the design of a new bioprocess (Li *et al.*, 2016).

The extraction and purification of externally produced organic acids from a fermentation broth requires multiple stages which are outlined in Figure 37. The process can vary depending on the organic acid but usually involves two main stages, extraction and refinement (Li *et al.*, 2016). Initially the organic acid is extracted from the fermentation broth through treatment, separation of the biomass from the broth containing the organic acid and then purification to remove the organic acid from the broth. After extraction of the organic acid from the fermentation cells and broth, refinement of the product occurs through additional purification steps such as crystallisation and chromatography to maximise the purity and yield of the organic acid.

When a bioprocess is scaled up to the level required for economic bio-production of an organic acid all of these above processes can become a significant expense. Separation provides one of the costliest steps and can be up to 30% of the total downstream processing cost (Murali, Srinivas and Ahring, 2017). As an extracellular organic acid is contained within the bioprocess broth separation of the broth and cells in an essential step for recovery. This is a disadvantage when designing a large scale bioprocess as the separation process must

therefore be carried out in a batch format ,common for carboxylic acid production (Saboe *et al.*, 2018), or a continuous extraction system must be developed.

The implementation of a continuous extraction system which would allow a continuous bioprocess has economic favourability over a batch or fed batch bioprocess for a variety of reasons. A continuous process allows equipment downtime to be reduced as there is no stop between batches and can eliminate the need for a significant number of inoculum stages (Li *et al.*, 2011). Along with improving the efficiency of the equipment, a continuous bioprocess may allow smaller reactors to be used, reducing the capital cost, while also producing a similar quantity of product to a batch bioprocess due to the continuous flux of substrates in and products out of the vessel (Brethauer and Wyman, 2010). The product is continuously removed from the bioprocess which can improve production through reducing end product inhibition in the process and providing a more concentrated source of product in comparison to processing the entire bioprocess broth (López-Garzón and Straathof, 2014a).

For organic acids such as lactic acid and succinic acid ,which have applications as esters, the integration of esterification into the bioprocess has been shown to streamline product recovery (López-Garzón and Straathof, 2014b). As esters are often insoluble in water an ester phase can form above the bioprocess broth and therefore extraction of the product from the broth can occur simply without disruption of the producing biomass. This eliminates the need for isolation and purification of the product from the broth reducing costly downstream processing steps within the bioprocess. Changing the bio-produced MMA precursor from mesaconate to a compound with favourable separation properties such as an ester may improve the economic viability of MMA bioproduction while also reducing the number of downstream processing steps and simplifying the infrastructure required.

Along with envisioned economic improvements, by limiting downstream processing and optimising the bioprocess, it was thought that the production of an ester closer in chemical structure to methyl methacrylate would reduce the complexity of the synthetic chemistry required to transform the precursor into methyl-methacrylate. As methyl-methacrylate is unlikely to be able to be biologically produced due to the extreme cytotoxicity of methanol (Leßmeier and Wendisch, 2015) a pathway was devised by Ingenza Ltd for the production of

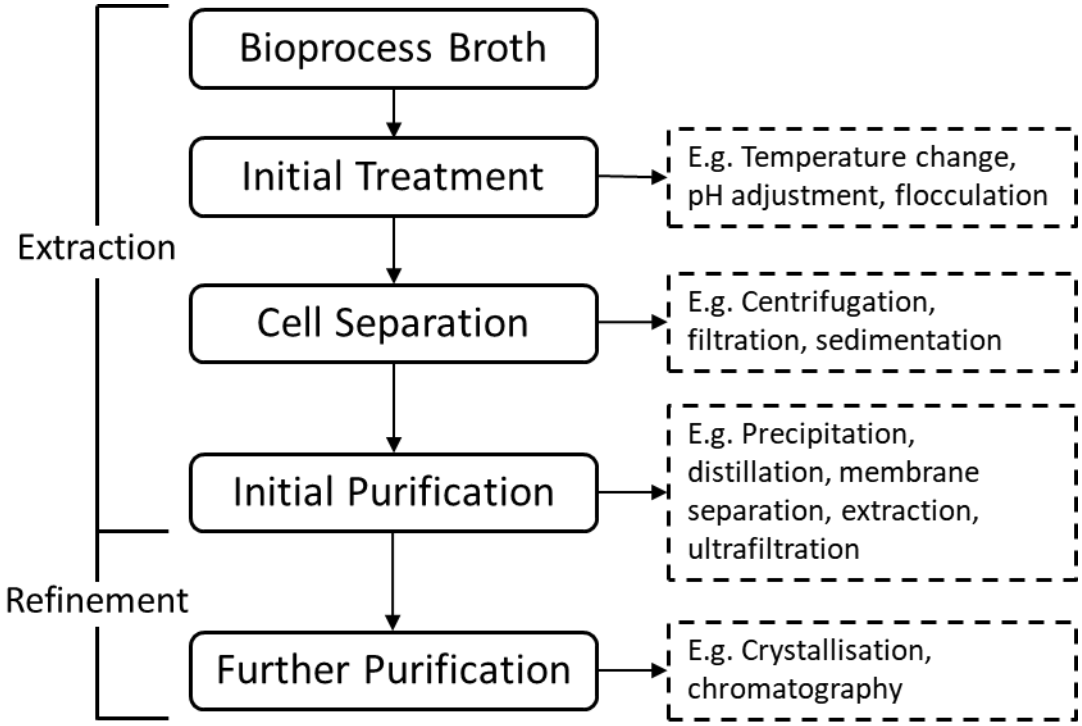


Figure 37: An outline of the downstream processing involved in the extraction and refinement of an extracellular organic acid. (Adapted from Li *et al.*, 2015*).*

an alternate methacrylate ester butyl-methacrylate (BMA) within *E. coli*. BMA was selected as the optimal ester product as the length of the carbon chain ensured insolubility in water and therefore ease of recovery was envisioned in the future large scale bioprocess.

4.1.1. The BMA production pathway

The pathway for BMA production devised by Ingenza Ltd and Lucite International (Eastham, Stephens and Yiakoumetti, 2015) is shown in Figure 38 and requires two substrates α-ketoisovalerate (KIV) and n-butanol. The pathway contains three steps catalysed by three separate enzymes, a branched chain ketoacid dehydrogenase complex (*BCKD*), an acyl-coenzyme A oxidase (*Acx4*) and an alcohol acyltransferase (*AAT*).

The first step in the pathway is the conversion of KIV to isobutyryl-CoA which is carried out by the BCKD complex. The BCKD complex is composed of three enzymatic units E1, E2 and E3 (Li

et al., 2003). The E1 unit is thiamine phyrophosphate (TPP) dependant and consists of two subunits α-E1 and β-E1 and is responsible for the decarboxylation of the branched chain α-keto acid (Wynn *et al.*, 1998). The E2 unit of the BCKD complex is a dihydrolipoyl transacylase which catalyses the transfer of the acyl group to a covalently bonded CoA molecule forming an acyl-CoA molecule (Isobutyryl-CoA). The E3 unit of the BCKD complex is a dihydrolipoamide dehydrogenase which oxidises the remaining lipoyl group from the E2 unit using the cofactors flavin adenine dinucleotide (FAD) and NAD^+ (Ævarsson *et al.*, 2000). The E1 and E2 units are unique to the BCKD complex but the E3 unit is shared by the pyruvate and α-ketoglutarate dehydrogenase complexes (Su *et al.*, 2017).

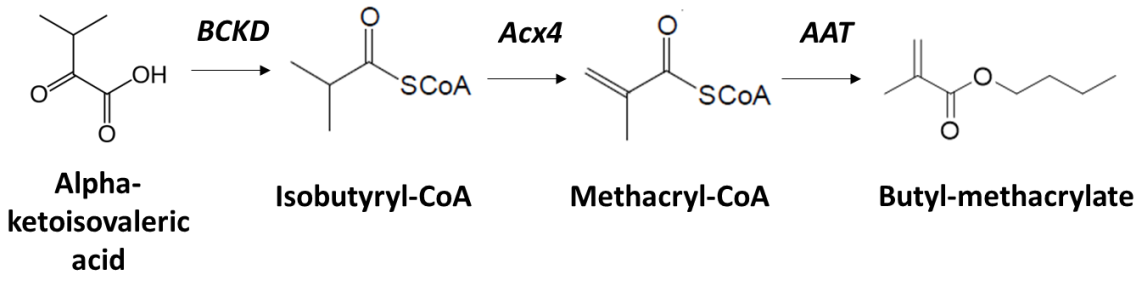


Figure 38: The pathway for the production of butyl-methacrylate (BMA) from α-ketoisovaleric acid (KIV) using a branched chain ketoacid dehydrogenase complex (BCKD), an acyl-coenzyme A oxidase (Acx4) and an alcohol acyltransferase (AAT).

In mammals the BCKD complex is located within the mitochondria (Kadota *et al.*, 2013) and is the rate limiting component of the branched chain amino catabolic pathway responsible for the oxidative decarboxylation of branched chain α-keto acids (Matsumoto *et al.*, 2010). The BCKD complex is well studied in mammals as mutations in the four subunits α- E1, β- E1, E2 and E3 can lead to the development of maple syrup urine disease (Cheng *et al.*, 2017). In prokaryotes the BCKD complex has been identified in *Pseudomonas aeruginosa* (McCully, Burns and Sokatch, 1986), *Pseudomonas putida* (Hester *et al.*, 1995) and *Bacillus subtilis* (Lowe, Hodgson and Perham, 1983). In *Pseudomonas* species the complex allows the catabolism of L-leucine, L-valine and l-isoleucine. In *Bacillus* the complex is essential for growth as a component of the pathway for producing cell membrane lipids.

The second step in the BMA production pathway is the conversion of isobutyryl-CoA to methacryl-CoA using an acyl-CoA oxidase. Acyl-CoA oxidases are a component of the β-oxidation pathway and catalyse the conversion of acyl-CoA thioesters to their equivalent trans-2-enoyl-CoA molecule (Sztajer, Wagner and Schmid, 1993). The oxidation reaction utilises FAD as a cofactor. Upon oxidation of the acyl-CoA thioester FAD is reduced to $FADH^-$ before reoxidisation with molecular oxygen, this process forms hydrogen peroxide as a by-product (Mackenzie *et al.*, 2006). An outline of the isobutyryl-CoA to methacryl-CoA pathway is shown in Figure 39.

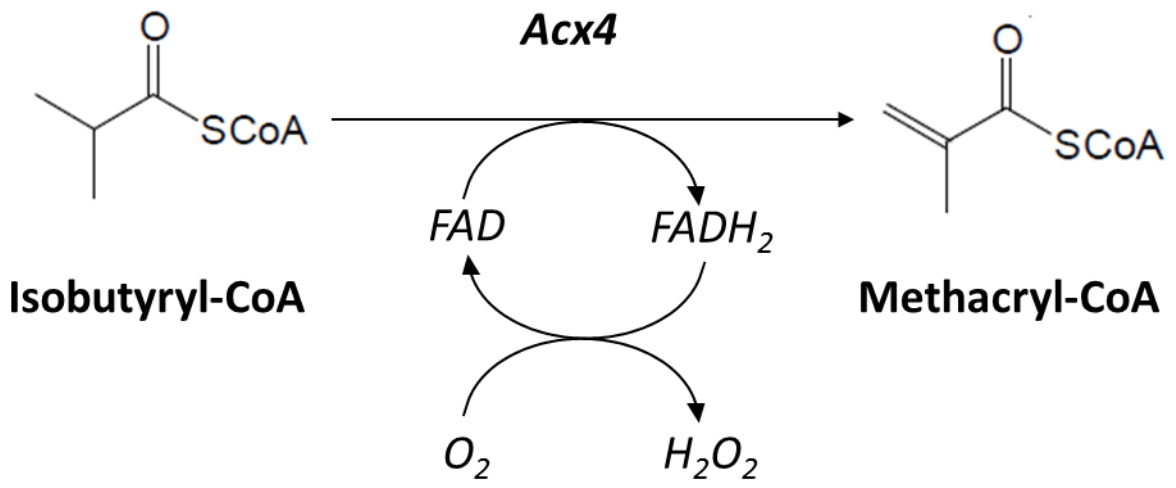


Figure 39: An outline of the operation of the acyl-CoA oxidase Acx4.

Acyl-CoA oxidases have been shown to be present in a variety of mammals (Battaile *et al.*, 2004) , plants (De Bellis *et al.*, 2000) and microorganisms (Ruprecht *et al.*, 2015) as β oxidation of fatty acids is a common biological process. Although common in biology a factor which differentiates acyl-CoA oxidases is the high specificity towards acyl-CoA thioesters with different chain lengths. Acyl-CoA oxidases are divided into activity on long chain, medium chain and short chain acyl-CoAs. In mammals, such as rats, activity is only available towards long and medium chain molecules, yeasts have activity to long and short molecules although plants such as *Arabidopsis thaliana* have the most studied enzymes with specific activity towards all length of acyl-CoAs (Hiltunen and Qin, 2000). The acyl-CoA oxidase present within *Arabidopsis thaliana* (Acx4) was selected for use within the BMA production pathway due to the specificity for only short chain acyl-CoAs an aspect which is unique to plants (Mackenzie

et al., 2006). This form of acyl-CoA oxidase will allow binding of isobutyryl-CoA for the production of methacryl-CoA (Lucas *et al.*, 2007).

The final step required for the production of BMA is the esterification of methacryl-CoA to butyl-methacrylate using an alcohol acyltransferase (AAT). Alcohol acyltransferases are prevalent in fruits where the production of esters and volatiles contribute to flavour and aroma (Galaz *et al.*, 2013). AAT's have been identified in a variety of fruits including apples (Souleyre *et al.*, 2014), melon (Shalit *et al.*, 2001), grapes (Kalua and Boss, 2009), strawberries (Pérez *et al.*, 1996) and bananas (Olias *et al.*, 1995) all of which produce a variety of esters and volatiles. Alcohol acyltransferases catalyse the transfer of the acyl group from the acyl-CoA to an alcohol present at the enzyme active site. This results in the production of an ester and the release of free coenzyme A (Shalit *et al.*, 2001). This esterification process for the production of BMA is outlined in Figure 40.

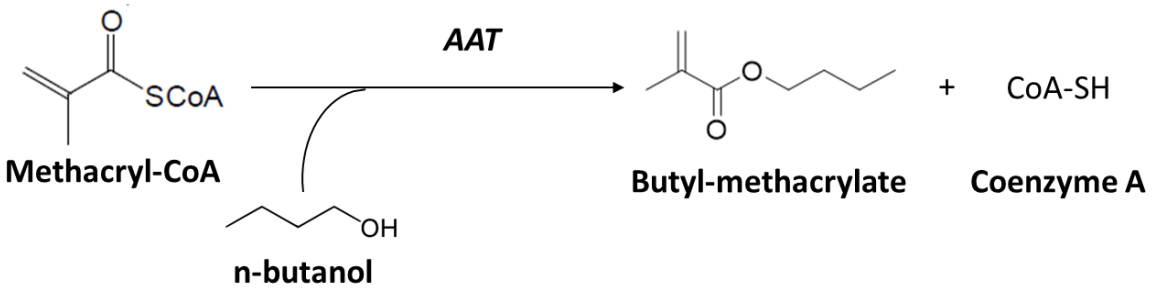


Figure 40: The alcohol acyltransferase (AAT) mechanism of action for BMA production.

The AAT from the apple species *Malus pumila* also known as the royal gala apple (mpAAT1) was selected for use in the BMA production pathway. The majority of esters produced within *Malus pumila* are butyl esters with butyl acetate and 2-methylbutyl acetate dominating the flavour of the ripe fruit. It was demonstrated by Souleyre et al. (2005) that in *E. coli* the mpAAT1 facilitated the production of butyl esters. As butanol would be the alcohol used in the production of BMA the mpAAT1 was identified as an appropriate AAT.

The combination of the BCKD complex, Acx4 and AAT will allow the production of BMA from KIV. At the time of writing this was the first demonstration of butyl methacrylate bio-production within a microbial expression system.

4.1.2. The KIV production pathway

As the BMA production pathway utilised KIV as the initial substrate a second pathway was required to produce KIV from glucose. In *E. coli* KIV cannot be produced at sufficient quantities to be utilised for BMA production therefore external supplementation of KIV was required. This was sufficient for initial investigation into BMA production but in order for the bioprocess to be economically viable BMA production from a sugar would be required. As such, a pathway was developed for the production of KIV from glucose. The KIV production pathway is outlined in Figure 41.

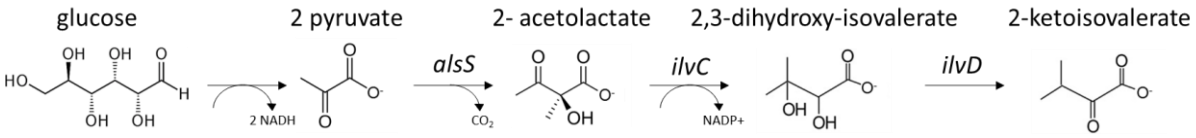


Figure 41: An outline of the KIV production pathway.

The KIV production pathway required the overexpression of three enzymes, an α-acetolactate synthase (*alsS*), a acetohydroxy acid isomeroreductase (*ilvC*) and a dihydroxy acid dehydratase (*ilvD*). Glucose is converted to two molecules of pyruvate through the Embden-Mayerhof-Parnas glycolytic pathway (Hollinshead *et al.*, 2016). This pathway was the basis for pyruvate accumulation which is required for the first enzymatic step. In *Bacillus subtilis* acetolactate production occurs via two separate enzymes. An acetohydroxy acid synthase (AHAS), isozymes of which are also present in *E. coli*, catalyses the condensation of two pyruvate molecules to form acetolactate. The second enzyme is an α-acetolactate synthase (*alsS*) which can produce acetolactate directly from two pyruvate molecules (Renna *et al.*, 1993). The acetolactate synthase *alsS* is homologous AHAS present in *E. coli* although has a higher affinity for pyruvate (Atsumi, Hanai and Liao, 2008).

In order to convert acetolactate to ketoisovalerate two enzymes *ilvC* and *ilvD* which are native to the L-valine biosynthetic pathway in *E. coli* were utilised. *IlvC* is an acetohydroxy acid isomeroreductase which catalyses the conversion of acetolactate to 2,3-dihydroxy-isovalerate. The reaction utilises the cofactor NADPH as a hydrogen donor as well as a

concerted carbon-carbon bond rearrangement. *IlvD* is a dihydroxy acid dehydratase which catalyses the conversion of 2,3-dihydroxy-isovalerate to 2-ketoisovalerate and water (Park *et al.*, 2007). It has been shown by Atsumi et al. (2008) that as part of an isobutanol production pathway within an *E. coli* host overexpression of *alsS* from *B.subtilis* along with *ilvC* and *ilvD* from *E. coli* allowed 22 g/L of isobutanol to be produced. The KIV concentration was not directly measured but as part of a linear pathway at least 22 g/L of KIV was produced. It was hypothesised that this should be able to be replicated for the incorporation into the BMA production pathway.

4.1.3. Bioprocess Design

Initially it was decided that research into BMA production would be carried out in batch biotransformations with supplementation of KIV and n-butanol. The biotransformation method was preferred to the bioreactor based research used for mesaconate production due to the toxicity of butanol which was used as a substrate and BMA which was the product of the biotransformation. In *E. coli* the presence of butanol has been shown to disrupt the cell membrane while inhibiting membrane transport systems. As a result the presence of 1% of butanol in a growth medium was shown to inhibit the growth of three *E. coli* strains (DH5a, K12 and W3110) by between 30% to 80% with complete inhibition of growth at 2% (Knoshaug and Zhang, 2009). In *E. coli BW25113,* the host used for the majority of BMA production strains, grown in M9 media with 0.8% butanol (v/v) a 0.26 difference in OD600/ml was observed at 4 hours (Rutherford *et al.*, 2010). As growth may not be possible in the presence of butanol a biotransformation allowed the separation of the inoculum growth and the BMA production phase in which butanol would be present.

In combination with the toxicity of butanol the produced BMA was also thought to be toxic to *E. coli*. It has been demonstrated by Ward et al. (2006) that in the presence of concentrations up to 1.5 mg/ml of ethyl/ butyl methacrylate copolymers no inhibitory effect on growth is demonstrated. This demonstrates the external tolerance of *E. coli* to hydrophobic polymers but does not account for internal production of butyl methacrylate and the damage hydrophobic molecules can exerted on the cell membrane. As BMA has not

previously been produced in *E. coli,* or any microorganism, no data is available on the effect of internal BMA on *E. coli* viability. BMA is a hydrophobic molecule (Georgiou and Patrickios, 2006) and it has been demonstrated that hydrophobic molecules can damage cell membranes through integration into hydrophobic lipid membranes (Palermo, Sovadinova and Kuroda, 2009). This integration can cause cell lysis, enzyme inactivation and obstruction of membrane transportation (Palermo and Kuroda, 2010). Therefore it was hypothesised that BMA production would have a detrimental effect on *E. coli* viability through lysis of the cell membrane.

As butanol and BMA have both been shown to have the potential to damage the cell membrane of *E. coli* a biotransformation approach to experiments was necessary to allow sufficient growth and production of the required enzymes before exposure to either toxic compound. Survival of the *E. coli* inoculum was not expected after internal production of BMA occurred. Thus, the use of small scale biotransformations allowed greater control to be exerted over each step of the bioprocess. As butyl methacrylate production in *E. coli* was a novel bioprocess the biotransformation method facilitated the accurate interpretation of results which may not have been possible in a bioprocess combining growth and production.

4.2. Strain Construction

The production and engineering of all strains used in this chapter was carried out in house at Ingenza Ltd. Strains were developed in synchronisation with the presentation and analysis of results from biotransformations resulting in a large quantity of strains being produced. An overview of the strains used within this chapter is shown in Table 13. The pathway required for BMA production was contained on plasmids entitled pMMA. The KIV production pathway was contained on plasmids entitled pKIV.

The BMA production pathway present on the pMMA plasmids contained a trc promotor which allowed expression without induction. The KIV production plasmid, pKIV, contained an inducible lac promotor and therefore induction was required using IPTG. Antibiotic resistance was included on all plasmids for strain selection. The BMA production plasmids used in this

Table 13: The overview of all strains used in the chapter.

Strain Name	***E. coli* Host Strain**	**Plasmids**	**BMA Genotype**
LUC0548	*BL21(DE3)*	pET21b	*Amp^R : (BCKD, Acx4, ECH, HCH)*
LUC0585	*BW25113*	pMMA 044	*Amp^R : (BCKD, Acx4, AATm4)*
LUC0615	*BW25113*	pMMA 050	*Amp^R : (BCKD, Acx4, AATm4)*
LUC0669	*BW25113*	pMMA 044	*Amp^R : (BCKD, Acx4, AATm4), ΔyciA*
LUC0683	*BW25113*	pMMA 050	*Amp^R : (BCKD, Acx4, AATm4), ΔyciA*
LUC0685	*BW25113*	pMMA 050	*Amp^R : (BCKD, Acx4, AATm4), ΔyciA, ΔtesB*
LUC0710	*BW25113*	pMMA 070	*Amp^R : (BCKD, Acx4, AATm5)*
LUC0711	*BW25113*	pMMA 071	*Amp^R : (BCKD, Acx4, AATm6)*
LUC0713	*BW25113*	pKIV	*$Camp^R$: (alsS, ilvC, ilvD, katE)*
LUC0714	*BW25113*	pMMA 050, pKIV	*Amp^R : (BCKD, Acx4, AATm4), $Camp^R$: (alsS, ilvC, ilvD, katE), ΔilvE, ΔilvA, ΔpanB, ΔleuA*
LUC0720	*BW25113*	pMMA 050, pKIV	*Amp^R : (BCKD, Acx4, AATm4), ΔyciA, ΔtesB*
LUC0721	*BW25113*	pKIV	*$Camp^R$: (alsS, ilvC, ilvD, katE)*
LUC0723	*BW25113*	pMMA 050, pKIV	*Amp^R : (BCKD, Acx4, AATm4), $Camp^R$: (alsS, ilvC, ilvD, katE), ΔpoxB, ΔaceE*
LUC0724	*BW25113*	pMMA 050, pKIV	*Amp^R : (BCKD, Acx4, AATm4), $Camp^R$: (alsS, ilvC, ilvD, katE), ΔidhA, ΔpflB, ΔaceE*
LUC0729	*BL21(DE3)*	pMMA 071	*Amp^R : (BCKD, Acx4, AATm6)*
LUC0730	*JM109*	pMMA 071	*Amp^R : (BCKD, Acx4, AATm6)*
LUC0731	*Top10*	pMMA 071	*Amp^R : (BCKD, Acx4, AATm6)*
LUC0746	*RNM5*	pMMA 050	*Amp^R : (BCKD, Acx4, AATm4), pspA*
LUC0747	*RNM5*	pMMA 050	*Amp^R : (BCKD, Acx4, AATm4), pspA/B/C/D/E*
LUC0748	*RNM5*	pMMA 050	*Amp^R : (BCKD, Acx4, AATm4)*
LUC0749	*BW25113*	pMMA 050, pKIV	*Amp^R : (BCKD, Acx4, AATm4), $Camp^R$: (alsS, ilvC, ilvD, katE)*

chapter, pMMA 044, pMMA 050, pMMA 070 and PMMA 071, all contained an ampicillin resistance marker. The plasmid containing the genes required for KIV production from glucose, pKIV, contained a chloramphenicol resistance marker.

4.2.1. Previous work by Ingenza Ltd

It had been shown by Ingenza Ltd in house that BMA could be produced in a biotransformation when using KIV as a substrate and a pathway containing a BCKD complex (*Pseudomonas aeruginosa*), an Acx4 enzyme (*Arabidopsis thaliana*) and an AAT enzyme. Work was carried out to determine that the AAT (*MpAAT1*) originating from Royal Gala apples was the optimum AAT for BMA production. These enzymes were contained on the pMMA 044 plasmid which was included in the current best production strain LUC0585. The biotransformation method was developed to produce a method in which reproducible results could be obtained. Work was carried out to try and produce a high throughput screening process for new strains and work was beginning into determining the factors which limited BMA production

4.2.2. Aims

It had been identified, by Lucite International Ltd, that a BMA production rate of 14 mM/hour was required for the creation of a commercially viable biological methyl-methacrylate production process. This was therefore deemed to be the main aim of this research. The experiments within this chapter aimed to improve the BMA production capacity of the biotransformation. As the biotransformation process was in its infancy, experiments were designed to increase the understanding of the biotransformation, improve the biotransformation method and to devise, create and test possible improvements to the production strain used. This should allow the BMA production rate to be improved while also providing insights for future research.

4.3. Determining the effect of oxygen availability on biotransformations of LUC0585

The previous work carried out by Ingenza Ltd (Section 4.2.1) demonstrated that LUC0585 ,which consisted of *E. coli* BW25113 containing the pMMA 044 plasmid (Section 4.2), was the current best strain for the production of BMA from KIV. This strain was shown to produce 0.2-0.3 mM of BMA in batch biotransformations and was the basis from which improvement in BMA yield would be evaluated.

At the time of entry into the BMA production project at Ingenza Ltd the current hypothesis was that BMA production by LUC0585 was limited by oxygen availability during the biotransformation process since the biotransformations were carried out in sealed Schott bottles (Section 2.2.2.1). The Acx4 enzyme ,originating from *Arabidopsis thaliana* contained within the pMMA 044 plasmid is an oxidase (Mackenzie *et al.*, 2006) and therefore has an O_2 requirement as an electron acceptor.

In order to determine whether oxygen availability was a factor which contributed to limited BMA production in the biotransformations of LUC0585 two biotransformations were set up. Both biotransformations were set up following the method for batch biotransformations (Sections 2.2.1.1 and 2.2.2.1). Both biotransformations were started from the same inoculum which was split into the two vessels. One biotransformation was sealed after inoculation, the other was sparged with oxygen above the surface of the media for 1 minute before being sealed. Oxygen was not sparged below the surface of the media as the volume was only 30 ml. The dramatic elevation of the oxygen concentration within the low volume of media may have induced oxidative stress response in *E. coli* (Baez and Shiloach, 2014). This may have been detrimental to BMA production and cell viability therefore sparging above the surface was selected. The biotransformations were both sampled at 4 hours. The samples were processed as outlined in section 2.2.3.6 and analysed for BMA production by HPLC (Figure 42).

Strain Genotypes
LUC0585- *E.coli BW25113 AmpR : (BCKD, Acx4, AATm4)*

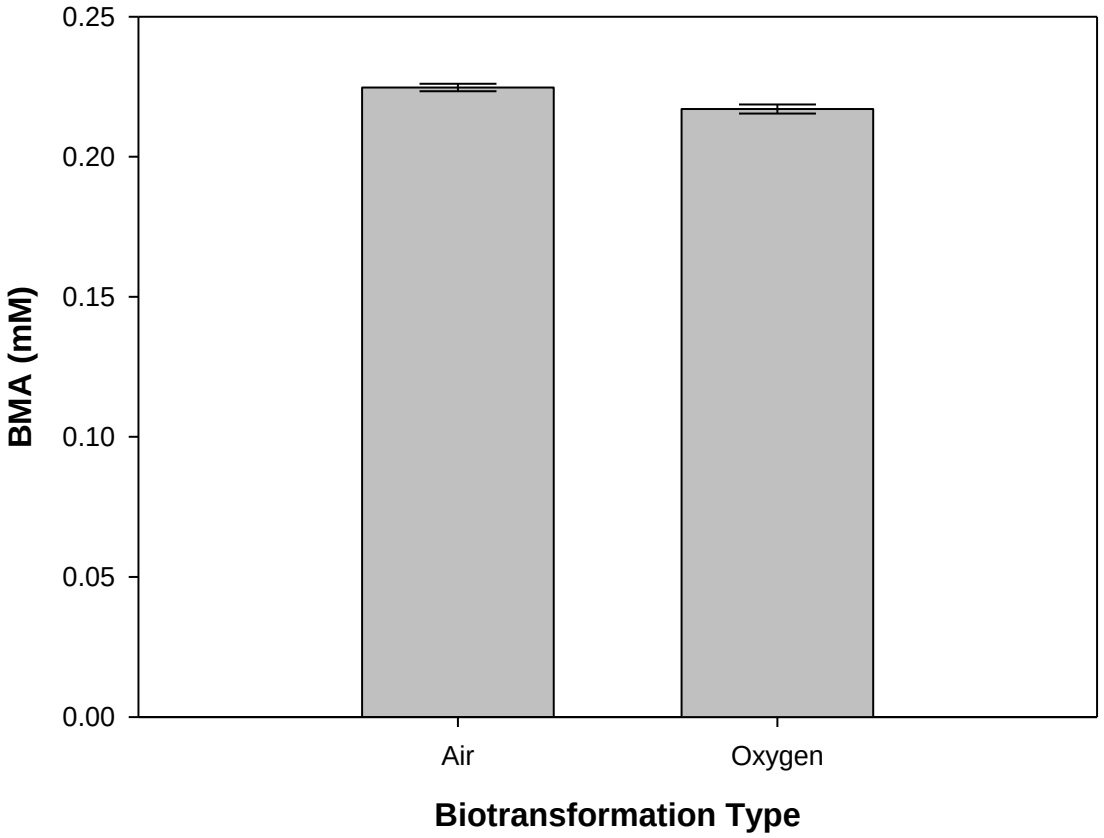


Figure 42: The BMA concentration measured by HPLC for the 4 hour sample from each LUC0585 biotransformation. The air biotransformation was set up as standard, the oxygen biotransformation was sparged with oxygen before incubation. The analysis was carried out in triplicate and the error bars represent the standard deviation of the measurements.

When looking at Figure 42 it could be seen that no increase in BMA production was observed in the biotransformation with increased oxygen present in the vessel. A slight decrease in BMA titre was observed in the increased oxygen biotransformation (0.2170 mM) in comparison to the standard biotransformation (0.2247 mM). It is possible that no increase in BMA titre was observed as the oxygen was not sparged below the surface of the biotransformation media which in combination with the un-baffled Schott bottle vessel means the oxygen transfer rate was likely to be low.

4.4. Determining the effect of a direct air sparge and alternative vessels on biotransformations of LUC0585

To determine if increasing the oxygen transfer rate had an effect on BMA production biotransformations were set up using various vessel types and sizes, with and without a direct air sparge into the biotransformation media throughout. Ten biotransformations were set up as outlined in Table 14. Biotransformations 1 and 2 used large shake flasks with and without baffles to determine if a larger surface area would benefit BMA production. Biotransformations 3 and 4 used the standard Schott bottles with biotransformation 3 a control and 4 incubated without rotation. The final 6 biotransformations utilise 3 smaller vessels, a serum bottle (similar in shape to a Schott bottle), a shake flask and a baffled shake flask. Of each biotransformation pair one was sealed and one had an air sparge within the vessel. It was hypothesised that these biotransformations would allow it to be seen whether a constant 1 vvm air sparge into the vessel would improve BMA production through increased oxygen availability.

The inoculum for all biotransformations were set up as outlined in section 2.2.1.1. Biotransformations 1-4 were set up as outlined in section 2.2.2.1 for batch biotransformations with only the vessel changed as shown in Table 14. The final six biotransformations were set up as outlined in section 2.2.2.2. The biotransformations were sampled at 0, 4 and 24 hours with the samples analysed for BMA production by HPLC. The resulting BMA concentrations measured are shown in Figure 43.

Table 14: The outline of the biotransformation conditions for each vessel in Figure 43. All biotransformations were incubated at 30°C and 180 RPM except biotransformation 4 which was only incubated at 30°C.

Biotransformation Number	Vessel	Biotransformation Volume	Air Sparging
1	500 ml baffled shake flask	30 ml	No
2	500 ml shake flask	30 ml	No
3	250 ml Schott bottle	30 ml	No
4	250 ml Schott bottle (Static)	30 ml	No
5A	100 ml baffled shake flask	20 ml	Yes
5B	100 ml shake flask	20 ml	Yes
5C	100 ml serum bottle	20 ml	Yes
6A	100 ml baffled shake flask	20 ml	No
6B	100 ml shake flask	20 ml	No
6C	100 ml serum bottle	20 ml	No

From Figure 43 it can be seen that the BMA concentration in biotransformations 1, 2, 5A, 5B, 5C, 6A, 6B and 6C all dropped over time from the 4 hour time point to 24 hour time point. The only biotransformations which did not display this drop in BMA concentration are biotransformations 3 and 4. These biotransformations were carried out in sealed Schott bottles which is the same vessel which was used in the previous experiment (Figure 42). All experiments using Schott bottles were sealed with polypropylene caps which are known to have high chemical resistance (Roberts and Constable, 2003), as such no BMA loss was observed in either experiment. The BMA loss observed in this experiment is thought to be due to the fact that all of the other vessels were sealed with silicone rubber Suba-seals which have a lower level of chemical resistance than polypropylene caps. As the vessels are glass

Strain Genotypes
LUC0585- *E.coli BW25113 AmpR : (BCKD, Acx4, AATm4)*

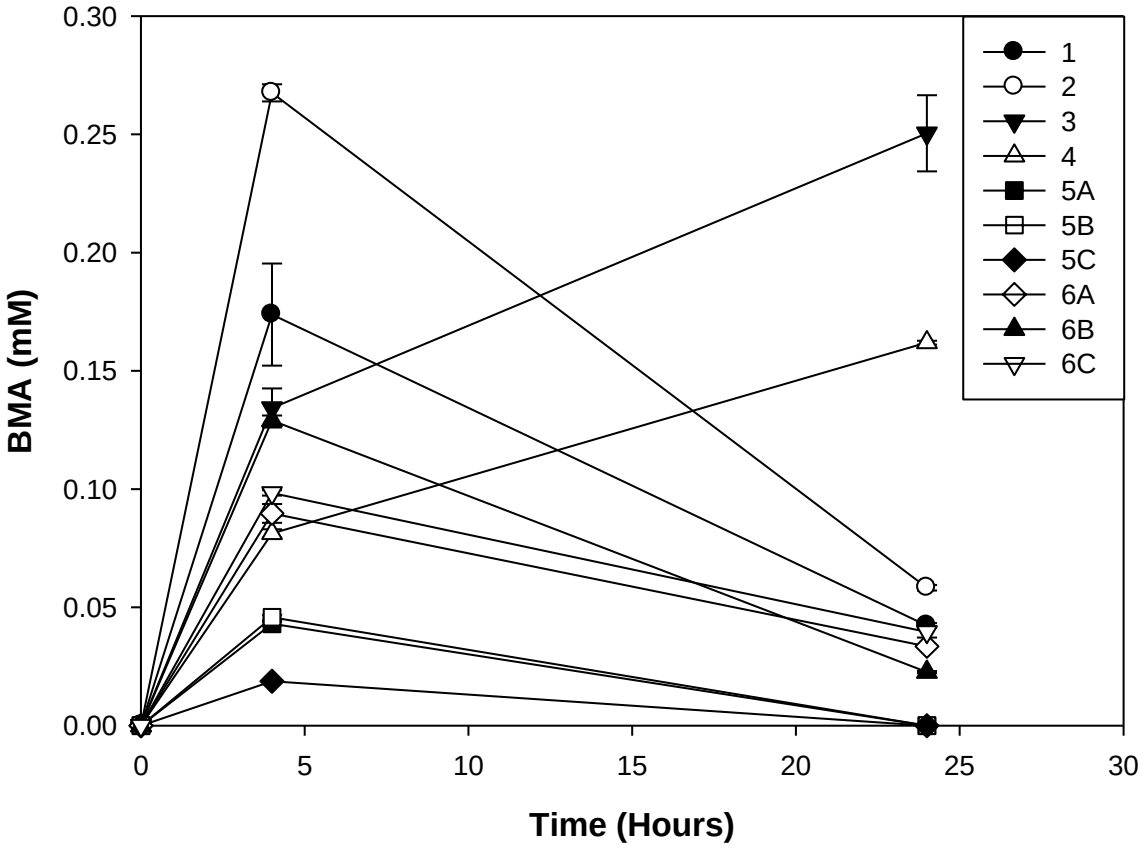


Figure 43: Multi vessel biotransformations with and without air sparging. All biotransformations were incubated at 30°C and 180 RPM except for biotransformation 4 which was incubated at 30°C and 0 RPM. The analysis was carried out in triplicate by HPLC and the error bars represent the standard deviation of the samples.

BMA would not permeate the vessel, therefore the loss must be due to the nature of the seals.

The vessels which had air sparged into the media using needles through the Suba-seals showed a complete loss of BMA at 24 hours suggesting that continuous air sparging may also cause BMA to leave through the off gas, adding additional complexity to the analysis of the BMA production process. As BMA was likely to be leeching into the seals on the vessels the BMA titres observed in all biotransformations barring 3 and 4 in Figure 43 cannot be used to accurately show BMA titre or production rates.

Biotransformation 3 and 4 can be compared and analysed as they were carried out in Schott bottles and did not show the BMA loss seen in the other biotransformations. Biotransformation 3 was incubated at 180 RPM and 30^{o}C after inoculation and number 4 was incubated at 30^{o}C with no shaking to compare the effect of mixing on BMA production. It can be seen from Figure 43 that the mixing has a positive effect on the biotransformation with a BMA titre of 0.25 mM produced in biotransformation 3 and 0.16 mM produced in biotransformation 4 at 24 hours. This result is likely due to less contact between the biotransformation enzymes (BCKD, Acx4 and AAT) and the substrates (KIV and butanol). The titre observed in biotransformation 3 is less at 4 hours (0.1343 mM) than the air biotransformation in Figure 42 (0.2247 mM), as these biotransformations are the same this suggests that there may be variation between biotransformation sets based on the inoculum.

In this biotransformation set all biotransformations had individual inocula although the OD600 of the inocula is standardised at 25 for all biotransformations upon starting. It was therefore concluded that variation in the sparged and non sparged biotransformations may have been due to the inocula and not the experimental conditions. It was also concluded that Schott bottles were the optimum vessel for the biotransformations as they did not cause BMA loss and that rotation was required while the biotransformations were incubated. In order to determine whether sparging had an effect on BMA production the final 6 biotransformations required repetition using a single inoculum.

4.4.1. Repetition of the sparged biotransformations

As the aim of the previous experiment was to accurately determine the difference between vessel type and the effect of sparging on the BMA yield a more accurate comparison would be achieved using a single inoculum split between all the vessels. Therefore the previous experiment was altered and repeated to remove inoculum variation from the biotransformation set. The new experiment set consisted of three biotransformations pairs. Each pair had a biotransformation with and without air sparging and a different vessel. The biotransformations were set up as outlined in sections 2.2.1.1 and 2.2.2.2.

Three separate inoculum flasks were grown before being mixed prior to the setup of the biotransformations. Three inoculum flasks were needed to produce sufficient biomass for all 6 biotransformations. This ensured that all of the biotransformations had the same inoculum and provided a more accurate comparison of the biotransformation vessels than the previous experiment where the inocula differed. The biotransformations were sampled at 3, 20 and 24 hours. The samples were analysed for BMA production by HPLC analysis, the resulting BMA quantification results are shown in Figure 44.

From the BMA quantification results in Figure 44 it can be seen that all vessels show BMA loss over time confirming the result of the previous experiment which showed BMA loss in vessels sealed with silicone rubber Suba-seals. As observed previously the vessels with a subsurface air sparge have a lower BMA titre at 3 hours and no BMA at later sample points. This is consistent with the hypothesis that BMA is being lost in the off gas released from the vessels. It may also be possible that the sparge facilitated further absorption of BMA into the Suba seal vessel cap. This hypothesis was also backed up by the strong odour of BMA which was observed in the incubator when sparged vessels were present.

As BMA was lost due to leeching the titres produced may not be accurate and therefore cannot confidently be analysed. The titre of the 3 hour samples could be compared to determine the superior vessel type as the experiment conditions were identical for each vessel. From Figure 44 it can be seen that in the sealed vessels the shake flask had the highest BMA titre at 3 hours of 0.43 mM this may have been because the shake flask had a larger surface area than the serum bottle which would provide better mixing. Although this does

Strain Genotypes
LUC0585- *E.coli BW25113 AmpR : (BCKD, Acx4, AATm4)*

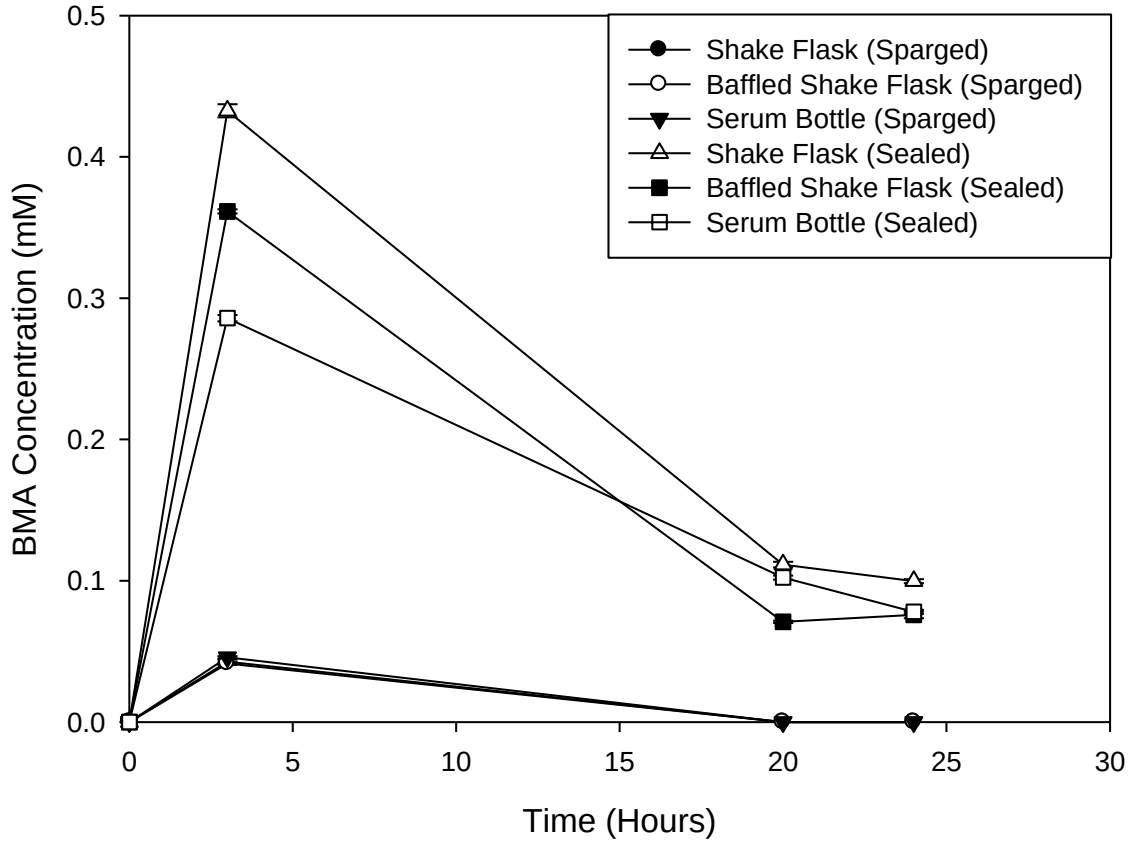


Figure 44: The BMA concentration measured from 6 biotransformations of LUC0585 in different vessels with and without air sparging. The samples were measured in triplicate and the error bars represent the standard deviation of the samples.

not appear to be the case as the baffled shake flask would then have been expected to produce the highest BMA titre as that vessel had the most turbulent mixing.

It is possible that BMA production compromised the membranes of the *E. coli* cells and the turbulent mixing from the baffled shake flask was detrimental to the cells and therefore BMA production. It appears that the larger surface area and non-turbulent mixing of the shake flask made it superior for BMA production through increasing mixing but not exerting a greater shear force on the *E. coli* cells. The sparged vessels all had similar BMA titres at 3 hours and as a large quantity of the BMA produced appeared to be lost in the off gas the vessels are not able to be compared.

It can be concluded from the above experiments that vessels which have silicone rubber seals are not suitable for use in BMA production. Therefore even though using a shake flask produced higher BMA concentrations, Schott bottles were used for future experiments as they were the only vessel which did not show loss of BMA during the biotransformations. Air sparging of the biotransformation vessels was implemented in order to provide additional oxygen for the Acx4. At the current levels of BMA production it appears that oxygen is not a limiting factor (*Figure 42*), therefore due to the complications which arise upon the addition of sparging i.e. BMA off gas loss, this feature was not incorporated into future experiments.

There was the possibility that the observed BMA loss in Figure 44 was due to variation in the sampling process (Section 2.2.3.6). As BMA is an ester it may not have uniform distribution within the biotransformation media and therefore the samples may not contain the accurate quantity of BMA in the biotransformation. Steps within the sampling method were in place to reduce this error, such as mixing the vessels before sampling and suspending in acetonitrile which should facilitate dissolution of the BMA. In order to determine the accuracy of the sampling process six separate samples were taken from the sealed serum bottle at 3 hours. All six samples were initially processed the same until the shaking stage at which three samples were shaken for 20 minutes as outlined in the method (Section 2.2.3.6) while the remaining three samples were sonicated for 20 minutes. The samples were mixed in two different ways to determine if the 20 minute shaking protocol was the optimum method for extracting the BMA from the cell membrane. After the mixing stage the samples were analysed by HPLC, the resulting BMA concentrations are shown in Figure 45. It can be seen from the figure that with the low standard deviation, 0.002 mM for the shaken samples and

0.003 mM for the sonicated samples, that the sampling process is accurate and that the concentration measured is reproducible.

Sonication of the samples was attempted as it was hypothesised that BMA was sequestered in the cell membrane upon production which could reduce the observed BMA concentration. It can be seen from Figure 45 that sonication does not cause an increase in concentration of BMA in the samples. From this result it was concluded that the current shaking method was sufficient for extraction of BMA from cell membranes and therefore suitable for HPLC sample preparation.

Strain Genotypes

LUC0585- *E.coli BW25113 AmpR : (BCKD, Acx4, AATm4)*

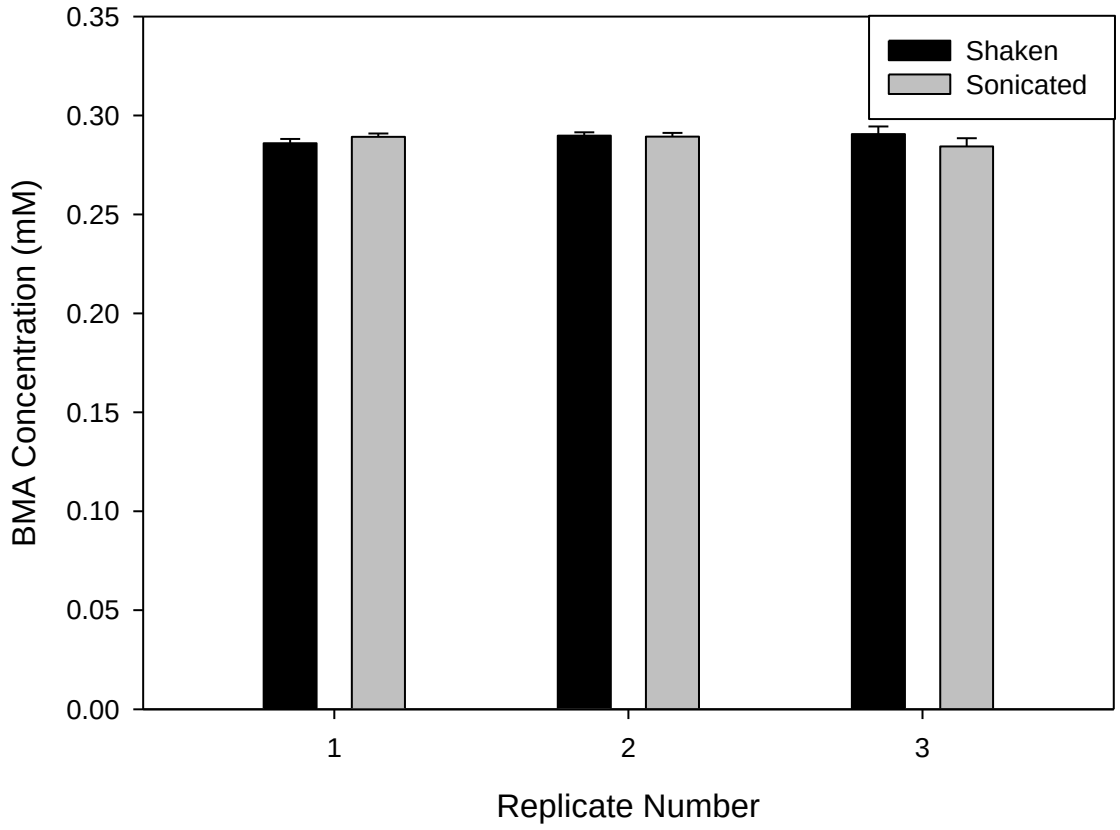


Figure 45: The BMA concentrations measured in the sample of LUC0585 processed through shaking with acetonitrile and sonication with acetonitrile. The sample was split into three replicates for processing. Each sample was measured in triplicate and the error bars represent the standard deviation of the samples.

4.5. Determining the effect of butanol concentration on LUC0585 biotransformations

Changing the vessel type and adding an air sparge to the biotransformations could not be implemented due to the limitation of materials which are resistant to BMA, hence further investigations were carried out on the standard batch biotransformation.

Butanol is known to be toxic to microorganisms including *E. coli* (Abdelaal *et al.*, 2015), therefore a set of biotransformations was carried out altering the level of butanol in order to see what effect butanol had on cell viability and BMA yield.

Four biotransformations were set up each with a different starting concentration of butanol i.e. 60 mM (Control), 30 mM, 15 mM and 5 mM. The control was 60 mM as that was the concentration used as standard for batch biotransformations. No concentration lower that 5 mM was used as the aim of the biotransformations is to produce a high titre of BMA and the pathway requires 1 mM of butanol per 1 mM of BMA. A lower concentration would therefore prove limiting regardless of any benefit which use of a low concentration may provide. LUC0585 was the strain used and the same inoculum was used for all four biotransformations to ensure variation is due to the butanol concentration only. Samples were taken from each biotransformation at 0, 2 and 4 hours for BMA analysis by HPLC and viability plating on LB + Ampicillin plates. The biotransformations were not sampled beyond 4 hours as the highest production rate was observed between 0 and 4 hours.

The result of the HPLC analysis for BMA is shown in Figure 46 and the result of the cell viability is shown in Table 15. From the viability plating it can be seen that at 60 mM there are no viable cells within the biotransformation at 4 hours. The 15 mM and 30 mM biotransformations also had a large reduction in viable cells in comparison to the 5 mM biotransformation. The 5 mM biotransformation showed no significant reduction in viability from 0 hours to 4 hours suggesting that butanol concentrations higher than 5 mM has a negative effect on cell viability. It is possible that the observed viability result is linked to BMA production rather than butanol due to the hydrophobic nature of esters, such as BMA, which can damage the cell membrane during production and thus affect the cell viability.

Table 15: The cell viability result from plating samples taken from the biotransformations with altered initial butanol concentrations. C= Confluent growth, T= Too many to count.

	Colony count at each dilution						
Time and butanol concentration	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**
0H - 5 mM	C	C	C	C	C	1100	185
0H - 15 mM	C	C	C	C	T	1308	270
0H - 30 mM	C	C	C	C	T	812	138
0H - 60 mM	C	C	C	C	C	764	141
2H – 5 mM	C	C	C	C	T	976	147
2H – 15 mM	T	601	88	4	0	0	0
2H – 30 mM	49	8	1	0	0	0	0
2H – 60 mM	515	87	12	0	0	0	0
4H – 5 mM	C	C	C	C	T	1064	116
4H – 15 mM	3	0	0	0	0	0	0
4H – 30 mM	7	0	0	0	0	0	0
4H – 60 mM	0	0	0	0	0	0	0

Strain Genotypes
LUC0585- *E.coli BW25113 AmpR : (BCKD, Acx4, AATm4)*

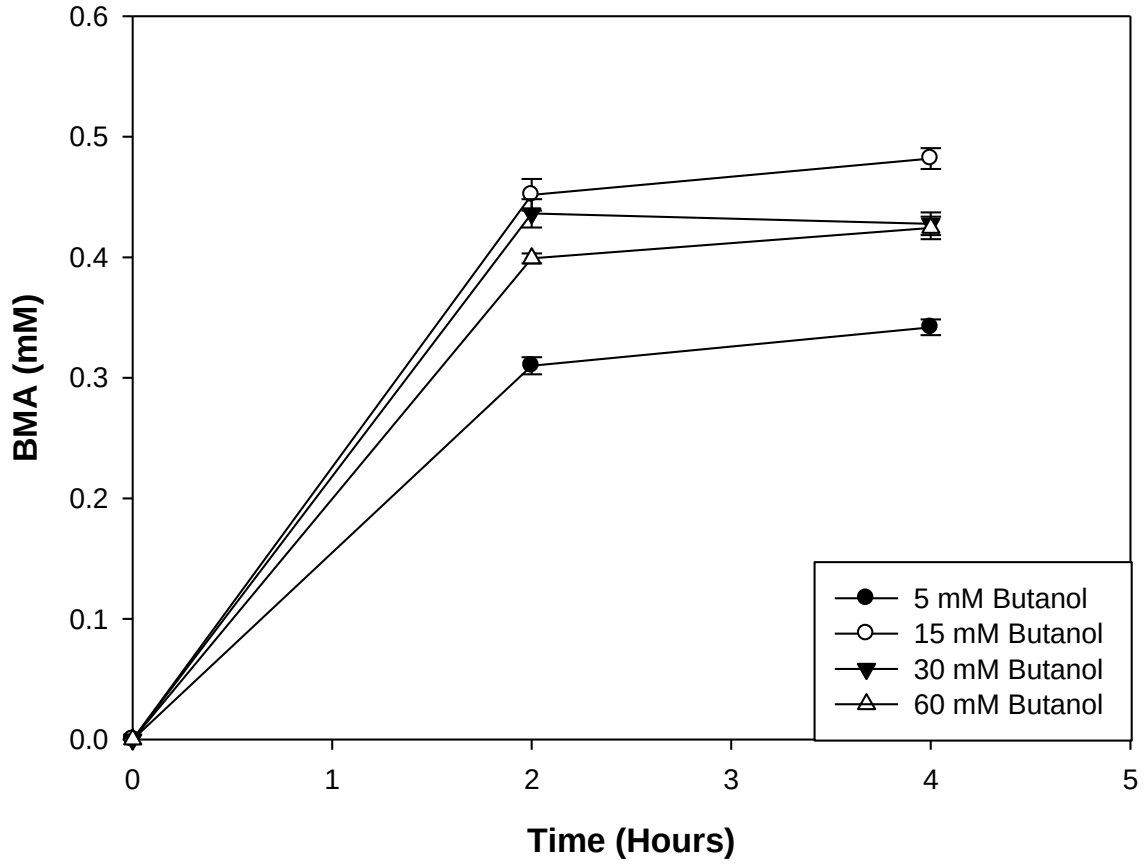


Figure 46: The BMA concentration measured in the samples taken from the four biotransformations of LUC0585 with different initial butanol concentrations. Each sample was measured in triplicate and the error bars represent the standard deviation of the samples.

The BMA result in Figure 46 shows that the 5 mM biotransformation produced approximately 25% less BMA than that other three biotransformations. The lower concentration of observed BMA suggests that a concentration of 5 mM of butanol has had a limiting effect on the production pathway. Interestingly only 0.342 mM of BMA was produced and not the theoretical maximum of 5 mM which may mean that a large portion of butanol is diverted from the desired pathway towards undesirable by-product formation. This result also demonstrates that viability does not appear to be linked to BMA productivity in the biotransformation process and confirms the hypothesis that increasing the butanol concentration can negatively effect viability. It has been shown in literature that butanol can increase permeability of cell membranes and disrupt the function of embedded membrane proteins. These effects can cause limit the growth of *E. coli* in the presence of butanol (Abdelaal *et al.*, 2015).

The 5 mM biotransformation had a stable viability throughout the biotransformation (Table 15) but as the biotransformation is carried out in buffer containing substrates and not growth media the viability may be irrelevant. It was possible that the proteins required for BMA production were produced during the inoculum growth stage and production ceased upon transfer of the cells to a nutrient devoid medium (sodium phosphate buffer (pH 7)). This result supports this theory and demonstrates that the proteins may function regardless of the integrity of the host cell as the 15 mM, 30 mM and 60 mM biotransformations produced more BMA at much lower cell viability counts.

The 15 mM, 30 mM and 60 mM biotransformations all had comparable viability counts at each sample point although varied slightly in the BMA concentration produced. There appears to be slight increases in the BMA concentration as the butanol concentration was reduced, 0.482 mM at 15 mM of butanol, 0.428 mM at 30 mM of butanol and 0.424 mM at 60mM of butanol. This may be due to increased by-product formation which uses other substrates required for BMA production. Thus, it can be concluded that viability does not appear to have an effect on BMA production and as the 60 mM butanol biotransformation does not have a substantial decrease in BMA production reducing the total butanol was not necessary for future batch biotransformations.

4.6. Development of a fed-batch biotransformation

It was shown in the biotransformations where butanol was varied (Figure 46) that reducing the initial butanol, could cause a small increase in BMA concentration and improve cell viability. The biotransformation containing 5 mM of butanol did not produce as much BMA as the other biotransformations which was hypothesised to been due to a lack of butanol availability. It could be seen that in all biotransformations regardless of the butanol concentration BMA production slowed after the 2 hour timepoint. It was possible that as a high concentration of both substrates was present in the batch biotransformation initially, 40 mM of KIV and 60 mM of butanol, that the slow production rate of BMA facilitated the production substrate utilising by-products. Depending on the production rate of the by-products one or both of the substrates may have become limited in the batch biotransformations.

A fed batch biotransformation method would eliminate this problem as the continual addition of substrates would ensure constant availability. A fed-batch biotransformation method would also improve cell viability as a lower concentration of butanol would be present in the media. Although cell viability was not thought to currently impact BMA production improved viability may have relevance in a future bioprocess.

In order to determine if this was the case a fed-batch biotransformation method was produced (Sections 2.2.1.3 and 2.2.2.3) to determine the effect the new method had on cell viability and BMA production. Biotransformations were set up starting with a maximum initial concentration of 5 mM butanol and KIV and a feed of additional substrates each hour. To get a more complete understanding of what the substrate levels were within the biotransformations KIV and butanol were measured at each sample point along with BMA. Along with the substrates iso-butyric acid (IBA) was also analysed over this biotransformation set. IBA was identified as a potential source of KIV diversion within the biotransformations. The first step in the BMA production pathway is the production of IBA-CoA from KIV by the BCKD complex. IBA-CoA can be hydrolysed to produce IBA if this was occurring this would limit the availability of IBA-CoA to the acx4 enzyme therefore limiting BMA production. It was possible that, as no increase in BMA production was seen when increasing butanol concentration (Figure 46), there was a lack of isobutyryl-CoA to utilise the additional butanol.

As the enzyme activity rates were unknown for the Acx4 and AAT, KIV may indeed have been diverted towards by-products such as IBA. As IBA could be analysed on the same HPLC method as KIV and butanol it was easily incorporated into the analysis.

The biotransformation set consisted of six biotransformations labelled 1-6. Each biotransformation had a different starting concentration of substrates and had a different feed of substrates each hour. The outline of all six biotransformations is shown in Table 16, all additions were made after each sample was taken to maintain a steady biotransformation volume. All six biotransformations contained the same inoculum to ensure that variation in BMA concentration was due to the substrate feeding. Samples were taken hourly until 6 hours after which a 23 hour sample was taken. All samples were processed as outlined in section 2.2.3.6 for HPLC analysis and 100µl of the 6 hour sample was spread onto LB + Amp plates to test for cell viability. The cell viability result is shown in Table 17 and the BMA concentration measured is shown in Figure 47.

Table 16: An outline of the six fed batch biotransformations detailing the initial substrate concentrations and subsequent feeds.

Biotransformation Number	**Initial KIV Concentration (mM)**	**Initial butanol Concentration (mM)**	**Addition / hour**
1	0	5	0.05 M Buffer
2	5	0	5 mM KIV
3	5	5	5 mM KIV
4	5	5	5 mM butanol
5	5	5	5 mM KIV + 5 mM butanol
6	1	1	1 mM KIV + 1 mM butanol

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

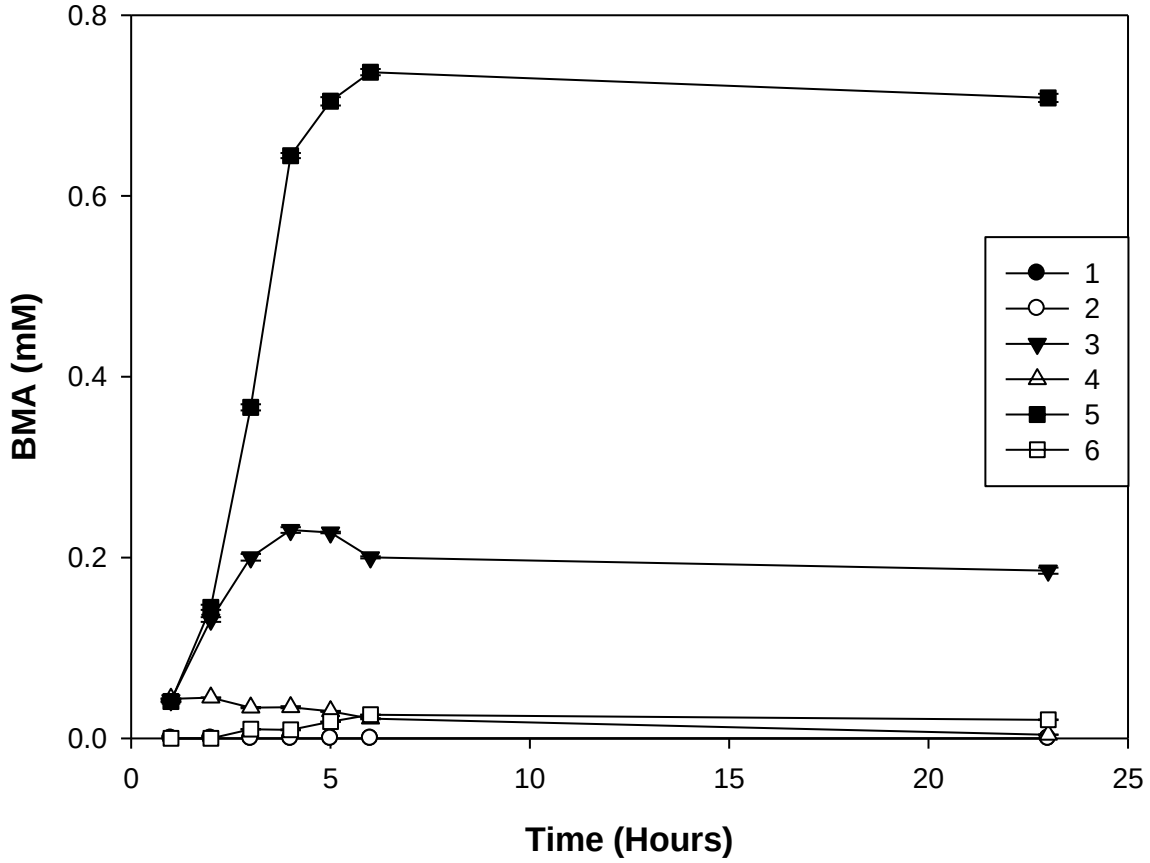


Figure 47: The BMA concentration measured in the samples taken from the fed batch biotransformations 1-6. Each sample was measured in triplicate with the error bars representing the standard deviation of the samples.

From Figure 47 it can be seen that the different substrate feed rates can have a large effect on the BMA concentration produced. The highest BMA titre is seen in biotransformation 5 where 0.74 mM of BMA was produced by 6 hours. This was the highest concentration of BMA achieved so far within a biotransformation. It can also be seen from this biotransformation set when looking at the viability table (Table 17) that the biotransformation which produced the most BMA had the lowest viability count. As biotransformation 5 and 4 both had the same butanol addition profile of 5 mM/ hour this suggested that BMA production may indeed impact cell viability.

Table 17: The cell viability of the 6 fed batch biotransformations. All samples were collected from the 6 hour timepoint.

Biotransformation Number	1	2	3	4	5	6
Colony Count at 10^{-1}	Confluent Growth	Confluent Growth	Confluent Growth	Confluent Growth	213	Confluent Growth

As the KIV, butanol, IBA and BMA levels were all analysed within the biotransformations the relation of the substrates (KIV and butanol) to products (IBA and BMA) can be compared in each biotransformation. The full analysis of the biotransformations is shown in the following figures, biotransformations 1 and 2 in Figure 48, biotransformations 3 and 4 in Figure 49 and 5 and 6 in Figure 50.

Biotransformation 1 (Figure 48) contained 5 mM of butanol initially with a 0.05 M sodium phosphate buffer (pH 7) addition each hour. No KIV was present in this biotransformation. It can be seen that as expected there was no BMA produced in this biotransformation. The butanol concentration drops over time suggesting that a butanol by-product is formed in the biotransformation in the absence of KIV. It was possible that this by-product was butyl acetate, this can be formed in the absence of KIV through the pathway of pyruvate to acetyl-CoA to butyl acetate. This could not be confirmed as butyl ester analysis was not carried out for this biotransformation set as the aim was to determine the effect of substrate feeding on BMA production.

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

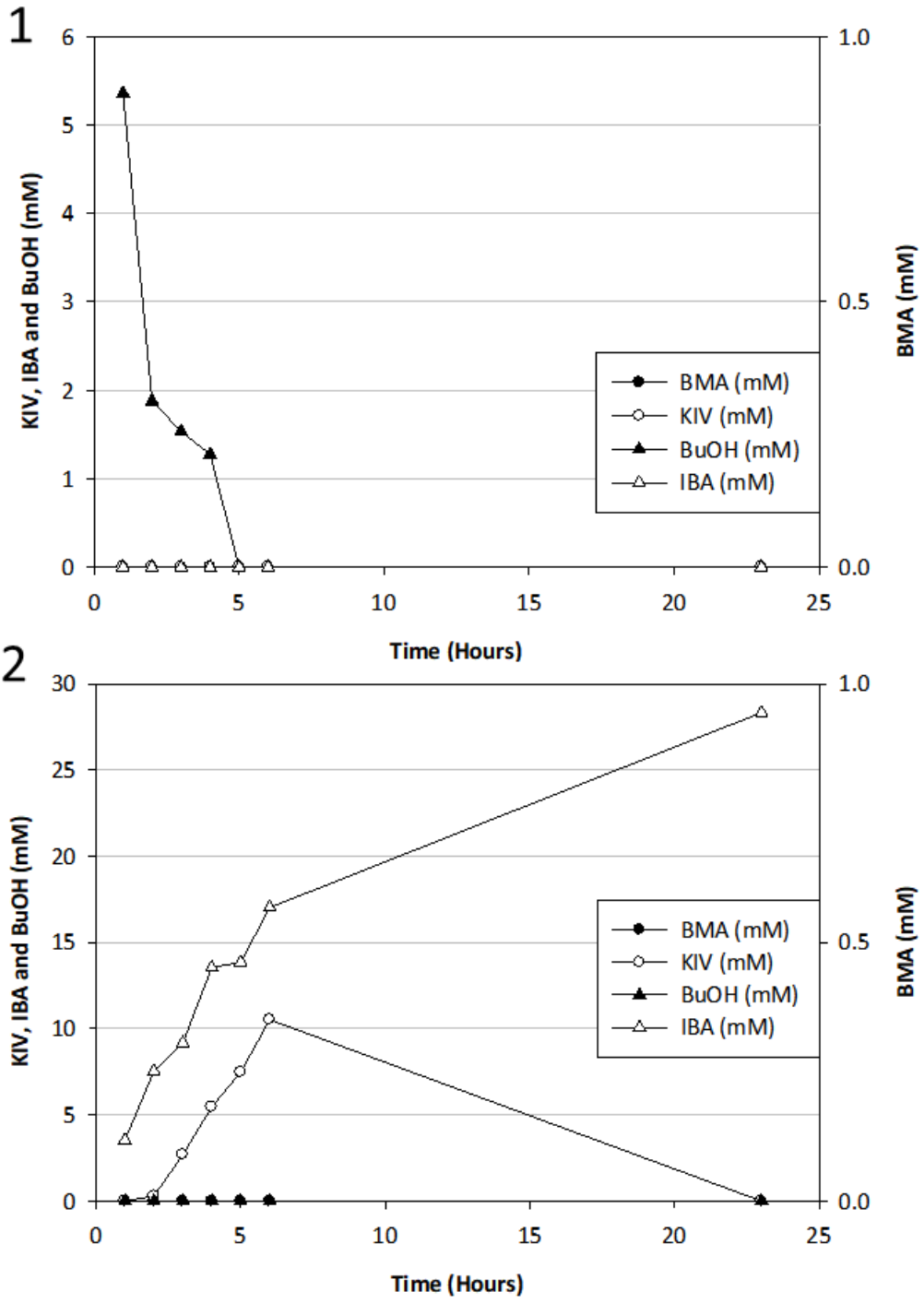


Figure 48: The full HPLC analysis of fed batch biotransformations 1 and 2 using LUC0585. Each sample was measured in triplicate and the error bars represent the standard deviation of the samples.

Biotransformation 2 (Figure 48) contained no butanol with a 5 mM initial concentration of KIV and a 5 mM feed of KIV being added each hour. As no butanol was present in this biotransformation no BMA was produced. It can be seen that IBA is continuously produced during the biotransformation utilising the KIV feed of 5 mM/ hour. This confirmed the hypothesis that IBA can be produced within LUC0585 when KIV is available in the biotransformation. The IBA titre reached 28.34 mM at 23 hours, at this time point all of the KIV in the biotransformation was depleted. As a total of 40 mM of KIV was added by 23 hours and was completely depleted it can be seen that IBA accounts for 70.9 % of the KIV utilised and is the largest by-product from KIV. The fate of the last 19.1% of KIV is unknown. It is possible that as the cells within this biotransformation had good viability that the KIV was metabolised for valine, leucine and iso-leucine production.

Biotransformation 3 (Figure 49) contained initially a 5 mM concentration of KIV and butanol with only a 5 mM concentration of KIV added each hour. This biotransformation was designed to investigate the impact a small quantity of butanol and a steady supply of KIV had on BMA production. It can be seen from the biotransformation that butanol is utilised slowly in the biotransformation with the initial 5 mM of butanol taking 4 hours to be depleted. In comparison to the butanol the KIV was utilised quickly with the initial 5 mM of KIV completely utilised in the first hour. A low concentration of BMA (0.23 mM) was produced in this biotransformation which was expected as only 5 mM of butanol was available. The BMA production continued until 3 hours after this timepoint butanol became depleted and therefore BMA production stopped.

The slow butanol utilisation rate suggested that either the AAT enzyme had a slow reaction rate as the available butanol is not being utilised quickly or that the Acx4 enzyme had a slow reaction rate limiting the availability of methacryl-CoA for the AAT. As observed in biotransformation 2 when KIV is fed into a biotransformation a high titre of IBA was produced, in biotransformation 3 25.18 mM of IBA was produced by 23 hours. As 40 mM of KIV was available by this point in the biotransformation the IBA production accounts for 63% of the KIV utilised. As seen in biotransformation 2 a large portion of the KIV fed into the biotransformation is unaccounted for.

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

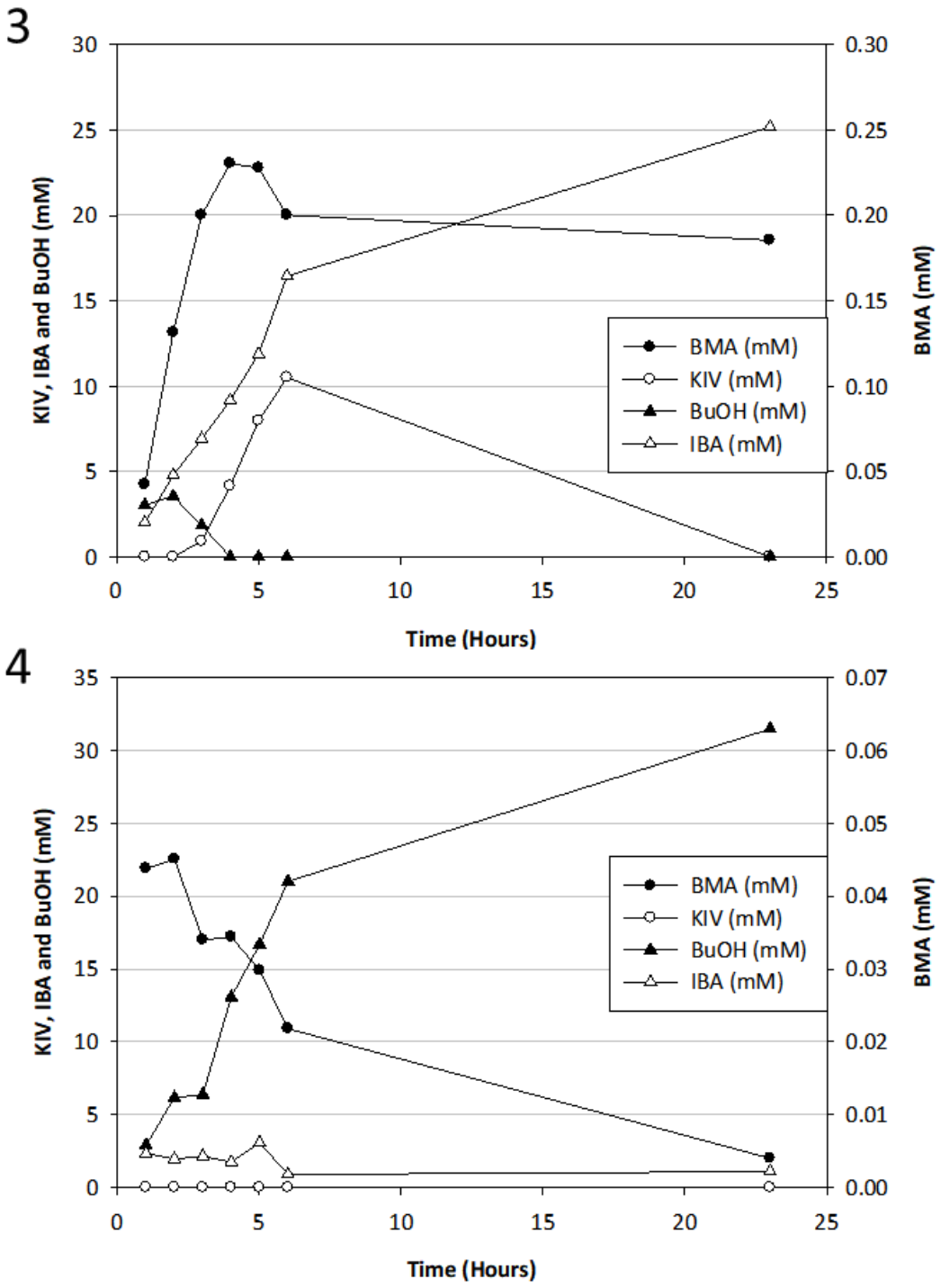


Figure 49: The full HPLC analysis of fed batch biotransformations 3 and 4 using LUC0585. Each sample was measured in triplicate and the error bars represent the standard deviation of the samples.

Biotransformation 4 (Figure 49) was the opposite of biotransformation 3, again the initial concentration of substrates was 5 mM of both KIV and butanol but the feed was 5 mM of butanol each hour. This biotransformation was designed to investigate the impact a small quantity of KIV and a steady supply of butanol had on BMA production. From the biotransformation it can be seen that the 5 mM of KIV was utilised within the first hour with all the BMA and IBA being produced in this hour. This demonstrates that the BCKD complex can utilise at least 5 mM of KIV/ hour. As IBA is produced this suggests that the acx4 and AAT pathway enzymes operate at a slower rate allowing IBA-CoA to be hydrolysed to IBA. A small quantity of BMA was produced in the first hour ,0.044 mM, which when combined with the IBA produced in the first hour ,2.33 mM, produced a total of 2.37 mM of product from 5 mM of KIV. Again a large quantity of KIV is unaccounted for suggesting that there may be by-products other than IBA which have a demand on KIV in the biotransformation. As 5 mM of butanol was added to the biotransformation each hour a concentration of 35 mM would have been expected at 6 hours had no butanol been utilised. A butanol concentration of 21.01 mM was measured at 6 hours and as only 0.0438 mM of BMA was produced either butanol was leaving the vessel during sampling or that butanol by-products were being produced.

Biotransformations 5 and 6 (Figure 50) were similar in design as they both had feeds of KIV and butanol every hour. Biotransformation 6 had a 1mM initial concentration of both butanol and KIV along with a 1 mM feed of both at each hour after sampling. The aim of this biotransformation was to determine if a lower concentration of both substrates fed over time resulted in less by-products and a higher BMA yield. It can be seen from Figure 50 that biotransformation 6 produced 0.026 mM of BMA at 6 hours which is low in comparison to biotransformation 5. In biotransformation 6 the butanol concentration increased over time showing that it was not limited, the KIV concentration was measured at 0 mM throughout. This suggested that the cause of the low BMA concentration measured was the lack of available IBA-CoA which was not diverted to by-product such as IBA. The IBA concentration measured in biotransformation 6 was 3.19 mM contributing to the KIV depletion and low BMA yield in the biotransformation. Biotransformation 6 demonstrated that in strain LUC0585 1 mM of butanol/ hour is sufficient to allow a build up to occur after 2 hours. Before the 3 hour sample point no BMA was produced therefore butanol must have been

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

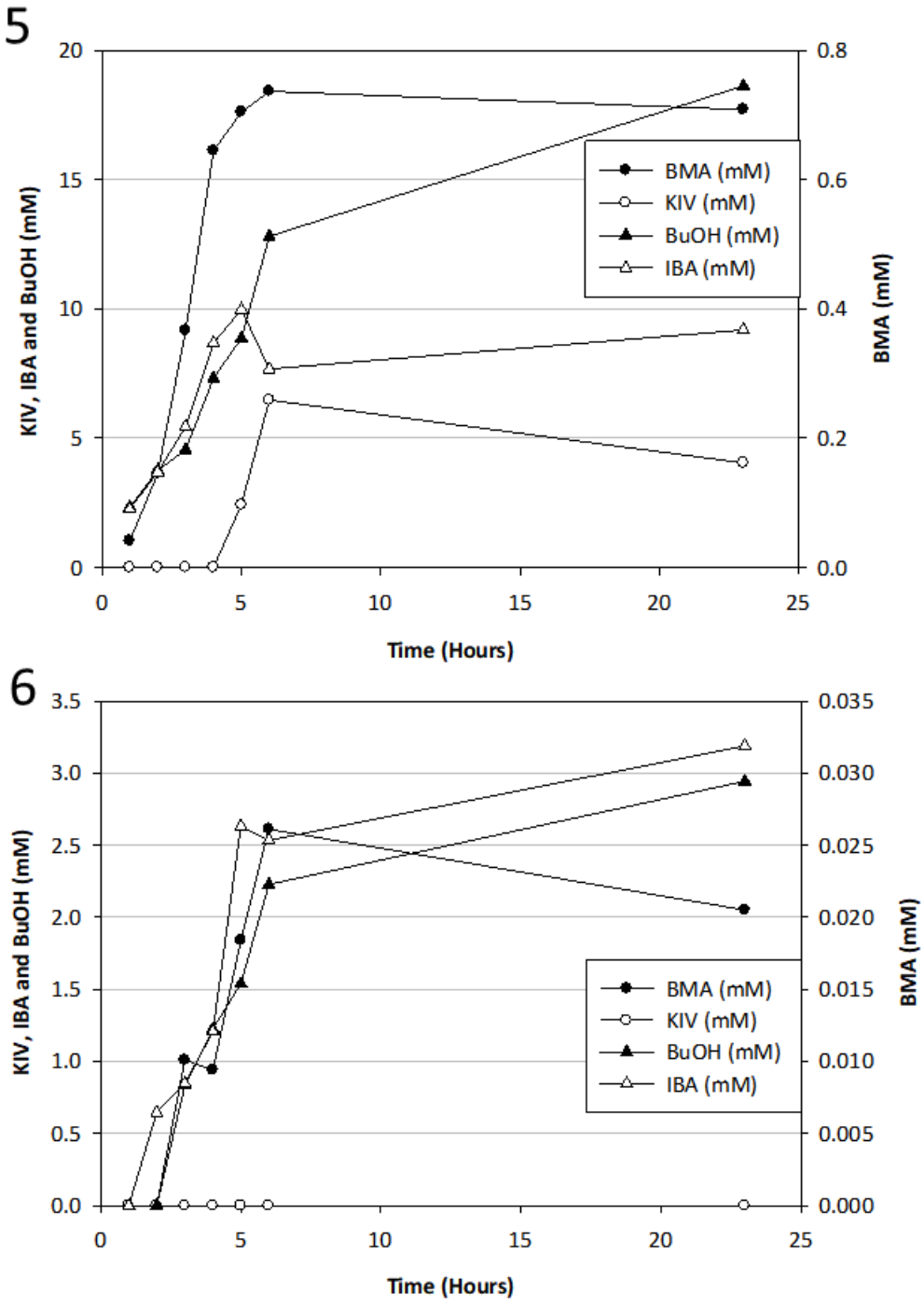


Figure 50: The full HPLC analysis of fed batch biotransformations 5 and 6 using LUC0585. Each sample was measured in triplicate and the error bars represent the standard deviation of the samples.

utilised for by-products. This suggested that butanol by-product formation occurs from the beginning of the biotransformations.

Biotransformation 5 had an hourly feed of 5 mM of KIV and butanol, this resulted in the highest BMA concentration observed in this set of 0.737 mM at 6 hours. This BMA titre was 66.03% higher than the batch biotransformation of LUC0585 (Figure 43) and 34.6% higher than the previous highest titre of 0.482 mM in the batch biotransformation with 15 mM of butanol and 40 mM of KIV (Figure 46). As suggested in the biotransformations using lower concentrations of butanol a higher BMA yield was observed when the available butanol in the biotransformation was lowered. The IBA concentration produced was lower than that observed in biotransformations 2 and 3 with only 10 mM being produced. Unusually the IBA production in biotransformation 5 slows when BMA production slows and although KIV is available it is not converted to IBA as previously observed in biotransformations 2 and 3. This may have been due to either the higher concentration of BMA produced or the cell viability as this biotransformation was the only one which did not have confluent growth at 6 hours. Whether this was either of the factors which was responsible was not immediately apparent.

The full analysis of all six fed batch biotransformations provided several conclusions. Biotransformation 1 and 2 demonstrated that each substrate had by-products associated with it as both were utilised in the biotransformations. In biotransformation 2 the by-product was identified as IBA. Biotransformation 3 demonstrated that butanol is utilised slowly with 5 mM being utilised in 4 hours. Biotransformation 4 demonstrated that KIV is utilised at a rate of at least 5 mM/ hour with all IBA and BMA being produced in the first hour. The IBA and BMA concentrations combined did not equate to the KIV utilised therefore KIV is also utilised by another pathway.

Biotransformation 6 demonstrated that a 1 mM feed of butanol was sufficient beyond 2 hours but, as shown by biotransformation 4, 1 mM of KIV/hour was not a sufficient feed and limited BMA production. Biotransformation 5 produced the highest concentration of BMA at 0.74 mM and as such the fed batch biotransformation method was concluded to improve BMA production which was initially hypothesised. A 5 mM feed/ hour of KIV was insufficient with KIV limited for the first 4 hours of the biotransformation, by comparison the 5 mM feed/ hour of butanol was sufficient to prevent limitation. KIV was not fully utilised for IBA production in

this biotransformation which may have been due to the low cell viability or the higher concentration of BMA.

In order to improve the BMA concentration, it was hypothesised that the feed of KIV would need to be increased above 5 mM/ hour for the first 4 hours of the biotransformation. As the concentration of BMA produced in biotransformation 5 was the highest observed at this point this experiment required repetition to confirm the result.

4.6.1. Repetition of the superior fed batch biotransformation

In order to confirm if the high BMA concentration observed in the previous experiment (Section 4.6, biotransformation 5) was reproducible two replicate biotransformations were set up. Both replicates were set up from the same inoculum and used the same stocks as the original biotransformation. Samples were taken hourly until 6 hours at which point the biotransformations were stopped. No 23 hour sample was taken as the original result showed that BMA production had stopped by 6 hours. There should have been no BMA present in the biotransformations at 0 hours as no butanol is present during the inoculum growth process in order to confirm this 0 hour samples were taken for BMA analysis. The result of the BMA analysis is shown Figure 51. From the figure it can be seen that the two replicates (2 and 3) had nearly identical BMA profiles. This confirms that replicates within a set of biotransformations are reproducible and that there is little variation associated with sampling analysis of BMA samples.

When comparing the original biotransformation with the two replicates it can be seen that the original biotransformation produced a lower titre of BMA than the replicates. At 6 hours the original biotransformation had a titre of 0.737 mM which is 21.4% lower than the BMA titre of 0.938 mM observed the highest producing replicate. From the figure it can be seen that the difference in the final titre of BMA is due to the production rate of the original over the first 2 hours of the biotransformation. The BMA production rate of biotransformation 1 over the first 2 hours was 0.073 mM/h. Biotransformation 2 and 3 had BMA production rates of 0.162 mM/h and 0.159 mM/h both of which are more than double that of biotransformation 1. Although the initial production rates differ the production rate between

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

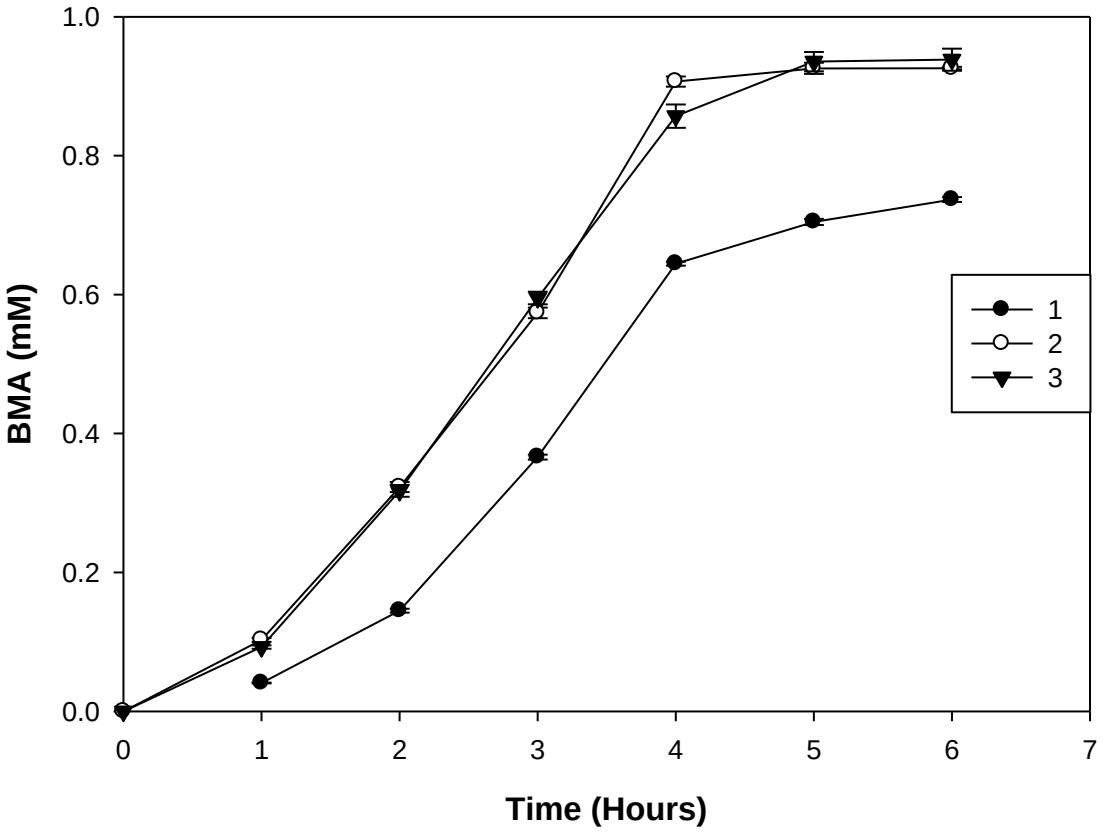


Figure 51: The BMA concentration measured in the samples collected from the original biotransformation (1) and the two replicate experiments (2 and 3). The samples were analysed in triplicate and the error bars represent the standard deviation of the samples.

2 and 4 hours in all three biotransformations are comparable at 0.25 mM/h (1), 0.29 mM/h (2) and 0.27 mM/h (3). This shows that the enzymatic activity in the original biotransformation was unaffected from the initial lag. The biotransformations all display the same production profile with BMA production slowing after 4 hours.

As the biotransformations were identical and used the same substrate stocks the most probable cause of the variation is the inoculum growth phase of the biotransformations in which the inoculum is grown, concentrated, resuspended in buffer and transferred into the biotransformation vessel.

A standardised method was used for this step which is outlined in section 2.2.1.3 which should have kept the variation at this stage in the process to a minimum. It is possible that the OD600 of the overnight inoculum may have been the cause of the variation observed. The OD600 of the original biotransformation inoculum was 6.49 and the OD600 of the replicates was 8.09 for replicate 2 and 6.86 for replicate 3. As the two replicates have a similar BMA production profile while having different inoculum OD600's it does not appear that this factor was the source of the variability. As the BMA titre at this point in time was much lower than necessary for commercial viability the possibility of the inoculum OD600 causing variation in final titre was not investigated further at this point with bioprocess and strain variation the priority.

It was concluded that the high BMA concentration observed in the previous experiment was reproducible and that the fed batch biotransformation method with a 5 mM feed/ hour of KIV and butanol improved the BMA concentration produced. The original experiment had a lower initial production rate of BMA causing the final concentration to be lower the reason for this was not clear, it was noted that variation may occur between identical experiments carried out in separate biotransformation sets.

4.6.2. LUC0585 fed-batch biotransformation feed optimisation

The biotransformation with a 5 mM feed of KIV and butanol produced the highest concentration of BMA also showed that KIV was limited for the first 4 hours of the biotransformation (Section 4.6-Biotransformation 5). In order to determine whether this KIV limitation had an effect on BMA production a biotransformation was set up using a 10 mM initial concentration of KIV and butanol and a 10 mM/ hour feed of both substrates. 10 mM of each substrate was selected for the feed concentration as it was hypothesised that increasing the KIV availability would increase the BMA concentration produced and therefore would require more butanol. The biotransformation was set up as outlined in section 2.2.1.3 and 2.2.2.3 for fed batch biotransformations.

Samples were taken periodically for BMA, IBA and KIV analysis. The butanol data for this biotransformation was inaccurate and not able to be used as the samples required a higher dilution factor than previous experiments due to the high levels of KIV and IBA. Butanol, KIV and IBA were analysed on the same HPLC method on which butanol had the lowest peak intensity. The increased dilution of the samples thus caused neighbouring peaks to interfere with the butanol peak, resulting in large error values and inconsistent readings between injections. Previous results had shown that butanol was not limited in 5 mM / hour fed batch biotransformations (Section 4.6) therefore it was not expected to be limited when the feed was doubled. Previous experiments have shown that the fate of some butanol is unknown in the biotransformations and thus an accurate mass balance would not have been possible using the butanol data. For these reasons the butanol data was not included in this dataset.

The IBA, KIV and BMA concentration measured in the samples is shown in Figure 52. From the figure it can be seen that a lower BMA concentration was produced in this biotransformation compared to the previous 5 mM / hour feed experiments as only 0.49 mM of BMA was produced by 24 hours in this experiment. This was a 34% reduction in BMA concentration compared to the 5 mM/ hour feed experiment. The reduction observed may be due to a higher level of butanol in the biotransformation from the 10 mM/ hour feed although this could not be confirmed due to the absence of the butanol data.

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

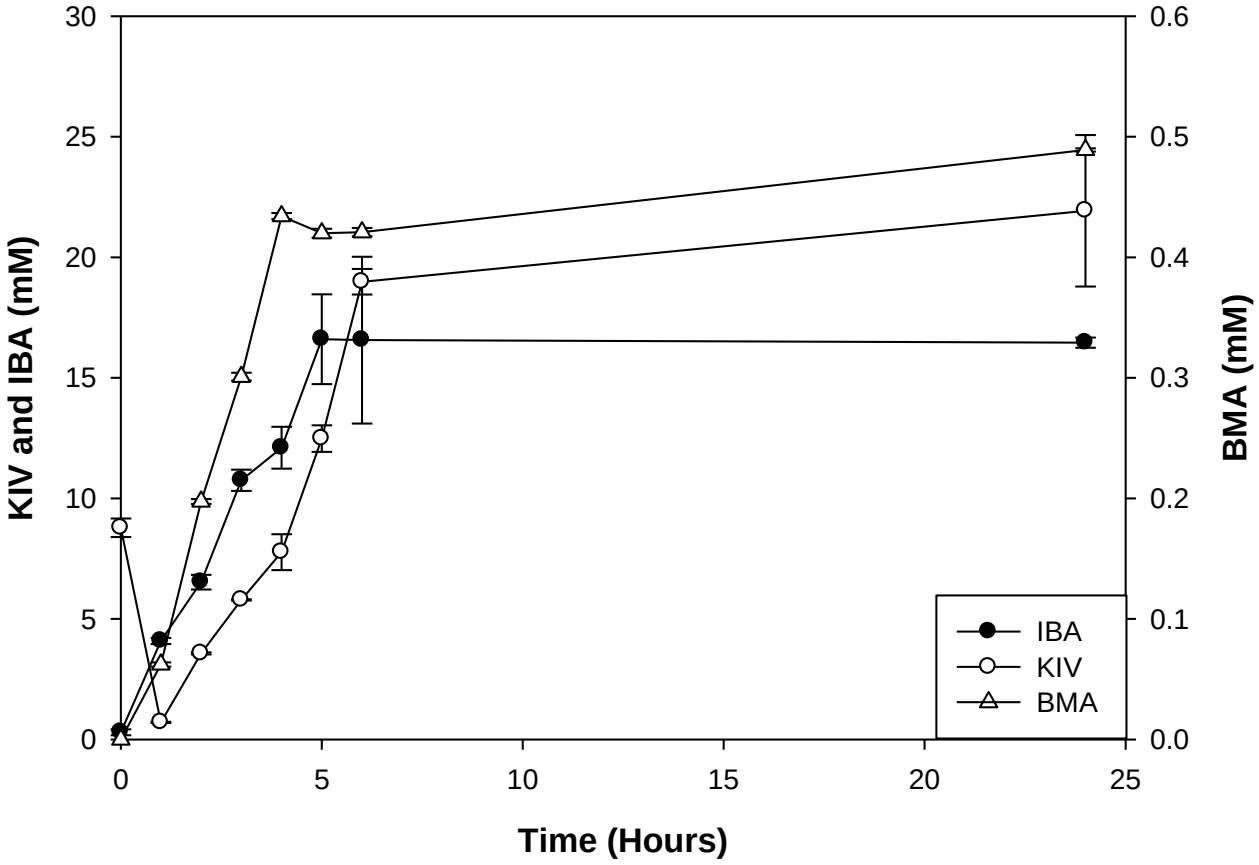


Figure 52: The BMA, IBA and KIV concentrations measured in samples from the fed batch biotransformation of LUC0585. The samples were measured in triplicate and the error bars represent the standard deviation of the samples.

The 10 mM/ hour KIV feed in this biotransformation prevented KIV from becoming limited with the KIV concentration dropping to 0.7 mM at 1 hour before rising as the biotransformation continued. The IBA concentration within the biotransformation reached 16.6 mM at 5 hours at which point the production of IBA stopped. IBA by-production remains the largest drain of KIV in the process although as KIV levels did not reach 0 mM IBA production did not limit the KIV available to the BMA production pathway. The final concentration of KIV was 21.93 mM, the final concentration of IBA was 16.46 mM and the final concentration of BMA was 0.49 mM totalling 38.88 mM of KIV accounted for. As 70 mM of KIV was added to the biotransformation in total 44.5% of the KIV in the biotransformation is unaccounted for suggesting additional KIV metabolism possibly for valine, leucine and iso-leucine production.

As a higher BMA concentration was not observed when KIV was no longer limited in the fed batch biotransformation it was concluded that substrate limitation may not be responsible for preventing high concentrations of BMA from being reached. As it was possible that the higher butanol feed was responsible for the lower concentration of BMA observed in this biotransformation further feed refinement was required.

4.6.3. Further biotransformation feed optimisation

The previous experiment (Section 4.6.2) showed that a 10 mM/ hour KIV feed could prevent limitation of KIV in the biotransformation. This, however did not result in a higher BMA titre. It was possible that the higher butanol feed of 10 mM/ hour had a negative effect on production as it may have accumulated in the biotransformation. Therefore a biotransformation set was produced with four different substrate feed patterns to determine the effect the different feeds of each substrate had on BMA production. The 4 biotransformations were set up as outlined in Table 18.

Table 18: The outline of the four fed batch biotransformations with different initial concentrations and feed concentrations of KIV and butanol.

Biotransformation Number	**Initial KIV (mM)**	**Initial butanol (mM)**	**Substrate feed (mM/h)**
1	5	5	5 (KIV) 5 (butanol)
2	10	10	10 (KIV) 10 (butanol)
3	5	10	5 (KIV) 10 (butanol)
4	10	5	10 (KIV) 5 (butanol)

The biotransformations were set up and carried out as described in section 2.2.1.3 and 2.2.2.3 for fed batch biotransformations. The same inoculum was used for all four biotransformations which helps to minimalise variation in this set. The BMA analysis for the set was carried out by HPLC. In contrast to the previous results the HPLC analysis for BMA was carried out using a gradient method (Section 2.2.3.3) as opposed to the isocratic method (Section 2.2.3.4) used

previously, both methods were developed by Ingenza Ltd. The gradient method was developed because in the isocratic method the BMA peak was closely integrated with other peaks resulting from the biotransformation media which may have altered the peak size and therefore effected the BMA concentration measured. The gradient method provided a cleaner chromatogram for analysis resulting in an accurate value for BMA measurements.

The BMA concentration measured in the 4 biotransformations is shown in Figure 53. It can be seen from Figure 53 that the new HPLC method provided an accurate lower BMA concentration. Biotransformation 1 in Figure 53 had a BMA concentration of 0.46 mM at 6 hours which is lower than all of the previous replicates (1, 2 and 3), Figure 51, which had BMA concentrations of 0.74 mM, 0.93 mM and 0.94 mM respectively. It has been shown previously that the BMA concentration can vary between sets although as the chromatograms show a clear improvement in the separation of the BMA peak from others, in the gradient method, the improved HPLC method was the likely cause of the BMA concentration drop observed.

When the 4 biotransformations within this set were compared it could be seen that all four biotransformation followed a BMA production profile which had been observed previously, with the highest rate of BMA production occurring in the first 3 hours and the production rate slowing down thereafter. The two biotransformations with the 10 mM KIV feed (2 and 4) had production rates of 0.136 mM/h and 0.141 mM/h over the first 3 hours which was higher than that of the biotransformations with the 5 mM feed of KIV (1 and 3) which had production rates of 0.119 mM/h and 0.118 mM/h over the same period. The biotransformations with a higher KIV feed also end at a higher BMA titre over the counterparts with the same butanol feed, 4 has a higher final titre at 0.68 mM than 1 at 0.56 mM and 2 has a higher final titre at 0.55 mM than 3 at 0.43 mM.

In conclusion the different butanol feeds of 5 mM/ h and 10 mM/h appeared to alter the BMA production rate after 3 hours with the 5 mM butanol biotransformations (1 and 4) having a higher production rate between 3 and 5 hours than biotransformations 2 and 3. This may explain why the fed batch biotransformations of LUC0585 had a higher BMA titre than batch biotransformations as the higher butanol concentration has a negative effect on the production rate after 3 hours. Why this negative effect occurred is not obvious, but it may be linked to cell viability as higher butanol levels were linked to lower viability (Figure 46).

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

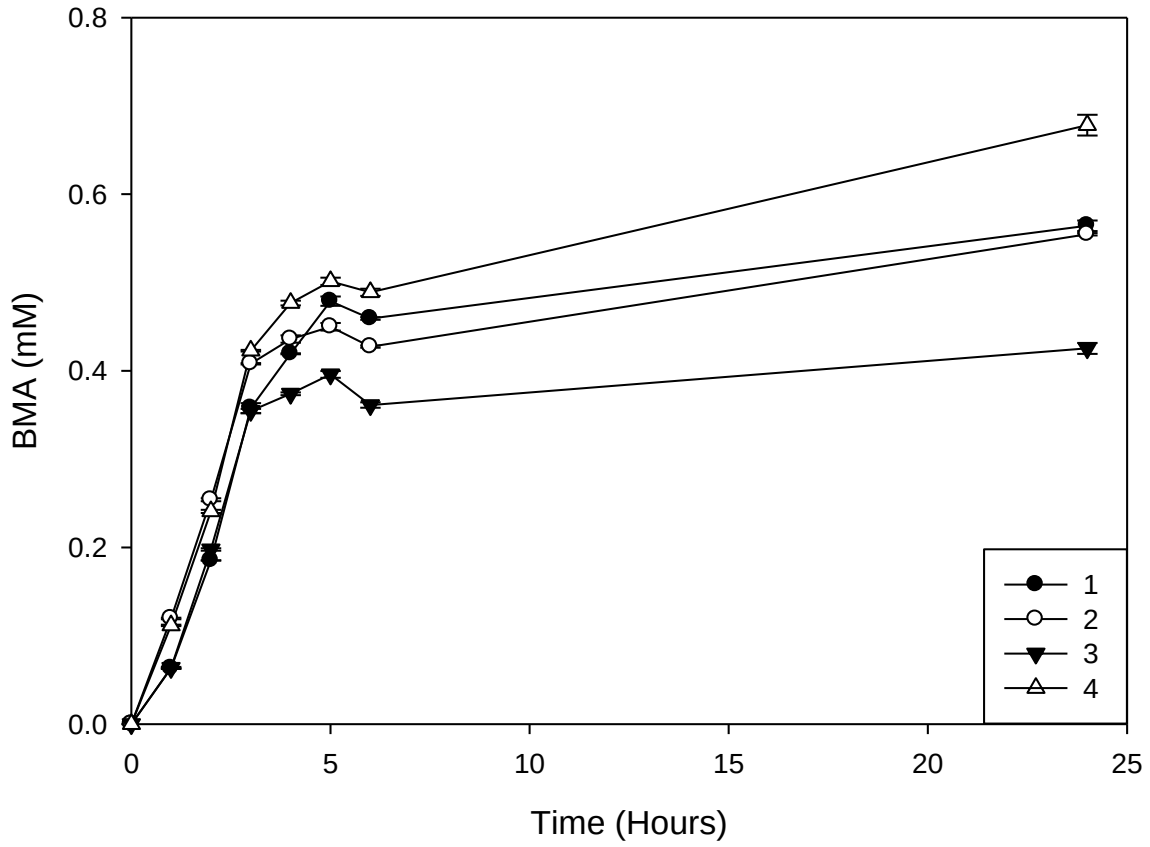


Figure 53: The BMA concentration measured from the four fed batch biotransformations analysed using the gradient HPLC method. All samples were measured in triplicate with the error bars representing the standard deviation of the samples.

In this biotransformation set increasing the KIV feed from 5 mM/ hour to 10 mM/ hour was shown to increase the final BMA concentration by 16.81% over the control (1) which had previously been shown to have the highest concentration. This was due to KIV no longer being limited in the biotransformation, which had been shown previously to occur in the 5 mM feed biotransformation (Section 4.6). As neither substrate should be limited in biotransformation 4 BMA production would be expected to continue at a high production rate. As this was not the case further investigation was needed to determine the factor which limits BMA production.

4.7. Biotransformation of LUC0585 with Lund media

Adaptations to the biotransformation method for LUC0585, changing the butanol concentration and altering the KIV and butanol to a fed batch process provided insight into the process with an observed improvement to the BMA concentration. These adaptations were unable to prolong production of BMA beyond 4 or 5 hours even when both substrates were available. This suggested that one or more of the enzymes responsible for the BMA production pathway, on the pMMA 050 plasmid (Figure 54), were becoming inactive in the biotransformation. It was possible that as the biotransformation media consisted only of sodium phosphate buffer (pH 7), KIV and butanol that a more comprehensive media may increase the length of BMA production through continued enzyme production.

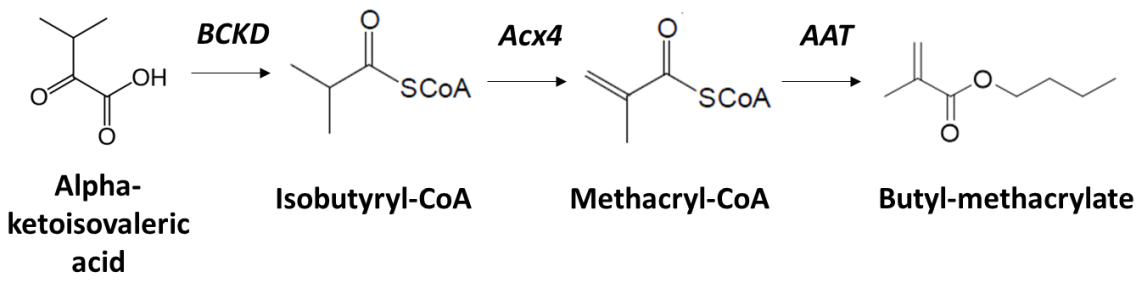


Figure 54: The three enzymes on the pMMA 050 plasmid required for the BMA production from KIV in E. coli.

In order to determine if keeping the biomass active in the biotransformation would improve the BMA concentration produced a biotransformation was set up where the overnight inoculum was grown in Lund media and the biotransformation was carried out in Lund media. Lund media was selected as it is a more complete media which can facilitate the growth of *E. coli* and has been used by Ingenza Ltd for the development of large scale bioprocesses (50,000 L). It was hypothesised that a growth media may allow the enzymes to continue to function beyond 5 hours. The composition of the Lund media is outlined in the methods (Section 2.2.4.1). The overnight inoculum in Lund media was supplemented with ampicillin which is required for plasmid retention in LUC0585. The Lund media was supplemented with 40 mM of KIV and 60 mM of butanol in order to have been comparable to previous batch biotransformations of LUC0585.

Samples were taken hourly for BMA analysis by HPLC to determine if more BMA was produced in the Lund biotransformation media. The result of the BMA analysis is shown in Figure 55. It can be seen from the figure that a low concentration of 0.08 mM was produced in the Lund biotransformation by 4 hours. This was less than previous batch biotransformations (Figure 46) where 0.42 mM of BMA was produced at 4 hours when the same quantity of butanol was available at 60 mM. The hypothesis was that having a more comprehensive media may allow BMA production to continue beyond 4-5 hours as the factor limiting the enzymes may have continued to be produced in the presence of a carbon source.

This was not the case with an initial lag in production followed by a low level of BMA production in the biotransformation. There are multiple reasons why BMA production may have been altered so. It was possible that the Lund biotransformation media improved the integrity of the cell membranes which have been shown to become damaged in the standard biotransformation process due to osmotic stress from the BMA ester and butanol toxicity. This would in turn limit butanol and KIV availability to the pathway enzymes and limit the export of BMA through the membrane for detection. It is also possible that as Lund media contains the substrates required for growth that the KIV was metabolised for valine, leucine or iso- leucine and therefore not available to the branched chain keto acid dehydrogenase (BCKD) to produce IBA- CoA for BMA production.

From this biotransformation (Figure 55) it was seen that the original biotransformation method of resuspending the cells in sodium phosphate buffer (pH 7) was superior to a biotransformation in Lund media for BMA production (Sections 2.2.1.3 and 2.2.2.3). The biotransformation was unable to produce BMA at a high rate beyond 4-5 hours the reason for which was still not known. As it did not appear that further BMA concentration and rate improvements could be made while only altering the biotransformation process the future experiments focused on adaptation of the LUC0585 strain to remove by-products with the aims of improving the BMA production rate towards the target of 2 g/L/h.

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

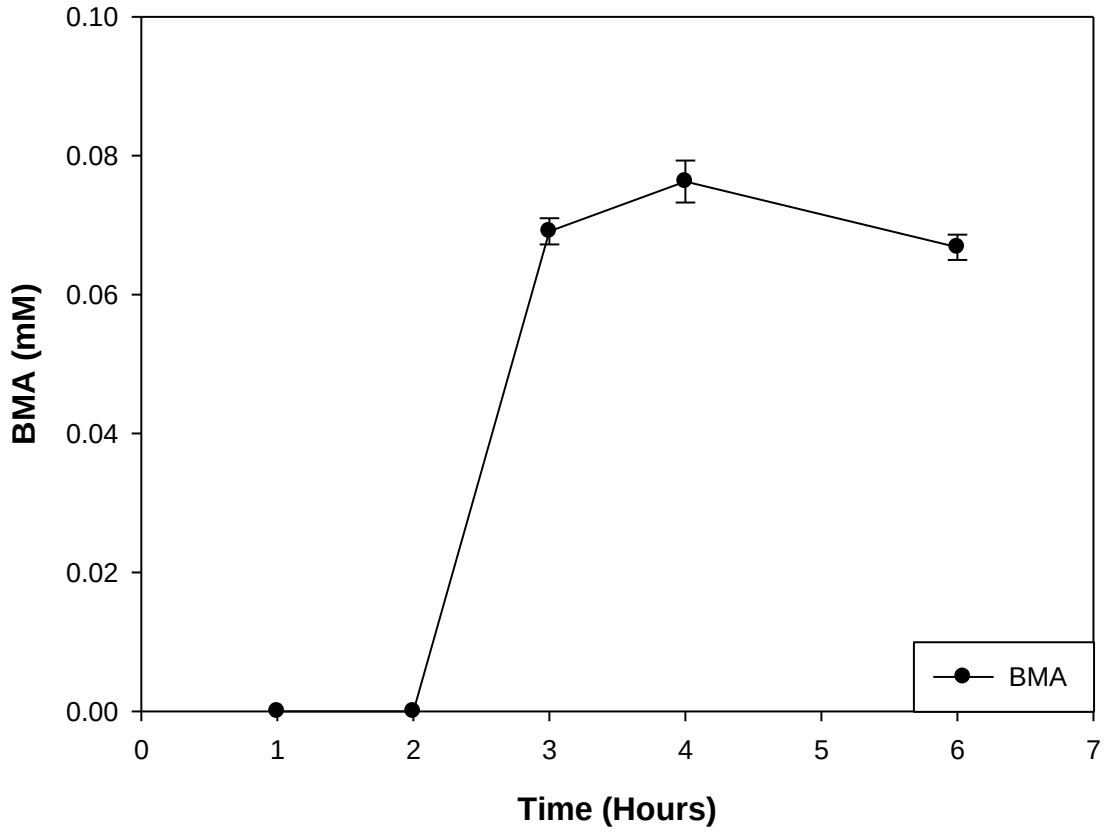


Figure 55: The BMA concentration produced in the batch biotransformation carried out in Lund media. The samples were measured in triplicate with the error bars representing the standard deviation of the samples.

4.8. By-product elimination from the biotransformation

It had been shown previously that changing the biotransformation to a fed batch method improved BMA production although this caused limitation of KIV within the biotransformation (Section 4.6). When the feed was increased to prevent substrate limitation a slight increase in BMA production rate was observed although the maximum BMA concentration produced was low in comparison to the quantity of KIV utilised (Section 4.6.2). As IBA had been shown to be a significant by-product in the fed batch biotransformation process with a concentration of 16.6 mM being produced when neither substrate was limited subverting a large portion of the available KIV (Section 4.6.2). It was therefore hypothesised that reducing or eliminating IBA production may improve the BMA yield through a greater availability of KIV during the initial production period. Therefore LUC0585 was subject to further modification with the aim of improving the BMA titre.

To attempt to limit or eliminate the IBA production by LUC0585 a Δ*yciA* strain was created. The *yciA* gene was selected for deletion because in *E. coli yciA* encodes an Acyl-CoA thioester hydrolase (Sonntag *et al, 2014*). It was hypothesised that IBA in the biotransformations was produced by hydrolysis of isobutyryl CoA which is produced from KIV by the BCKD enzyme Figure 56. Deletion of *yciA* has been shown previously to reduce butyrate formation from butyryl- CoA (Volker *et al.*, 2014). Therefore it was possible that the deletion of *yciA* would reduce the hydrolysis of IBA-CoA to IBA in the biotransformations. It was possible that this would result in a higher BMA concentration as more IBA-CoA would be available to proceed through the BMA production pathway via the Acx4 enzyme.

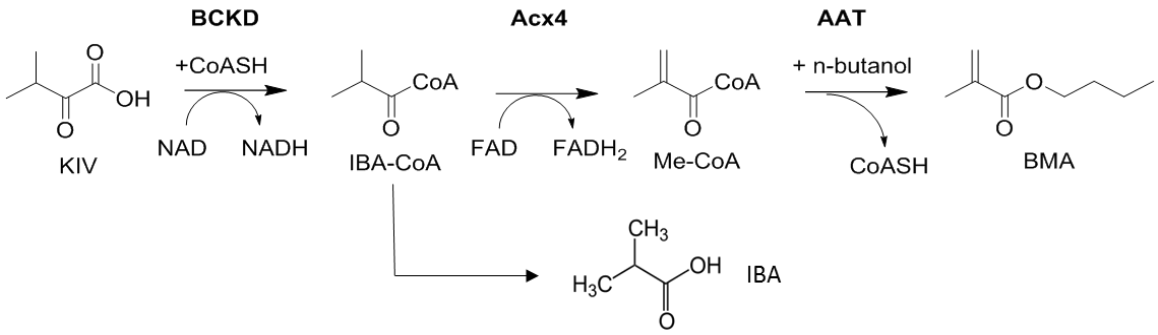


Figure 56: The BMA production pathway in the biotransformations showing IBA production from IBA-CoA.

The new strain was labelled LUC0669 and was tested for IBA and BMA production. The KIV concentration was also monitored. As less KIV was expected to be utilised the fed batch biotransformation with a 5 mM feed of both KIV and butanol was used for this experiment. The inoculum preparation and fed-batch biotransformation method is outlined in the method sections 2.2.1.3 and 2.2.2.3. Samples were taken at each time point before the KIV and butanol feed was added. Samples were analysed by HPLC for BMA, IBA and KIV concentrations. The result of the HPLC analysis is shown in Figure 57.

The fed batch biotransformation of LUC0585 (Figure 50, biotransformation 5) produced 0.74 mM of BMA, 9.98 mM of IBA and had 6.48 mM of KIV available. LUC0669 in Figure 57 had in comparison 0.51 mM of BMA, 8.67 mM of IBA and 0.64 mM of KIV available at 6 hours. It was seen that in LUC0669 the deletion of *yciA* did not result in the elimination of IBA from the biotransformation with LUC0669 producing only 13 % less. This suggested that there may be other hydrolases which were still active within the cells and may need to be deleted to reduce IBA production.

When comparing the KIV usage in both strains LUC0585 had more KIV available at 6 hours which suggested that less KIV was being utilised during the biotransformation. Why the deletion of *yciA* caused a greater quantity of KIV to be utilised is not known. A lower concentration of BMA was produced in LUC0669 demonstrating that the deletion of *yciA* did not have a positive effect on BMA production. Further work was required in order to eliminate IBA production, it is likely that another hydrolase is present which would need to be identified and deleted in order to prevent the breakdown of IBA-CoA.

As KIV was continuously added in both LUC0585 and LUC0669 BMA should have continued to be produced. As this was not the case it was possible that the bottleneck in the biotransformation was either the Acx4 or the AAT enzymes as we know large quantities of IBA-CoA is produced by the BCKD indicated by the high concentration of IBA produced.

Strain Genotypes
LUC0669- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4) ΔyciA*

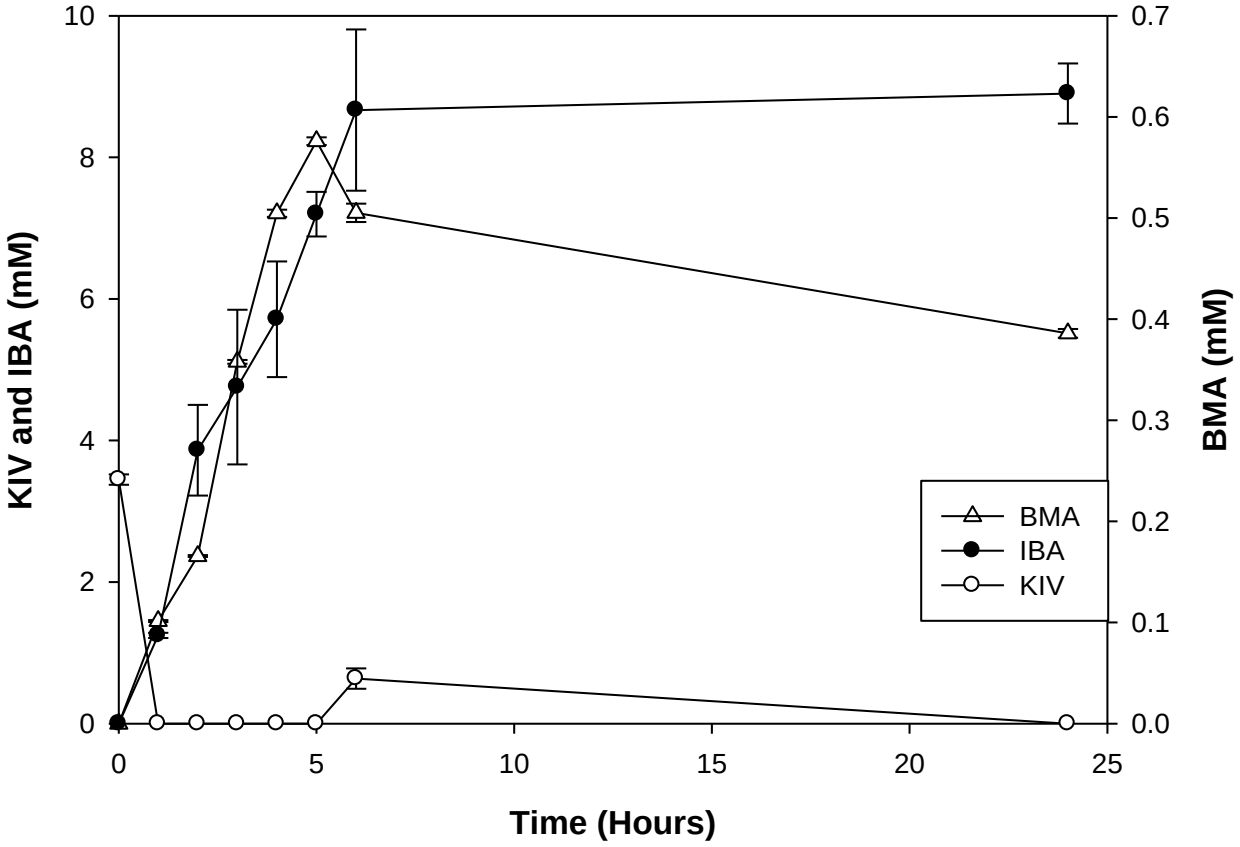


Figure 57: The IBA, BMA and KIV concentrations measured in samples collected from the fed batch biotransformation of LUC0669. All measurements were carried out in triplicate with the error bars representing the standard deviation of the samples.

4.9. Determination of Acx4 activity through 3- HIBA production

It has been seen in previous experiments that when KIV is available in the biotransformations that larger quantities of IBA are produced than BMA. This suggests that IBA- CoA produced by the BCKD may be accumulated due to a low activity of the Acx4 enzyme. This would lead to the formation of IBA which is an undesirable by-product in the biotransformation. In order to determine if the Acx4 was the limiting reaction step in the BMA production pathway a biotransformation was carried out using a strain which can produce 3-hydroxyisobutyric acid (3-HIBA) which uses the Axc4 enzyme in the production pathway. 3-HIBA occurs naturally as an intermediate in the valine degradation pathway in microorganisms such as *Pseudomonas putida* (Marshall and Sokatch, 1972) and in humans (Wanders, Duran and Loupatty, 2012). As a compound 3-HIBA has potential applications in the production of pharmaceuticals such as tocopherol which is commonly known as vitamin E (Lang, Buehler and Schmid, 2015).

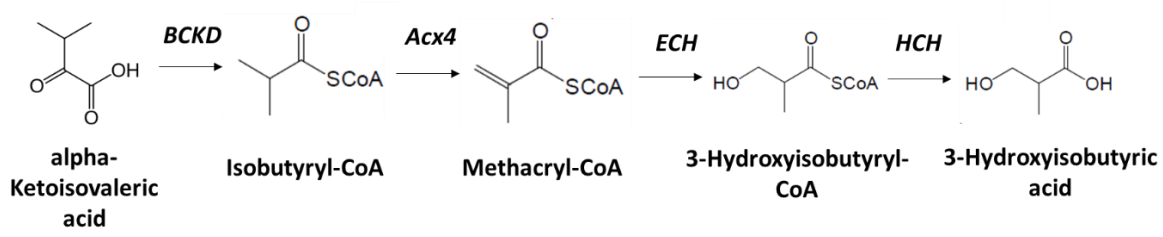


Figure 58: The 3-HIBA production pathway constructed within E. coli.

The pathway constructed to produce 3-HIBA in *E. coli* is shown above in Figure 58. All 4 enzymes were contained within an IPTG inducible plasmid containing an ampicillin resistance marker for selection and the strain was entitled LUC0548. In this strain 3-HIBA was to be produced from KIV initially following a similar pathway to the BMA production pathway used in LUC0585. As shown in Figure 58, KIV was first converted to isobutyryl CoA by BCKD then dehydrogenated by Acx4 to produce methacryl- CoA. Both of those steps are also contained within the BMA production pathway in LUC0585. An enoyl–CoA hydratase (ECH) then hydrates methacryl- CoA to produce 3-hydroxyisobutyryl-CoA. The final step is the removal of the CoA group by 3- hydroxyisobutyryl-CoA hydrolase (HCH) to produce 3-hydroxyisobutyric acid (3-HIBA). If the 3-HIBA production rate in the biotransformation was

higher than the BMA production rate of 0.109 mM/h, previously observed in Figure 52, when KIV was always available, it would indicate that the Acx4 enzyme was not currently the limiting factor for BMA production.

The biotransformation was set up as outlined in section 2.2.2.1 for inducible biotransformations with an IPTG induction and growth phase in addition to the standard biotransformation method. The biotransformation was carried out in the biotransformation buffer with a batch addition of 40 mM of KIV. Samples were taken every 2 hours for 6 hours for analysis by HPLC. The biotransformation was only run for 6 hours as the majority of BMA production in previous biotransformations occurred during the first 4-5 hours. The 3-HIBA, IBA and KIV concentrations measured in the samples by HPLC are shown in Figure 59.

From Figure 59 it can be seen that 3-HIBA was successfully produced in LUC0548. A final concentration of 28.8 mM was measured at 6 hours with a production rate of 4.8 mM/hour. This production rate of 3-HIBA is 4303 % higher than the BMA production rate of 0.109 mM/ hour which was observed in Figure 52. This result suggested that the BCKD and Acx4 enzymes which are present in the BMA production pathway are able to produce IBA-CoA at a rate of at least 4.8 mM/hour. This meant that it was likely that the AAT enzyme was responsible for the low production rate of BMA within the biotransformations. This was further supported by the fact that high concentrations of IBA were produced in previous biotransformations which was likely facilitated by the build-up of IBA-CoA.

In this biotransformation 7.79 mM of IBA was produced with production beginning after the 2 hour sample. The reason for the delay in IBA production was likely due to a build-up of IBA-CoA resulting from the Acx4 operating at a faster rate than the ECH in the biotransformation. In contrast to previous BMA production biotransformations in which a large portion of KIV is unaccounted for this biotransformation was close to a mass balance. At 6 hours, 37.7 mM of KIV was utilised while 36.59 mM of 3-HIBA and IBA was produced. This resulted in 97% of the utilised KIV being accounted for in the biotransformation mass balance. The KIV utilisation rate over the first 4 hours of the biotransformation was 7.67 mM/hour which was similar to the KIV utilisation rate in Figure 52 which was 8.05 mM/hour. This result shows that the in both biotransformations the BCKD operated at a similar rate demonstrating that the BCKD is not a limiting step in the production of BMA.

From the 3-HIBA biotransformation it was determined that the Acx4 enzyme is not responsible for the low BMA concentrations produced as it has been shown to operate at a much higher rate than the BMA production rate observed previously. As the KIV utilisation rates were similar it is likely that the Acx4 enzyme is operating at a similar rate in the BMA biotransformations. The AAT was therefore identified as the next target for improvement of the BMA production process. If the AAT is the limiting step in the biotransformation it is possible that the KIV which was unaccounted for in the BMA biotransformation was sequestered as IBA-CoA which was not analysed in the HPLC method.

Strain Genotypes
LUC0548- *E.coli BL21(DE3) Amp^R : (BCKD, Acx4, ECH, HCH)*

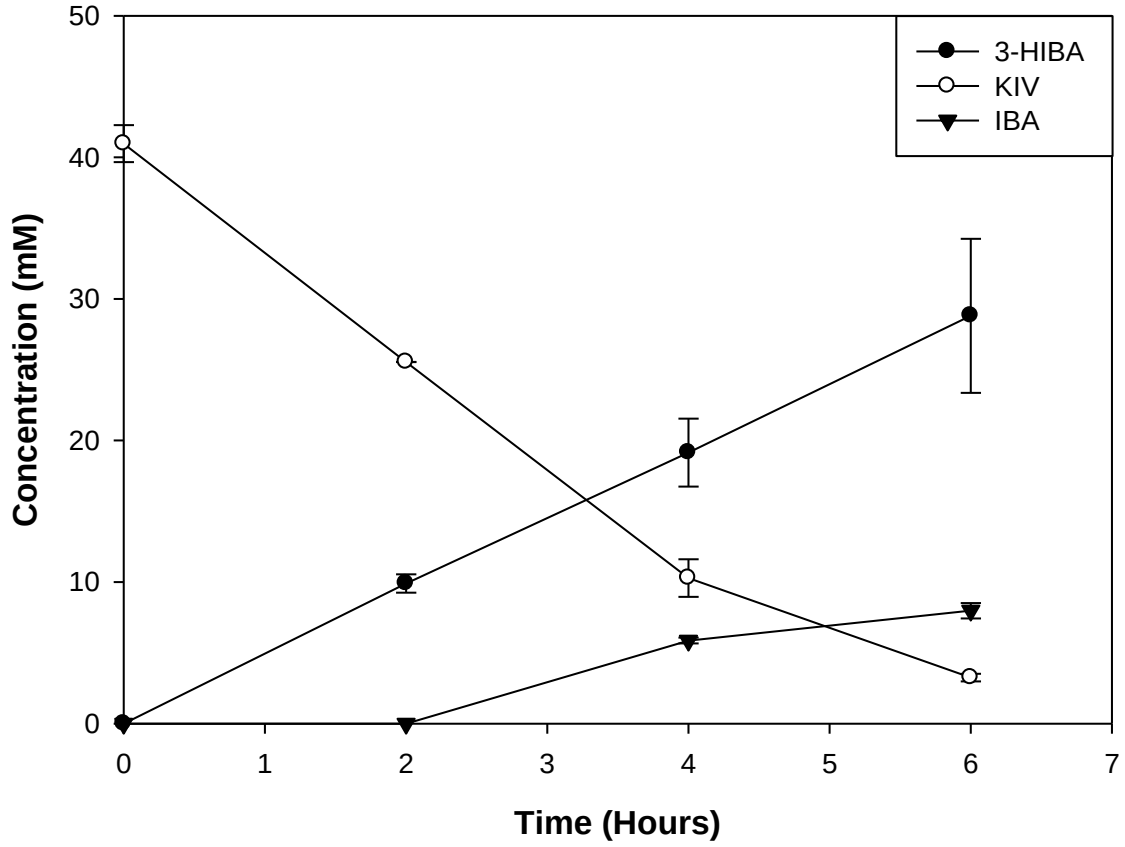


Figure 59: The IBA, KIV and 3-HIBA concentrations measured in the samples taken from the biotransformation of LUC0548. The samples were measured in triplicate with the error bars representing the standard deviation of the samples.

4.10. Upregulation of the alcohol acyltransferase (AAT)

It was hypothesised from the 3-HIBA production biotransformation (Section 4.9) that the AAT enzyme was the rate limiting step in the BMA production pathway due to the activity displayed by the Acx4 enzyme. In order to test this hypothesis an *E. coli* strain was created which contained the same enzymes with up regulation of the AAT gene expression through alteration of the ribosome binding site. This plasmid was entitled PMMA 50 and the strain LUC0615. To determine if up regulation of the AAT would increase the BMA concentration observed in a biotransformation a batch biotransformation was carried out with LUC0615.

As fed batch biotransformations were shown to produce the highest concentration of BMA in LUC0585, five fed batch biotransformations were set up to determine if upregulating the AAT had a positive effect on BMA production. Three had a 5 mM/ hour feed of both KIV and butanol, the original feed used for LUC0585. Biotransformations with a 10 mM feed/ hour of KIV and butanol and 10 mM feed/ hour of KIV and a 5 mM/ hour feed of butanol respectively completed the set. The biotransformations with higher feeds of substrates were included as it was hypothesised that potentially more BMA would be produced and thus there would be a greater demand on the substrates. The outline summary of the five biotransformations is shown in Table 19. The biotransformations were set up as outlined in the fed batch biotransformation method (Section 2.2.1.3 and 2.2.2.3). Samples were taken each hour for BMA analysis by HPLC the results of which are shown in Figure 60.

From Figure 60 it can be seen that biotransformations 1,2,3 and 5 had near identical BMA production profiles. Biotransformations 1,2 and 3 were replicates therefore the BMA production profile was expected to be similar, and hence it can be said that biotransformations using LUC0615 are reproducible. The 3 replicate biotransformation had an average peak BMA concentration of 0.82 mM at 5 hours and an average production rate of 0.183 mM/ hour between the 1 hour and 5 hour samples.

Table 19: The outline of the five biotransformations that were run in this set including the KIV and butanol initial concentrations and hourly feeds.

Biotransformation Number	**Initial KIV (mM)**	**Initial butanol (mM)**	**Feed / hour**
1	5	5	5 mM KIV and 5 mM butanol
2	5	5	5 mM KIV and 5 mM butanol
3	5	5	5 mM KIV and 5 mM butanol
4	10	10	10 mM KIV and 10 mM butanol
5	10	5	10 mM KIV and 5 mM butanol

By comparison LUC0585 in a fed batch biotransformation with a 5 mM / hour feed of both butanol and KIV produced a maximum BMA concentration of 0.48 mM by 5 hours. This biotransformation had a production rate of 0.105 mM/ hour between the 1 and 5 hour samples. From this it could be seen that upregulating the AAT, as hypothesised, did indeed improve BMA production with a 71% increase in BMA concentration and a 74% increase in production rate by 5 hours.

Biotransformation 5 followed a very similar BMA production profile to biotransformations 1,2 and 3. A BMA concentration of 0.81 mM was produced at 5 hours and the biotransformation had a production rate of 0.175 mM/ hour. As biotransformation 5 had a 10 mM/ hour feed of KIV and a 5 mM/ hour feed of butanol the result suggested that either KIV is not a limiting substrate initially or that at a 10 mM/ hour feed the KIV level was still insufficient to prevent it being a limiting factor for BMA production. This could be investigated by using LUC0615 in a batch biotransformation which contained an initial concentration of 40 mM of KIV.

It was also possible that butanol availability was limiting the BMA production as the AAT is being overexpressed in the pMMA 050 plasmid and therefore there may be a greater butanol requirement. Biotransformation 4 had a 10 mM/ hour feed of both KIV and butanol. It can be seen from Figure 60 that when the butanol feed was doubled the BMA concentration at 1 hour was higher at 0.16 mM and the highest production rate of 0.23 mM/hour was observed

between the 1 and 3 hour samples. Although there was a higher initial production of BMA and a higher production rate a lower concentration was produced by 5 hours with only 0.75 mM being produced. These results indicate that a higher butanol concentration at the start of the biotransformation increases the initial production of BMA and that butanol is not a limiting factor in fed batch biotransformations of LUC0615.

In conclusion the fed batch biotransformations of LUC0615 revealed that upregulation of the AAT produced the highest concentration of BMA at 0.83 mM at the 5 hour sample in biotransformation 2 and the highest production rate of 0.226 mM/ hour in biotransformation 4. The biotransformations with increased feeds demonstrated that it may have been possible that both KIV and butanol are limited when feeding LUC0615.

Strain Genotypes
LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

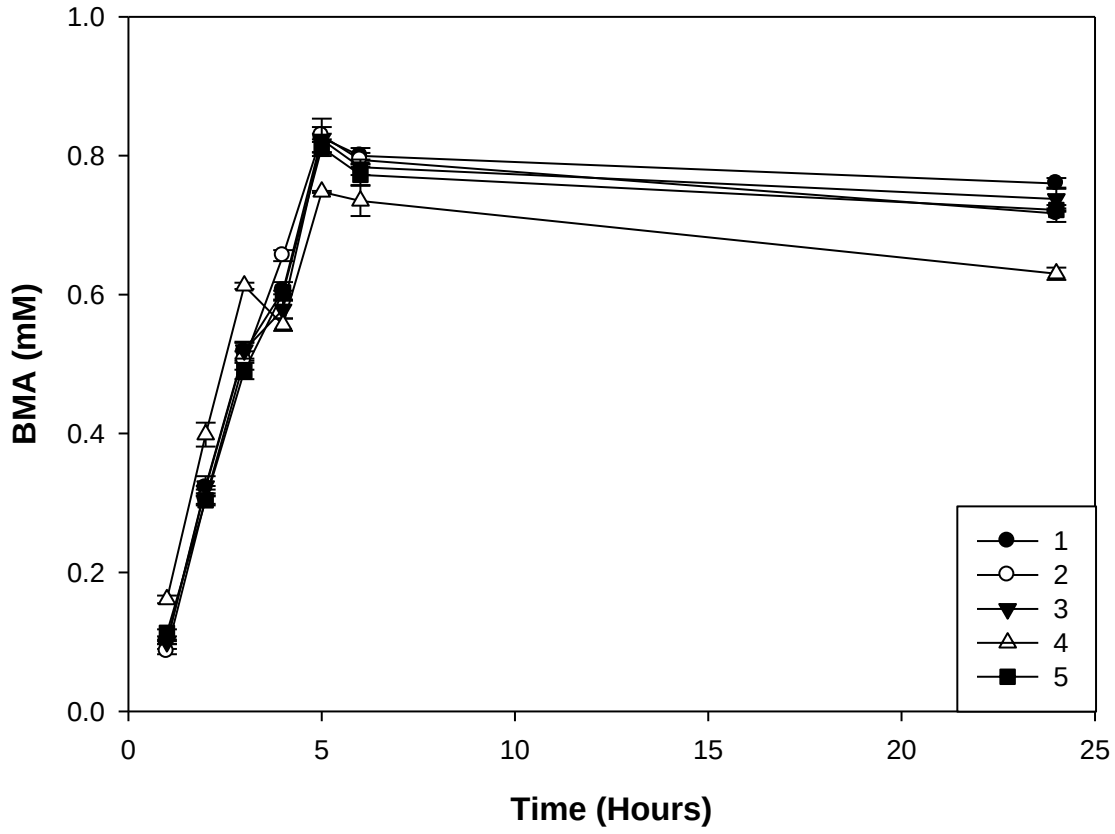


Figure 60: The BMA concentration produced by the five fed-batch biotransformations of LUC0615.The outline of each biotransformation is shown in Table 19. The error bars represent the standard deviation of the sample measurements.

4.10.1. Full analysis of LUC0615

As LUC0615 had been shown to produce a higher titre of BMA at a faster rate than LUC0585 it was important to determine how the substrates were being utilised in the biotransformation. The greater yield of BMA suggested that there may be greater use of the substrates and therefore one may have been limiting in the biotransformation. It was also possible that the IBA concentration may be lower as more KIV may have been utilised for BMA.

In order to determine the concentration of the substrates and to monitor IBA production a fed- batch biotransformation was run with samples taken for both BMA analysis and substrate/by-product analysis. The biotransformation feed was 5 mM of both KIV and butanol fed each hour after each sample was taken. The biotransformation was set up as outlined in section 2.2.1.3 and 2.2.2.3 with samples taken each hour for analysis. The BMA, KIV, IBA and butanol concentrations measured by HPLC are shown in Figure 61.

In Figure 61 1.16 mM of BMA was produced in the biotransformation which is higher than the concentration previously observed for fed- batch biotransformations of LUC0615 (Figure 60). The analysis for this biotransformation was run before the HPLC method was changed to the gradient method. The isocratic method of analysis had been shown in LUC0585 to provide higher values for BMA due to greater peak interference from the media. Therefore this BMA concentration was thought to be inflated and will not be used for comparison. As the aim of this biotransformation was to analyse the KIV, IBA and butanol concentrations which were produced, from a separate HPLC method, the discussion will focus on these compounds.

Throughout the biotransformation the butanol concentration within the media increased showing that butanol was not a limiting factor on BMA production. As the butanol concentration increased over time butanol was being utilised slower than 5 mM/ hour which was the hourly feed. The butanol increased at a rate of 1.4 mM/ hour therefore approximately 3.6 mM/ hour was utilised during the biotransformation. As only a small amount of the butanol would have been necessary to produce approximately 1.2 mM of BMA the majority of the butanol is unaccounted for. As butanol was not limiting the butanol by-products were not immediately of interest.

Strain Genotypes
LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

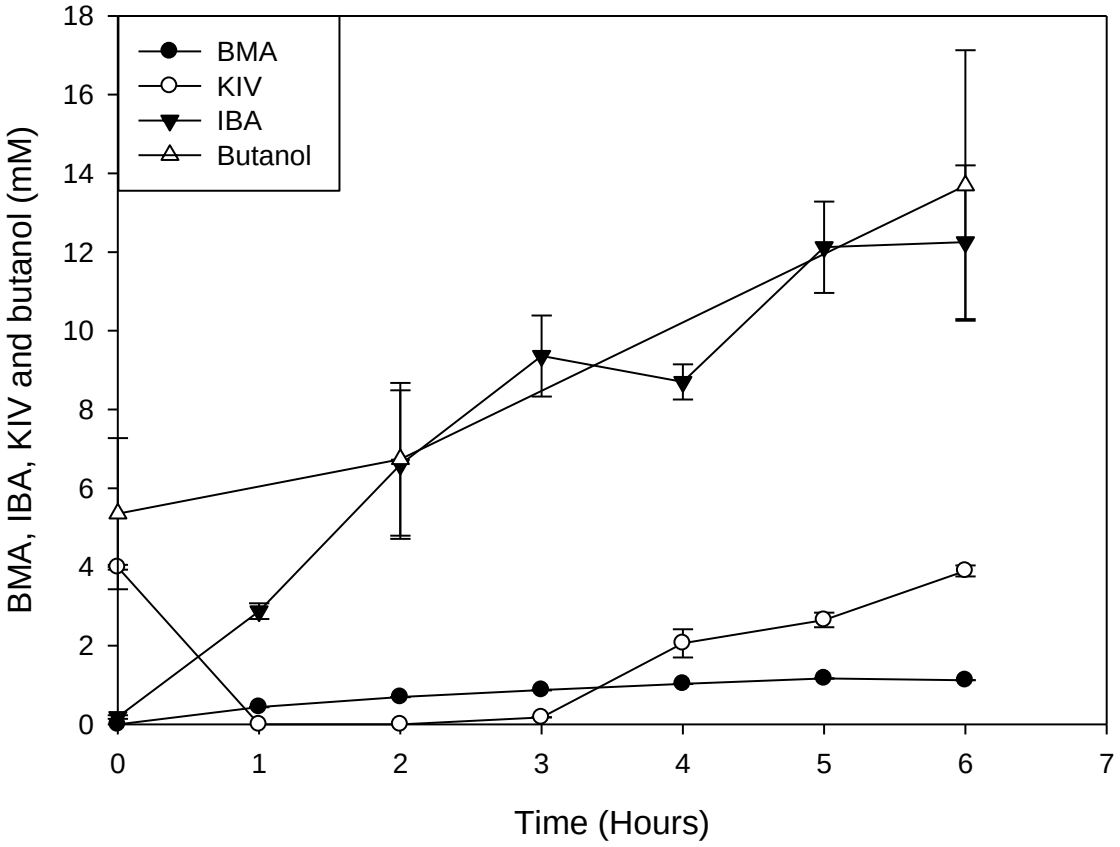


Figure 61: The BMA, IBA, butanol and KIV concentrations measured in the fed- batch biotransformation of LUC0615 by HPLC analysis. The feed consisted of 5 mM of KIV and butanol each hour added after a sample was taken. The error bars represent the standard deviation of the measurements.

As seen in LUC0585 IBA was a major by-product of the biotransformation with 12.3 mM produced by 6 hours. Similar to LUC0585 this by-product most likely contributed to the KIV limitation observed in the first 3 hours of the biotransformation. It has already been shown that a higher KIV feed of 10 mM/ hour did not improve the BMA titre produced in Figure 60. As the first 3 hours of the biotransformation was when the BMA production rate was highest it is possible that LUC0615 may produce a higher titre of BMA if IBA production was eliminated reducing the competition for IBA-CoA in the pathway.

In conclusion LUC0615 was not limited on butanol but was limited on KIV during the BMA production phase. IBA was produced at a high titre which may have been competing with the acx4 for IBA- CoA in the biotransformation limiting BMA production.

4.10.2. IBA elimination in LUC0615

It had been seen in previous strains, and in LUC0615, that IBA is a major by-product of the BMA production pathway with 12.3 mM produced by 6 hours in the fed-batch biotransformation of LUC0615 (Figure 61). Previously IBA elimination was attempted in LUC0669 which involved the deletion of *yciA*, an acyl-CoA thioesterase, from LUC0585 which contained the previous version of the BMA production plasmid.

IBA elimination was not successful in this strain and as LUC0615 produced a greater concentration of IBA than LUC0585 it is possible that this was the cause of the limited KIV shown in Figure 61. It was hypothesised that IBA elimination would increase the IBA- CoA available to the Acx4 as it is no longer converted to IBA. This may in turn reduce the KIV utilised for IBA-CoA production preventing the KIV limitation observed. In the previous experiment using LUC0669 it was concluded that that the elimination of *yciA* was not sufficient to eliminate IBA production and that another hydrolase may be present. The enzyme *tesB* is a second acyl- CoA thioesterase which is known to be present in *E. coli* and can cleave 3- hydroxyacyl – CoA esters such as IBA- CoA (Zheng *et al.*, 2004).

To determine if IBA by-production could be prevented in LUC0615 two strains were produced, LUC0683 and LUC0685. LUC0683 contained the same BMA production plasmid as LUC0615 with a *yciA* deletion. LUC0685 contained the BMA production plasmid PMMA 050 with deletion of both thioesterases, *yciA* and *tesB*. The strains and the deletions are shown in Table 20. To see if the deletion of *yciA* and *tesB* could prevent the production of IBA both strains were run in a fed batch biotransformation with 5 mM of KIV and 5 mM of butanol fed every hour. The experiment was set up as outlined in sections 2.2.1.3 and 2.2.2.3 for a fed-batch biotransformation.

Table 20: An outline of LUC0615 and the two strains containing thioesterase deletions.

Strain Name	**BMA production plasmid**	**Deletions**
LUC0615	PMMA 050	None
LUC0683	PMMA 050	*yciA*
LUC0685	PMMA 050	*yciA*, *tesB*

Samples were taken from the two biotransformations every hour for 6 hours and the feed, 5 mM of KIV and butanol/ hour, was added after each sample was taken. The resulting BMA, IBA, KIV and butanol concentrations are shown in Figure 62 for LUC0683 and Figure 63 for LUC0685. It can be seen from the biotransformation of LUC0683 (Figure 62) that the deletion of only *yciA* did not prevent the production of IBA as 4.7 mM was produced by 5 hours. This result confirms what was seen in LUC0669 (Figure 57) where when using the previous production plasmid deletion of only *yciA* did not prevent IBA production. In comparison to LUC0669 less IBA was produced and the production was delayed until after the 3 hour timepoint this may have been caused due to the improved production plasmid in LUC0683.

Butanol was limited throughout the biotransformation with the 5 mM feed being utilised fully before every sample. The KIV feed was similarly utilised until after 5 hours when the production rate of IBA and BMA slowed. Although the IBA concentration produced was much lower than that produced in LUC0615 Figure 61 it was still present which limits IBA- CoA available to the acx4 enzyme. This result was expected as *yciA* deletion alone had not previously stopped IBA production.

Strain Genotypes

LUC0683- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), ΔyciA*

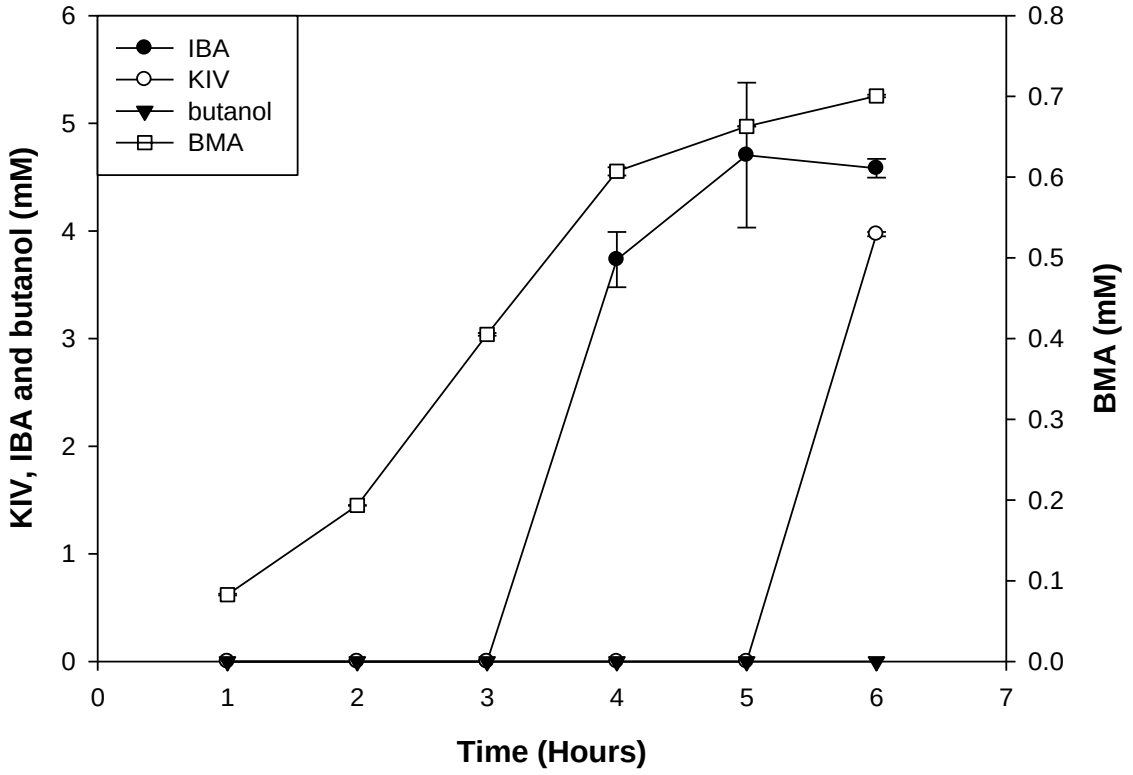


Figure 62: The IBA, KIV, BMA and butanol concentrations measured by HPLC from the fed batch biotransformation of LUC0683. The feed for this experiment was 5 mM of KIV and 5 mM of butanol added after each sample. The error bars show the standard deviation of the samples.

Strain Genotypes

LUC0685- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), ΔyciA, ΔtesB*

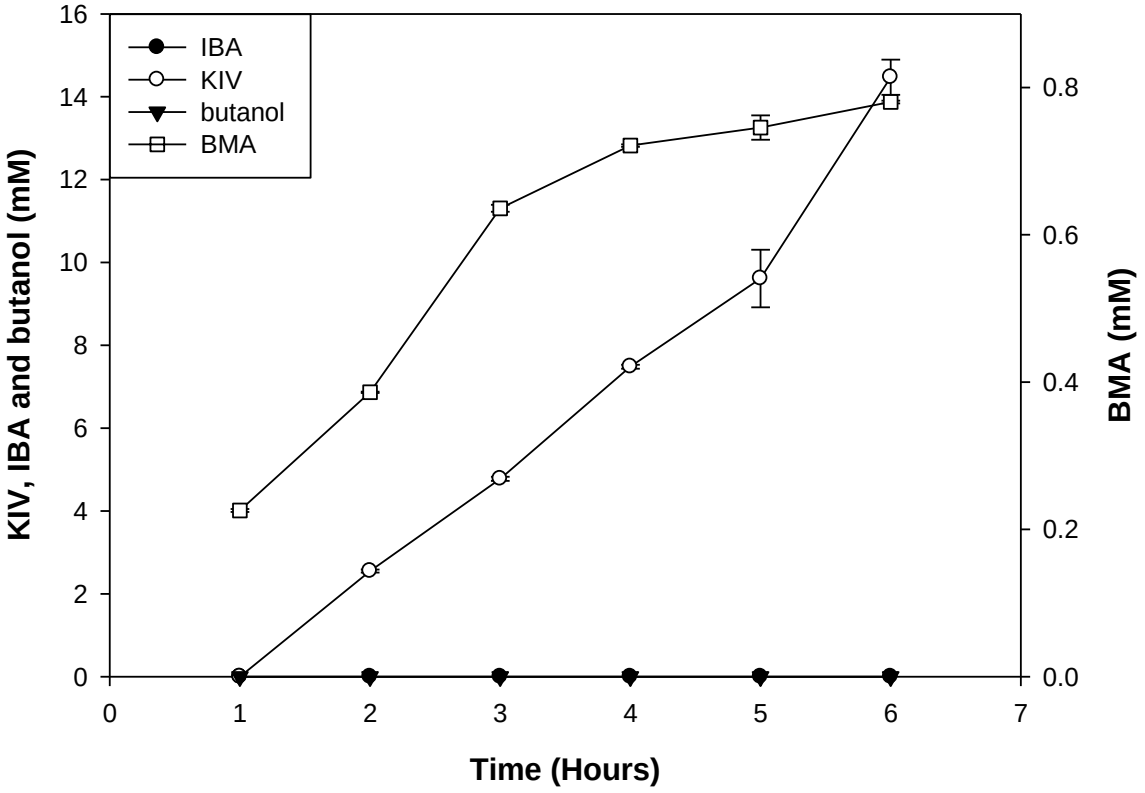


Figure 63: The IBA, KIV, BMA and butanol concentrations measured by HPLC from the fed batch biotransformation of LUC0685. The feed for this experiment was 5 mM of KIV and 5 mM of butanol added after each sample. The error bars show the standard deviation of the samples.

When looking at the fed- batch biotransformation for LUC0685 (Figure 63) it can be seen that the deletion of both *yciA* and *tesB* successfully eliminated the production of IBA. This confirmed the hypothesis that the deletion of more than one thioesterase is required and suggests that these are the only two thioesterases involved in IBA production. It can be seen that the elimination of IBA prevents KIV from being limited throughout the biotransformation as after the 1 hour timepoint the KIV concentration increases over time. KIV accumulates at a rate of 2.9 mM / hour which suggests that the BCKD enzyme in the pathway either has an activity of 2.1 mM / hour or that there may be end product limitation on the enzyme in the presence of IBA- CoA. As Figure 62 shows that KIV can be utilised at 5 mM / hour end product limitation of the BCKD is the more likely option.

As IBA is no longer produced it was expected that the BMA concentration produced would be increased due to a greater availability of IBA -CoA for the acx4 enzyme. As a similar BMA concentration was produced in the fed batch biotransformation of LUC0685 and LUC0615 this would suggest that the acx4 is the rate limiting step in the production pathway. We know this not to be the case as the 3-HIBA production experiment showed that the acx4 can operate at a higher activity rate. The only difference observed between the BMA concentration in LUC0683 and LUC0685 is that LUC0685 had a faster production rate over the first 4 hours of 0.18 mM / hour compared to 0.15 mM / hour in LUC0683. As LUC0685 had a higher BMA production rate and titre but a lower KIV utilisation rate the bottleneck in the pathway is more likely to be further up the pathway.

In both biotransformations butanol was not detected suggesting that it is absent or only a small quantity is left at each sample point. We know that butanol was not missing from the feed as BMA is unable to be produced in its absence. It is possible that butanol is limiting in the biotransformation and is preventing a higher concentration of BMA from being produced. As 35 mM of butanol was added in total to each biotransformation and only 0.7- 0.8 mM of BMA was measured a butanol by-product must be produced, whether this is limiting BMA production is unknown. It is possible either that the AAT utilises the maximum amount of butanol before by- product formation or that butanol byproducts are limiting the activity of the AAT. Therefore the next step was to investigate the fate of the butanol in the pathway to determine if there is a major by-product reducing the availability of butanol to the AAT enzyme.

4.11. GC-MS analysis to determine the presence of butyl- esters

It was shown in the previous experiment that when IBA was eliminated from the biotransformation the butanol was limited throughout. As LUC0685 only produced 0.78 mM of BMA and utilised 35 mM of butanol it was hypothesised that a large quantity of butanol was diverted towards by-products. The most likely butanol by-product in this strain is butylisobutyrate (BIB). As the engineered pathway requires an alcohol O- acyltransferase (AAT) which converts methacryl- CoA to BMA it is possible that the AAT is not specific enough to methacryl -CoA and has activity on isobutyryl- CoA, in the presence of butanol this would result in the production of BIB. As LUC0685 eliminated a pathway for IBA-CoA towards IBA it is possible that the potential build-up of IBA -CoA allows the AAT to produce BIB. This in turn would utilise butanol which may explain why the IBA elimination strains are limited on butanol.

In order to determine if BIB is being produced a biotransformation of LUC0685 was designed for headspace analysis by GC-MS. GC-MS analysis was selected to identify any butyl esters as headspace analysis allows for the removal of volatile substances from the media through heating (Hbschmann, 2008). This allows for targeted detection of the butyl esters without interference from other media compounds. The method conditions for the GC-MS analysis are outlined in section 2.2.3.1. Standards were prepared to quantify BMA, butanol and BIB in the samples. In order for headspace analysis to be carried out the biotransformation needed to be carried out in sealed headspace vials therefore the biotransformation was required to be a batch biotransformation. This should not have a negative effect on the biotransformation as the headspace vials were similar in shape to Schott bottles which were normally used. The biotransformation volume was adjusted to 2 ml to accommodate for the change in vessel size.

As the aim of the experiment was to analyse butyl esters which may be present in low concentrations 10 mM was selected for the initial butanol concentration and 20 mM for the initial KIV concentration. This meant that the vials could be run on the GC-MS without dilution. The biotransformation inoculum was set up as outlined in the batch biotransformation method in section 2.2.1.1. The three biotransformation vials were analysed by GC-MS headspace analysis at 6 hours and the resulting concentrations of BMA, butanol and BIB is shown in Table 21.

Table 21: The analyte concentrations measured by GC-MS at 6 hours in the batch biotransformation of LUC0685.

Analyte	Concentration (mM)
Butanol	0.068
BMA	1.36
BIB	1.13
Butanol Deficit	7.44

From the GC-MS analysis it can be seen that the 10 mM of butanol was almost completely utilised with only 0.068 mM remaining. It appears that as hypothesised the AAT is not only specific to methacryl- CoA as 1.13 mM of BIB was produced. It appears that BIB is a major by-product in the biotransformation as nearly as much BIB was produced as BMA. Due to the pathway being used the only way to reduce the production of BIB would be to increase the specificity of the AAT to methacryl- CoA. The highest concentration of BMA so far was observed at 1.36 mM. This may have been either due to their being a higher initial concentration of butanol at 10 mM or the difference in the sample preparation before analysis.

When samples were prepared for HPLC analysis they go through a lysis step using acetonitrile and shaking whereas for the GC-MS headspace analysis the samples are heated to 90^{o}C. It may have been possible that heating samples was a superior method for the release of BMA from the *E. coli* within the media as the vapour pressure of n- butyl methacrylate increases with temperature (National Center for Biotechnology Information., 2018). Although this method appears to be superior for BMA extraction the volatility of BMA means that heating before HPLC analysis would result in atmospheric loss of the BMA from the sample. This does suggest that more BMA may have been produced than had been previously measured by HPLC analysis, regardless HPLC analysis was used for future experiments to allow for previous data to be compared to future strain improvements.

As 10 mM of butanol was initially present in the biotransformation 7.44 mM of butanol is still unaccounted for after measuring residual butanol, BMA and BIB. As no other significant peaks were detected on the sample chromatograms for this experiment the fate of the 7.44 mM of butanol is unknown. It may not be possible to eliminate butanol by-products as they could not be identified therefore to prevent butanol limitation in the biotransformations it may be of advantage to use the batch method in future experiments. As it was shown in Figure 46 that higher initial butanol concentrations had an adverse effect on viability improving the viability may improve the BMA yield from the biotransformations.

4.12. Optimisation of the LUC0615 biotransformation

LUC0615 was shown in fed-batch biotransformations to increase the BMA concentration and production rate compared with LUC0585 through upregulation of the AAT enzyme on the BMA production plasmid. From the data provided from the full analysis of LUC0615 it could be seen that KIV was limited for the first three hours of the biotransformation which was the main BMA production period. Therefore it was hypothesised that returning to a batch biotransformation with higher initial concentrations of butanol and KIV may allow a higher concentration of BMA to be produced.

In order to test this hypothesis a batch biotransformation of LUC0615 was carried out. The inoculum was prepared and the biotransformation was set up as according to the batch biotransformation method (Sections 2.2.1.1 and 2.2.2.1). The biotransformation contained 40 mM of KIV and 60 mM of butanol. Samples were taken each hour to determine the BMA concentration using HPLC analysis. As the aim of the experiment was to determine if the upregulation of AAT had a positive effect on BMA production the BMA production profile of a batch biotransformation of LUC0585 was included in Figure 64 for comparison to LUC0615.

From Figure 64 it could be seen that a batch biotransformation improved the BMA production by LUC0615. A final concentration of 0.96 mM of BMA was produced by LUC0615 in the batch biotransformation, this was a 129% increase on the final BMA concentration in the batch biotransformation of LUC0585 which was 0.42 mM and a 17% increase over the fed batch biotransformation average. This was also the highest BMA concentration produced by a

biotransformation analysed using the HPLC gradient method which had a greater accuracy. Figure 51 which had the previous highest BMA concentration was analysed using the isocratic HPLC method which had poor BMA peak separation leading to inflated BMA concentrations.

The BMA production rate in both biotransformations was nearly identical for the first 2 hours at 0.19 mM/ hour with BMA production continuing in LUC0615 until 5 hours. At 5 hours the BMA production by LUC0615 reduced and the concentration slowly increased over the remainder of the biotransformation. This initial production rate was 19% higher than the production rate seen in the fed-batch biotransformations of LUC0615 over the first two hours and confirmed the theory that a greater availability of substrates would increase the production rate.

Although a higher concentration of BMA was produced by LUC0615 the yield was much lower than the maximum possible BMA titre of 40 mM as 40 mM of KIV was present in the biotransformation. This demonstrated that although upregulation of the AAT more than doubled the BMA yield further improvement is necessary to reach a high conversion rate of KIV to BMA. The result from this biotransformation appeared to confirm the hypothesis that the AAT is the limiting factor in the biotransformation process. It was also possible that as the biotransformation method had returned to a batch biotransformation that the high initial concentration of butanol had a negative effect on BMA production which had been observed in LUC0585.

Strain Genotypes
LUC0585- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*
LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

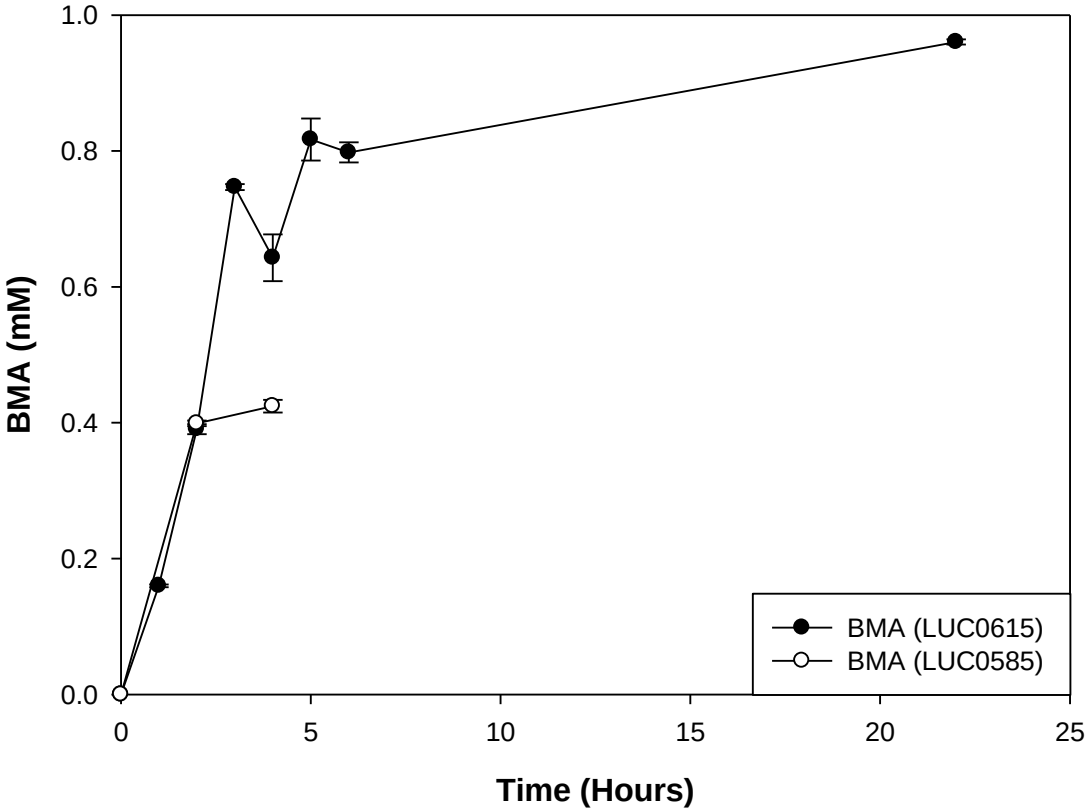


Figure 64: The BMA concentration produced by LUC0615 in a batch biotransformation. For comparison the batch biotransformation of LUC0585 is also included in the plot. The error bars represent the standard deviation of the measurements.

4.13. Improving cell viability in batch biotransformations

As batch biotransformations had been shown to be able to produce more BMA than fed-batch biotransformations for LUC0615 it was possible that the higher initial concentration of butanol may now be limiting BMA production in a similar way to that observed in LUC0585 (Table 15) when higher initial concentrations of butanol had a detrimental effect on cell viability which was thought to be due to damage to the cell wall of *E. coli*.

Toxic solvents such as butanol are known to permeate cell membranes and disrupt the function of embedded membrane proteins which has a detrimental effect on growth (Abdelaal *et al.*, 2015). Although growth is not of interest in the biotransformation, as the growth of the inoculum occurs before the addition of butanol, the damage to the membrane may alter the capacity to produce BMA. This may occur though prevention of the substrates entering the cell or loss of cofactors to the media from within the cell.

Along with butanol the BMA produced in the biotransformations was thought to have a detrimental effect on the integrity of the cell membrane. As BMA is a hydrophobic ester it is thought to embed in the membrane, again causing structural damage to the cell. Along with substrate considerations the viability of the cell may also prove important to the downstream processing of the biotransformation at a larger scale. With whole cells present in the biotransformation the separation and removal of BMA in downstream processing would be simpler and therefore more efficient.

In order to determine if the viability of the cells within the biotransformation could be improved two changes to the production strain was made. The host *E. coli* strain used for the strain construction was changed from BW25113 to RNM5 which was a strain which had been grown over time in a BMA rich media. This strain had demonstrated an improvement to external BMA tolerance as a result of this exposure and was therefore used, as this may help to improve viability in the biotransformation.

In addition to a more tolerant host, phage shock proteins were introduced to the strains. In *E. coli* the phage shock protein operon is induced in response to infection by a filamentous phage and contributes to the survival of the cell through mitigation of inner membrane permeability (Flores-Kim and Darwin, 2016). It was therefore hypothesised that these

proteins may improve the cell viability in the biotransformation. Phage shock protein A (PspA) is part of this phage shock protein operon. The function of PspA is not fully understood but it is thought to repair proton leakage of damaged phospholipid membranes (Kobayashi, Suzuki and Yoshida, 2007). It can also be produced in *E. coli* in response to the presence of hydrophobic organic solvents. It has previously been shown when over expressed to improve the survival of *E. coli* cells in response to exposure of n-hexane (Kobayashi, Yamamoto and Aono, 1998). Due to this function the protein PspA was selected for overexpression in a biotransformation strain alone and as part of the whole phage shock protein operon.

Three strains were produced for testing by batch biotransformation and viability testing which are shown in Table 22. The phage shock proteins were under the control of a constitutive λ promoter. The biotransformations were set up as outlined in sections 2.2.1.1 and 2.2.2.1 for batch biotransformations. Samples were taken every 2 hours for BMA analysis via HPLC and viability testing on LB agar plates (Section 2.2.3.8). Undiluted samples were used for viability testing as it has previously been shown that reduced viability was observable at this concentration for BMA biotransformations. The result of the BMA analysis is shown in Figure 65 and the viability of the samples is shown in Table 23.

Table 22: The strains used for improved cell viability biotransformations.

Strain Name	**Host *E. coli* strain**	**Production plasmid**	**Addition plasmid**
LUC0748	RNM5	PMMA 050	Empty
LUC0746	RNM5	PMMA 050	PspA
LUC0747	RNM5	PMMA 050	Psp(A/B/C/D/E)

It can be seen from Figure 65 that the introduction of phage shock proteins improved the BMA concentration of both strains at 6 hours. LUC0746 had a 68% increase over the control LUC0748 and LUC0747 had a 12% increase over LUC0748. It appears that the addition of multiple phage shock proteins reduced the BMA production which may have been due to an additional demand from the production of multiple extra proteins or a limitation of substrates and BMA export due to the strength of the membrane. All strains showed a large decrease in BMA concentration over previous strains containing PMMA 050. This was thought to have

been caused by the addition of a second plasmid. As previously observed BMA production appeared to stop overnight with less BMA present in the 24 hour sample.

When looking at the viability table (Table 23) it can be seen that the RNM5 control strain LUC0748 had loss of viability after 4 hours which showed that external BMA tolerance alone was not enough to improve viability. Both LUC0746 and LUC0747 had confluent growth on the agar plates showing that the introduction of phage shock proteins did improve viability. This confirms the hypothesis that the loss of cell viability was due to damage to the cell membrane in the biotransformations from butanol and BMA. Although a lower titre of BMA was produced in these biotransformations this result demonstrated that it will be possible to improve cell viability if viability proves to be a problem for downstream processing in a large scale BMA production bioprocess.

In conclusion the introduction of phage shock proteins was shown to improve the viability of the cells within the biotransformation with confluent growth occurring in both strains which had the proteins. The addition of PspA alone improved the BMA titre by 68% compared to the control but when multiple proteins were added this increase was reduced to 12%. Although the addition of phage shock proteins improved the BMA concentration produced over the control the addition of the second vector appeared to reduce the BMA production capacity of the PMMA 050 plasmid with only 0.18 mM produced in the control strain. Although the findings of this experiment will have significance when the biotransformation is increased in scale the core aim was to improve the BMA production capacity to a level at which the process could be commercially viable. Therefore as a significant decrease was observed in the BMA concentration experimental focus was shifted from viability to improvement of the AAT within the production pathway.

Strain Genotypes

LUC0746- *E.coli RNM5 Amp^R : (BCKD, Acx4, AATm4) pspA*

LUC0747- *E.coli RNM5 Amp^R : (BCKD, Acx4, AATm4) pspA/B/C/D/E*

LUC0748- *E.coli RNM5 Amp^R : (BCKD, Acx4, AATm4)*

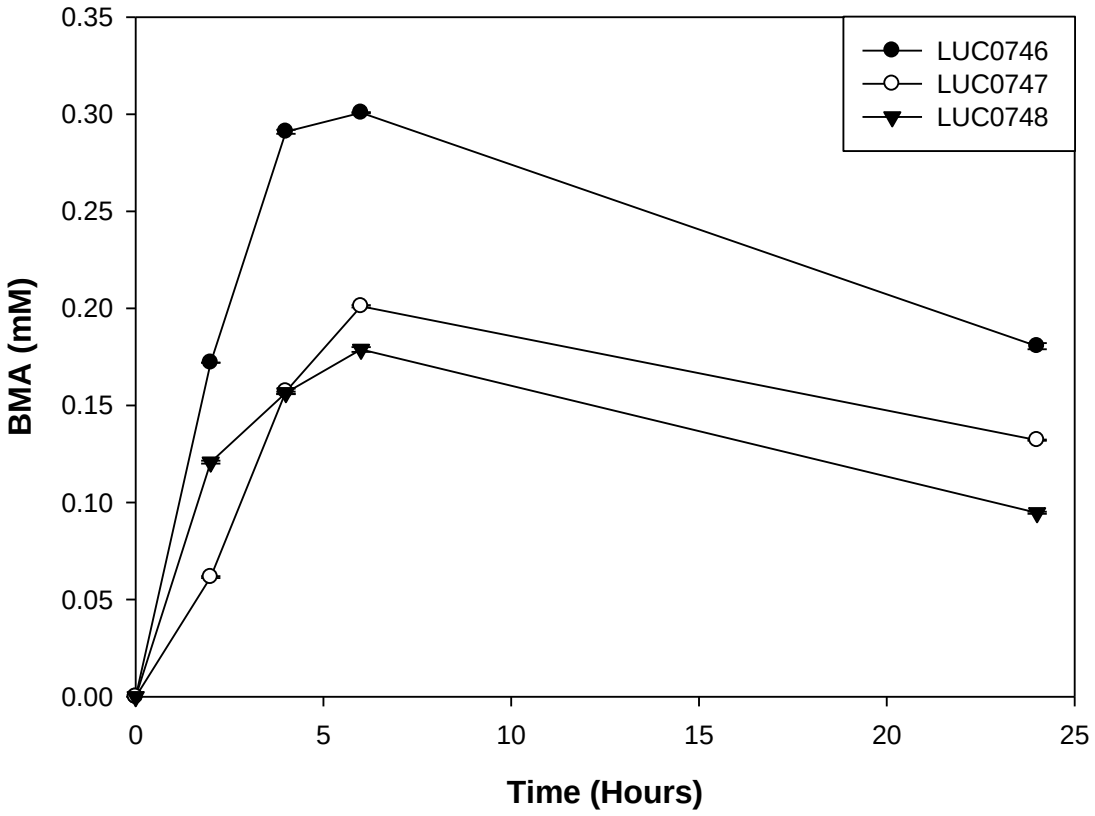


Figure 65: The BMA concentration measured in the biotransformation of LUC0746, LUC0747 and LUC0748. All samples were analysed by HPLC and measured in triplicate. The error bars represent the standard deviation of the samples.

Table 23: The growth of each sample from LUC0748, LUC0746 and LUC0747 on an agar plate and grown at 30°C for 24 hours. Each sample was spread onto LB agar plates containing ampicillin and kanamycin. This was performed in triplicate for each sample.

Time	**LUC0748 (Control)**	**LUC0746**	**LUC0747**
0	Confluent Growth	Confluent Growth	Confluent Growth
2	Confluent Growth	Confluent Growth	Confluent Growth
4	Low Growth	Confluent Growth	Confluent Growth
6	No Growth	Confluent Growth	Confluent Growth

4.14. Improvement of the alcohol acyltransferase (AAT)

As it was shown in previous experiments that overexpression of the AAT caused an improvement in the BMA production rate and titre observed (section 4.10). It was also hypothesised from the butyl ester analysis (section 4.11) that the AAT was not specific to methacryl- CoA but also could convert isobutyryl-CoA to butyl isobutyrate. It was possible that this lack of specificity had a negative effect on BMA production therefore two more AAT's were developed by Mitsubishi Chemical Corporation and incorporated into the production plasmid for BMA production.

The two additional AAT's entitled M5 and M6 had mutations which increased the activity of the AAT. Point mutations were used to increase the solubility of the AAT protein resulting in a higher activity, two mutated AAT's were created this way by Mitsubishi Chemical Corporation. The specifics of the mutations were not disclosed to Ingenza Ltd and are therefore not available. The resulting AAT's were incorporated into the BMA production plasmid, replacing AAT M4, producing PMMA 070 containing AAT M5 and PMMA 071 containing AAT M6. These plasmids were added to *E. coli* BW25113 to produce two new strains, LUC0710 containing PMMA 070 and LUC0711 containing PMMA 071. These new strains are outlined in Table 24.

Table 24:The strains created using the mutated AAT's. ↑ indicates upregulation within the plasmid.

Strain Name	**Host Strain**	**Plasmid**	**AAT**	**Other Plasmid Contents**
LUC0615	*E. coli* BW25113	PMMA 050	M4 (↑)	BCKAD, acx4 (↑), Amp (Ampicillin resistance marker)
LUC0710	*E. coli* BW25113	PMMA 070	M5 (↑)	BCKAD, acx4 (↑), Amp (Ampicillin resistance marker)
LUC0711	*E. coli* BW25113	PMMA 071	M6 (↑)	BCKAD, acx4 (↑), Amp (Ampicillin resistance marker)

In order to determine if these mutations to the AAT would have a positive effect on BMA production three batch biotransformations were run using each of the strains. The biotransformations were set up as outlined in section 2.2.2.1 for batch biotransformations. Samples were taken from each biotransformation each hour for 6 hours to determine the BMA concentration produced. The result of the HPLC analysis is shown in Figure 66.

It can be seen from Figure 66 that both mutated AAT enzymes increase the production rate of BMA within the biotransformations. The greatest difference in production rate was seen in the first hour of the biotransformation. In the first hour LUC0615 had a production rate of 0.14 mM/ hour, LUC0710 had a production rate of 0.19 mM/ hour and LUC0711 had the greatest production rate of 0.75 mM/ hour which was the highest production rate observed from a BMA biotransformation. Although a high production rate is observed initially it is reduced to 0.11 mM/hour. Why the production rate dropped after the first hour is not clear although it may be possible that the higher activity of the AAT caused a limitation on butanol or KIV. As a result the final BMA titre was not able to reach a level higher than that previously observed by LUC0615 with LUC0711 reaching 0.82 mM at 6 hours.

This therefore demonstrated that the AAT may be a rate limiting step in the biotransformation as the mutations increased the activity of the enzyme which as a result improved the production rate. Although the production rate of BMA was improved by the mutations the biotransformation of LUC0711 was not able to reach a higher final titre than that previously observed in LUC0615. In order to determine why the production rate drops further experiments were needed using LUC0711.

Strain Genotypes

LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

LUC0710- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm5)*

LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

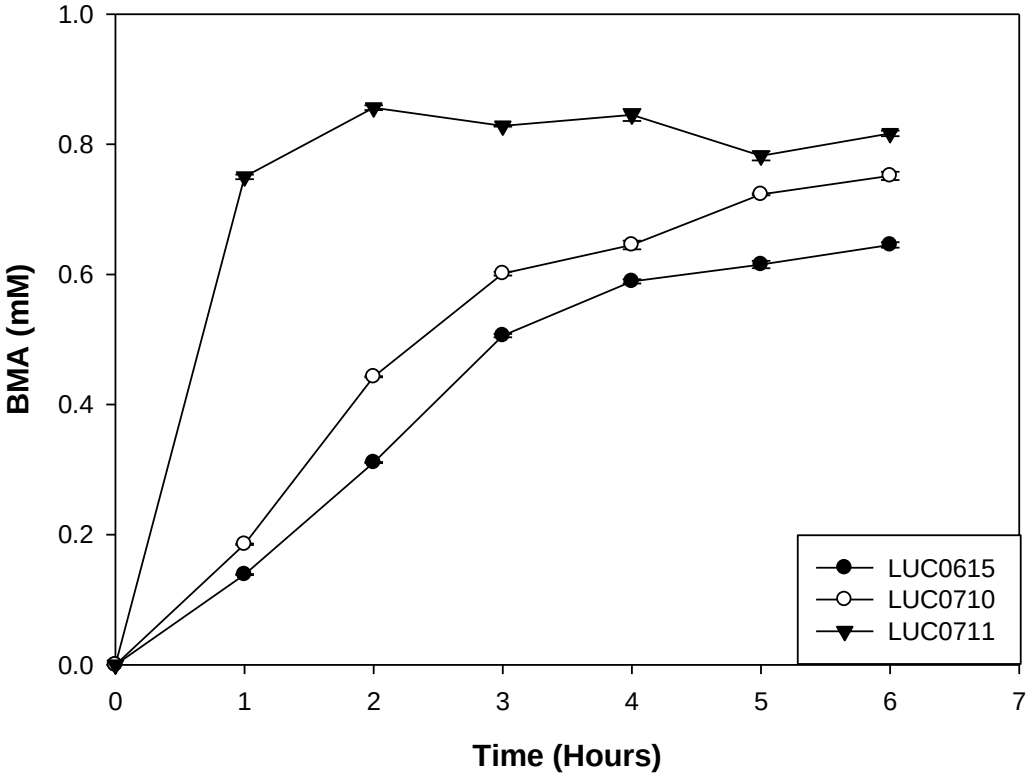


Figure 66:The result of the HPLC analysis of the strains containing mutated AAT's. The error bars show the standard deviation of the samples.

4.14.1. Full comparison of AAT m4 (LUC0615) and AAT m6 (LUC0711)

It was shown previously in Figure 66 that increasing the activity of the AAT improved the BMA production rate to the highest level observed in a BMA biotransformation of 0.75 mM/ hour. Although this production rate was initially high the final titre between LUC0615 with the old AAT and LUC0711 with the improved AAT was comparable as the production rate dropped off after the first hour. In order to determine why the production rate of BMA drops in comparison to LUC0615 full analysis of the by-products and substrates was required.

Two batch biotransformations were set up using LUC0615 and LUC0711 as outlined in the batch biotransformation method (section 2.2.1.1 and 2.2.2.1). Both biotransformations contained 40 mM of KIV and 60 mM of butanol and had an inoculum OD600 of 25. Samples were taken at 0,1,2,4 and 6 hours for analysis by HPLC to measure the BMA, KIV, IBA and butanol concentrations. The result of the BMA analysis is shown in Figure 67 and the result of the KIV, IBA and butanol analysis is shown in Figure 68.

From Figure 67 it can be seen that the BMA profile of both biotransformations followed what had previously been observed with BMA produced in LUC0615 for 4 hours before production stopped and the production rate in LUC0711 slowing after 1 hour. Both biotransformations produced a greater concentration of BMA than previously observed with LUC0615 producing 1.1 mM and LUC0711 producing 1.54 mM. Although the batch biotransformation and sampling method had been kept constant throughout the chapter variation in BMA titre had been seen between biotransformation sets. It was hypothesised that this may have been due to differences in the OD600 of the inoculum before concentration to an OD600 of 25 which was standard for the method. As the aim of the BMA biotransformations was to reach a higher BMA production rate of 14 mM/ hour these deviations in BMA concentration between replicates was not immediately investigated but was noted. The BMA concentration of 1.54 mM and production rate of 1.25 mM/ hour observed initially in LUC0711 were both the greatest observed so far in a BMA biotransformation.

It was hypothesised that the high production rate of BMA observed in LUC0711 stopped after 1 hour due to limitation of either butanol or KIV. From Figure 68 it could be seen that this was not the case. In both biotransformations the butanol concentration did not drop to a

Strain Genotypes

LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

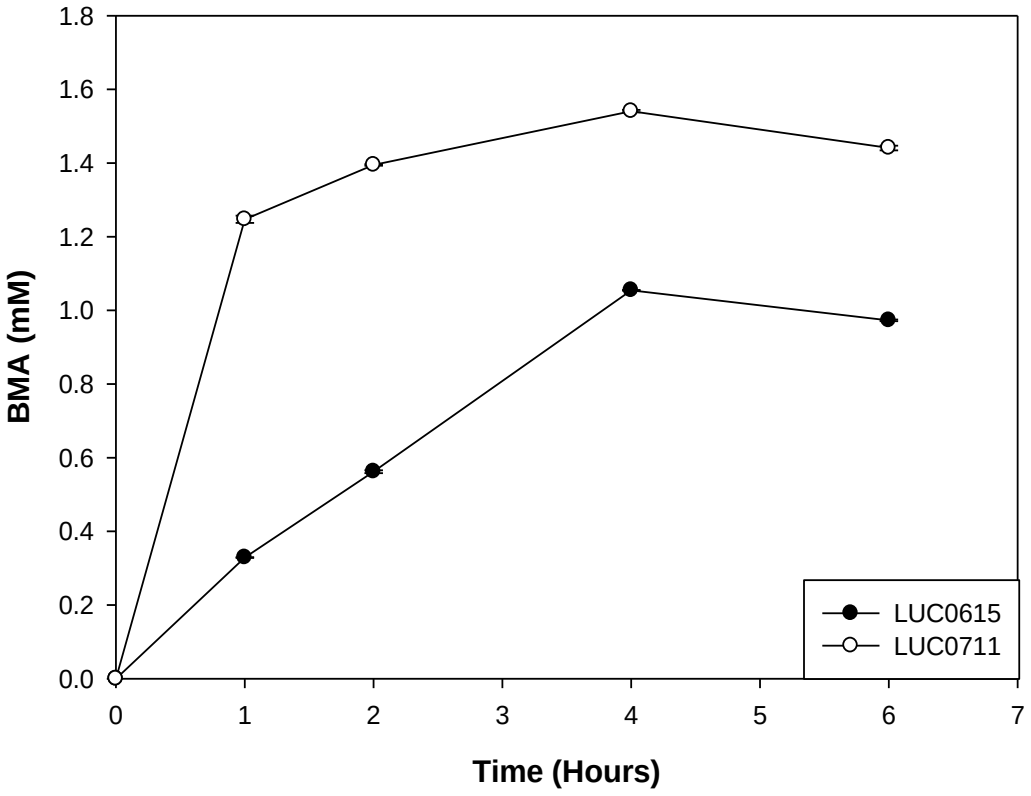


Figure 67: The BMA concentration present within the samples from the biotransformation of LUC0615 and LUC0711 as measured by HPLC. The error bars represent the standard deviation of the samples.

Strain Genotypes

LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

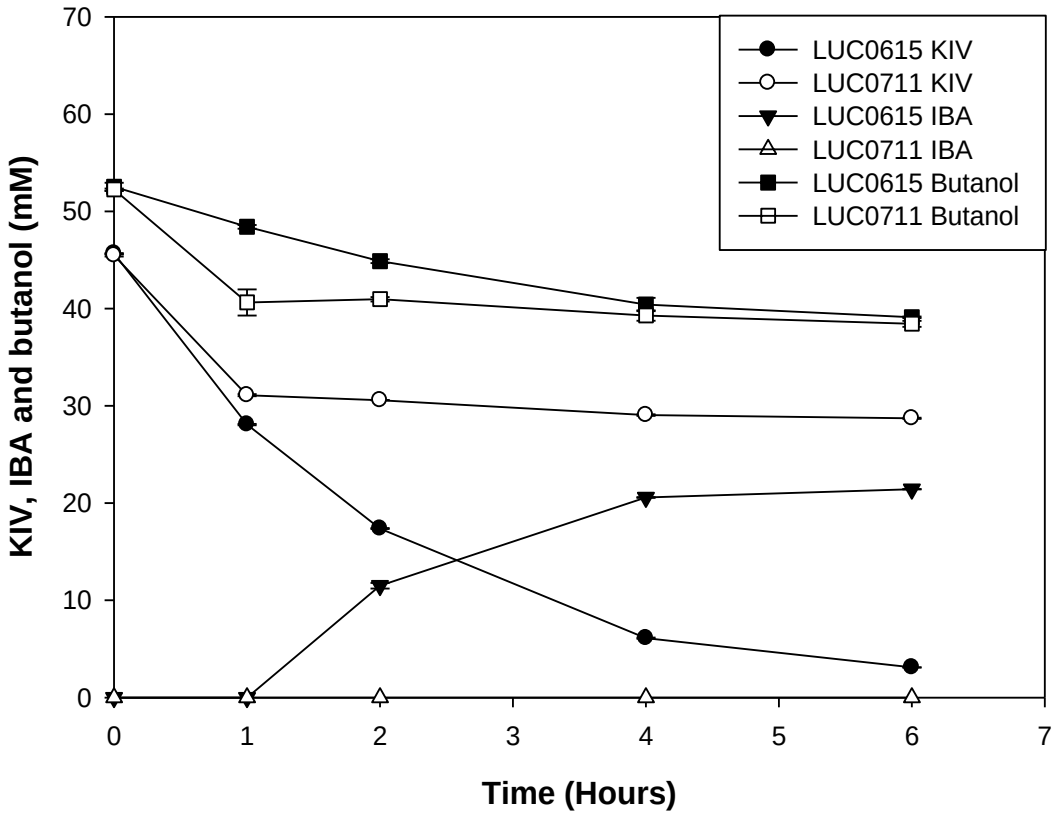


Figure 68: The KIV, IBA and butanol concentration present within the samples from the biotransformation of LUC0615 and LUC0711 as measured by HPLC. The error bars represent the standard deviation of the samples. The black markers represent the analytes from LUC0615 samples and the white markers represent the analytes from LUC0711 samples.

limiting level with 39.1 mM remaining at 6 hours in the LUC0615 biotransformation and 38.4 mM remaining at 6 hours in LUC0711. As expected due to the difference in BMA production rate in the first hour LUC0711 uses more butanol. In the first hour LUC0615 used 4.1 mM of butanol while in LUC0711 11.6 mM of butanol was used in the first hour. In LUC0711 as only 1.25 mM of BMA was produced in the first hour there must be an additional butanol by-product which is produced along with BMA as there should be no other way for butanol to leave the vessel. It is possible that butyl-isobutyrate may account for some of this as it had already been shown to be produced in BMA biotransformations (Table 21).

The LUC0711 biotransformation utilised 16.75 mM of the 45.5 mM total of KIV over 6 hours with the majority (14.37 mM) utilised in the first hour during BMA production. In comparison the LUC0615 biotransformation utilised 42.6 mM of the available 45.7 mM of KIV available. The increase in KIV utilisation seen in LUC0615 was due to the production of IBA which began at 1 hour and continued at a rate of 4.3 mM / hour reaching a concentration of 21.4 mM at 6 hours. As the KIV utilisation rate from 1 to 6 hours was 5 mM/ hour IBA production accounted for 86% of the KIV utilised beyond 1 hour again demonstrating that IBA was the major drain on KIV in the biotransformation. The biotransformation of LUC0711 produced no IBA which was unexpected.

It appeared that increasing the activity of the AAT in LUC0711 prevented accumulation of IBA-CoA and therefore IBA-CoA could not hydrolysed to produce IBA. It was possible that this occurred due to increased flux through the pathway to BMA but as BMA production slowed after 1 hour IBA-CoA accumulation should have begun. The second possible explanation was that in LUC0711 the activity of the BCKD stopped after 1 hour which conserved KIV and prevented the production of IBA-CoA. As KIV utilisation slowed dramatically in the biotransformation after 1 hour it appeared that BCKD deactivation was the most likely theory. Why the BCKD became inactive was not immediately apparent although it was hypothesised that it was due to an intracellular limitation of LUC0711. In conclusion, LUC0711 was again shown to have a higher initial production rate of BMA over LUC0615. Along with a higher concentration of BMA and a faster production rate LUC0711 produced no IBA which was thought to be due to inactivation of the BCKD as KIV was not limited. Butanol and KIV within the batch biotransformation of LUC0711 was not limited therefore substrate limitation was not responsible for the cessation of BMA production after 1 hour.

4.15. Determining the effect on BMA production of changing the E. coli host strain.

It could be seen from previous biotransformations of LUC0711 that regardless of the production rate the BMA concentration produced was always below 1.6 mM. It was hypothesised that this concentration limit was due to an intracellular factor and the biotransformations were not substrate limited. It was possible that this was due to a limitation of the host *E. coli* strain BW25113. In order to determine if this was the case the PMMA 071 BMA production plasmid was inserted into three alternative *E. coli* hosts. It was hypothesised that changing the host strain may allow the high BMA production rate produced by PMMA 071 to continue for longer than 1 hour.

The stains constructed for the biotransformations are outlined in Table 25. The batch biotransformations were set up as outlined in sections 2.2.1.1 and 2.2.2.1. Samples were taken from each biotransformation at 0,1,2,3 and 6 hours to analyse the BMA concentration produced. All analysis was carried out by HPLC. The resulting BMA concentrations measured are shown in Figure 69.

Table 25: The strains produced using alternative E. coli host strains.

Strain Name	**Host *E. coli* Strain**	**BMA Production Plasmid**
LUC0711	BW25113	PMMA 071
LUC0729	BL21(DE3)	PMMA 071
LUC0730	JM109	PMMA 071
LUC0731	TOP10	PMMA 071

Strain Genotypes

LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

LUC0729- *E.coli BL21(DE3) Amp^R : (BCKD, Acx4, AATm6)*

LUC0730- *E.coli JM109 Amp^R : (BCKD, Acx4, AATm6)*

LUC0731- *E.coli TOP10 Amp^R : (BCKD, Acx4, AATm6)*

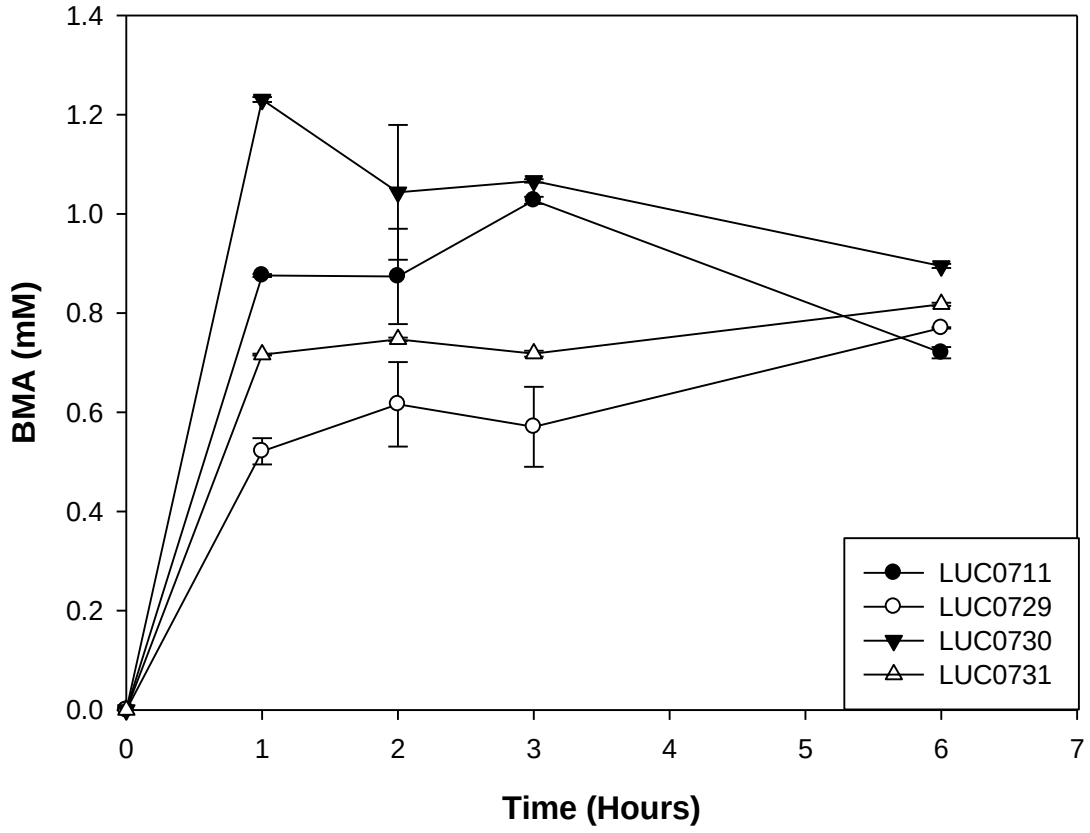


Figure 69: The BMA concentration produced by LUC0711, LUC0729, LUC0730 and LUC0731. The error bars represent the standard deviation of the samples.

It can be seen in Figure 69 that changing the host *E. coli* strain did have an effect on BMA production but did not allow the BMA production rate to continue beyond 1 hour as was hypothesised. In all four strains the BMA production rate dropped after 1 hour as had been observed previously in LUC0711. Of the four strains LUC0730 had the highest BMA production rate of 1.23 mM/ hour over the first hour, this was comparable to the production rate observed previously by LUC0711 (Figure 67). The host for LUC0730 was JM109 which is a commercial strain of *E. coli* used as competent cells, why this host would have a higher BMA production rate in this experimental batch was unknown. During the inoculum growth phase of the biotransformation LUC0730 had the lowest OD600 at the time of concentration of 2.84. As the other strains all had an OD600 greater than 4.75 it was possible that the generation of the inoculum could have an effect on the BMA production rate of the strain.

It was concluded from these biotransformations that the high production rate observed in the first hour by strains containing PMMA071 could not be continued by changing the host *E. coli* strain. The only strain which had a higher production rate than LUC0711 was LUC0730. It was thought that this higher production rate was due to the lower OD600 of the inoculum prior to concentration.

4.16. Determining the effect of inoculum growth time on LUC0711 biotransformations

It has been seen previously that when replicate biotransformations were run in different batches that the BMA concentration produced varied. This was seen particularly in LUC0615 and LUC0711. For example LUC0615 in identical batch biotransformations has been shown to produce maximum BMA concentrations at 6 hours of 1.1 mM (Figure 67), 0.65 mM (Figure 66) and 0.8 mM (Figure 64). LUC0711 showed similar variation with BMA concentrations of 1.54 mM (Figure 67), 1.03 mM (Figure 69) and 0.85 mM (Figure 66). As all of these biotransformations were identical but run at different times it was possible that the variation was due to the volatility of BMA which may make the sampling process less accurate but it was also possible that as the inoculum growth time was not standardised that this may have an effect on BMA production.

For batch biotransformations, the inoculum was always grown overnight but the inoculum growth time was not standardised as the goal was to produce enough biomass to have a concentrated OD600 of 25 in the biotransformation. Therefore it was hypothesised that this difference in BMA concentration observed may have been due to variation in the length of time the inoculum was grown. This growth time was not standardised due to equipment availability but was approximately 18 hours. Therefore in order to determine if this was a factor, batch biotransformations of LUC0711 were carried out using inocula concentrated at different timepoints.

Initially a growth curve of both LUC0615 and LUC0711 was produced in order to determine what inoculum timepoints to use. An inoculum flask was set up for each strain as outlined in the method for inoculum production (Section 2.2.1.1). Samples were taken at each timepoint and the OD was measured at 600nm in triplicate. The resulting growth curves are shown in Figure 70.

The growth curve of LUC0711 allowed 4 inoculum timepoints be identified which could be tested in batch biotransformations. The timepoints that were selected were 18 hours which was used as the control, 16 hours which due to working hours was the minimum time an overnight inoculum could be left, 8 hours which was when inoculum growth was at the mid exponential stage and 25 hours which was a late timepoint when growth was slowing. These four inoculum timepoints should allow it to be seen whether the inoculum growth time influenced BMA production. Four batch biotransformations of LUC0711 were set up as outlined in the batch biotransformation method (Section 2.2.2.1) with the inoculum growth stopped and concentrated at the timepoints described above. The inoculum conditions for the biotransformations are outlined in Table 26. Samples were taken from the biotransformations at various timepoints for BMA analysis by HPLC the result of which is shown in Figure 71.

It could be seen from Figure 71 that the length of time the initial inoculum was grown had a large effect on the BMA concentration produced especially in the first hour of production. The control biotransformation, biotransformation 3, which had the inoculum growth time of 18 hours produced 0.76 mM of BMA in the first hour which is similar to what has been observed previously.

Strain Genotypes

LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

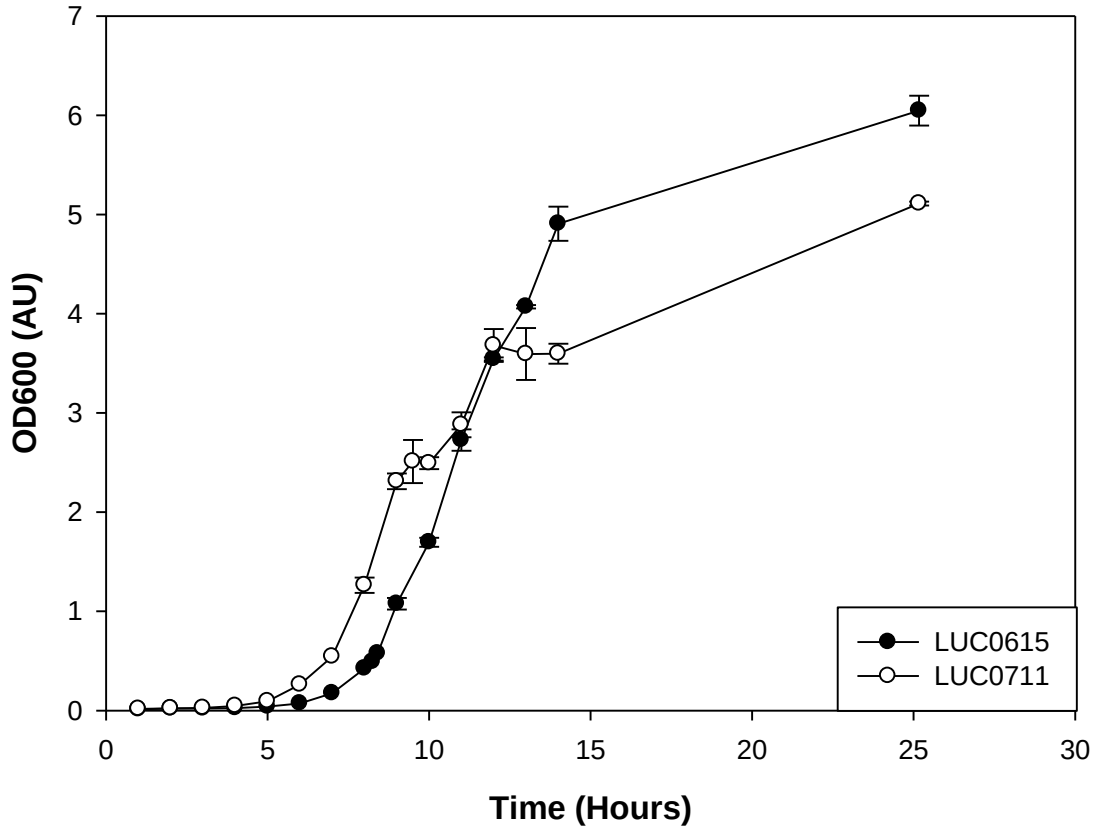


Figure 70: The growth curve of both LUC0615 and LUC0711 measured at 600 nm. The OD was measured in triplicate at each timepoint. The error bars represent the standard deviation of the measurements.

Table 26: The overview of the four batch biotransformations set up to determine the effect of initial inoculum OD600 on BMA production.

Biotransformation	Strain	Inoculum OD600 (AU)	Inoculum growth time (Hours)
1	LUC0711	1.07	8
2	LUC0711	3.88	16
3	LUC0711	4.34	18
4	LUC0711	5.11	25

Strain Genotypes
LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

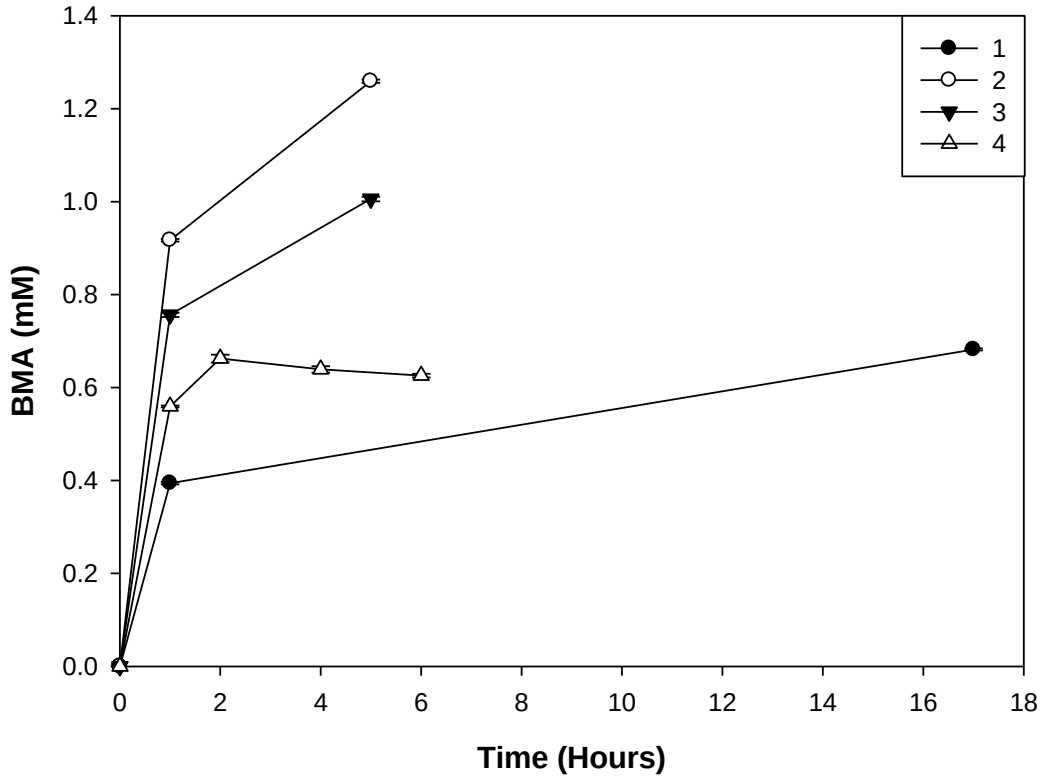


Figure 71:The BMA concentration profile of four batch biotransformations of LUC0711 at various inoculum timepoints before concentration to an OD600 of 25. The error bars represent the standard deviation of the measurements.

In comparison the two biotransformations with the lowest BMA production rate in the first hour had the highest and lowest inoculum growth times before concentration. The biotransformation with the inoculum concentrated at 8 hours only produced 0.39 mM of BMA in the first hour and the biotransformation with the inoculum concentrated at 25 hours produced only 0.56 mM of BMA in the first hour. Both of these values are lower than the concentration produced by the control at 0.76 mM in the first hour. The biotransformation which had the highest production rate of BMA was biotransformation 2 which produced 0.92 mM of BMA in the first hour. This production rate was 21% higher than the control biotransformation.

From the result of the biotransformations it can be seen that the time the inoculum was grown before concentration had a significant effect on the BMA production rate. As the AAT and acx4 are overexpressed in LUC0711 it could have been possible that the longer the inoculum was grown the more BMA was able to be produced due to greater enzyme availability although this was shown not to be the case with the oldest two inoculums producing less BMA. It was hypothesised in the batch biotransformations of LUC0615 and LUC0711 (Section 4.14.1) that there was an intracellular factor which was limiting BMA production as in the previous experiment substrates were shown not to be limited. It appeared that the length of time the inoculum was grown may alter the availability of this intracellular factor as all the biotransformations had the same quantity of cells and same concentration of substrates.

In conclusion, the variation in BMA concentration seen between identical biotransformations could indeed have been caused by the length of time the inoculum was grown before concentration. Why this variation occurred may be due to an intracellular factor which may be present at different levels during growth. As a result of this experiment the inoculum growth time was monitored closer in future experiments to try and limit variation in BMA concentration observed.

4.17. Determining the limiting factor in LUC0711 batch biotransformations

Changing the host strain and altering the OD600 of the starting inoculum before concentration did not extend BMA production beyond the first hour of the biotransformation. It had previously been hypothesised that an intracellular factor may be responsible for the lack of BMA production observed after the first few hours when both substrates were available. If this was the case when more cells were present in the biotransformation a higher concentration of BMA should be observed proportional to the quantity of cells.

In order to determine if BMA production would indeed follow this theorised model, two biotransformations of LUC0711 were set up with different inoculum OD600's. In a standard batch biotransformation the inoculum was grown to give an OD600 of around 5, the inoculum growth media was then removed and the cells were resuspended to give an OD600 of 25 in sodium phosphate buffer (pH 7) before the addition of KIV and butanol. One biotransformation was set up in this way. In the other biotransformation the inoculum was resuspended to give an OD600 of 50 before the addition of KIV and butanol. In both biotransformations the volume was 30 ml and the KIV and butanol concentration was the same at 40 mM of KIV and 60 mM of butanol. Therefore both biotransformations were identical apart from the quantity of cells.

Samples were taken from each biotransformation at 0, 1, 2, 3 and 6 hours for BMA analysis by HPLC. It was hypothesised that doubling the quantity of cells within the biotransformation would double the quantity of BMA produced. The result of the BMA analysis of both biotransformations is shown in Figure 72.

In Figure 72 it could be seen that the biotransformation with an OD600 of 25 had a BMA production rate of 0.88 mM/ hour for the first hour and reached a maximum concentration of 1.03 mM. When the OD600 was doubled to 50 the BMA production rate over the first hour was 1.6 mM/ hour and the maximum concentration reached was 2.01 mM at 2 hours. This was a 82% increase in production rate and a 95% increase in maximum concentration reached.

Strain Genotypes
LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

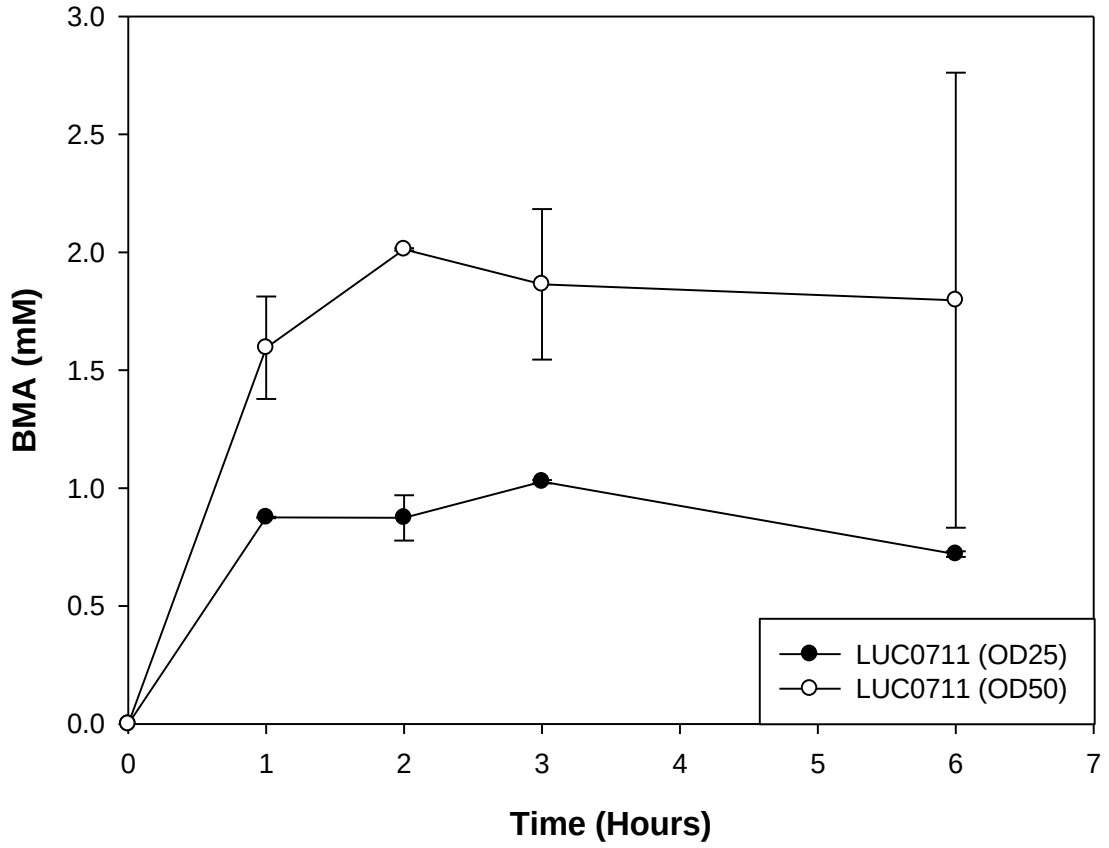


Figure 72: The BMA concentration measured by HPLC for both biotransformations of LUC0711. The error bars represent the standard deviation of the samples.

The 3 hour and 6 hour samples from the biotransformation with an OD600 of 50 had a very high standard deviation between the triplicate HPLC injections therefore the 2 hour sample was used to determine the maximum concentration produced. Why the error was so high in these samples was not apparent but may have been caused by either an air bubble in the HPLC sample vial or a mechanical error in the auto injector. Due to the volatility of BMA, samples were unable to be re-run. This was due to the initial piercing of the vial by the HPLC needle which allowed BMA to evaporate from the vial changing the concentration of subsequent sample analysis.

This result appears to indicate that the production rate and concentration of BMA produced is directly linked to the quantity of cells in the biotransformation. As a greater concentration of BMA was able to be produced while using the same concentration of KIV and butanol it could be concluded that it was indeed an intracellular factor and not an extracellular factor (e.g. media/BMA) which was responsible for the loss in production rate seen after 1 hour. The most likely candidate was co-factors such as coenzyme-A which are required for the BMA production pathway to function.

Although the 6 hour sample from the biotransformation with an OD600 of 50 had a very high margin of error between the injections the sample was unable to be re-run as BMA loss occurs in HPLC vials which have been pierced. As the aim of the experiment was to determine if a higher quantity and rate of BMA production was observed in the first hour this sample was not integral to the aim.

In conclusion, doubling the quantity of cells within the biotransformation caused a 82% increase in BMA production rate and a 95% increase in the maximum BMA concentration. This showed that an intracellular component is limiting the production of a higher quantity of BMA in batch biotransformations of LUC0711 as doubling the quantity of cells caused almost double the concentration of BMA to be produced. It was possible that this limitation was caused by a lack internal compounds, such as co-factors, which are required for the activity of the pathway enzymes.

4.18. The effect of cofactors on BMA production biotransformations

From the previous experiments and analysis of LUC0711 it was thought that an internal factor was responsible for the limitation of BMA production as the substrates (KIV and butanol) had been shown to be available when BMA production stops. As it was concluded after the full analysis of LUC0711 (Section 4.14.1) that it was most likely the BCKD which was inactive in the pathway as no IBA was produced. The branched chain ketoacid dehydrogenase complex consists of three subunits (E_1,E_2 and E_3) and in order for the BCKD to produce IBA-CoA it requires three cofactors. Coenzyme A (CoA) is required for reception of the acyl group within the E_2 active site, NAD^+ which plays a role in reoxidisation of the lipoyl moiety in the E_3 active site and thiamine pyrophosphate (TPP) which initially combines with KIV in the E_1 active site (Berg, Tymoczko and Stryer, 2002). It was therefore thought that the addition of these three cofactors to the biotransformation may allow the BCKD to continue producing IBA-CoA and as a result BMA production to continue.

In order to determine if this hypothesis was correct initially four biotransformations were set up consisting of two batch biotransformation of LUC0615 and LUC0711 along with two batch biotransformations of LUC0615 and LUC0711 including cofactors. The concentration of cofactors used was initially decided to be 1 mM of CoA, 1 mM of NAD^+ and 0.2 mM of TPP. The biotransformations were set up according to the method for batch biotransformations with cofactors (Section 2.2.2.4). Samples were taken at various timepoints for BMA analysis by HPLC. An overview of the four biotransformations is shown in Table 27 and the result of the HPLC analysis is shown in Figure 73.

Table 27: The outline of the four initial cofactor biotransformations.

Biotransformation	Strain	Initial substrates	Initial cofactors
1	LUC0615	40 mM KIV, 60 mM butanol	None
2	LUC0711	40 mM KIV, 60 mM butanol	None
3	LUC0615	40 mM KIV, 60 mM butanol	1 mM CoA, 1 mM NAD^+, 0.2 mM TPP
4	LUC0711	40 mM KIV, 60 mM butanol	1 mM CoA, 1 mM NAD^+, 0.2 mM TPP

Strain Genotypes

LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

LUC0711- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm6)*

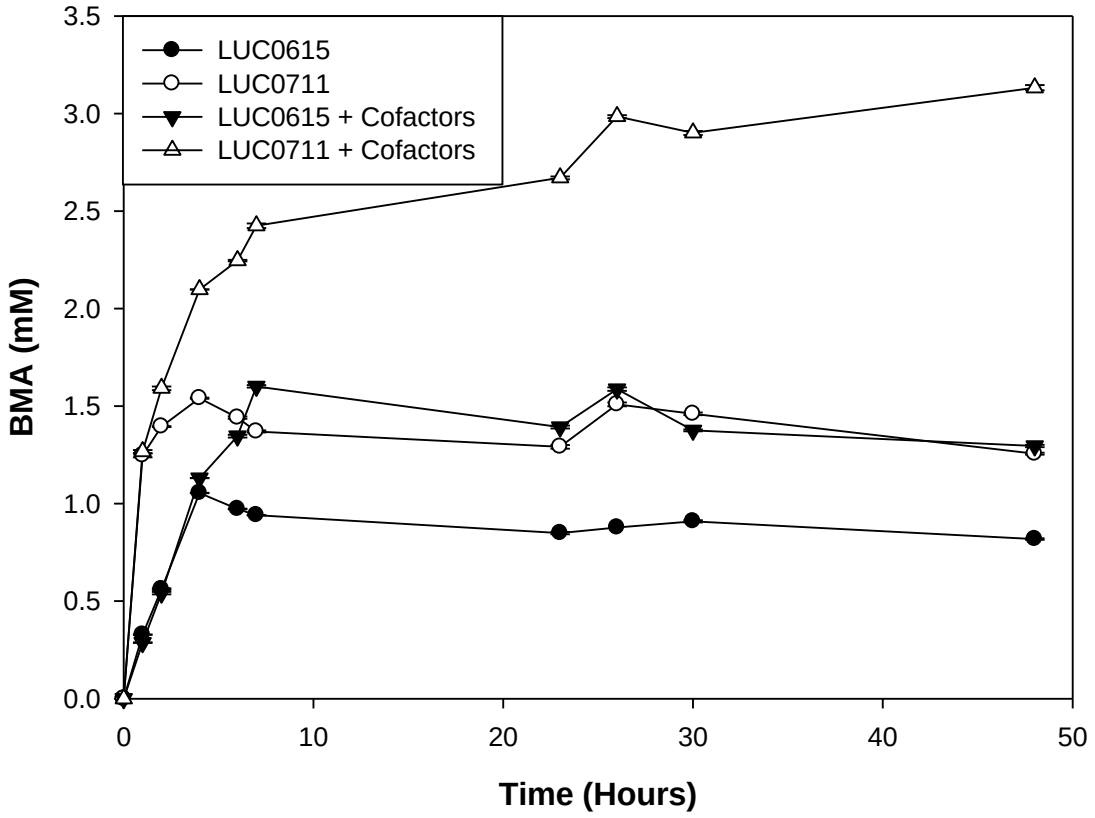


Figure 73: The BMA concentration produced by the batch biotransformations of LUC0615 and LUC0711 with and without cofactor addition at 0 hours. Analysis was carried out by HPLC. The error bars represent the standard deviation of the samples.

It can be seen from Figure 73 that both biotransformations in which cofactors were added produced a greater concentration of BMA than the controls. In LUC0615 the control batch biotransformation produced 0.94 mM of BMA by 7 hours at a production rate of 0.13 mM/ hour. By comparison when cofactors were added at the start of the biotransformation LUC0615 produced 1.6 mM of BMA by 7 hours at a production rate of 0.23 mM/ hour. This was a 70% increase in BMA titre and a 77% increase in BMA production rate. After 7 hours the BMA production stopped in both biotransformations.

LUC0711 which had previously been shown to be the superior BMA production strain also improved in effectivity when cofactors were added to the biotransformation. The control of LUC0711 produced 1.37 mM of BMA by 7 hours at a production rate of 0.19 mM / hour which when cofactors were added LUC0711 produced 2.43 mM of KIV by 7 hours and had a production rate of 0.35 mM/ hour. This was a 77% increase in BMA concentration and an 84% increase in production rate. Unlike LUC0615 BMA production continued in LUC0711 until the final sample at 48 hours by which point the BMA concentration was 3.13 mM. This was the highest BMA concentration produced to date.

This result shows that the earlier hypothesis that a deficiency of coenzymes was responsible for the limitation of BMA production in the biotransformation was correct. It also appears that it was indeed the BCKD complex which was responsible for the limitation of BMA production and that this was due to limitation of cofactors available for its operation. As it had been seen previously that LUC0615 produced a large quantity of IBA during biotransformations it was possible that KIV limitation was the causing LUC0615 to produce less BMA than possible with extra cofactors.

To test whether it was substrate limitation, cofactor limitation or a combination of both which was limiting the production of BMA in LUC0615 after 7 hours three further biotransformations were set up where in one additional substrates were added after 6 hours, in another additional cofactor was added after 6 hours and in the final biotransformation both were added at 6 hours. Samples were taken at each time point for BMA analysis by HPLC. The overview of the biotransformations is shown in Table 28 and the result of the BMA analysis is shown in Figure 74.

Table 28: Overview of the biotransformations to determine the limiting factor of BMA production by LUC0615.

Biotransformation	Strain	Initial Substrate	Initial Cofactors	6 Hour Addition
1	LUC0615	40 mM KIV, 60 mM butanol	1 mM CoA, 1 mM NAD^+, 0.2 mM TPP	40 mM KIV, 60 mM butanol
2	LUC0615	40 mM KIV, 60 mM butanol	1 mM CoA, 1 mM NAD^+, 0.2 mM TPP	1 mM CoA, 1 mM NAD^+, 0.2 mM TPP
3	LUC0615	40 mM KIV, 60 mM butanol	1 mM CoA, 1 mM NAD^+, 0.2 mM TPP	40 mM KIV, 60 mM butanol, 1 mM CoA, 1 mM NAD^+, 0.2 mM TPP

From Figure 74 it can be seen that the addition of extra cofactors and substrates altered the BMA production profile in the biotransformations. The additional substrates provided in biotransformation 1 allowed BMA production to continue until 24 hours where the concentration produced was 1.67 mM of BMA.

It could be concluded from this result that it was a substrate, most likely KIV due to IBA production, which was limited and prevented the maximum concentration of BMA being produced from the original cofactor addition. This conclusion was backed up by the result of biotransformation 2 where only cofactors were added at 6 hours. In this biotransformation the addition had no effect on the BMA concentration after 6 hours.

The biotransformation in which both additional substrates and cofactors were added at 6 hours had the best BMA production profile with BMA production continuing until the 26 hour sample where 2.41 mM of BMA was measured. This biotransformation had a production rate of 0.9 mM / hour until the 26 hour sample. When compared to the biotransformation of LUC0711 with only cofactors added at 0 hours it can be seen that LUC0711 is a superior strain for BMA production as at 26 hours 2.98 mM of BMA was produced at a rate of 0.12 mM/ hour. As LUC0711 achieved a higher concentration and a faster production rate while using

Strain Genotypes
LUC0615- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4)*

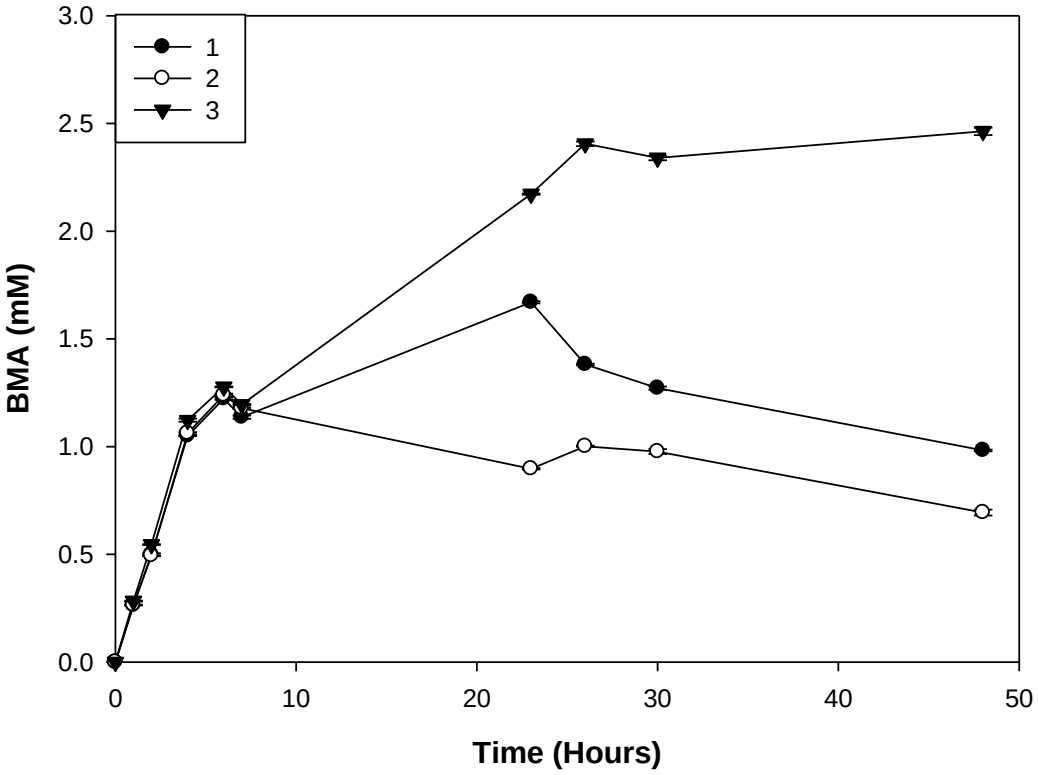


Figure 74: The BMA concentration produced by the 3 biotransformations of LUC0615. The analysis was performed by HPLC and the error bars represent the standard deviation of the samples.

half as much substrates and cofactors it can be concluded that it is the superior strain for BMA production.

In order to maximise the BMA production potential of LUC0711 future cofactor experiments could be performed with additional substrates and cofactors while analysing all possible compounds. This should allow for a higher concentration of BMA to be produced while minimising the costs associated with the process.

4.19. The production of BMA from glucose

4.19.1. The production of KIV from glucose

In order for the bioproduction of BMA to become economically feasible it is important for the pathway to begin from simple sugars such as glucose rather than KIV which has a greater cost. As it has already been shown that BMA can be produced from KIV it was important to demonstrate that it is possible for *E. coli BW25113* to produce KIV at a sufficiently high rate. To demonstrate this a KIV production plasmid entitled pKIV was for addition into *E. coli BW25113*. The pKIV plasmid contained a chloramphenicol resistance marker along with *ilvC*, *ilvD* and *katE* from *E. coli* and *alsS* from *B.subtilis*. It had been shown by Atsumi, Li and Liao in 2009 that these enzymes could be used in *E. coli* to produce KIV from glucose with the aim of isobutanol production. The KIV production pathway for the pKIV plasmid is outlined in Figure 75.

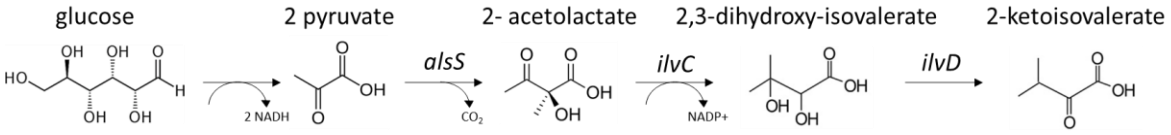


Figure 75: The KIV production pathway contained within the pKIV plasmid.

Along with the enzymes for KIV production *katE* was also overexpressed in the pKIV plasmid. Catalase HPII (*KatE*) functions as a catalase to decompose hydrogen peroxide to prevent damage occurring to the cell. It is possible for the acx4 enzyme to produce hydrogen peroxide therefore catalase HPII was added as a preventative measure to reduce potential damage at higher levels of BMA production. Along with the pKIV plasmid, a second plasmid entitled $pKIV_{NADH}$ was produced which was identical to the pKIV plasmid except the *ilvC* enzyme had been adapted to require NADH for operation as an alternative to NADPH. This second plasmid was created as it was hypothesised that NADPH generation within the biotransformation may be limited due to flux of glucose away from the pentose phosphate pathway. Therefore two strains, LUC0713 and LUC0721, were produced to determine if KIV could be produced from

glucose and if changing the specificity of *ilvC* altered the KIV yield. An outline of both strains is shown in Table 29.

Table 29: An overview of the two strains created to produce KIV from glucose.

Strain Name	**Host**	**Plasmid**
LUC0713	*E. coli BW25113*	pKIV (*ilvD*, *alsS*, *ilvC*, *katE*)
LUC0721	*E. coli BW25113*	$pKIV_{NADH}$ (*ilvD*, *alsS*, $ilvC_{NADH}$, *katE*)

To determine of LUC0713 and LUC0721 could produce KIV from glucose two biotransformations were set up using the batch biotransformation method outlined in section 2.2.2.1. The only change from the method was that after the inoculum was grown, the biotransformation media consisted of Lund media which contained 56 mM of glucose replacing the sodium phosphate buffer (pH 7), KIV and butanol. Lund media was selected for the biotransformations as it is a cheap media suitable for large scale bioprocesses. Therefore Lund media was a candidate for the future bioprocess and was used for glucose biotransformations. Samples were taken from each biotransformation at 0 hours and 6 hours for analysis by HPLC to determine the glucose and KIV concentrations. The result of the HPLC analysis is shown in Table 30.

Table 30: The result of the KIV production biotransformation in Lund media supplemented with 56 mM of glucose. The values shown are from the 6 hour sample.

Strain	**Glucose Remaining (mM)**	**KIV Produced (mM)**
LUC0713	0	46.68
LUC0721	0	32.08

From Table 30 it can be seen that both of the strains produced KIV and utilised all of the available glucose. LUC0713 produced the highest concentration of KIV at 46.68 mM which is comparable to the concentration of KIV used in batch biotransformations of 40 mM. As LUC0721 produced less KIV when *ilvC* utilised NADH it can be concluded that NADPH was not limited in the biotransformation and that *ilvC* had greater efficacy in its original form. As the KIV concentration produced was above 40 mM it was concluded that the pKIV plasmid pathway functioned correctly and BMA production from glucose could be attempted.

4.19.2. BMA production from glucose

As both the KIV production plasmid (pKIV) and the BMA production plasmid (PMMA 050) have been shown to work independently the next step was to attempt production of BMA from glucose within a biotransformation. As KIV had been successfully produced from the pKIV plasmid and BMA had been produced from the PMMA 050 plasmid, the combination of both within a strain was hypothesised to yield BMA from glucose. The PMMA 050 plasmid was selected as even though it produced less BMA the BCKD complex was active for longer in biotransformations and therefore was thought to have a greater potential to produce BMA from glucose if even just a small quantity.

Five strains were produced to attempt BMA production from glucose which are outlined in Table 31. The control strain LUC0749 contained both the pKIV plasmid and the PMMA 050 plasmid. The other four strains were variations on the control with various deletions and auxotrophies to prevent byproducts. LUC0714 was a valine, leucine, isoleucine and pantothenate auxotroph containing deletions of *ilvE, ilvA, panB* and *leuA*. The deletion of *panB* and *leuA* was to prevent the production of pantothenate and leucine both of which use KIV as a substrate. The deletion of *ilvE* and *ilvA* prevented the production of isoleucine and valine which would reduce the pyruvate and KIV available in the pathway.

LUC0720 contained, along with both plasmids, deletion of *yciA* and *tesB* which had previously been shown to eliminate IBA production. The final two strains LUC0723 and LUC0724 were acetate auxotrophs due to the deletion of *aceE*. This was to prevent butyl acetate formation.

The other deletions in these strains *idhA, pflB* and *poxB* prevent alternative pathways for pyruvate. It was hypothesised that this would improve KIV production.

Table 31: The outline of the strains produced for BMA production from glucose.

Strain Name	***E. coli* Host**	**Plasmids**	**Deletions**	**Brief Description**
LUC0749	BW25113	pKIV, PMMA 050	None	Control
LUC0714	BW25113	pKIV, PMMA 050	*ΔilvE, ΔilvA, ΔpanB, ΔleuA*	Control + valine, leucine, isoleucine and pantothenate auxotroph
LUC0720	BW25113	pKIV, PMMA 050	*ΔyciA, ΔtesB*	Control + IBA deletion
LUC0723	BW25113	pKIV, PMMA 050	*ΔpoxB, ΔaceE*	Control + Pyruvate conservation + Acetate auxotroph
LUC0724	BW25113	pKIV, PMMA 050	*ΔidhA, ΔpflB, ΔaceE*	Control + Pyruvate conservation + Acetate auxotroph

It was hypothesised that all 5 strains would produce BMA from glucose but at different rates due to the deletions included. The inoculum of LUC0723 and LUC0724 was supplemented with 10 mM of acetate to help facilitate growth. The inoculum was grown as outlined in section 2.2.1.2 with the addition of acetate for the strains described previously. The batch biotransformations were set up as outlined in section 2.2.2.1 with addition of 90 mM of glucose instead of KIV. Samples were taken each hour for BMA and KIV analysis, and samples were taken at 0 and 6 hours for glucose analysis. The result of the HPLC analysis is shown in Figure 76.

Strain Genotypes

LUC0714- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), Camp^R: (alsS, ilvC, ilvD, katE), ΔilvE, ΔilvA, ΔpanB, ΔleuA*

LUC0720- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), ΔyciA, ΔtesB*

LUC0723- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), Camp^R: (alsS, ilvC, ilvD, katE), ΔpoxB, ΔaceE*

LUC0724- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), Camp^R: (alsS, ilvC, ilvD, katE), ΔidhA, ΔpflB, ΔaceE*

LUC0749- *E.coli BW25113 Amp^R : (BCKD, Acx4, AATm4), Camp^R: (alsS, ilvC, ilvD, katE)*

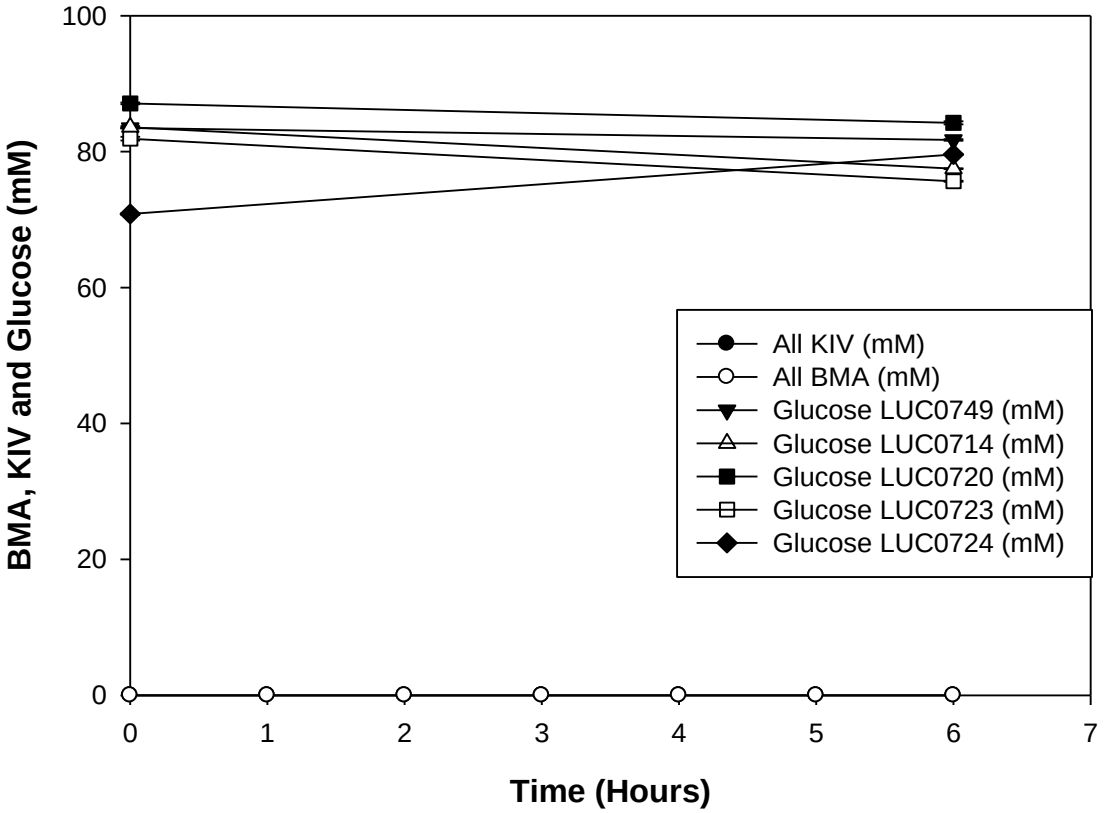


Figure 76: The BMA, KIV and Glucose concentration measured from the biotransformation of LUC0749, LUC0714, LUC0720, LUC0723 and LUC0724. The error bars represent the standard deviation of the samples.

From the analysis of the biotransformation samples in Figure 76 that no BMA was produced in any of the biotransformations. This result was unexpected as each of the plasmids had been shown individually to function in the production of KIV from glucose and BMA from KIV. Why no BMA production occurred was unknown it was possible that, as the inoculum was not grown in the presence of glucose, it was unable to be utilised in the biotransformation.

It appears from the glucose samples that very little glucose was utilised in any of the biotransformations. The slightly different starting concentration of glucose seen in each biotransformation was likely due to pipetting error. LUC0724 had a lower starting concentration of glucose than the rest which as the error is small may have been caused by error in the dilution of the sample. As glucose is produced which is not possible this is likely the case. It was possible that no KIV was measured as it was utilised as soon as it was produced but as so little glucose was used in the biotransformations it was more likely that no KIV was produced.

It appears from analysis of the samples that the pKIV plasmid was not functioning correctly in the biotransformations and was therefore what caused no BMA to be produced. In conclusion BMA was not able to be produced directly from glucose and the deletions made to the control strain did not alter this. Further experiments are required to determine why the pKIV plasmid was inactive.

4.20. Conclusions

The original BMA production strain LUC0585 containing the pMMA 044 production plasmid had previously been shown to produce BMA in a batch biotransformation, although the concentration produced was below the theoretical maximum of 60 mM due to 60 mM of butanol being available. Increasing the oxygen available in the biotransformation was shown to have no effect on the BMA concentration produced, while sparging the biotransformation vessels resulted in BMA loss from the vessel when compared to similar experiments in sealed biotransformations.

Changing the biotransformation vessel type resulted in variation in the BMA concentration although no alternative vessel was suitable for future biotransformations since the rubber seal absorbed BMA from the medium. Therefore Schott bottles were used for future biotransformations.

The concentration of butanol used within biotransformations of LUC0585 was shown to have a minor effect on BMA production although viability plating showed that at butanol concentrations of 15 mM, and above, complete cell death was observed beyond 2 hours. Due to the toxicity of the butanol within the biotransformations, a fed-batch biotransformation method was developed which resulted in a greater concentration of BMA being produced. Full analysis of these biotransformations showed that isobutyric acid (IBA) was a major by-product and that the substrate KIV was limited during the BMA production phase. Butanol was shown not to be limited during the biotransformations.

When the KIV feed within the biotransformation was increased until it was no longer limiting a small increase in BMA concentration was observed, although BMA production still stopped after 6 hours when both substrates were present indicating that another factor was responsible for the low BMA production rate. In order to determine if a more comprehensive biotransformation media allowed BMA production to continue for longer a biotransformation was set up with Lund media although this resulted in a low concentration of BMA being produced.

IBA elimination was attempted with the deletion of a thioesterase (*yciA*) although this did not reduce the IBA produced.

A biotransformation carried out using LUC0548 containing the 3-HIBA production plasmid (pET21b) demonstrated that the acx4 enzyme can operate at a much higher rate than observed in the BMA biotransformations. acx4 was therefore not the bottleneck in the production process.

LUC0615 was produced which had a plasmid with upregulation of the AAT enzyme (pMMA 050). This resulted in an increase in BMA concentration and production rate over the original strain suggesting that the AAT may be the rate limiting enzyme in the biotransformation process.

IBA elimination was again attempted while using the upregulated AAT plasmid and the deletion of two thioesterases (*yciA* and *tesB*). This resulted in the elimination of IBA which reduced KIV usage in the biotransformation but did not increase the BMA concentration produced. Butanol was limited during the biotransformation but could not be accounted for. GC-MS analysis determined that butyl-isobutyrate production was a major butanol by-product although the majority of butanol remained unaccounted for.

Changing the biotransformation of LUC0615 back to a batch biotransformation was shown to improve the BMA production rate of the strain over the fed batch biotransformation. Increased viability was shown not to improve the BMA production in BMA resistant strains. Viability was therefore not a factor which limited BMA production in the biotransformation. Improving the activity of the AAT enzyme within the BMA production plasmid (pMMA 071) was shown to improve the BMA production rate with LUC0711 producing the highest production rate observed in a biotransformation. Although the production rate was higher, the overall BMA concentration was similar to LUC0615 suggesting that another pathway limitation existed No IBA was produced by LUC0711 and therefore the BCKD complex was thought to become inactive in the biotransformation.

Changing the *E. coli* host strain did not allow BMA production to continue beyond 1 hour and was therefore not thought to be the limiting factor in the biotransformation. The initial OD600 of the inoculum which was previously variable was shown to have an effect on the initial BMA production rate but not the length of time the production rate continued for with the rate again dropping after 1 hour.

Doubling the quantity of cells within the biotransformation doubled the BMA produced, it was therefore concluded that an intracellular limitation was responsible for the BMA production rate slowing after 1 hour. This showed that BMA toxicity and external concentrations of substrates was not responsible for the slowing production rate.

Addition of cofactors to the biotransformation prolonged the duration of the high BMA production rate in LUC0615 and LUC0711 resulting in higher BMA concentrations over the control. This indicated that the BCKD complex was the cause of the limitation as the cofactors added were cofactors required by the BCKD complex. In LUC0615 it was shown that addition of cofactors and substrates at 6 hours prolonged BMA production further.

KIV was shown to be able to be produced from glucose using the pKIV plasmid within the control *E. coli BW25113* host. It was also concluded that NADPH was not limited in the biotransformation. BMA was unable to be produced directly from glucose in a variety of strains containing both the pKIV and BMA production plasmids. In all strains glucose was not utilised and it was concluded that the pKIV plasmid was not operating correctly in the dual plasmid strains. This was confirmed by the lack of KIV in the biotransformation. As each plasmid works independently but not together further work is required to produce BMA directly from glucose within a biotransformation.

LUC0711 containing the pMMA 071 BMA production plasmid demonstrated the highest BMA production rate with no IBA production. Both of these aspects made LUC0711 the optimum strain for BMA production and should therefore be used for future work.

4.21. Future work

- The HPLC analysis of LUC0711 showed that more KIV and butanol was utilised than can be accounted for by the BMA produced. As LUC0711 produced the highest BMA concentration understanding the utilisation of KIV and butanol would allow the biotransformation to be refined. In order for the biotransformation to be as economical as possible limiting unnecessary utilisation of the substrates butanol and KIV is required. Identification of butanol by-products and KIV by-products would allow LUC0711 strain optimisation to take place allowing more KIV and butanol to be available for BMA production. GC-MS analysis of samples may allow butyl-esters to be identified as had previously been carried out for LUC0615. In LUC0615 this analysis still resulted in a large quantity of unaccounted for butanol. Complete lysis of the biotransformation broth may further reduce unaccounted for butanol which may be sequestered within the cells. NMR spectroscopy may be required if a large quantity of butanol remains unaccounted for.

  Further analysis of the metabolism of KIV within *E. coli* and the possible products from the enzymes contained within the BMA plasmid may allow further KIV by-products to

be identified. NMR spectroscopy may again be required to identify further KIV by-products which can then be eliminated from the biotransformation.

- Butyl isobutyrate was shown to be a major butanol by-product in LUC0615 and as such elimination of the by-product may improve the efficiency of the biotransformation due to a greater butanol availability. An alcohol acyl-transferase (AAT) is required to produce butyl-isobutyrate from IBA-CoA. The AAT is also used for the production of BMA from methacryl-CoA therefore engineering superior chemoselectivity in the AAT would prevent IBA-CoA being accepted by the enzyme binding site and reduce or eliminate butyl-isobutyrate production.

- Batch biotransformations of LUC0615 and LUC0711, in which CoA, TPP and NAD^+ were added, showed an increase in the length of BMA production which resulted in a higher final concentration of BMA being produced. This prolonged BMA production, in comparison to control biotransformations without the addition of cofactors, was thought to be due to prolonged activation of the BCKD complex. The BCKD complex utilises all three coenzymes (Ævarsson *et al.*, 2000) during activation although it is not known if all of the additional coenzymes were required. In order to further optimise BMA production repetition of these biotransformations would allow quantification of each cofactor by HPLC.

  Coenzyme A (Shurubor *et al.*, 2017), TPP (Lu and Frank, 2008), and NAD^+ (Yoshino and Imai, 2013) can be analysed by HPLC which would allow quantification of each compound to be monitored over the course of the biotransformation.

- As coenzymes are expensive additions to a bioprocess it may be possible that all coenzymes are not required or could be added at a lower concentration. It had been shown in LUC0615 that a second addition of cofactors at 6 hours did not prolong production further suggesting that the coenzymes were no longer limiting after the first addition. The biotransformation of LUC0615 could be repeated using incrementally lower concentrations of coenzyme which could help to determine the

optimum concentration of all three. It may also be possible that all three cofactors are not required. To determine if this was the case biotransformations could be carried out using only each cofactor individually at different concentrations to see what effect each alone has on BMA production.

- BMA production directly from glucose was not successful, with no glucose being utilised and no KIV being produced. As both the pMMA 050 plasmid and the pKIV plasmid had been shown to work independently the reason for this was not immediately apparent. It may be possible that either the presence of butanol in the biotransformation or the presence of both plasmids has had a detrimental effect on the activity of the pKIV plasmid. In order to determine if this was the case, the KIV production biotransformation should be repeated in the presence of butanol to determine if this was a factor. A strain should also be produced containing the pKIV plasmid and an empty plasmid to simulate the presence of the pMMA 050 plasmid. This new strain could then be run in a biotransformation with and without butanol to determine if the presence of the second plasmid was detrimental to the operation of the pKIV plasmid. These experiments would allow insight into the inactivity of the pKIV plasmid to be gained for future process development.

- If BMA production from glucose was demonstrated to be successful in a strain containing the BMA production plasmid pMMA 050 and the KIV production plasmid, the next step would be incorporation of the pMMA 070 plasmid. The pMMA 070 plasmid was shown to produce BMA at a higher production rate with no by-production of IBA. The replacement of pMMA 050 with pMMA 070 may therefore improve the BMA yield from glucose.

Chapter 5

Summary of conclusions

This thesis contains results outlining the novel production of both the organic acid mesaconate, in *Corynebacterium glutamicum,* and the ester butyl methacrylate (BMA) in *Escherichia coli*. The global attitude in mass market polymer production is shifting towards a sustainable future and as such polymers with unique properties, like PMMA, require alternative methods of production. Both chemicals have the potential to be incorporated into the industrial pathway for the production of poly methyl methacrylate (PMMA) providing an avenue for the production of this polymer from biomass, which would eliminate the current reliance on petrochemically derived precursors. Several important conclusions can be drawn this early work into the biological production of both chemicals which is discussed below.

Firstly, through a multitude of bioprocesses, it was shown that mesaconate production is possible within *C. glutamicum* with a potential for yield improvement due to the availability of large quantities of the precursor glutamate. The primary aim of this portion of work was to effectively demonstrate mesaconate production from glucose in *C. glutamicum* which was achieved. The overarching goal for the mesaconate production research was to maximise the glutamate utilisation rate and as such maximise the mesaconate production rate.

It was concluded that the glutamate mutase activity was the limiting factor for mesaconate production with the addition of a glutamate mutase reactivatase increasing the mesaconate titre. In addition, it was concluded that oxygen was the main driver for glutamate mutase deactivation since reducing the dissolved oxygen within the bioprocess improved the mesaconate titres in all strains. The glutamate mutase with reactivatase from *Desulfosporosinus meridiei* had a higher production rate than the glutamate mutase with reactivatase from *Clostridium tetanomorphum* which had been shown to produce a high mesaconate titre in *E. coli* (Wang and Zhang, 2015). It was therefore concluded that the glutamate mutase from *D. meridiei* was the optimum glutamate mutase for future work on mesaconate production. Although a higher production rate was observed when using this glutamate mutase mesaconate production stops at a much earlier timepoint. It was hypothesised that a lack of coenzyme B12 may have been responsible for this although this was not tested.

The novel research into butyl methacrylate production in engineered strains of *E. coli* provided several key conclusions. The aim of the butyl methacrylate research was to achieve a production rate of 14 mM/ hour. This production rate was not able to be reached but

incremental gains towards this target were achieved. It was initially concluded that the alcohol acyltransferase (AAT) was the limiting factor for BMA production from KIV and butanol. Engineered strains either demonstrating upregulated AAT expression (LUC0615) or containing mutated AAT's with increased activity (LUC0710/LUC0711) were shown to produce higher titres than their predecessors.

Although higher titres were seen in these strains the production of BMA stopped when both substrates (KIV and butanol) were shown to be available indicating the presence of another bottleneck. It was concluded from the biotransformations in which additional cofactors were added that the branched chain ketoacid dehydrogenase (BCKD) complex was the bottleneck. The cofactors which were added were required by the BCKD complex for activity and their addition prolonged BMA production. It was also concluded that a major by-product IBA could be eliminated from the biotransformation through deletion of two thioesterases *yciA* and *tesB*, although this by-product was shown not to be present in the biotransformations of the best producing strain LUC0711. It was concluded from the biotransformations in which BMA production was attempted from glucose that the pKIV plasmid was not functioning as expected and KIV was not being produced. As such BMA production directly from glucose was not successful.

Neither chemical was able to be produced at a level to which it could currently be integrated into the production pathway for PMMA. Although this was expected as the production of both of these chemicals is still in the early stages of development. As such there is considerable future work to be carried out on the production of both chemicals. The main conclusions outlined above provide guidance as to what the priority for the future work into the production of both chemicals should be.

For mesaconate production:

- Reducing the dissolved oxygen concentration resulted in a yield improvement. Determination of the optimum dissolved oxygen concentration within the bioprocess may limit the deactivation of the glutamate mutase and as a result maximise mesaconate production before further strain improvement.
- A coenzyme B12 synthesis and regeneration pathway should be incorporated into the production strain IL5.0.1(G2).2(F1-F2-F3) to determine if limitation of coenzyme B12 was responsible for mesaconate production stopping when glutamate is available.

For butyl methacrylate production:

- Further cofactor experiments are required to determine exactly which cofactors were important for prolonged BMA production. After this strain engineering would allow the concentration of the required cofactors to be increased within the biotransformation.
- Further improvement in the activity of the AAT should be explored as this was seen to have a large impact in the production rate of BMA.
- BMA production directly from glucose is required as a process using KIV would have a higher cost. Production of a strain using the BMA production plasmid from LUC0711 and the pKIV plasmid should be carried out and biotransformations run to determine the bottleneck in KIV production.
- The fate of butanol within the biotransformation should be explored further as butanol availability may become a bottleneck upon achieving higher production rates of BMA.

Chapter 6

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