Sustainable production of platform chemicals using novel synthetic biology and microbial fermentations

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A thesis submitted to the University of Strathclyde in part fulfillment of the regulations for the degree of Doctor of Philosophy.

September 2015

Copyright Declaration

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Acknowledgements

First and foremost I would like to thank my supervisors Professor Linda Harvey, Professor Brian McNeil and Dr Alison Arnold for their continued support and advice over the course of my PhD. I would like to thank Ingenza Ltd for their assistance throughout this project. In particular, I would to thank Dr Reuben Carr and Dr. Ian Archer for their support and advice as well as Dr Scott Baxter for his expertise in the genetic engineering of all the strains involved in this project. A thanks also to Michael Bott for supplying the Bol-3 strain of *C. glutamicum*

Thank you also to all my friends and colleges within SIPBS most importantly those in the fermentation centre: Brian Griffin, Scott Parker, Joana Faustino, Ellis Robb, Wan Bin Mohtar, Nurzilla Binti Ab Latif and Dr Chiara Viegelmann. I would like to pay particular appreciation to Dr Mariana Fazenda and Dr Peter Gardner for their support and guidance throughout my project. As well as Dr Alison MacFadyen for her technical assistance in acquiring qPCR and LC-MS results in this project.

A special thanks to my parents for their unbelievable support and encouragement as well as their continued belief in my abilities over my entire academic career. Thank you also to my brother and the rest of family for their support. And to Andrew I would like to thank him for his patience, understanding and his incredible ability to keep me positive through the latter stages of writing.

I am lastly grateful for the studentship that was provided by SULSA BioSKAPE as well as for the supplementary funding supplied by Ingenza Ltd.

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List of Abbreviations

ACN	Aconitase
ALDH	Acetaldehyde dehydrogenase
ADH	Alcohol dehydrogenase
CER	Carbon evolution rate
CFE	Cell Free extract
CGG	*C. glutamicum* glucose growth medium
CGXII	*C. glutamicum* XII growth medium
CS	Citrate synthase
DAD	Diode Array Detector
DCW	Dry cell weight
DOE	Department of Energy (US)
EMA	Ethyl methacrylate
EMB	Ethambutol
Fum	Fumerase
Glm	Glutamate mutase
h	hour
HPLC	High Performance Liquid Chromatography
iBMA	Isobutyl methacrylate
IDH	Isocitrate dehydrogenase
iPMA	Isopropyl methacrylate
KGS	α-ketoglutarate synthetase

LDH	Lactate dehydrogenase
tMAAL	Methylaspartate ammonium lyase from *Clostridium tetanomorphum*
MeOH	Methanol
Mex	4-Methoxyphenol
MAA	Methacrylic acid
MMA	Methyl methacrylate
MQO	Malate: quinone oxidoreductase
MV	Methyl viologen
ODHC	Oxoglutarate dehydrogenase complex
OPA	o-phthalaldehyde
OUR	Oxygen uptake rate
PC	Pyruvate carboxylas
PDA	Potato Dextrose Agar
PDHC	Pyruvate dehydrogenase complex
PEPC	Phophenolpyruvate carboxylase
PHA	Polyhydroxyalkanoates
PPG	Polypropylene glycol
PPP	Pentose phosphate pathway
PTS	Phosphotransferase transport system
PYK	Pyruvate kinase
RI	Refractive index
RPM	Revolutions per minute

RQ	Respiratory coefficient
SCS	Succinyl CoA synthetase
SQO	Succinate: menaquinone oxidoreducase
TCA	Tricarboxyclic acid cycle
TTA	Total titrable acid

Abstract

Fluctuating oil prices and environmental concerns have led to a directed effort by industry to develop sustainable production of high value chemicals. Mesaconate, an industrially relevant platform chemical used in the production of methyl methacrylate, can be synthesised through the L-glutamate degradation pathway present in certain anaerobic organisms. This study investigates the potential of genes derived from this pathway to be expressed in *Corynebacterium glutamicum*, as a cellular factory to produce mesaconate in the first instance, with future scope to produce methyl methacrylate. *C. glutamicum* was chosen as a primary organism due to its existing industrial use as a microbial workhorse for the production of L-glutamate.

Firstly, as the proposed process incorporated the initial over production of L-glutamate, various induction methods were investigated to establish an optimal process. Genetically engineered strains were then constructed and examined for their ability to produce mesaconate. Further process development was carried out to develop an efficient induction protocol. While the accumulation of mesaconate remains unclear, novel expression of the desired glutamate mutase and methylaspartate ammonia-lyase within *C. glutamicum* ATCC 13869 was established.

A secondary approach where production of desired chemicals under oxygen limited conditions was examined. High cell densities of several mutant strains of *C. glutamicum* were investigated during a bioconversion process. Significant levels of succinic acid were achieved, however, further conversion to α-ketoglutarate, the precursor to L-glutamate was not observed. Co-factor regeneration appeared to be crucial for successful application of this process.

Finally, toxicity of potential products and feedstocks were analysed to identify detrimental or toxic effects. While many of the compounds appeared to have little effect on the organisms examined, presence of methyl methacrylate, methacrylic acid and methanol significantly decreased growth and/or production. Design of future processes will be significant in maintaining these compounds at non-inhibitory levels to ensure efficiency.

Chapter 1 Introduction

1.1 **Current state in the production sustainable platform chemicals**

As petroleum stocks decrease and the barrel price for crude oil continues to fluctuate, it is essential for industry to reduce its dependency on these finite reserves and utilise more sustainable resources (Figure 1-1). Scrutiny of the impacts of crude oil derived compounds on the environment have also raised concerns in recent years and has added urgency for the development of alternative routes towards fuels and chemical building blocks. It has been proposed that biological production of high value chemicals could serve as an abundant reservoir for sustainable manufacture.

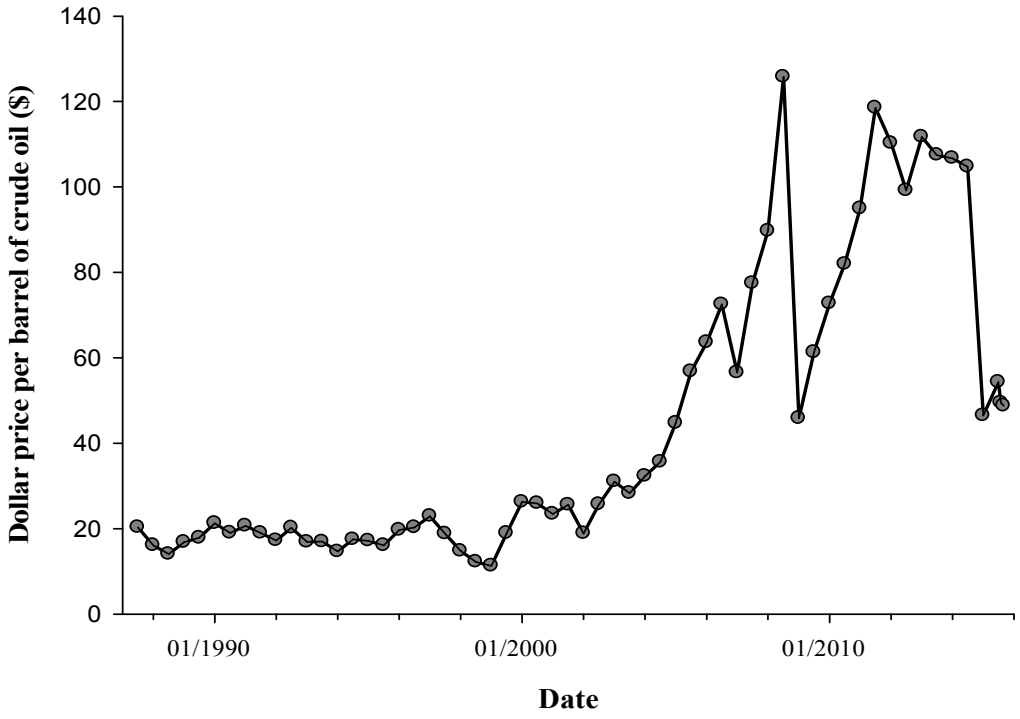


Figure 1-1 European Brent Spot Price - Crude oil prices from 1987-2015
Data obtained from the Energy Information Administration, US Department of Energy (US Energy Information Administration 2015b).

Industrial production of biofuels has already been exploited as an alternative method for fuel and energy production. Development for the production of ethanol, biodiesel and advanced biofuels such as butanol and algal biodiesel are starting to address the energy demand in the US and have several major advantages over conventional petroleum based fuels. Firstly, they are produced from renewable biomass sources. For example, ethanol can be produced from corn stover, sugar beet and sugar cane (Cardona *et al.*, 2010). Biodiesel can be produced from vegetable oil, waste fats and grease (Phan and Phan, 2008). Secondly, these fuels are generally nontoxic, biodegradable and burn cleaner compared to their petroleum based counterparts. They can also be blended with conventional petroleum and diesel to be used in unmodified automobile engines. Again using ethanol as an example, almost all of the gasoline sold in the US contains ethanol at a concentration of 10 % v/v. Higher concentrations of ethanol require engine modification to obtain successful fuel consumption. Biodiesel on the other hand, is chemically similar to that of petroleum derived diesel and therefore can be directly substituted into a diesel powered engine without loss of fuel economy. Ease of adaption of biodiesel has seen its commercial use in the US dramatically increase from 10 million gallons in 2001 to 1.4 billion gallons in 2013/14 with a worldwide consumption of 6.44 billion gallons observed in the same period (US Energy Information Administration 2015a).

Despite the beneficial advantages of using biofuels and their ability to be produced at high volume from biomass, these compounds are relatively low value commodities and therefore exhibit low profitability making it an unfavourable option for many investors. Integrated biorefineries producing both fuel and high value chemicals could increase profitability and productivity that would make this sector more attractive to both industry and investors.

Biological synthesis of organic acids for commercial use has been performed for over a 100 years. For example; production of citric acid, an organic acid formed in the Tricarboxcylic acid (TCA) cycle, is one of the most recognisable compounds that has been made exclusively in industry through fungal fermentation since the early 20^{th} century (Currie, 1917). The original use for theses acids were primarily in the food and beverage industry as acidifiers. However, over the years many more uses

have been associated with them including components of metal cleaning agents, pharmaceutical additives and recently, their use as building block molecules in the production of polymers which have become a topic of interest (Sauer *et al.*, 2008).

Before successful integration of these bio-based compounds into a biorefinery setting can be realised, several challenges must be overcome. Firstly, the technology on how to produce these building blocks has to be refined and developed to provide an efficient process that can compete with the chemically produced counterparts. Secondly, the abundance of chemicals that can be obtained from biological metabolism is vast. Therefore, identification of potential candidates that would fulfil the economic goal of the biorefinery would be required in order to target the most desirable compounds.

In 2004 the US department of Energy released a paper outlining a list of compounds that could be obtained from carbohydrates through microbial bioprocesses (Werpy and Petersen, 2004). In consultation with the National Renewable Energy Laboratory and the Pacific Northwest National Laboratory, they created an initial list of 300 compounds that would economically and technically be able to "support the production of fuels and power in an integrated biorefinery" (Werpy and Petersen, 2004). A shortlist of 15 compounds was then established that consisted of candidates that could be derived from sugar compounds and are outlined in Table 1-1. These compounds also possessed a high chemical functionality which increased their potential use as a platform chemical for multiple products. By publishing this list, the Department of Energy provided an outline for more targeted research into these particular avenues highlighting an importance for target chemical structures that are highly desirable.

Table 1-1 The Department of Energy top chemical opportunities from carbohydrates

Compound	Potential use
Succinic, fumaric and malic acids	Solvents, Polyesters
2,5-Furan dicarboxylic acid	Polyesters
3-Hydroxypropionic acid	Acrylic acid
Aspartic acid	Chelating agent, Sweeteners
Glucaric acid	Solvents, Adpic 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

Table adapted from (Werpy and Petersen, 2004, Choi *et al.*, 2015)

Since the release of this paper, it has inspired a considerable volume of research identifying and developing the technologies required for the bio-production of the compounds identified. In 2010, Bozell and Petersen (2010) released an update on the advances of the list created by the DOE in 2004 and re-valuated the potential of the compounds. In some cases, several of the compounds were disregarded due to the lack of market growth that was initially expected. In addition to the 2004 list, lactic acid and ethanol were added since the potential in these compounds had grown, with ethanol in particular no longer considered just a commodity for biofuel.

By examining the literature, advances in several of the compounds that appear on both lists, as well as others not mentioned that exhibit potential, will be outlined in this chapter. Particular attention will be paid to organic acid and amino acids involved in central carbon metabolism, as exploiting this pathway to produce high value chemicals is a major subject that will be covered in this study. The various

compounds outlined are produced by a variety of microorganisms under both aerobic and anaerobic conditions. Process methods for their production range from batch, fed-batch and continuous culture. The latter two processes provide significant advantages over a batch process, such as increased product yields and reduction in time between batches which ultimately leads to increased cost efficiency of the process.

1.2 **Organic acids**

1.2.1 **Succinic acid**

Succinic acid production has been widely studied within the literature as it has the potential as a precursor compound to a variety of industrially important chemicals including; tetrahydrofuran, γ-butyrolactone, 1,4-diaminobutane and 1,4-butanediol (Bozell and Petersen, 2010, Almeida *et al.*, 2012). These building blocks can then be used by industry to manufacture green solvents, pharmaceutical products and biodegradable plastics. According to the DOE report to achieve an economically competitive processes, productivity of the fermentation would need to reach a minimum of 2.5 $gL^{-1}\cdot h$ with a bioprocess cost of $0.25/lb.

Meynial-Salles *et al.* (2008) achieved this level of production by harnessing the potential of the obligate anaerobe *Anaerobiospirillum succiniciproducens*. Using a continuous cell cycle bioreactor they achieved a productivity of 14.8 $gL^{-1}\cdot h$. However, end-product inhibition was observed. To overcome this undesirable effect, a process was established that coupled an electro dialysis system to the reactor. This removed the organic acids present, returning the free medium back to reactor. This additional step did result in a reduction of productivity to 10.4 $gL^{-1}\cdot h$ (83 gl^{-1} succinic acid titre) however; product recovery was made simpler as highly concentrated solutions of succinic acid were obtained. These high productivity results were obtained directly from glucose which the author did not disclose as the primary carbon source which is not desirable. Ideally, to obtain cost efficient

processes, incorporation of sustainable carbon sources would be beneficial. In this case, hydrolysate from starch based materials could be incorporated as an alternative carbon source instead of refined glucose.

A. succiniciproducens has been observed to produce succinic acid using renewable sources such as glycerol and whey, albeit with less success. A maximum production rate of $1.35 gL^{-1} \cdot h^{-1}$ for both carbon sources was observed (Lee *et al.*, 2000, Lee *et al.*, 2001).Other organisms have been examined for the production of succinic acid exhibiting varying degrees of success.

Several strains of *Escherichia coli* have been developed to improve the yield of succinic acid which similar to *A. succiniciproducens*, are achieved utilising an anaerobic process. Liu *et al.* (2013) designed a strain which overexpressed the phosphoenolpyruvate carboxykinase gene from *Bacillus subtilis* and lacked genes for pyruvate formate lyase, lactate dehydrogenase, pyruvate carboxylase and *pts*G, required during glucose uptake. Deletions of the above mentioned genes have all been previously reported to increase the production of succinic acid in *E. coli* but only with a limited yield (Wu *et al.*, 2007). With the over expression of the genes required for phosphoenolpyruvate carboxykinase (PEPCK), the strain was capable of utilising xylose and glucose, obtained from sugarcane bagasse hydrolysates, resulting in an end titre of 39.3 gl^{-1} of succinic acid.

Other than whey and sugarcane, microalgal hydrolysate has also been shown to be a carbon source for engineered *E. coli* strains to produce succinic acid to a concentration of 1.24 ± 0.08 mol/mol total sugar content (Bai *et al.*, 2015). Therefore, there is potential to use *E. coli* as a producer of succinic acid using renewable resources which is in line with the overall view to construct a sustainable process.

As well as the organisms indicated above, metabolic engineered strains of *Mannheimia succiniciproducens* have also displayed their potential as succinic acid producers. By exploiting knowledge of the strains genome sequence, Lee *et al.* (2006) identified key pathways leading to by-product formation. Deletion of the corresponding genes for lactate, pyruvate and acetate production resulted in a strain

capable of end succinic acid titre of 13.4 gl^{-1} during batch culture. Accumulation was further improved with the introduction of a glucose feed which increased the yield to 52 gl^{-1} after 29 h cultivation. Productivity however remained lower than achieved by *A. succiniciproducens* with a yield of only 1.8 $gL^{-1}h^{-1}$.

Corynebacterium glutamicum is another organism of interest for the production of succinic acid (Jojima *et al.*, 2013, Choi *et al.*, 2015). Similar to *A. succiniciproducens*, the process to achieve succinic acid accumulation was under anaerobic conditions, which were not favourable for cellular growth. Therefore, a two phase process is generally implemented where cultures are grown aerobically to obtain a high cell density whereupon the cells are harvested. Cells are then resuspended in the production medium under oxygen deprived conditions for the accumulation of succinic acid (Okino *et al.*, 2008a, Litsanov *et al.*, 2012). Currently, Litsanov *et al.* (2012) have achieved one of the highest yields of succinic acid using engineered *C. glutamicum*. Deletion of genes required for the synthesis of lactate and acetate and the over expression of genes that enhance the reductive TCA cycle lead to the creation of a strain that yielded 1.67 mol/mol succinic acid/glucose (genetic manipulation of this strain to produce succinic acid is discussed in Chapter 5). Although this was an exceptional yield, the process required the addition of a large quantity of formic acid for the regeneration of cofactors. This extra cost would make its feasibility in industry unlikely.

Continuous cell cycling with glucose as the sole carbon source appeared to exhibit conditions where upon maximum succinic acid yields were obtained. Improvement in the utilisation of renewable carbon sources is required in order to obtain a commercially efficient process as many require breakdown of the biomass source before use. Further addition of electrodialysis like system described by Meynial-Salles *et al.* (2008) has the potential to prevent product inhibition and therefore increase productivity further.

1.2.2 **Fumaric acid**

Fumaric acid, like succinic acid, is a naturally occurring organic acid which can be produced by a variety of organisms. It is a versatile compound which can be used as a raw material in the production of unsaturated polyesters, alkyd and paper resins, plasticizers, pharmaceuticals and as additives in the food and drink industry (Roa Engel *et al.*, 2008). Current production of fumaric acid is through chemical conversion of maleic anhydride, replacing the fermentation method that was historically used in the 1940's (Goldberg *et al.*, 2006).

In recent years, the fermentative pathway towards fumaric acid has had a resurgence in interest due to its independence from the petrochemical industry. Historically, species of *Rhizopus* were utilised for their high accumulation of fumaric acid and therefore several processes were patented (Goldberg and Stieglitz, 1986, Ling and Ng, 1989). Current developments focus on metabolic engineering of this organism to utilise sustainable resources such as crude glycerol and xylose in an attempt to create a more sustainable process that can be linked to an integrated biorefinery (Huang *et al.*, 2015, Liu *et al.*, 2015a).

As *Rhizopus oryzae* is naturally inhibited by glycerol, Huang *et al.* (2015) employed evolutionary engineering of the strain to improve utilisation. This resulted in a strain that exhibited an increase in glycerol dissimilation and pyruvate consumption when compared with the parental strain. Significantly higher levels of oxaloacetate and malate as well as fumarate were also present in the residual media compared to the parental strain. This suggests carbon flux was more favourable towards the reductive pathway rather than the oxidative arm of the TCA cycle. This increased flux is obviously beneficial for the production of fumaric acid as less carbon is lost to by-products. During glycerol feeding an end yield 25.5 gl^{-1} of fumaric acid was achieved which was in significant contrast to the 1.2 gl^{-1} that the parental strain achieved. Further improvements of the strain could have been made as levels of ethanol and lactic acid were also observed due to oxygen limitations. By genetically engineering the strain to be defective in the pathways leading to the production of these anaerobic

analytes, further improvement could be made to the product yield as again, carbon would not be lost to by-products.

1.2.3 **Malic acids**

Malic acid is another C_4 carbon compound that exhibits potential as a platform chemical and has applications in the food, drink and nutrient supplements industry as well as being an intermediate in the production of polymers (Zhang *et al.*, 2013). Current production of this compound is mainly by the chemical conversion of maleic anhydride or fumaric acid under high pressure to produce a racemic mixture of both the D and L sterisomers of malic acid (Goldberg *et al.*, 2006). Enzymatic conversion of fumaric acid has also been used to obtain malic acid where the reaction is catalysed by fumarase from immobilised *Brevibacterium flavum*. Both of the aforementioned techniques rely on unsustainable feedstock chemicals that are currently produced through petrochemical means and require costly catalysts.

As malic acid is an intermediate of the TCA cycle it can be produced by a variety of organisms. Despite being over two decades old the study by Battat *et al.* (1991) with *Aspergillus flavus* ATCC 13697 remains, to date, the most efficient microbial process for the production of L-malic acid. A high titre of 113 gl^{-1} of L-malic acid was achieved from 120 gl^{-1} of glucose after 190 h of cultivation. A maximum production rate of 0.59 gl^{-1} h was achieved when optimum process conditions were implemented, where agitation/aeration and medium components had significant influence on the accumulation rate of L-malic acid.

Despite the high productivity of this strain, an industrial process was never implemented due to the strains well documented co-production of highly toxic components known as aflatoxins (Gqaleni *et al.*, 1997). As the principal use of L-malic acid in the 1990's was directed at the food industry, presence of aflatoxins prevented its use due to safety concerns and alternative potential organisms were sought.

Metabolic flux analysis (MFA) has been employed to understand the optimal pathway towards L-malic acid of recombinant strains of *Escherichia coli* (*E. coli*) (Moon *et al.*, 2008). By understanding the metabolism of the organism it was anticipated that significant enzymes that related to the overproduction of L-malic acid would be identified. Interestingly, Moon *et al.* (2008) observed that optimal conditions were under aerobic conditions and identified phosphoenolpyruvate caboxykinase (PEPC) as an enzyme with potential. By over expressing the native *E. coli pck*A gene responsible for PEPC, the mutant strain exhibited accumulation of L-malic acid where previously no productivity was observed. Over expression of *pck*A from *Mannheimia succinicproducens* MBEL55E resulted in an increase to the end yield of L-malic acid significantly from 1.42 gl^{-1} to 9.25 gl^{-1}, exhibited a productivity rate of 0.75 $gL^{-1}h^{-1}$ and conversion ratio of 0.75 mol/mol glucose. Although this strain exhibited a lower overall yield than achieved by *A. flavus* an increase in production rate is positive. Further improvement of yield was not achieved by deletion of key genes responsible for the production of by-products. Improvement in the process is still required to obtain the yield of L-malic acid attained by the current method of enzymatic conversion.

End product inhibition on *E. coli* remains problematic to achieve high yields as metabolism has been observed to be hindered when malic acid is present in concentrations above 60 gl^{-1} (Cao *et al.*, 2011). This is due to the compounds low acid dissociation constant (pKa) value of 3.4 which is not an optimal pH for growth of organism such as *E.coli (Zou et al., 2013)*.

Production of pure L-malic acid is not the only route being examined in more detail within the literature. Production of poly (β-L-malic acid) (PMA) by strains of *Aureobasidium pullulans* not only offers an alternative route towards malic acid through hydrolysis of the polymeric compound with sulphuric acid but also a desirable product in itself. PMA is a natural bio-polyester which is biocompatible, biodegradable and water soluble. Use of this compound has already been implemented in biomedical devices and primarily as a drug carrier. In addition, PMAs water insoluble derivatives have the potential to replace petrochemical

derived compounds as a sustainable building block in the production of detergents and biodegradable plastics.

PMA production by the *A. pullulans*, a polymorphic fungus, was first described by Nagata *et al.* (1993) when screening strains of black yeast using selective medium which contained mannitol as its primary carbon source. Unlike malic acid, PMA is nontoxic to the cells and can be easily removed from the production broth by ethanol precipitation. In 2012, a study of PMA production by a diverse group of *A.pullulans* stains was performed (Manitchotpisit *et al.*, 2012). Fifty-six strains, compromising of 30 newly isolated and 26 previously reported strains, were analysed in a medium containing 5% w/v glucose. After 7 days culturing, accumulation of PMA ranged from 2.6 ± 0.2 to 11 ± 0.9 gl^{-1} between the isolates. This considerable variability between isolates indicates genetic diversity within the subset of *A.pullulans* and the importance of screening in order to obtain isolates capable of the highest productivities.

Zou *et al.* (2013) implemented a screening process that characterised isolates based on the colour of the colony formation of *A.pullulans* isolates on potato dextrose agar (PDA). It was noted that isolates that produced un-pigmented colonies exhibited higher PMA productivity compared to those that contained pigment. By-product formation would not only affect overall productivity of the strain but increase downstream processing to isolate PMA from the production broth. Sub-culturing the un-pigmented strain resulted in the purification of the strain which was then examined in submerged culture under both batch and fed batch conditions. An increase in productivity from 0.49 $gL^{-1}h^{-1}$ to 0.61 $gL^{-1}h^{-1}$ was observed when the process was converted from a batch to a fed-batch process. Increase in production when moving to this process is a reasonable outcome as it allows an initial production of a high cell density. This can then be exploited to produce the desired compound whilst maintaining the process within optimal conditions that provide the highest yields, for example limiting on carbon source.

Interestingly, by using immobilised *A. pullulans* cells in a fibrous-bed bioreactor, this productivity was increase further to 0.74 $gL^{-1}h^{-1}$ with a 0.55 g/g conversion ratio from glucose. It was hypothesised by the author of the paper that this was due to the

reduction of cellular growth that was observed in this particular environment compared to the submerged culture. Although this does improve the productivity, the implementation of such a system in an industrial setting presents its own challenges This includes low flow rates of materials, reduced mass transfer and potential blockage of the bed matrix with solid debris (Willaert, 2006). Nevertheless, this productivity presents a promising route to PMA and L-malic acid as it lacks the inhibition of cellular metabolism observed in systems producing pure L-malic acid.

As the highest yields of both L-malic acid and PMA were produced using glucose as the primary carbon source, further work in this area remains to develop an efficient process that utilises a more sustainable and cost effective raw materials such as wheat straw pre-treated with alkaline hydrogen peroxide (Leathers and Manitchotpisit, 2013)

1.2.4 **Itaconic acid**

Itaconic acid, also known as methylenesuccinic acid, is a C_5 carbon compound that is used in the plastics industry as a co-monomer in the production of acrylic fibres, rubbers and reinforced glass fibre (Werpy and Petersen, 2004, Okabe *et al.*, 2009). Two such copolymers that can be constructed from itaconic acid are poly(acrylic acid-*co*-itaconic acid) which has desirable characteristics as a superabsorbent material (Katime and Rodríguez, 2001) and poly(itaconic acid-*co*-*N*-vinylpyrrolidone) which can be use in the manufacturing of oral drug delivery systems (Koetting and Peppas, 2014). Similar to succinic acid, it can also be used as a precursor to compounds such as 3 and 4-methyl γ-butyrolactone, 3-methylpyrrolidine, and 3-methyltetrahydrofuran. As well as the aforementioned applications, itaconic acid has also been proposed as a potential precursor in the production of methyl methacrylate (MMA). MMA is a highly sought after monomer that is used in the production of Plexiglas (Choi *et al.*, 2015).

Due to the wide and varied applications of itaconic acid, it was considered as a commercially lucrative acid in the 2004 DOE report. To make this compound

commercially viable, the report stated that production rates would be required to be improved to a minimum of 2.5 gl^{-1} and have a bioprocess cost <$0.25/lb, the same as succinic acid, in order to compete with current production of the commodity chemicals. However, since the report was released, market growth of itaconic acid has been slow, leading to its exclusion from the updated paper in 2010. Therefore, improvement is still required to accomplish the true potential of this compound.

Itaconic acid is traditionally produced by certain strains of *Aspergillus*. It was first produced by using *Aspergillus itaconicus* (Kinoshita, 1931). Since then, it has been mainly produced by *Aspergillus terreus* for industrial purposes (Bentley and Thiessen, 1956). Even though it has been industrially produced for many years, the precise pathway that leads to the production of itaconic acid is still debated (Bonnarme *et al.*, 1995).

Until recently, attempts to improve efficiency of itaconate production have been rare. It is know that the presence of itaconic acid itself is inhibitory towards the growth of *A. terreus* (Kobayashi and Nakamura, 1964). A knock on effect of this is that further production of the acid is halted. Improved efficiency of the organism could be achieved by identifying itaconate resistant strains. Yahiro *et al.* (1995) used N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) as a mutagenesis agent to colonies of *A. terreus* with improved production of itaconic acid. Compared to the wild type control, *A. terreus* IF0 6365, production was increase by 30 %. However, with a maximum yield of only 85 gl^{-1}, it still does not compete with some strains of *A. niger* which can produce in excess of 100 gl^{-1} under favourable conditions (Papagianni *et al.*, 1998).

Itaconic acid is produced through the citric acid branch of the TCA cycle. Firstly, citric acid is dehydrated to cis-aconitic acid from which a decarboxylation reaction is necessary to acquire itaconic acid. The cis-aconitic acid decarboxylase (CAD) enzyme, regulated by the *cad*A gene, has been identified in *A. terreus* and is essential for the production of itaconic acid as its deletion renders production inoperable (Dwiarti *et al.*, 2002, Kanamasa *et al.*, 2008). Li *et al.* (2011) isolated the *cad*A from *A .terrus* and overexpressed the gene in *A. niger* AB 1.13, a major producer of citric acid. Albeit in low levels, inclusion of this gene resulted in accumulation of 0.07 gl^{-1}

itaconic acid which was not observed with the parental strain. However, it should be noted that production of citric acid by the strain used was relatively low, 0.7 gl^{-1} in the parental strain and 0.15 gl^{-1} by the itaconic producer. This is in stark contrast to other strains of *A. niger* which can produce 100 gl^{-1} citric acid. Therefore, by expressing the *cad*A gene within a strain optimised in citric acid production may increase the overall yield of itaconic acid.

As yet, no successful process has been implemented into full industrial production. Further work on the post production polymerisation of this organic acid is still required to make the process commercially viable as it remains less efficient than current methods. (Choi *et al.*, 2015).

1.2.5 **Lactic acid**

Lactic acid appeared in the top 30 list in the original DOE report and recently gained further attention as a platform chemical due to its potential application in a variety of products. Currently, 400,000 tonnes of lactic acid is produced per year worldwide with this market expected to continue to grow due to its use in the manufacturing of polylactic acid (PLA) (Choi *et al.*, 2015). This bio-polymer is used in the USA, EU and Japan for the manufacture of food packaging. Lactic acid can undergo esterification to the corresponding lactic ester. This in turn can be catalytically reduced to produce polypropylene glycol or dehydrated to produce acrylic acid albeit with low yield ability (Varadarajan and Miller, 1999, Bozell and Petersen, 2010). Electrospinning of PLA has also shown the potential of production of the compound on a nanoscale to create scaffolding that can be used in neural tissue engineering (Yang *et al.*, 2005).

Currently, lactic acid is mainly produced by biological fermentation by lactic acid bacteria such as *Lactobacillus helveticus* (Kylä-Nikkilä *et al.*, 2000) or by the fungal strains such as *Rhizopus oryzae* **(**Abdel-Rahman *et al*., 2010). As commodity chemicals, the individual L and D isomers of lactic are more valuable than that of the racemic DL form which is formed during the chemical synthesis of lactic acid.

Biological production is therefore advantageous as the L form is more abundantly produced by microorganisms.

Strains of *C. glutamicum* have been created that lack their native L-lactate dehydrogenase (L-LDH) and overexpress D-lactate dehydrogenase gene (*ldh*A) from *Lactobacillus bulgaricus* in order to over produce the D-isomer (Okino *et al.*, 2008b, Jia *et al.*, 2011). By utilising a two phase production process similar to that used in the production of succinic acid under oxygen deprived conditions, Okino *et al.* (2008b) observed high end yields of 120 gl^{-1} D-lactic acid and a production rate of 4 $gL^{-1}h^{-1}$ using this modified strain. This is a significantly higher yield than has been achieved when growth of the organism has taken place during the same phase as organic acid accumulation. Improvements like these highlight the benefits of using the two phase method to obtain high yields of organic acids under oxygen deprived conditions.

Additionally, production of lactic acid using sustainable resources has also been examined thoroughly throughout the literature. Engineered strains of *E. coli* have been designed that enable utilisation of xylose, glycerol, sugarcane and corn steep liquor (Zhao *et al.*, 2013, Mazumdar *et al.*, 2013a, Wang *et al.*, 2013). Continued work in this area to ultimately reduce production costs and improve yields would be beneficial.

1.3 **Biopolymers**

As well as microbial synthesis of building block chemicals for the production of plastics, direct production of polymeric compounds by various organisms has also been investigated. Unlike conventional petroleum based plastics, microbial produced polymers have been shown to exhibit complete degradation under both aerobic and anaerobic conditions. Of the various compounds that have been investigated, the polyester polyhydroxyalkanoate (PHA) has shown potential as an alternative candidate (Chanprateep, 2010).

Microbial production of PHA has been examined extensively as an alternative route in the production of biodegradable thermoplastics. These compounds are synthesised by a variety of bacteria from over 75 different genres, in which they accumulate intracellularly and act primarily as carbon and energy reserves under nutrient stress. PHAs also have non-storage related roles in which they are crucial constituents of the cytoplasmic membrane required for DNA transport, protection from degradative enzymes and in the construction of calcium channels. Their molecular mass is very much dependent on the microbial producers and range between 50,000 and 1,000,000 Da (Keshavarz and Roy, 2010). This is sufficiently high enough to exhibit similar polymeric characteristics to conventional plastics such as polypropylene.

In nature, the pathway towards PHA varies depending on the environment that the organism inhabits. Three pathways for synthesis occur via the de novo pathway towards fatty acids, the β-oxidation pathway and through chain elongation reactions where condensation of acetyl CoA moieties leads to the production of 3-hydroxyacl-CoA. While PHA's exhibit potential, large scale production of these compounds remain expensive. Natural producers of PHA generally have long generation times, grow at low temperatures, are difficult to lyse and contain pathways that are capable of degrading PHA. Therefore, in order to develop the production of this compound into a more viable process, metabolic engineering of several bacteria that are not natural producers has been investigated. By inserting the PHA synthase gene, *phaC1*, from *Pseudomonas sp*. LDC-5, into a strain of *E. coli*, Sujatha and Shenbagarathai (2006) successfully induced the synthesis of PHA from 0 to 3.4 gl^{-1}. The advantage of using *E. coli* compared to the natural producer is that many of the undesirable characteristics mentioned above are eliminated and a much more efficient process could be developed.

However, even with successful expression of PHA synthases genes in various organisms, production costs of PHA remained a limiting factor in the integration of an industrial process. Therefore, a major area of investigation is the potential use of waste by-products as feed stocks. These have included plant oil (Ciesielski *et al.*, 2015), waste water (Kourmentza *et al.*, 2015), waste activated sludge (Jiang *et al.*, 2009), agricultural waste (Koller *et al.*, 2005) and waste glycerol from biodiesel

production (Zhila *et al.*, 2015). Continued process development in this area has led to a reduction in process cost from €10-12 per kg^{-1} in 2006 (Kosior *et al.*, 2006) to €1.50 per kg^{-1}in 2010 (Chanprateep, 2010). With the market for bioplastics projected to increase from 2 to 5 million tons by 2020, the dramatic decrease in price has put PHA in contention as being the potential alternative candidate to petroleum based plastics.

1.4 **Alcohols**

1.4.1 **Ethanol**

Ethanol was not included in the original top compound list due to its status as a supercommodity (Werpy and Petersen, 2004). However, the use of a variety of alcohols as feedstock chemicals has increased the potential of bioethanol to be utilised as a building block compound (Rass-Hansen *et al.*, 2007, Bozell and Petersen, 2010).

As discussed previously in this chapter (Section 1.1), the primary route for this compound is directed at the fuel market with over 86 million tons produced per year. Bioethanol is currently produced using strains of *Saccharomyces cerevisiae* as a biological catalyst to convert sugarcane and corn starch into the desirable compound (Tesfaw and Assefa, 2014). Compared to other microorganisms, this yeast exhibits favourable characteristics that are advantageous during ethanol production. One of the most desirable characteristics is the organism's tolerance to the alcohol, making higher end yields possible. Nevertheless, this tolerance level still requires development. Further improvement of ethanol tolerance has been achieved in recent years through a variety of genetic engineering techniques including, genome shuffling (Shi *et al.*, 2009), resulting in strains with tolerance at 25 % (v/v). Metabolomic analysis of the organism has also identified key metabolites involved with ethanol stress, which could be targeting to improve tolerance levels (Ohta *et al.*, 2015).

Genes of importance in the production of ethanol have been identified as pyruvate decarboxylase (PDC) and acetaldehyde dehydrogenase (ALDH) Incorporation of the genes associated with these enzymes, *pdc* and *adh*B in *C. glutamicum* R resulted in an accumulation of ethanol with a rate of $30gL^{-1}h^{-1}$ under oxygen deprived conditions which was a 3 fold increase compared to the wild type (Inui *et al.*, 2004a). This was achieved after the deletion of the *ppc* and *ldh*A, insinuating by-product elimination is crucial in obtaining high yields of the desired product. It is also interesting to note that inhibitory effects exhibited under aerobic growth and production condition were prevented when growth arrested production was employed.

Although, the bioethanol production process is well established, only approximately a fifth of the amount produced each year is used in the production of non-fuel related products. Alternative uses of ethanol include in the production of ethylene, synthesised during ethanol dehydration, as well as, for the production of acetic acid and ethyl acetate during ethanol oxidation over various catalysts (Christensen *et al.*, 2006). Continued process development is required to establish alternative pathways for platform chemicals where ethanol can be substituted as sustainable building block compound as a feedstock.

1.5 **Amino acids**

1.5.1 **L-Aspartic acid**

Appearing in the 2004 DOE report, aspartic acid is an essential amino acid that is produced by all organisms and is required for protein synthesis. Its primary use as a commodity chemical is in the food and beverage industry in the production of the artificial sweetener aspartame. Similar to succinic acid it can also be used as a building block in the production of 1,4-butanediol, tetrahydrofuran and γ-butyrolactone which are used in the both the manufacturing of polymers and as chemical solvents (Werpy and Petersen, 2004). Current production of this amino acid

is through enzymatic conversion of fumaric acid with aspartase in the presence of ammonia. It can be synthesised by all organisms and therefore can also be produced through biological fermentation. As yet, no method has been developed that is more efficient than the enzymatic conversion currently in use. However, as fumaric acid is its precursor compound in the enzymatic production process, improvement in its production through fungal fermentation (Section 1.2.2) would also improve costs associated with the production of L-aspartic acid.

1.5.2 **L-Glutamic acid**

L-glutamic acid remains one of the highest produced amino acids with over 2.3 million tons produced each year (Choi *et al.*, 2015). Currently, this compound is already produced in high concentration industrially using microbial bioprocesses. *Corynebacterium glutamicum* is the industrial workhorse in this area under aerobic conditions. Development of fed-batch processes have led to increased productivities and high end yield titres of in some cases over 100 gl^{-1}. Further examination of *C. glutamicum* will appear throughout this thesis.

Until now, the majority of L-glutamate produced worldwide is directed for use as a flavouring agent in the food and drink industry. However, this amino acid has the potential to be used as a building block chemical with a variety of applications such as the production of polyesters and polyamides and has a variety of derivatives that add to its potential.

As of yet, no industrial process is in existence that utilises the glutamate pathway to produce high value chemicals. In this thesis we will examine the early ground work to develop a viable microbial bioprocess which will see the *in vivo* conversion of *de novo* mesaconate, utilising the glutamate production pathway. Mesaconate can be converted to the monomer molecule methyl methacrylate used in Plexiglas, through a two-step process. Therefore, the production of mesaconate through microbial process would be desirable as a sustainable route towards this high value chemical.

1.6 **Strategic Aim of this programme of work**

As stated in the previous section, industry is striving for replacement sustainable routes feedstocks for polymer synthesis which currently derive from petrochemical sources. As discussed in this chapter, the potential of using *C. glutamicum* as a biological producer of platform compounds has recently gathered more momentum. Several methods have already been developed to utilise existing pathways in order to produce high yields of potential chemical building block. Since genomic sequencing of *C. glutamicum*, major genes involved with metabolism as well as the production of organic and amino acids have led to a better understanding of the organism itself. Carbon metabolism and L-glutamate production by this organism has been well documented in the literature and will be examined further in Chapter 3.

Although L-glutamate production is well known for its use as a flavouring agent, its potential as a building block chemical has still to be realised. The compound has various derivatives that can be used as potential starter compounds for monomers that can then be used for polyesters and polyamides. However, one relatively untapped area of investigation is the potential to use *C. glutamicum* as a direct producer of mesaconate, a compound which is produced in the glutamate degradation pathway in *Clostridum tetanomorphum*. When polymerised with acrylamide, mesaconate can be converted to produce a hydrogel that has multiple applications (Baris and Karadag, 2005). It can also be converted to methyl methacrylate used in the production of Plexiglas which has a current market of 3.2 million tons per year (Choi *et al.*, 2015).

To the knowledge of the author, metabolic engineering to produce mesaconate in a host organism has only been successful once in *E. coli* (Wang and Zhang, 2015). However, the use of this pathway has not been exploited in a natural producer of L-glutamate. Therefore, using *C. glutamicum*'s long history in the overproduction of L-glutamate as a platform, it was anticipated that this could confer to over production of mesaconate. As this project was in conjunction with Ingenza Ltd this process has significant industrial interest.

1.7 **Objectives**

1. Investigate bacterial production of organic and amino acids as a potential renewable source of feedstocks for chemical polymerisation processes
2. Identify the most efficient production process for the production of building block chemicals
3. Implement genetically engineered strains into the optimised process to convert amino/organic acid production to platform chemicals
4. Determine potential effects of end products on the growth and productivity of the organism

1.8 **Hypothesis**

We hypothesise that by exploiting process development and molecular engineering, bio-production of valuable platform chemicals, such as mesaconate, could be achieved.

Chapter 2 Materials and Methods

2.1 *Aspergillus niger*

2.1.1 **Organism and culture maintenance**

Aspergillus niger A60, engineered strain Il-EAN-C19 and *Aspergillus terreus* were obtained from Ingenza Ltd., Edinburgh, UK. Master cultures were produced by plating spore suspension onto potato dextrose agar (PDA) plates that were incubated at 30°C for 7 days. PBS-Tween 0.1 % v/v solution was used to remove spores from the plate. To remove debris, the spore suspension was filtered using a sterile Pal-filter and Buchner conical flask. Miracloth (Merck Millipore, Hertfordshire, UK) was used as a filter, allowing the spores to pass through while cell debris attached to the fibres. With a pure spore suspension a spore count was performed using a hemoacytometer and then stored in 1ml volumes at -80°C in cryovials containing 10% glycerol. Working cell banks were created in the same manner.

2.1.2 **Growth medium**

In all studies the medium composed of 140 gl^{-1} sugar (glucose/maltose/sucrose where specified), 2 gl^{-1} $(NH_4)_2SO_4$, 2 gl^{-1} KH_2PO_4, 60 mgl^{-1} $MgSO_4$, 0.2 mgl^{-1} $(NH_4)_2Fe(SO_4)_2.12H_2O$, 0.1 mgl^{-1} $ZnSO_4$, 0.06 mgl^{-1} $CuSO_4$. During batch fermentation 0.01% v/v polypropylene glycol (PPG) was added to the medium before sterilisation to act as an antifoam agent. In all cases, all cultures were grown at 30°C unless otherwise stated.

In experiments where comparisons of carbon source in shake flasks were performed, 140, 70, 50 and 35 gl^{-1} concentration of glucose, sucrose or maltose were used. All other elements of the growth medium were unchanged.

2.2 *Corynebacterium glutamicum*

2.2.1 **Organism and culture maintenance**

Various strains of *C. glutamicum* outlined in Table 2.1 were used throughout to examine production of, in the first instance, L-glutamate and laterally intermediate platform chemicals under both aerobic and anaerobic conditions. All master cultures were received from Ingenza Ltd, Edinburgh, UK.

To produce working cell banks, 0.05 mL of the master culture was aseptically transferred to 50 mL of growth medium (CGG) containing 5 gl^{-1} yeast extract, 10 gl^{-1} soya peptone, 5 gl^{-1} NaCl and 5 gl^{-1} glucose. If required, appropriate antibiotics were also added to the medium. The culture was incubated on a rotary shaker (Minitron Incubator Infors UK, Reigate, UK) at 30 °C and 250 rpm until an optical density of 10 at 600 nm was achieved. At this point cells were harvested at 7000 rpm for 10 minutes using a Heraeus megafuge 8R centrifuge (Thermo Scientific Inc., MA, USA), washed once in 0.9 % w/v NaCl, resuspended in fresh medium and stored in 10 % v/v glycerol at -80 °C. In the cases of *C. glutamicum* ATCC 13869 engineered strains, cell banks were used as they were received from Ingenza Ltd. to reduce the risk of losing activity through subculture.

2.2.1 **Solid state growth**

Where solid state growth was required, *C. glutamicum* was inoculated onto CGG agar (5 gl^{-1} yeast extract, 10 gl^{-1} soya peptone, 5 gl^{-1} NaCl, 5 gl^{-1} glucose and 15gl^{-1} bacteriological agar) containing the required antibiotics as and when required. The culture was then incubated at 30 °C for 24 h. Single cell colonies were used to inoculate pre-cultivation medium in some instances in place of the working cell bank to examine variability in production levels.

Table 2-1 *C. glutamicum* strains for aerobic production of L-glutamate

Strain	Previous work	Notable genes	Origin	Ingenza ID	Antibiotic used
C. glutamicum ATCC 13032	Kalinowski *et al.* (2003)	N/A	ATCC	*C.glut* WT(1)	N/A
C. glutamicum 13032 Δ *ldh*A	New engineered strain	*ΔldhA*	Ingenza Ltd.	*C.glut ΔldhA*	N/A
C. glutamicum Δ *ldh*A empty vector	New engineered strain	*ΔldhA*	Ingenza Ltd.	IL.1.1.7.0	25 μgl^{-1} kanamycin
C. glutamicum ATCC 13869	Shirai *et al.* (2007) Asakura *et al.* (2007)	N/A	ATCC	IL.5.0.0.0	N/A
C. glutamicum ATCC 13869 engineered – 1	New engineered strain	Glutamate mutase Ammonium lyase	Ingenza Ltd.	IL.5.0.2(G2).1.(F1F2)	200 μgl^{-1} spectinomycin 25 μgl^{-1} kanamycin
C. glutamicum ATCC 13869 engineered - 2	New engineered strain	Glutamate mutase Ammonium lyase	Ingenza Ltd.	IL.5.0.1(F1.F2-F3).2(G2)	200 μgl^{-1} spectinomycin 25 μgl^{-1} kanamycin
C. glutamicum ATCC 13869 engineered - 3	New engineered strain	Glutamate mutase Ammonium lyase	Ingenza Ltd.	IL.5.0.1(G2).2(F1.F2).	200 μgl^{-1} spectinomycin 25 μgl^{-1} kanamycin

2.2.2 Investigation of L-glutamate production methods

2.2.2.1 Ethambutol addition

To 50 mL of propagation medium (LB broth), 50 µL of the stated *C. glutamicum* working cell bank was added. The culture was then incubated overnight in an rotary incubator at 30 °C and 250 rpm. This propagation culture was used to inoculate between 0.05-10 L of CGXII biotin-sufficient medium ($(NH_4)_2SO_4$ 20 gl^{-1}; Urea 5 gl^{-1}; $CaCl_2$ 10 mgl^{-1}; K_2HPO_4 1 gl^{-1}; KH_2PO_4 1 gl^{-1}; $MgSO_4.7H_2O$ 0.25 gl^{-1}; MOPS 42 gl^{-1}; glucose 44gl^{-1}; $FeSO_4.7H_2O$ 10 mgl^{-1}; $ZnSO_4.7H_2O$ 1 mgl^{-1}; $MnSO_4.H_2O$ 10 mgl^{-1}; $CuSO_4$ 0.2 mgl^{-1}; $NiCl_2x6H_2O$ 0.02 mgl^{-1}; protocatechuate 34 mgl^{-1}, biotin 0.2 mgl^{-1}) containing between 0-100 μgl^{-1} of ethambutal, to a starting optical density of 0.5 at 600 nm.

The required volume of the culture was harvested by centrifugation at 7500 rpm for 10 minutes, and cells washed once in 0.9 % w/v NaCl before being resuspended in the required volume of CGXII medium depending on the scale of the experiment. Cultures were then incubated at 30 °C in an orbital incubator at a speed of 250 rpm. Samples were acquired periodically and analysed for DCW, optical density, residual sugar, and analyte concentration.

2.2.2.2 Heat induction

For production of L-glutamate by heat induction the previously described experimental design was employed to establish a propagation culture (Section 2.2.2.1)

. This again was used to inoculate 50 mL of CGXII medium to an optical density to approximately OD_{600} = 1. The culture was incubated at 33 °C on an orbital incubator for the initial 10 h of growth. Thereafter, the temperature was increased to 39 °C to trigger L-glutamate production (Stansen *et al.*, 2005). Samples were acquired

periodically and analysed for DCW, optical density, residual sugar, and analyte concentration.

2.2.2.3 Tween 40® induction

For L-glutamate production by Tween 40® induction a modified protocol described by Shirai (Shirai *et al.*, 2007) was applied. A 400 mL inoculation medium was prepared in a 2 L baffled Erlenmeyer flask, consisting of; glucose 40 gl^{-1}; $(NH_4)_2SO_4$ 30 gl^{-1}; Na_2HPO_4 3gl^{-1}; KH_2PO_4 6 gl^{-1}; NaCl gl^{-1}; $CaCl_2$ 84 mgl^{-1}; $FeCl_3$ 3.9 mgl^{-1}; $ZnSO_4 \cdot 7H_2O$ 0.9 mgl^{-1}; $CuCl_2 \cdot H_2O$ 0.3 mgl^{-1}; $MnSO_4 \cdot 5H_2O$ 5.56 mgl^{-1}; $(NH_4)_6MO_7O_24.4H_2O$ 0.1 mgl^{-1}; $Na_2B_4O_7 \cdot 10H_2O$ 0.3 mgl^{-1}; $MgSO_4 \cdot 7H_2O$ 0.4 gl^{-1}; $FeSO_4 \cdot 7H_2O$ 40 mgl^{-1}; thiamine HCl 0.5 mgl^{-1}; EDTA 0.1 gl^{-1}; and biotin 10 μgl^{-1}. 0.8 mL of the *C. glutamicum* WCB was aseptically transferred and the culture incubated at 30 °C and 250 rpm on in an orbital incubator for 24 h.

Batch medium preparation was as above at a 9 L scale using a Braun Biostat C stainless steel bioreactor. The entire inoculum culture was used to inoculate the batch medium. Growth of the culture was monitored until an optical density of approximately OD_{600} = 10 was achieved where upon the induction with a Tween 40® solution (Tween 40® 0.8 mgl^{-1}; PPG 2 µl/L prepared in 100 mL of dH_2O) was performed. Samples were acquired periodically and analysed for DCW, optical density, residual sugar, and analyte concentration.

2.2.2.4 Biotin limitation production of L-glutamate

To 50 mL of either LB broth or pre-cultivation medium, 50 µL of the stated *C. glutamicum* working cell bank was added. The culture was then incubated overnight in an orbital incubator at 30 °C and 250 rpm. This propagation culture was used to inoculate between 50 - 400 mL of CGXII minus biotin medium ($(NH_4)_2SO_4$ 20 gl^{-1}; Urea 5 gl^{-1}; $CaCl_2$ 10 mgl^{-1}; K_2HPO_4 1 gl^{-1}; KH_2PO_4 1 gl^{-1}; $MgSO_4.7H_2O$ 0.25 gl^{-1};

MOPS 42 gl^{-1}; glucose 44 gl^{-1}; $FeSO_4.7H_2O$ 10 mgl^{-1}; $ZnSO_4.7H_2O$ 1 mgl^{-1}; $MnSO_4.H_2O$ 10 mgl^{-1}; $CuSO_4$ 0.2 mgl^{-1}; $NiCl_2x6H_2O$ 0.02 mgl^{-1}; protocatechuate 34 mgl^{-1}) containing 5 μgl^{-1} (biotin depletion medium) of biotin to a starting optical density of 0.1 at 600 nm. The required volume of the culture was harvested by centrifugation at 7000 rpm for 10 minutes, and cells washed once in 0.9 % w/v NaCl before being resuspended in the CGXII medium. Again the culture was incubated overnight or until an optical density of 10 at 600 nm was achieved. Once the cell density had been attained, the culture was used to inoculate the biotin limitation (the production medium) to a starting optical density of 1 at 600 nm. The limitation medium was prepared in the same manner as the depletion medium however contained a final concentration of either 0, 1 (optimum) or 200 μgl^{-1} biotin. As previously, cells were harvested and washed in 0.9 % w/v NaCl before being resuspended in the required volume of biotin limitation medium depending on the scale of the experiment.

2.2.3 **Shake flasks**

All shake flasks were carried out in 500 mL Erlenmeyer flasks with a working volume of 50 mL. Samples were taken aseptically on a regular basis and examined by various analysis methods as described below.

2.2.4 **4 L Batch Fermentations**

All 4 L batch and fed batch fermentations were carried out in a Bioflo® 110 bioreactor (New Brunswick Scientific, Enfield, CT) with a maximum working volume of 10 L. The vessel itself consisted of borosilicate glass with a stainless steel top plate with ports for pH, dissolved oxygen and temperature probes as well as a port for a condenser. Parameters and set-points were controlled by the internal digital control unit.

To the agitation shaft, two six-blade Rushton Turbines were attached to give vigorous mixing throughout the fermenter. Stainless steel baffles were also added to the fermenter to encourage more efficient mixing. A circular angular sparger was fixed below the bottom impeller to provide air. The sparger was positioned in such a way to ensure equal distribution of air bubbles in the fermenter and allowing the impeller to break down the air bubbles, increasing their surface area making oxygen transfer rates more efficient. The air flow was kept at a constant rate of 1 vvm.

The fermenter vessel was kept at a constant temperature of 30 °C by use of a heating jacket. Accurate temperature was monitored by the control unit. The pH was controlled to within ±0.05 of the set point of 7 using a reference electrode (Metter Toledo Ltd.., Leicester, UK). 2 M Sulfuric acid and 25 % v/v Ammonium hydroxide were used to maintain this and was introduced to the reactor using peristaltic pumps attached to the control unit. Dissolved oxygen tension was monitored using a dO2 probe (Broadley-James Ltd.., Bedford, UK). The probe was calibrated at the fermentation operating temperature and agitation speed 24 h after autoclaving to allow the electrode to stabilize. First, oxygen free nitrogen (OFN) was introduced into the fermenter system until the dO2 measurement reached 0 %. It was then calibrated to 100% using compressed air. Foam was controlled manually using a peristaltic pump attached to a reservoir of polypropylene glycol (PPG).

Off gas measurements were made using a Servomex 4100 gas purity analyser (Servomex, Netherlands). Oxygen at a concentration of 19.0 % (v/v) and carbon dioxide at 1.75 % (v/v) were flushed through the system to calibrate the system. Exhaust gas from the bioprocess could then be measured and used to calculate the carbon evolution rate (CER) and the oxygen uprate rate (OUR). These values could then be used to calculate the respiratory coefficient of the bioprocess (CER/OUR).

2.2.5 **10 L Batch Fermentations**

All 10 L batch fermentations were in a stainless steel bioreactor controlled by a Biostat C-DCU, (B.Braun Biotech International, Switzerland). This bioreactor had a

total volume of 22 L which enabled foaming issues to be contained. For agitation, the vessel contained three six-bladed Rushton turbines attached to the stirrer shaft in such a way as they would be submerged throughout the fermentation. Sterile air was introduced to the system through a circular angular sparger located at bottom of the fermenter. Stainless steel baffles were also present to ensure efficient and even distribution of the bubbles. To maintain the temperature within ±0.1 °C of the set point, the vessel encompassed a double walled heat exchanger that allowed cool or warm water to flow when appropriate. This was controlled through control loops programed in the DCU. Dissolved oxygen tension was monitored using a dO_2 probe (Broadley-James Ltd.., Bedford, UK). Similar to the previous section (Section 2.2.4) the probe was calibrated at the fermentation operating temperature and agitation speed 24 hours after autoclaving to allow the electrode to stabilize. First, oxygen free nitrogen (OFN) was introduced into the fermenter system until the dO2 measurement reached 0 %. It was then calibrated to 100 % using compressed air. When oxygen was limiting dO_2 was maintained at 30 % through increasing agitation. A pH probe (Metler Toledo Ltd., Leicester, UK) was used to monitor and control the pH. The pH was controlled to within ±0.05 of the set point (pH 7) by automatic addition of either 25 % v/v ammonium hydroxide or 2 M sulfuric acid. Foam was controlled manually using a peristaltic pump of attached to a reservoir of PPG.

2.2.6 **Optimisation of fed batch bioprocess using biotin limitation induction**

To increase productivity, an optimised feeding strategy was devised. Employing the glucose utilisation rate over the first 20 h of the achieved during the batch process in Section 3.8.2, under biotin limitation, an hourly consumption rate was calculated. A feed profile that introduced 1.4 $gL^{-1}h^{-1}$ glucose and 0.14 $gL^{-1}h^{-1}$ ammonium sulphate to the culture broth over a 20 h period was developed. Addition of further trace elements and chelating agents were detrimental to growth therefore not included in future feed processes.

2.2.7 **Development of the mesaconate induction process**

A series of processes were developed in order to understand which condition would elicit the production of mesaconate using the engineered strains created by Ingenza Ltd. In cases where induction was required, a 1 mM/L solution of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was employed. All processes were carried out under normal biotin limitation process at a scale of 4L with deviations detailed in Table 2.2 and discussed further in Chapter 4.

Table 2-2 Process outline for engineered strains of *C. glutamicum*

Process no.	***C. glutamicum* ATCC 13869 engineered strain**	**Induction**	**Method**
1	1, 2, 3	No	No induction
2	1, 2	Yes	IPTG at 0h of batch
3	1, 2	Yes	IPTG at 25 h of batch
4	1, 2	Yes	ITPG at 25 h of batch immediate switch to micoraerobic environment

For all processes, samples were acquired periodically for standard analysis previously described. In addition, where stated, samples were obtained for LC-MS, RNA-Seq and qPCR examination.

2.3 *Escherichia. coli*

2.3.1 Culture and organism maintenance

A liquid culture of *E. coli* BW25113 (Datsenko and Wanner, 2000) *Δpfl*B *ΔldhA* was received from Ingenza Ltd., Edinburgh, UK. All cultures were prepared from this single working cell bank. To 50 mL of Lund medium (glucose, 11.9 gl^{-1}; $(NH_4)_2SO_4$, 10 gl^{-1}; K_2HPO_4, 73 gl^{-1}; $NaH_2PO_4 \cdot 2H_2O$, 18 gl^{-1}; $HOC(CO_2H)(CH_2CO_2NH_4)_2$, 2.5 gl^{-1}; $MgSO_4 \cdot H_2O$, 0.24 gl^{-1}; $CaCl_2 \cdot 2H_2O$, 1 mgl^{-1}; $FeCl_3$, 20.06 mgl^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.36 mgl^{-1}; $Cu_2SO_4 \cdot 5H_2O$, 0.32 mgl^{-1}; $CoCl_2 \cdot 7H_2O$, 0.36 mgl^{-1}; $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, 11.15 mgl^{-1}; yeast extract, 5 gl^{-1}; kanamycin 100 μgl^{-1} and Carbenicillin 34 μgl^{-1}) 10 µL of the working cell bank was aseptically transferred. The culture was then place in an orbital incubator at 37 °C and 250 rpm and monitored over time for its cellular growth. Cells were harvested at various time points depending on cell density required. Once harvested, by centrifugation at 7000 rpm for 10 minutes, cells were resuspended in fresh Lund medium and used for anaerobic biotransformation or toxicity testing.

2.4 Anaerobic biotransformation of *C. glutamicum* and *E. coli* stains

Unless stated otherwise, all work was carried out by the author at Ingenza Ltd., Midlothian, UK, with molecular manipulation and cell banking prepared by the company to standard operating procedures. All strains used in anaerobic work are described in Table 2.3.

Table 2-3 Strain Construction for anaerobic bioconversion

Strain	Organism	Genotype	Plasmid	Enzyme of interest
IL.1.6.6.(K).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pAN6	Glyceraldehyde-3-Phosphate Dehydrogenase
IL.1.6.5.(A).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pIL-3 (pEKEx3 Ptuf)	Ketoglutarate synthase
IL.1.6.1.0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pEKEx3	Empty vector
IL.1.6.1.(A).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pEKEx3	Ketoglutarate synthase
IL.1.6.1.(ABC).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pEKEx3	Ketoglutarate synthase Pyruvate synthase, Ferredoxin
IL.1.6.1.(ADE).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pEKEx3	Ketoglutarate synthase Flavoprotein reductase, Flavoprotein
IL.1.7.0.0	*C. glutamicum* ATCC 13032	ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} *ΔldhA* ΔsucA	/pEKEx3	ODHC Eo1 subunit deletion

All genetic manipulation carried out by Ingenza Ltd. to company standard operating procedure

Table 2-3 Strain Construction for anaerobic bioconversion continued

Strain	Organism	Genotype	Plasmid	Enzyme of interest
IL.2.1.1.0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Empty vector
IL.2.1.1.(A).0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Ketoglutarate synthase
IL.2.1.1.(ABC).0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Ketoglutarate synthase Pyruvate synthase, Ferredoxin
IL.2.1.1.(ADE).0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Ketoglutarate synthase Flavoprotein reductase, Flavoprotein

All genetic manipulation carried out by Ingenza Ltd. to company standard operating procedure

2.4.1 **Cell preparation**

For *E. coli* strains, the organism was prepared as previously detailed (Section 2.3.1). A different preparation approach was required for *C. glutamicum* which is detailed as follows. A 20 mL starter culture containing BHI (Brain heart infusion media), glucose and 200 μgl^{-1} spectinomycin medium in 50 mL baffled conical flask was prepared. To the medium, 40 µL of stock cells were added and then the flask was placed in an orbital incubator at 30 °C at 250rpm. After 18 h growth, a 500 mL flask containing 50 mL CGXII (Keilhauer *et al.*, 1993) medium with 200 μgl^{-1} spectomycin was inoculated with 5 mL of the starter culture. The propagation flask was placed in an orbital incubator at 30 °C at 250rpm for approximately 7h. Based on the optical density of the propagation flask, the volume of culture required to be added varied to give a starting OD_{600nm} of 1.2 in 150 mL CGXII medium, containing 300 µL of a 200 μgl^{-1} spectomycin in all flasks. The culture was then grown overnight at 30 °C in an orbital incubator at 250 rpm. The cells were harvested by centrifugation at 7000 rpm at 4°C for 15 minutes. Cell pellets were then used immediately or stored at either 4 or -20 °C to test sustainability of cell activity.

An alternative cell preparation was developed when issues arose with the expression of certain inserted genes which were not observed using the CGXII medium. As with the pre-mentioned method, a 20 mL starter culture containing BHI, glucose and 200 μgl^{-1} spectomycin medium, in 50 mL baffled conical flask was prepared. To the medium 40 µL of stock cells were added and then the flask was placed in an orbital incubator at 30 °C at 250rpm. After 16 hour of incubation, the optical density of the culture was measured. This propagation flask was used to inoculate 50-500 mL CGG medium, containing 200 μgl^{-1} spectomycin, to a staring OD_{600nm} of 1.2. The culture was again grown in an orbital incubator at 30 °C and 250 rpm for 24 h. Cells were then harvested and stored as previously mentioned.

2.4.2 **150 mL Biotransformation**

A medium containing 154 mM sodium chloride, 111 mM glucose (Alpha Aeser, Lancashire, UK), 200 mM sodium bicarbonate (VWR, Leicestershire, UK) and with or without 220 mM ammonium formate was prepared in 150 mL dH_2O and filter sterilised. Gluconate was also used as an alternative carbon source to glucose. The medium was used to resuspend *C. glutamicum* or *E. coli* cell pellets. The mixture was then transferred to a sterile 2-necked round bottom flask which was constantly heated to 30 °C using a thermocoupled heating block and was stirred with a magnetic stirrer bar at 250 rpm. Suba-seals were placed on the openings of the flask to exclude oxygen from entering. In some experiments, nitrogen gas was sparged either directly into or above the medium to create an anaerobic environment. Alternatively, if low cell mass was achieved in the growth stage the volume was adjusted appropriately and the experiment carried out in sealed 50 mL Falcon tubes in an orbital incubator at 30 °C and 250 rpm. Preparation in this way was to ensure cell loading in each experiment was kept constant, with a switch to flacon tubes reducing the risk of medium evaporation. Standard cell loading volume was approximately 4g (wet cell weight) in 150 mL biotransformation medium. The pH was maintained above 6.9 by the addition of 2 M KOH and below 10 with 200 mM HCl. Samples were taken regularly and heat treated at 100 °C for 5 minutes to denature the enzymes. The supernatant was separated from the cells by centrifugation 13000 rpm 10 minutes and stored at -80 °C before being analysed for organic and amino acid concentrations by HPLC.

2.4.3 **Scale up of anaerobic biotransformation**

The aim of scale up was to increase regulation of both pH and the anaerobic state of the vessel. Firstly in an attempt to ensure all oxygen was purged from the vessel an inert atmosphere was created using the below set up (Figure 2.2). This arrangement consisted of a 1L 3-necked round bottomed flask with one port made securely air

tight, another allowed the insertion of a pH probe which again was made air tight and a final port for sampling. This last port also had the addition of a balloon filled with OFN_2 which allowed the creation of positive pressure ensuring an inert atmosphere. Medium was increased to 500 mL with the cell loading kept constant with previous work at 4 g per 150 mL. Cell preparation using CGG medium was performed using the aforementioned technique. Purging of the medium with filtered N_2, for at least 30 minutes prior to inoculation, was performed to eliminate oxygen before the biotransformation was initiated. To maintain culture temperature the vessel was suspended over liquid paraffin which was kept constant at 30 °C which with the culture broth was continuously mixed with a magnetic stirrer bar. pH was maintained between 7.4-7.7 with the manual addition of either 2 M potassium hydroxide or 2M sulphuric acid. To maintain sterility, the process was performed inside a laminar extraction hood. Samples were taken regularly and heat treated at 100 °C for 5 minutes to denature the enzymes. The supernatant was separated from the cells by centrifugation at 13000 rpm for 10 minutes and stored at -80 °C before being used in the process. During sampling, the vessel was purged with filtered N_2 in an attempt to exclude the introduction of oxygen.

To improve efficiency of this design the experimental process was scaled up to a 1.5L biotransformation performed in 2L DASGIP parallel bioreactor system (Eppendorf, Hamburg Germany). The vessel itself consisted of borosilicate glass with a stainless steel top plate with ports for pH, dissolved oxygen and temperature probes as well as a port for a condenser. Parameters and set-points were controlled by DASGIP software V4.0 (Eppendorf, Hamburg Germany). Agitation of the medium was by 2 Rushton impellers at 300 rpm and pH was controlled at 7 ± 0.05 by 2 M potassium hydroxide and 2 M sulphuric acid. After the dissolved oxygen probe was calibrated the medium was purged with N_2 for a further 1 h to ensure all oxygen was excluded from the reactor. During the biotransformation the N_2 was either allowed to continue to purge the vessel for the entirety of the process or the vessel was sealed in order to maintain an inert atmosphere. Samples were taken regularly and heat treated at 100 °C for 5 minutes to denature the enzymes. The supernatant was separated from the cells by centrifugation at 13000 rpm for 10 minutes and stored at -80 °C before being used in the process.

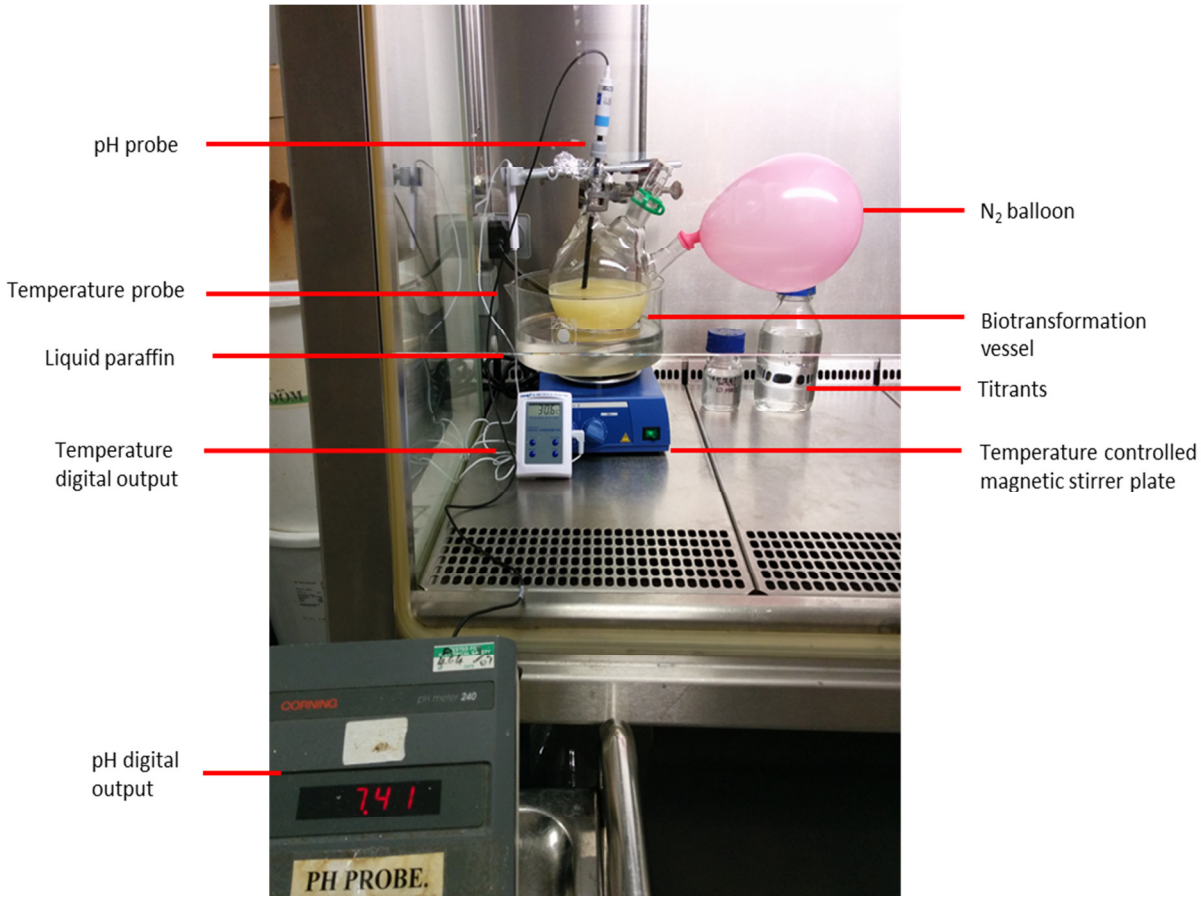


Figure 2-1 500 mL biotransformation under N2 inert atmosphere

2.5 Test for Toxicity

2.5.1 *Aspergillus niger* in solid state

To 75ml of molten PDA, concentrations of methacrylic acid and methylmethacrylate ranging from 0-10 gl^{-1} were added. The agar was then split between 3 plates. When set, 1.08 x 10^7 *A. niger* A60 spores were aseptically transferred to the plate. The plates were then incubated at 30°C in the dark for 7 days. Examination of the plates

was carried out every 24 h analysing colony growth and colour, spore colour and agar pigmentation.

2.5.2 *Aspergillus niger* in liquid culture

A. niger A60 was the only fungal strain used when examining the effects of MAA and MMA under liquid culture. Using a 500ml Erlenmeyer flask, 200 mL of liquid growth medium was prepared as previously stated (Section 2.1.2) with the addition of MAA or MMA at concentrations ranging from 0-2.5 gl^{-1} either into the initial medium or where stated added after 48h of growth. The cultures were incubated for 8 - 10 days on a rotary shaker at 180 rpm. Triplicate 5 mL samples were obtained every 24 h over a 7 day period and analysed for DCW, TTA, citrate accumulation and glucose consumption.

2.5.3 *C. glutamicum* and *E. coli* in liquid culture

Cultures of *C. glutamicum* ATCC 13869 and *E. coli* BW25113 Δ*pfl*B *ΔldhA* were prepared as described previously in sections for the Biotin limitation (Section 2.2.2.4) and *E. coli* (Section 2.3.1) growth. In each case, the organism was cultured to a pre-production medium point to cell densities between 0.5 and 5 gl^{-1}. Cells were then harvested and resuspended in the required volume of 0.9 % w/v NaCl. The growth of the organism was then examined in the presence of a number of chemicals at various concentrations to determine any visible detrimental effects. The chemicals in question were intermediates in the pathway to produce methyl methacrylate from L-glutamate. This included methyl methacrylate itself at 2.5, 5 and 7.5 gl^{-1}, ethyl methacrylate at 0.43, 0.9 and 1.35 gl^{-1}, iso-proyl methacrylate at 0.225, 0.4 and 0.675 gl^{-1}, iso-butyl methacrylate at 0.09, 0.18 and 0.27 gl^{-1} and methanol at 3, 6 and 9 gl^{-1}. As all of the above chemicals were purchased as pure components they all contained p-methoxy phenol as an inhibitor. The effect of this was also examined at 15, 30 and

45 ppm. Double strength stock solutions of the chemicals were prepared in double concentrated medium required for both *C. glutamicum* (CGXII biotin depletion) and *E. coli* (Lund). To a 96 welled plate, 100 µL of cell stock and 100 µL chemical stocks were added. This gave a twofold dilution of both stock solutions resulting in a final concentration of cells either of 0.25 or 2.5 gl^{-1}. Continuous monitoring of the culture was carried out using a Synergy™ HT multimode microplate reader (Biotek® Instruments, Inc. Vermont, USA). Optical density of the cultures was measured every 15 minutes for 24 h at 600 nm. Agitation of the plate was continuous between the samples in an attempt to simulate conditions in a reactor. Temperature was kept constant at 30 °C for *C. glutamicum* and 37 °C for *E. coli* experiments. The system was locally controlled using Gen5 software (Version 2.06 BioTek® Instruments, Inc. Vermont, USA) which incorporated the collection of the UV data.

2.6 **Enzymatic assays**

2.6.1 **Succinyl CoA synthetase(SCS) assay development**

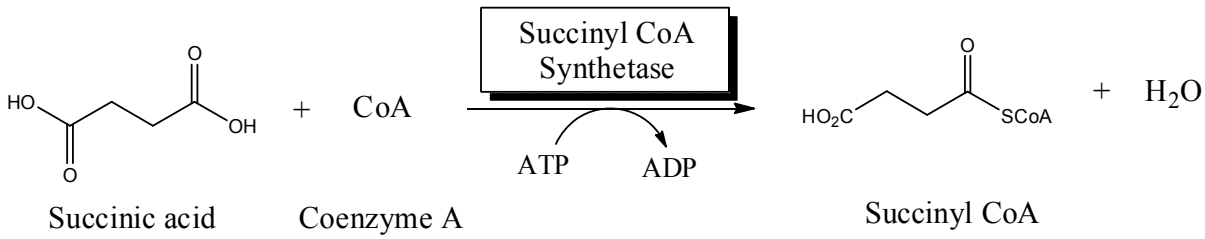


Figure 2-2 Biochemical representation of Succinyl CoA synthetase assay

2.6.1.1 **SCS HPLC assay**

A modified assay from Megazymes (Megazyme International, Wicklow, Ireland) was used to analyse the conversion of succinic acid to succinyl CoA using succinyl CoA synthetase purchased from Megazymes. To a 1.5 mL microcentrifuge tube, the

follow mixture was prepared: Tris-HCl pH 8.4, 34 mM; magnesium chloride, 3.4 mM; ATP, 1.2 mM; coenzyme A, 0.89 mM; sodium succinate, 5.8 mM. The reaction mixture was made up to a final volume of 1 mL by addition of dH_2O. The reaction was initiated by added 1 unit/mL of succinyl CoA synthetase and was incubated at room temperature for five minutes. To quench the reaction 0.2 mL of 0.2 M formic acid was added and immediately inverted to mix. The sample was then stored at -20 °C until analysed using a CoA gradient method by HPLC. Negative controls, where one component of the assay was eliminated with the deficit being replaced with deionized water, were prepared in the same manner as the positive reaction outlined above.

2.6.1.2 SCS Spectrophotometric assay

To determine whether the production of succinyl CoA from disodium succinate could be observed spectrophotometrically, an assay described by Gibson *et al* 1967 was replicated. To a quartz cuvette the follow mixture was prepared: Tris-HCl pH 7.2, 100 mM; magnesium chloride, 10 mM; β-mercaptoethanol, 2 mM; disodium succinate, 1 mM; ATP, 0.375 mM; coenzyme A, 0.1 mM. The reaction mixture was made up to a final volume of 1 mL with deionized water and inverted to mix. The initial absorbance was measured at 230 nm. To initiate the reaction, 0.02 unit/mL of succinyl CoA synthetase was added and immediately inverted to mix. After enzyme addition, the absorbance was continuously monitored until the reaction plateaued at 230 nm. The absorbance increase was based on the UV absorbance of the newly formed thioester bond of succinyl CoA. After a period of no further absorbance increase, the enzymatic reaction was quenched by addition of 0.2 mL of 0.2 M formic acid and inverting immediately to mix. The sample was then stored at -20 °C until analysed using a CoA gradient method by HPLC. Negative controls, where one component of the assay was eliminated with the deficit being replaced as deionized water, were prepared in the same manner as the positive reaction described above.

2.6.2 **Isocitrate dehydrogenase assay**

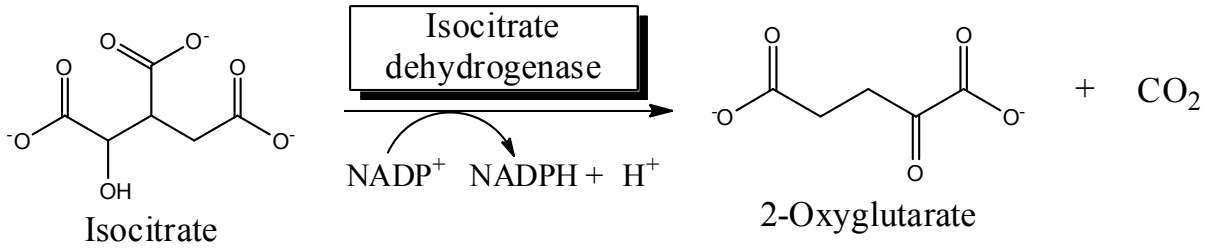


Figure 2-3 Biochemical representation of isocitrate dehydrogenase assay

An isocitrate dehydrogenase (IDH) assay was developed to determine activity of the enzyme in cell free extract. A 2 mL positive reaction was prepared in a quartz cuvette: Tris-HCl pH7.4, 62.5 mM; DL-Isocitrate acid trisodium salt, 0.66 mM; $NADP^+$, 0.45 mM; manganese chloride, 1.35 mM. The reaction mixture was made up to 2 mL with deionized water and mixed by inversion. The absorption at 340 nm was then monitored until constant after which 0.03 unit/mL IDH was added and inverted to mix, to initiate the reaction. The absorbance increase was based on the UV absorbance of the formation of NADPH.

2.7 **Analysis techniques**

2.7.1 ***A. niger***

2.7.1.1 Dry cell weight

Dry cell weight was determined by gravitational filtration of 5 mL culture sample through GFP filter paper. The filtrate acquired to be used in further analysis. The

filter was then dried in a microwave oven (Cookworks, Vancouver Canada) on the defrost setting for 20 minutes before measuring.

2.7.1.2 pH

The pH was measured using a corning pH meter 240 every 24hr when in submerged culture and every 48hr when in shake flask culture.

2.7.1.3 TTA (total titratable acid)

The total acid content of each sample was determined by titrating 0.7 mL of crude culture filtrate against 0.025N NaOH using a 1% w/v phenolphthalein solution as an indicator. The total acid was then calculated using the following equation and was assuming the acid forming was citrate where specified (Figure 2-4). The results were displayed in gl^{-1}.

$$\left(\frac{\text{Titre} \times \text{Normality of alkali} \times \text{No. moles of alkali required to neutralise 1 mole of acid} \times \text{MW}}{\text{Sample volume}} \right)$$

Figure 2-4 Total titratable acid equation

2.7.2 *C. glutamicum*

2.7.2.1 Optical density

Growth was measured by following the optical density of samples at an absorbance of 600 nm using a Biomate 5 spectrophotometer (Thermo Fisher Scientific Inc. Waltham, USA). Samples were diluted appropriately to ensure an absorbance value below 1 was obtained to comply with Beer Lambert law.

2.7.2.2 Dry cell weight

To determine dry cell weight, 1 mL samples of the culture medium were transferred to pre-weighed Eppendorf tubes. They were then pelleted by centrifugation for 10 minutes at 13000 rpm. The supernatant was removed and stored at -80°C to be examined later. The pellets were then washed in distilled water (dH_2O) and centrifuged again. The water portion was then discarded and the pellet and Eppendorf were dried in an oven at 100 °C for 24 h. After drying, the samples were cooled in a desiccator for a further 24 h before being weighed again.

2.7.2.3 pH

The pH of each sample was measured offline using a Corning 240 pH meter (Corning, New York, USA)

2.7.2.4 Ammonium and potassium ion concentration

Ammonium and potassium measurements were made using a YSI 2950 Select Biochemistry Analyzer (YSI incorporated, OH) that included an ion selective electrode (ISE) capable of measuring ion concentration in a dilute solution. Mechanism of its analysis is described within the manufacturers manual and, in brief, is accomplished as follows. The electrodes membrane contained an ionophore layer sandwiched between two layers of PVC matrixes. During sampling, the ions are able to cross through the membrane with the ions of interest being trapped in the layer of ionophores. To calculate the concentration of the specific analytes, the potential across the membrane is measured when equilibrium is achieved between the internal and external membrane solutions and is compared to a reference solution. The resulting millivolts measurement is then converted into concentration using several algorithms that are pre-programmed onto the system. Due to the similarities of their ions, ammonium and potassium measurements are acquired simultaneously by the system and by implementing the selectivity coefficient of the values were interpreted by the software.

2.7.2.5 Glucose consumption

Glucose measurements were made using a YSI 2950 Select Biochemistry Analyzer (YSI incorporated, OH). The system consisted of an electrochemical probe that was coupled with a membrane imbedded with glucose oxidase. When samples were introduced to the system, the enzyme interacted with the D-glucose within the sample and oxygen to produce D-Glucono-d-Lactone and hydrogen peroxide. Released hydrogen peroxide could then be detected by the platinum anode of the probe leading to the creation of a signal current. Experimental supernatant was diluted with dH_2O to bring them into the linear range of the machine approximately $< 9\ gl^{-1}$.

2.7.2.6 LC-MS sample preparation

Where stated, samples were acquired for analysis by liquid chromatography–mass spectrometry to fully understand the complete analyte composition during the mesaconate production process. Samples were either whole samples or were separated into cell and supernatant fragments by centrifugation at 7000 rpm for 10 minutes. The subsequent samples were plunged in liquid nitrogen and stored at -80 °C before analysis was performed by Dr Alison MacFadyen (University of Strathclyde).

2.7.2.7 RNA-SEQ and qPCR sample preparation

Where stated, samples were analysed using as RNA sequencing (RNA-Seq) or qPCR. In particular, this method was employed to identify difference in gene expression before and after induction of *C. glutamicum* strains during mesaconate production process. 6 mL samples were acquired before, immediately after and 10 minutes after induction and treated with RNAprotect (QIAGEN, Venlo, Netherlands) at a ratio of 1:2 sample to protectant. Following the manufactures recommendations, the sample was incubated at room temperature for 5 minutes before being pelleted to remove the supernatant. The liquid layer was removed in its entirety and pellets were stored at –80 °C until cell disruption and RNA-seq or qPCR were performed by Dr Alison MacFadyen (University of Strathclyde).

2.7.2.8 NMR sample preparation

Possible mesaconate samples to be analysed by NMR were obtained using a fraction collector connected to a Gilson HPLC (Section 2.8.1) during organic acid analysis (Section 2.8.3). Once collected, liquid from the sample was evaporated using a rotary evaporator (Rotavapor model R-3, Buchi Ltd., Oldham, UK). Precipitate from the sample was then resuspended in deuterium oxide (Sigma-Aldrich, Missouri, US) and

evaluated using proton (H^+) NMR spectroscopy (WestCHEM NMR Facility at University of Strathclyde).

2.7.2.9 Bradford Assay

Where determination protein concentration was required, a Bradford assay was employed (Bradford, 1976). Firstly, a 2 mg/mL bovine serum albumin (BSA) solution (Sigma Aldrich, Misspuri, US) was used to prepare a set of protein standards ranging for $0.25^{-1}.4$ $mgl^{-1}mL$, by serial dilution. For each standard, 1.5 mL of Bradford reagent (Sigma Aldrich, Misspuri, US), at room temperature, and 0.05mL of the standard was added to a semi-micro cuvette. The solutions were then vortexed to mix thoroughly and incubated at room temperature for 30 minutes before their absorbance was measured spectrophotometrically at 595 nm. This created a standard curve which was used to calculate the protein concentrations in samples from fermentations if and when required. Samples were prepared in the same manner as the standards.

2.8 HPLC analysis

2.8.1 Gilson HPLC modular system

All separation and quantification of organic acids and L-glutamate was carried out using a Gilson HPLC modular system (all components unless otherwise stated were acquired from Gilson, Middleton, USA). The system consisted of two model 360 pumps, a model 850 nanometric module, model 811C dynamic mixer, model 234 auto-injector fitted with a 20 µL volume sample loop. A model 832 temperature control unit was used to control the temperature of the sample rack. To maintain column at the desired temperature a Jones chromatography model 7971 column heater (Jones Chromatography, Hengoed, UK) was used. To measure the constituents

of the samples, two detector units were included in the system; a Gilson model 170 diode array detector (DAD) and also a Viscotek VE 3580 refractive index (RI) detector (Malvern Instruments, Malvern, UK). A Gilson 234 autosampler was also fitted. The entire system was centrally controlled using the Unipoint software application (Version 5.11) (Gilson, Middleton USA).

2.8.2 **Agilent HPLC 1100/1200 system**

For analysis performed at Ingenza Ltd. an Agilent 1100/1200 infinity HPLC system was used (Agilent, Berkshire, UK). This system consisted of inbuilt modules which include a RI and DAD, binary pump, thermostatted column compartment and a high performance autosampler with thermostat. The entire system was controlled using Agilent Chemstation software (version for LC 3D systems Rev. B. 04.03(16), Agilent, Berkshire, UK).

Individual methods were developed for the different molecules of interest.

2.8.3 **Organic acid and glucose**

Organic acids, and to a lesser extent glucose concentrations were quantified using a Rezex ROA column 8 μm particle size, 300 mm length, 7.8 mm diameter (Phenomenex, Cheshire, UK). Analytes were eluted using an isocratic mobile phase composed of 5 mM H_2SO_4 at 1 ml/min with a column temperature of 75 °C. Crude samples were filtered and diluted where necessary before a sample volume of 20 μL was injected in triplicate. Analyte measurements were detected both by UV absorption at 210 nm and refractive index detection.

During every analytical process, to determine linearity, a five point standard curve was created for each analyte of interest, with concentrations ranging from 2 - 200

mM depending on the compound. These included; citric acid, oxalic acid, alpha ketoglutarate, succinic acid, fumaric acid, malic acid, acetic acid, pyruvic acid, lactic acid, mesaconate and, in some cases where the YSI was not available, glucose. Triplicate injections of the solutions were used to create the calibration curve, with the area under the chromatography peak plotted to create a line of best fit, possessing an R^2 value of >95%. The corresponding equation of the line was then used to determine unknown concentrations within the experimental samples. Values were subsequently converted to gl^{-1} for future comparison

2.8.4 **Amino acid quantification**

To estimate amino acid production, a derivatisation process was applied to create a chromophore containing molecule allowing detection by the DAD detector. This was achieved by the derivatisation method (Section 2.8.4) involving the reaction between the amino acid, *ortho*-phthalaldehyde (OPA) and 2-mercaptoethanol (Figure 2-5). The reaction has been well documented in the literature and is proven to work on many amino acids (Roth, 1971, Galaev, 1980, Hanczkó and Molnár-Perl, 2003, Gardner *et al.*, 2013). However, due to instability of the compound created, the process was carried out in the sample loop, with the derivatisation taking place immediately prior to injection on to the column. The column used for this method was a reverse phase C_{18} chromatography column (Grace Davidson Discovery Sciences, Illinois, USA). Analytes were eluted, depending on the system employed, with either a gradient of formic acid solution and methanol (Table 2-4) or sodium phosphate solution and acetonitrile (Table 2-5) both with a flow rate of 1 mL/min using a Grace C18 column (Grace Davison Discovery Sciences). Column temperature was kept constant at 40 °C throughout the process. Analytes were examined by UV absorbance at 340 nm.

During every analytical process, to determine linearity, a five point standard curve was created for each analyte of interest, with concentrations ranging from 0.5-20 mM. These included; glutamic acid, alanine, valine, glutamine, lysine and β-

methylaspartate. Triplicate injections of the solutions were used to create the calibration curve, with the area under the chromatography peak plotted to create a line of best fit, possessing an R^2 value of >95%. The corresponding equation of the line was then used to determine unknown concentrations within the experimental samples. Values were subsequently converted to gl^{-1} for future comparison.

2.8.4.1 Derivatisation method for amino acid quantification

For the derivatisation, method two solutions were prepared; a 0.4 M borate buffer adjusted to pH 10 using 4 M potassium hydroxide and a reagent solution. The latter solution was prepared by dissolving 20 mg of OPA in 0.5 mL of methanol. 20 μL of 2-mercaptoethanol was then added and the regent made up to 5 mL with 0.4 M borate buffer. To attain derivatisation, a 1:2:2 ratio of sample to buffer and reagent was required. This was achieved using an existing injection programme (Gardner, 2012) that allowed for pre-column mixing of the sample with the reagents. With this method, consistency of sample treatment was ensured.

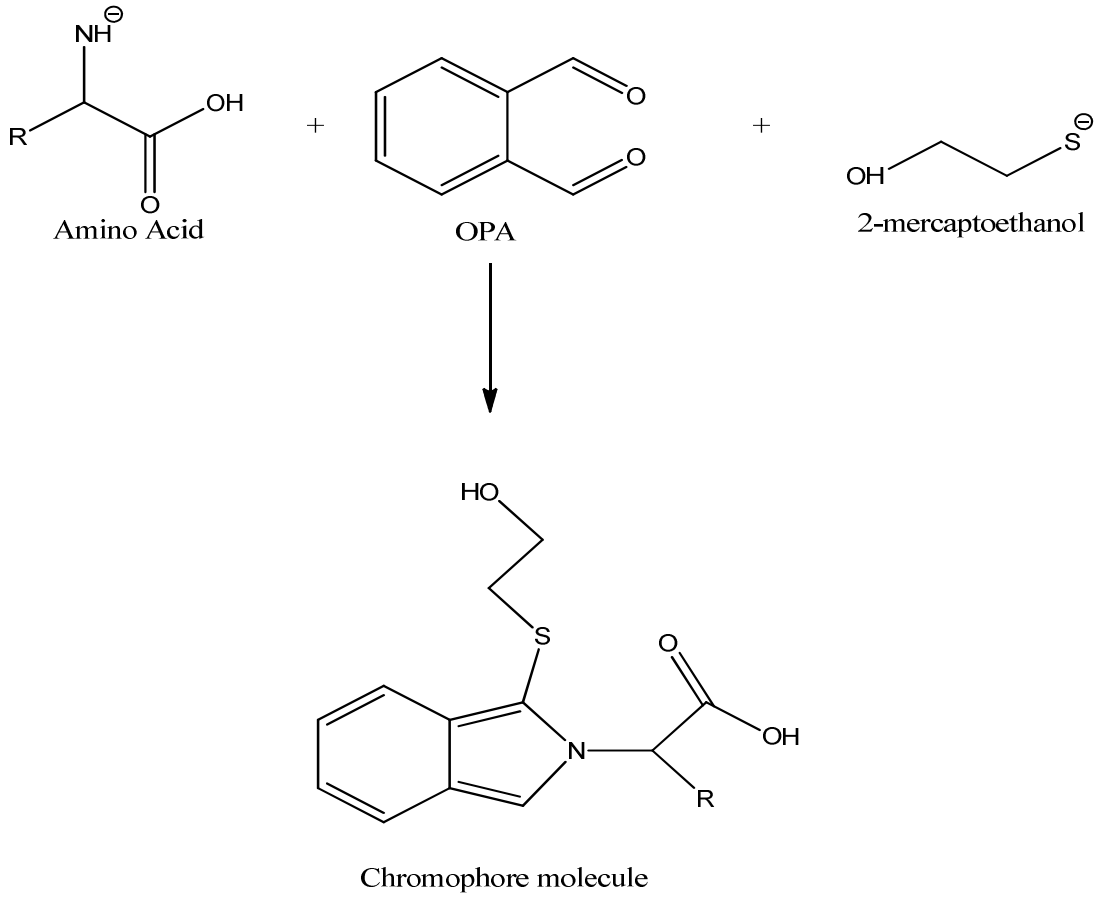


Figure 2-5 Chromophore production

Reaction between an amino acid, OPA and 2-mercaptoethanol to produce a chromophore allowing detection at 340 nm adapted from Gardner (2012)

Table 2-4 Gradient method for amino acid separation using Gilson HPLC modular system

Time (min)	**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

Table 2-5 Gradient method for amino acid separation using Agilent 1200 system

Time (min)	**5 mM $NaPO_4$ (pH7) (%)**	**MeCN (%)**
0	95	5
4	75	25
6	20	80
8	20	80
8.5	95	5
12	95	5

2.8.5 **Succinyl CoA qualitative analysis**

To qualitatively analyse succinyl CoA synthesis during the SCS-assay an HPLC method was developed using a Luna C_{18} 100A column (5 µm particle size, 250 mm length, 4.6 mm diameter; Phenomenex, Cheshire, UK). It was eluted with a gradient of using 10 mM sodium phosphate solution and methanol at a flow rate of 0.8 mL/min (Table 2-6). Column temperature was kept constant at 25 °C throughout the process. Crude samples were filtered before 20 µL of the sample was injected onto the column using an Agilent HPLC 1200 system (Agilent, Berkshire, UK). The following analytes were examined by UV absorbance at 260 nm; succinyl CoA and CoA.

Table 2-6 Gradient method for sucinyl CoA & CoA determination

Time (min)	**10mM $NaPO_4$ (pH4.3) (%)**	**MeOH (%)**
0	90	10
10	40	60
10.5	20	80
15.5	20	80
16	90	10
20	90	10

2.9 **Data recording statistical analysis**

SigmaPlot (version 10.0 Systat Software, Inc. SigmaPlot for Windows) was used to compile the experimental data. Appropriate X-Y scatter plots were created to illustrate trends in the production of specific production analytes, glucose

consumption, DCW and dO2 concentrations. Error bars on graphical outputs represent standard deviations of the sample values where n=3.

As the volume of data amassed was great, only analytes of interest were subjected to statistical analysis using Origin Pro software (9.0 64 Bit edition, OriginLab Corporation, Northampton, USA). However, it should be noted that as this was an early method of development, repetition of experiments was low. Also, results obtained whilst at Ingenza Ltd were only analysed with single HPLC chromatographs to comply with company protocol. Therefore, reliance on statistical analysis may be limited. Where significance was examined mean variance analysis such as the Tukey test permitted single-step multiple comparison of sample values.

Chapter 3

L-glutamate production protocols using strains of *C. glutamicum*

3.1 Introduction

Despite its historical use in the industrial sector, *Corynebacterium glutamicum* was not considered a primary candidate for the manufacture of platform chemicals (Jojima *et al.*, 2013). Recent developments in metabolic engineering of this strain, as well as a fuller understanding of its molecular physiology, have enhanced the potential use of this organism in the production of commodity chemicals and fuels.

3.1.1 *C. glutamicum* background

Currently, the industrial production of amino acids by microbial bioprocesses is essential to meet worldwide demand. Although other strains have been identified, *C. glutamicum* remains the industrial workhorse in the production of several amino acids under aerobic batch and fed-batch processes (Hermann, 2003). Of the amino acids produced, L-glutamate and L-lysine are dominant with demand only set to increase with the possible use of these molecules as platform chemicals (Becker and Wittmann, 2011).

C. glutamicum is a Gram-positive, nonsporulating, non-motile, slightly curved rod shaped bacterium that exhibits a high G+C content. It is widely distributed in nature including; soil, soil containing contaminants such as sewage and manure, as well as on vegetables and fruit (Liebl, 2005). It was first described for amino acid synthesis by Kinoshita *et al.* (1957) when screening organisms for the overproduction of glutamic acid under biotin limited conditions.

As well as for the accumulation of amino acids, genetic engineering of the organism has promoted investigation of the strain for the production of other high value compounds such as lactic acid and succinic acid and ethanol which were discussed in Section 1.2. Other uses include the production of polyhdroxyalkanoate for manufacturing of biopolyesters and cadavenie, a polyamine with similar structural properties required for building block to make nylon (Kind *et al.*, 2014). As well as

chemical compound production, *C. glutamicum* has been considered as an alternative organism for the expression of recombinant proteins (Liu *et al.*, 2015b). With the wide variety of applications, it is no surprise that an interest in developing the organism, both biochemically and genetically, as well as optimising process conditions has been observed over the past few decades.

Although, glutamic acid is the major product produced by *C. glutamicum*, its potential outside of the flavouring industry remains fairly unexploited. In this chapter we will examine the literature to identify key areas in central carbon metabolism, significant genes and induction methods required for glutamate production.

3.1.2 *C. glutamicum* central carbon metabolism

Due to the increasing importance of this organism, it has been sequenced and analysed by several individuals (Ikeda and Nakagawa, 2003, Kalinowski *et al.*, 2003, Yukawa *et al.*, 2007, Lv *et al.*, 2011). This has led to an increase, over the past decade, in attempts to improve productivity by genetic manipulation. Several key genes responsible for proteins involved in cellular metabolism and production of L-glutamate have been identified and examined which are discussed below.

3.1.2.1 Sugar uptake mechanism

C. glutamicum has the ability to utilise a variety of carbohydrate sources including glucose, sucrose and fructose as well as alcohols, and organic acids (Witthof *et al.*, 2013). Due to industrial reliance on molasses and starch hydrolysate as carbon sources, which contain varying amounts of the aforementioned sugars, the mechanism of its uptake has been investigated.

Transport of the preferred carbon source, across the cytoplasmic membrane, is mainly mediated through the phosphotransferase transport system (PTS). This

system consists of sugar specific membrane bound permease enzymes (EII^{glu} for glucose, EII^{fru} for fructose, and EII^{suc} for sucrose) as well as two shared cytoplasmic proteins, known as enzyme I (EI) and HPr (Moon *et al.*, 2007, Teramoto and Inui, 2013). The sugar specific permeases are encoded by the genes *pts*G, *pts*F and *pts*S for glucose, fructose and sucrose respectively and are positioned beside a Deo-R type transcriptional regulator known as SugR. Overexpression of SugR in *C. glutamicum* has been shown to reduce the utilisation of glucose when the organism is grown in the presence of the carbon source (Engels and Wendisch, 2007). Reduced expression of the *pts*G gene under these conditions suggests that the presence of the regulator acts as a negative feedback for the transcription of the major permease enzymes.

Although the PTS system is the major pathway for glucose uptake, mutants of *C. glutamicum* with defective *pts*G gene have been observed to remain metabolically active a low levels (Lindner *et al.*, 2011). The alternative pathway utilises inositol permeases that were capable of transporting glucose across the cellular membrane.

As well as gene regulation, it has also been suggested that osmotic stress, caused by product accumulation, can impact glucose uptake rates. Gourdon *et al.* (2003) proposed that under osmotic stress, biochemical modifications of the PTS transport system and not gene expression were responsible for a reduction in sugar uptake. This effect was shown to be reversible when as washed cell previously exposed to osmotic stress, reverted to high sugar uptake.

During the migration across the membrane, the sugars are subsequently phosphorylated which permits the continuation of metabolism into glycolysis or the pentose phosphate pathway which will be discussed in (Section 3.1.2.2).

3.1.2.2 Glycolysis and the Pentose Phosphate Pathway

Once the hexose sugar enters the cytoplasm, they are phosphorylated and then metabolised through either glycolysis or the pentose phosphate pathway (Figure 3-1).

Glycolysis is the major route through which carbon catabolism occurs and results in the step-wise conversion of glucose-6-phosphate to pyruvate. In the presence of glucose, expression of the major genes involved in glycolysis are observed *C. glutamicum* (Han *et al.*, 2007). On glucose depletion, expression is reduced with a switch in metabolism towards glycogenic production of organic acids observed.

Although several enzymes are involved in glycolysis, pyruvate kinase (PYK) is considered a major control factor of the pathway as it is both important in the production of energy as well as being a branch point into the TCA cycle. The enzyme catalyses the conversion of phosphoenolpyruvate to pyruvate and in the process yields one molecule of ATP. As phosphoenolpyruvate is also a precursor to oxaloacetate, an intermediate in the TCA cycle, action of pyruvate kinase reduces carbon through this pathway. The enzyme has been observed to be strongly inhibited by the presence of ATP owing to feedback regulation therefore it is significant in controlling energy metabolism within the organism (Jetten *et al.*, 1994).

Strains of *C. glutamicum* with defective PYK gene (*pyk*) have been created to examine the flux of carbon during glutamic acid production. Under biotin limiting conditions, Sawada *et al.* (2010) observed comparable growth rates between the *pyk*-mutant and the wild type. However, a 45 % increase in specific production rate of glutamic acid and an increased glucose utilisation rate was also observed. This provides some evidence of the potential bottlekneck of pyruvate kinase in the production of the amino acids. Interestingly, in the same strain under non glutamate producing conditions, i.e. biotin sufficient condition, biomass accumulation is increased compared to the wild type (Sawada *et al.*, 2015). This increase is also combined with metabolic disturbances that down regulate the respiration rate within the organism.

As for the pentose phosphate pathway, it serves as an alternate route for carbon sources at the glucose-6-phosphate branch and circumvents glycolysis. The pathway is split into two stages: an oxidative and a non-oxidative phase. Primary function of

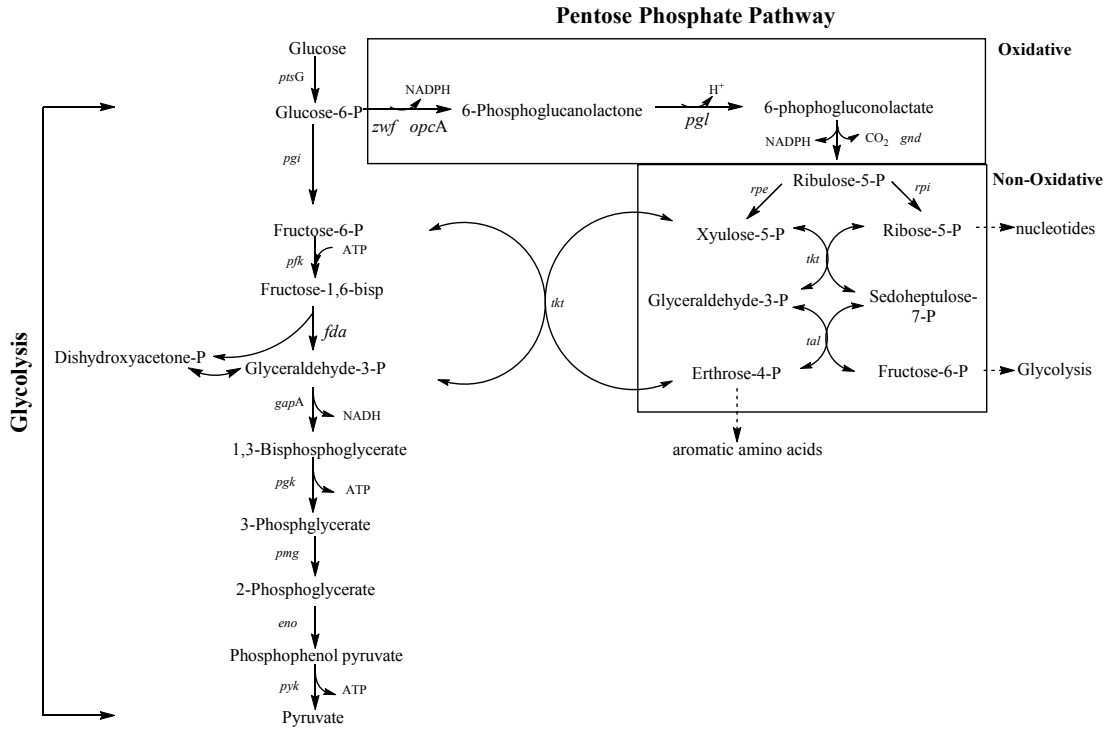


Figure 3-1 Glycolysis and the Pentose Phosphate Pathway
Adapted from Yokota and Lindley (2005)

this pathway is vital in the anabolic production reducing cofactors NADPH and precursor metabolites ribose-5-phosphate and erthose-4-phosphate. It also produces key intermediate compounds, glyceraldehyde-3-phosphate and fructose-6-phosphate that can replenish the glycolysis pathway. Metabolic analysis of *C. glutamicum* under glutamate production conditions indicate that flux towards this pathway is reduced compared to that of glycolysis (Shirai *et al.*, 2007). This is rational, as the requirement of NADPH for the conversion to glutamic acid from α-ketoglutarate can be met through the activity of isocitrate dehydrogenase, the precursor enzyme in the TCA cycle. However, with the potential to increase NADPH availability,

exploitation of the pentose phosphate pathway for increased production of the cofactor has received more attention as a means to improve yields.

3.1.2.3 Anaplerotic pathways towards Oxaloacetate

It has been established that *C. glutamicum* possesses two anaplerotic pathways that lead to glutamate production. The first is catalysed by pyruvate carboxylase (PC), controlled by the *pyc* gene, which converts pyruvate to oxaloacetate (Sauer and Eikmanns, 2005). The second is catalysed by phosphoenolpyruvate carboxylase (PEPC), controlled by the *ppc* gene, converting phosphoenolpyruvate to oxaloacetate (Sauer and Eikmanns, 2005). Sato *et al.* (2008) discovered that by disrupting the *pyc* gene, production of L-glutamate increased. Whereas when the *ppc* gene was disrupted, growth rate decreased and no L-glutamate was produced. This gave evidence that PEPC is essential for the production of the amino acid. There was also evidence that by overexpressing the *ppc* gene in the *pyc* knock-out strain productivity improved as suggested by an increased metabolic flux from phosphoenolpyruvate to oxaloacetate. This result was also observed by Shirai *et al.* (2007) thus outlining the importance of the *ppc* gene in the production of glutamate.

It may also be interesting to note that disruption of the gene involved in lactate production did not improve the accumulation of glutamate (Shirai *et al.*, 2007). Therefore, determining the reason behind this and then establishing a redirection of the flux from Lactate producing pathway towards glutamate production could help improve productivity.

3.1.2.4 The TCA cycle

Independent of the initial carbon source, the Tricarboxcylic (TCA) cycle if not parts of the cycle, are required to obtain energy in growing cells and is critical in the production of the precursor, α-ketoglutarate for the production of L-glutamate. A

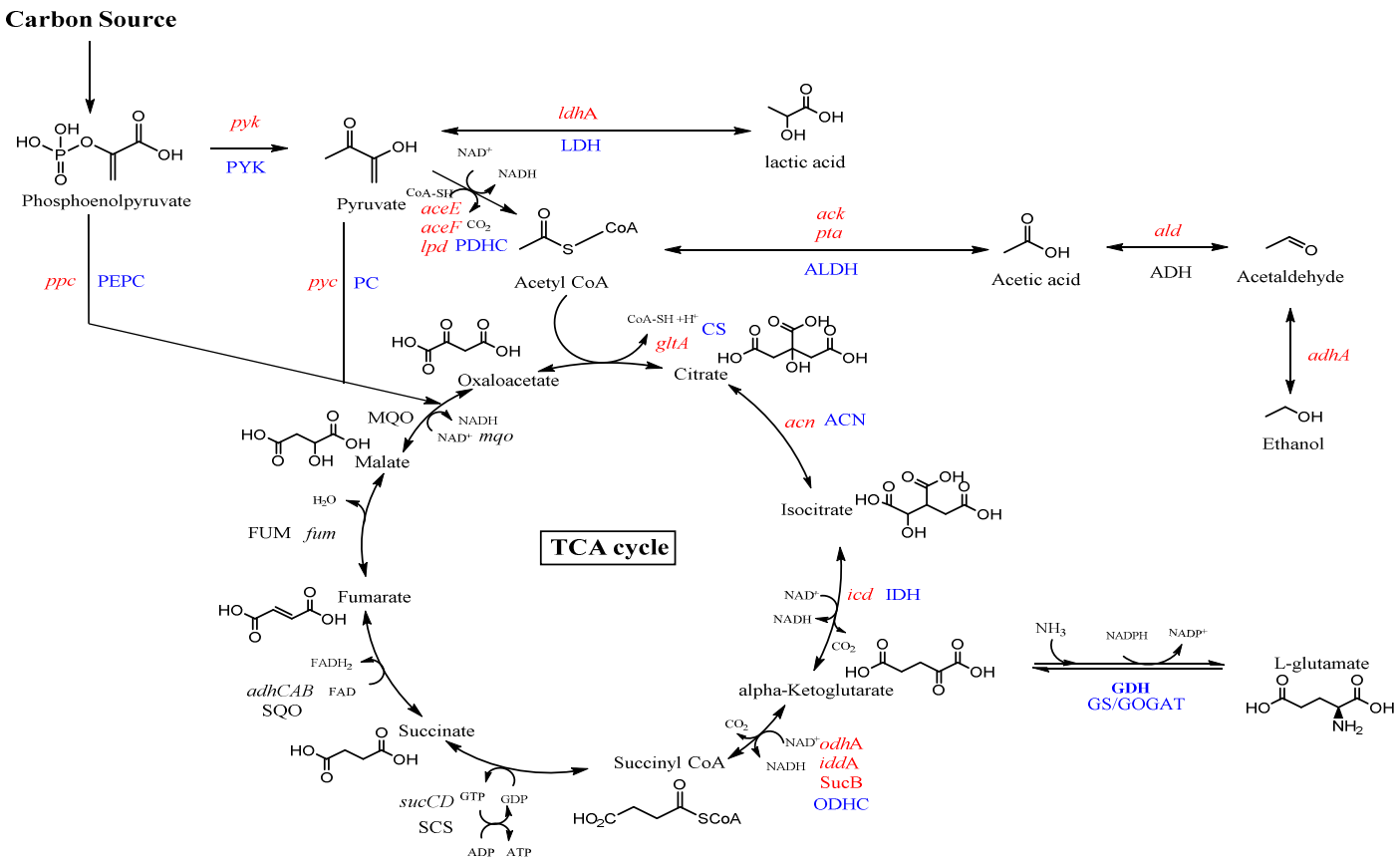
diagrammatical representation of this pathway is illustrated in Figure 3-2, indicating key enzymes and genes that are important for L-glutamate production.

Pyruvate, produced as the terminal compound in glycolysis, is decarboxylated by pyruvate dehydrogenase complex to produce acetyl CoA. Although it is an important enzyme that produces the primary substrate that fuels the TCA cycle, during glutamate producing conditions activity of this enzyme appears to decrease (Hasegawa *et al.*, 2008). These conditions appear to favour the flux of carbon source through the anaplerotic pathway towards oxaloacetate as opposed to the production of acetyl CoA.

Continuing the cycle, citrate synthase catalyses the condensation of oxaloacetate and acetyl-CoA to produce citric acid, which is converted to its isomer, isocitrate, by acontitase. The enzyme isocitrate dehydrogenase (IDC) then converts isocitrate to α-ketoglutarate with the simultaneous production of NADPH, vital for glutamate production through glutamate dehydrogenase. Deletion of the IDH gene, *icd*, completely halted production of glutamate in *C. glutamicum* however overexpression failed to increase accumulation (Eikmanns *et al.*, 1995). This suggests that although IDH is essential for glutamic acid production, it is not a limiting step in the pathway towards the amino acid.

The 2-oxoglutarate dehydrogenase complex (ODHC) comprises of the subunits Eo1 (OdhA), Eo2 (AceF) and Eo3 (dihydrolipoyl dehydrogenase) (Usuda *et al.*, 1996) and catalyses the conversion of α-ketoglutarate to succinyl CoA. As it plays a significant role in the flux of carbon towards glutamate analysis of its activity has received significant scrutiny.

A study by Shimizu *et al.* (2003) describing a metabolic reaction model, allowed an insight into the flux distribution around 2-oxoglutarate a key branch point. It was identified that overexpression of both isocitrate dehydrogenase and glutamate dehydrogenase, key enzymes in the production of glutamate, exhibited no significant effects on accumulation. However, by reducing the activity of the 2-oxoglutarate dehydrogenase complex (ODHC), a significant increase was observed in glutamate production. The reduction of ODHC activity has been observed in *Brevibacterium*



PYK	Pyruvate kinase
PEPC	Phophenolpyruvate carboxylase
PC	Pyruvate carboxylas
LDH	Lactate dehydrogenase
ALDH	Acetaldehyde dehydrogenase
ADH	Alcohol dehydrogenase
PDHC	Pyruvate dehydrogenase complex
CS	Citrate synthase
ACN	Aconitase
IDH	Isocitrate dehydrogenase
ODHC	Oxoglutarate dehydrogenase complex
SCS	Succinyl CoA synthetase
SQO	Succinate: menaquinone oxidoreducas
Fum	Fumerase
MQO	Malate: quinone oxidoreductase

Figure 3-2 *C. glutamicum* carbon metabolism towards L-glutamate

Key genes for L-glutamate production are highlighted in red whereas enzymes in key areas are highlighted in blue (adapted from Bott (2007), Teramoto *et al.* (2011))

lactofermentum, a related species to *C. glutamicum*, during glutamate production (Kawahara *et al.*, 1997). An overexpression of the *odh*A gene responsible for the Eo1 subunit of ODHC was observed to dramatically reduce the productivity of L-glutamate synthesis by *C. glutamicum* (Kim *et al.*, 2009).

In its phosphorylated state, the ODHC's activity is inhibited by the presence of OdhI attached to one of its subunits. The binding of protein kinase G (PknG) to the oxoglutarate dehydrogenase inhibitor protein (OdhI), elevates the inhibitory effects and allows phosphorylation of ODHC, increasing its activity. Therefore, since a reduced activity was observed to be beneficial to the production of L-glutamate, the genes controlling these molecules were analysed by Schultz *et al.* (2007). As expected, when OdhI was removed, L-glutamate production decreased compared to the wild type (*C. glutamicum* ATCC 13032). This was because inhibition of ODHC did not exist, allowing the conversion of α-ketoglutarate to succinyl CoA to take place. Therefore, it could be taken from this that OdhI is essential for glutamate production. However, when deletion of PknG was examined, mixed results were observed when different trigger conditions were introduced. Biotin limitation at a concentration of 1 μgl^{-1} increased productivity by 4.3 times compared to the wild type whereas induction by penicillin G exhibited a reduction in accumulation of 48%. The results of the PknG deletion from this study are uncertain and lead to the assumption that PknG plays various roles under different treatment methods.

Asakura *et al.* (2007) describes the production of a strain with a deletion of the *odh*A gene which lead to a dramatic reduction in ODHC activity. The strain was observed to have comparable production levels to an induced wild type strain (*C. glutamicum* ATCC 13869), without the need for any treatment conditions such as biotin limitation, penicillin and Tween 40 additions. When the induction methods were employed to the mutated strains, productivity was increased by approximately 10%.

Disruption of ODHC through mutations in the *odh*A gene does not always constitute increased production of L-glutamate. Nakamura *et al.* (2007) observed no significant improvement in productivity when *odh*A was disrupted in the same strain of *C. glutamicum.* Therefore, there is conflicting evidence regarding the importance of *odh*A deletion and the improvement in L-glutamate production.

3.1.2.5 Conversion of α-ketoglutarate to L-glutamate

For synthesis of L-glutamate, two pathways can be utilised (Figure 3-3). Firstly, the amino acid is generated through the reductive amination of α-ketoglutarate (2-oxoglutarate) in the presence of ammonia. This reaction is reversible and is catalysed by glutamate dehydrogenase (GDH). Secondly in low nitrogen conditions conversion of α-ketoglutarate can be accomplished using glutamine synthetase/glutamate synthase which is also known as L-glutamine:2-oxoglutarate aminotransferase (GS/GOGAT) (Bott, 2007). In this case, the ammonium is used to amidate L-glutamate catalysed by glutamine sythetase (GS) and utilising ATP to form L-glutamine. Reductive transfer of the amide group to α-ketoglutarate is then catalysed by glutamate synthase (GOGAT). This results in the conversion of α-ketoglutarate to L-glutamate.

As the latter system requires consumption of ATP, it is biologically less favourable compared to synthesis through GDH, therefore GDH is the primary pathway towards L-glutamate. To analyse the significance of GDH, its corresponding gene, *gdh*, was deleted for *C. glutamicum* 13032. No significant effect on growth or glutamate production was observed indicating that it was not essential for the organism (Börmann *et al.*, 1992). Muller *et al.* (2006) indicated that on deletion of the *gdh*. repression of the GOGAT pathway by the transcriptional regulator AMTr was reduced allowing for the pathway to intervene in the absence of GDH to produce glutamate.

Examination of the GS/GOGAT system by Schulz *et al.* (2001) indicated two of enzymes were regulated by carbon and nitrogen concentrations in the culture medium. During carbon sufficient and nitrogen limiting conditions, both enzymes and their corresponding genes are up-regulated. Glutamine synthase activity was dependent on the carbon concentration. During carbon limitation, the enzyme activity decreased and increased again when a carbon feeding regime was implemented. However, in both instances, glutamate synthase remained unchanged. This highlights the need for strict regulation of metabolites present in the culture medium to produce the most efficient process.

These routes to glutamic acid cannot be thought of as two completely separate pathways as they appear to be both active, as deletion of one pathway does not necessarily inhibit glutamate accumulation. However, regulation must occur, as accumulation of L-glutamine by the action of GS is essentially a by-product of L-glutamate production. Interestingly, increased accumulation of L-glutamine occurs at low pH's, therefore management of this will reduce this activity. In order to operate the process at low pH which would be desirable in industry (Werpy and Petersen, 2004), disabling this pathway would be necessary in order to prevent unwanted by products.

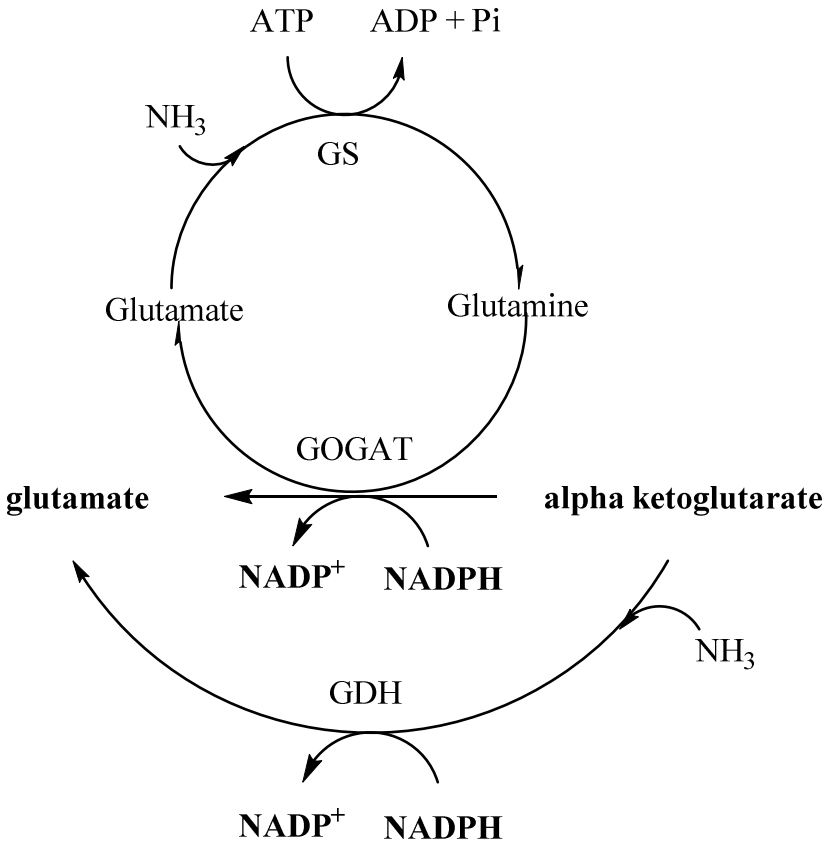


Figure 3-3 Synthesis of glutamate by glutamate dehydrogenase and glutamine sythetase/glutmate synthase (GS/GOGAT) system (adapted from Yuan *et al.* (2009))

3.1.3 **Glutamate exporter**

To date, only one known exporter of glutamate has been identified. Recently, discovery of the potential glutamate transporter YggB, encoded by the NCg11221 gene was identified by Nakamura *et al.* (2007) which appeared to be significant during production conditions. By examining *odhA* disrupted mutants, it was noted that additional mutations were observed in the NCg11221 gene which coded for a mechanosensitive ion channel homolog. Disruption of this gene appeared to completely deter glutamic acid secretion even under biotin limiting conditions. Intracellular examination of the cells, revealed the accumulation of glutamic acid, therefore reinforcing the compellingly theory that this gene was responsible for the creation of the YggB exporter essential for the secretion of the amino acid. Interestingly, amplification of NCg11221 did increase glutamate secretion under induction conditions. However, under non-induction conditions, no increase in secretion was observed, suggesting that the presence of the exporter itself was not independently sufficient for overproduction and requires the simultaneous reduction of ODHC activity for L-glutamate accumulation. It has been suggested that this exporter is activated under induction methods due to alterations in the membrane tension leading to the structural transformation of the protein and leading to L-glutamate exportation.

Further research is required to fully understand this mechanism as exploitation of the excretion systems could improve accumulation rates. This would be beneficial to industrial production.

3.1.4 **L-glutamate induction methods**

Excretion of L-glutamate into the fermentation broth generally requires a trigger to stimulate transfer across the membrane. Different conditions have been developed over the past 50 years which are surprisingly varied; Biotin limitation (Shiio *et al.*, 1962a), heat induction (Lapujade *et al.*, 1999), penicillin (Nunheimer *et al.*, 1970),

Tween® 40 (Kim *et al.*, 2009), and ethambutol addition (Radmacher *et al.*, 2005). All have some effect on cell wall integrity and possible interaction with some of the key enzymes or genes involved in the production of L-glutamate (Figure 3-4) (Schultz *et al.*, 2007). Further details regarding theories behind the mechanisms of these varied methods will be discussed throughout this chapter.

It is interesting to note that in producing the desired product of L-glutamate, growth of the organism could be affected (Nunheimer *et al.*, 1970). For instance, it has long been known that by inducing overproduction of L-glutamate under biotin limitation, biotin levels are sub optimum for growth and therefore growth is hindered (Shiio *et al.*, 1962b). Biotin is an essential nutrient for cellular growth as it is required as a cofactor in the biosynthesis of fatty acids. Therefore, modelling of *C. glutamicum* biosynthesis could prove useful in reaching a balance between growth and productivity (Sun et al., 2011). Further discussion of biotin importance in L-glutamate production will be cover in Biotin limitation Section 3.8.

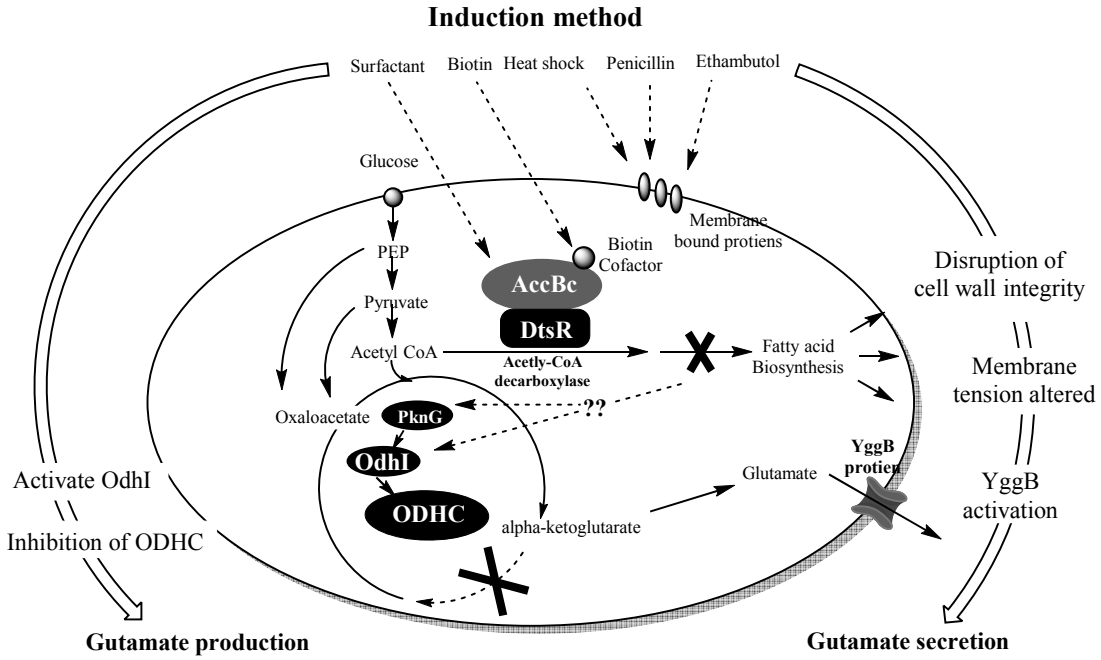


Figure 3-4 Proposed glutamate production mechanisms by induction methods

(Adapted from Ikeda and Takeno (2013))

3.2 **Process overview**

C. glutamicum has a rich history in the production of amino acids such as L-glutamate and L-lysine. However, strains utilised in industry are highly guarded secrets. Therefore, to determine the most efficient production process for the strains obtained from Ingenza Ltd., a screening of several production conditions was performed. The strains and the processes under which they were examined are detailed in Table 3.1

Table 3-1 Strain and process screening for high production of L-glutamate

Strain	**Reference**	**Notable genes**	**Biotin limitation**	**Tween 40® induction**	**Temp shift induction**	**Ethambutol induction**
C. glutamicum ATCC 13032	(Kalinowski *et al.*, 2003)	Wild type	Yes	No	Yes	Yes
C. glutamicum ATCC 13032 Δ *ldh*A	Ingenza Ltd. strain	*ΔldhA*	Yes	No	No	Yes
C. glutamicum ATCC 13032 Δ *ldh*A empty vector	Ingenza Ltd. strain	*ΔldhA*	Yes	Yes	No	No
C. glutamicum ATCC 13869	(Shirai *et al.*, 2007, Asakura *et al.*, 2007)	Wild type	Yes	Yes	No	No

In industry, it has become common practice to use Tween 40® and heat induction as the more cost efficient method of L-glutamate production (Eggeling, 2005).These methods as well as the classical method of biotin limitation and the more recent approach of inhibiting arabinogalactan synthesis by ethambutol were examined also. It was decided at the time not to investigate penicillin induction due to the expense of the lab grade compound compared to the cheaper alternative induction methods.

3.3 **Objective**

As efficiency of L-glutamate production is crucial for the success of the proposed pathway towards the desired platform chemical, all of the above techniques would be scrutinised in order to identify the trigger process method that would be carried forward to screen potential new engineered strains.

3.4 **Aims**

1. To identify the most appropriate inducer for the strains selected for this study
2. To produce an optimal protocol for the production of L-glutamic acid

3.5 **Temperature triggered induction of L-glutamic acid overproduction**

Secretion of L glutamic acid is observed when cultures of *C. glutamicum* are heat shocked, with some processes producing 85 gl^{-1} in just 24 h (Delaunaya *et al.*, 1999, Lapujade *et al.*, 1999). This method typically consists of a two phase procedure where the cultures are initially grown at 33 °C until a substantial biomass has been achieved. Cultures are then subjected to an increase in temperature to 39 °C whereupon L-glutamate secretion is observed. It is still not fully understood why under these conditions glutamate is excreted, however, analysis of key enzymes

involved in its synthesis provides some insight into its mechanism. When the culture temperature is shifted to 39 °C, ODHC activity is completely inhibited and pyruvate dehydrogenase activity is decreased by 35% compared to the values observed under growth temperature of 33 °C (Uy *et al.*, 2003).

This method has several favourable aspects as it can be used in conjunction with a variety of sustainable carbon sources as it does not require a low concentration of biotin in order to secrete amino acids. It has also been used in conjunction with other induction methods to improve production yields.

3.5.1 Shake flask analysis of temperature triggered L-glutamate production

At the point of this investigation, only *C. glutamicum* ATCC 13032 wild type and *C. glutamicum ΔldhA* strains were available for analysis. Therefore, to examine the viability of the process for future studies, the wild type strain only, was assessed in shake flask culture (Table 3-1).

In keeping with future experiments, the organism was cultivated in CGXII glucose minimal medium containing 200 $\mu g l^{-1}$ of biotin (Section 2.2.2.2). Conditions there after were derived from Boulahya *et al.* (2010). Cultures were cultivated at 33 °C for 10 h, corresponding to an approximate cell density of 1 $g l^{-1}$. At this point, the temperature was increased to 39 °C to elicit the production L-glutamate.

Similar to many studies in the literature examining temperature-triggered processes (Uy *et al.*, 1999, Uy *et al.*, 2003, Lapujade *et al.*, 1999), Boulahya utilised the capabilities of *C. glutamicum* 2262. It was expected that some differences would be observed between this and the 13032 wild type strain.

As exhibited in Figure 3-5, low productivity was observed during the temperature up-shock of the wild type strain. After the temperature was increased to 39 °C, the growth rate also decreased from 0.15 to 0.05 h^{-1} with a maximum DCW weight of only 2.2 $g l^{-1}$ achieved. The low biomass yield could go some way to explain the low

yield of L-glutamate, which only peaked at 0.2 gl^{-1}. This was in stark contrast to what has been observed in the published literature. Boulahya *et al.* (2010) reported achieving a final biomass of 6 gl^{-1} and a yield of 11 gl^{-1} of glutamate after 11h.

Admittedly, a slight medium variation was employed in the present study, which contained a 10 times lower concentration of biotin. This could explain the slower growth rate as lower levels of biotin has been displayed to decrease growth rate (Shiio *et al.*, 1962b). However, the concentration of biotin was above the level that would be necessary to be considered limiting as it is two hundred times higher than would be required to induce production of L-glutamate through biotin limitation (Eggeling and Reyes, 2005). The process was repeated in triplicate and no improvement in growth or productivity was observed.

Due to the poor efficiency of the organism under these conditions, time constraints and the promise of other induction methods, this line of investigation was discontinued at this point as its industrial relevance was not competitive.

Although this method has been observed to produce large quantities of L-glutamate, limitations do exist. Uy *et al.* (2003) observed over prolongation of the process, an adaption in the culture was observed that promoted growth over production. Interestingly, by increasing the process temperature further to 41 °C, production was again initiated. This issue could prove problematic in continuous culture as successive temperature increases would be required in order to maintain high production rates.

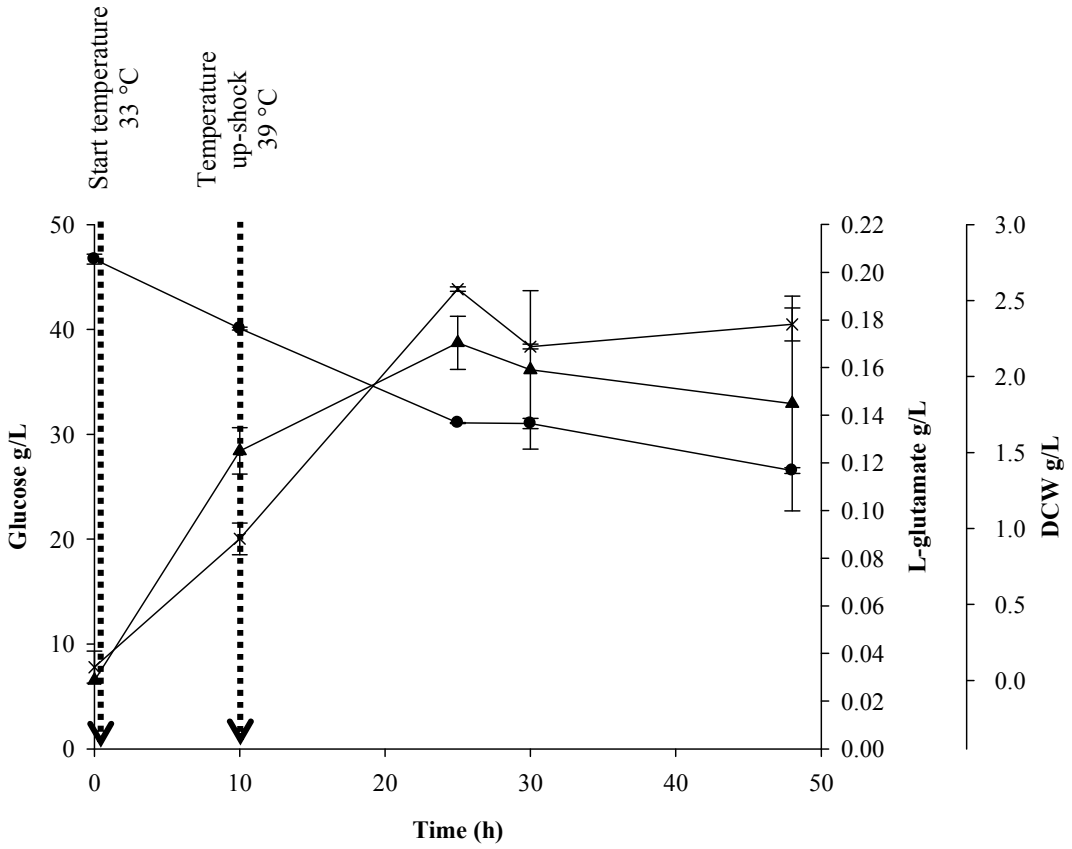


Figure 3-5 Temperature up-shock for the induction of L-glutamate production.
C. glutamicum ATCC 13032 cell banks were used to inoculate shake flasks containing biotin-sufficient CGXII media. Cultures were incubated at 33 °C, 250 rpm until an OD_{600nm} = 10 was achieved. Incubation temperature was then increased to 39 °C arrows denote where temperature increase occurred. Time profiles of the glucose utilisation (●), DCW (▲) and L-glutamate (x) accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.6 Tween 40® induction of *C .glutamicum* for the production of L-glutamic acid

Similar to heat induction, Tween 40® triggered induction has become a popular choice in industry as it allows the use of cheaper raw materials than other methods (Eggeling, 2005). Again, the method of induction is not fully understood but is presumed that disruption of cell membrane integrity as well as possible inhibition of the ODHC enzyme appear to be significant.

It was decided to evaluate the process using two *C. glutamicum* strains; ATCC 13869, which has been utilised as a wild type strain in various studies examining Tween induction (Shirai *et al.*, 2007, Asakura *et al.*, 2007) and *C. glutamicum* ATCC 13032 *ΔldhA*, a lactate dehydrogenase negative strain containing an empty vector to allow for future modifications engineered by Ingenza Ltd.. By using the same wild type as the mentioned papers it was expected that similar levels of glutamate would be achieved under the same conditions and therefore would give a direct comparison. As well as the wild type, an engineered *C. glutamicum* ATCC 13032 Δ *ldh*A empty vector strain was examined due to the industrial partner interest in using genetically modified organisms that had been created in house (Table 3-1).

To examine the efficiency of this process with the aforementioned strains, a modified protocol was developed from Shirai *et al.* (2007). Medium composition was unchanged to that used the experiments where no labelled glucose was utilised. However, the scale was increased to 10 L compared to 80 mL, and was performed in a 15 L Braun Biostat Q (Section 2.2.2.3). The increase in volume was selected as it was more applicable to the scale up that would be required if the process was to be adopted in industry.

According to Shirai *et al.* (2007), Tween 40® at a concentration of 0.8 mgl^{-1} elicited the greatest L-glutamate excretion. Therefore, during mid exponential phase of the process, approximately 3-4 gl^{-1} of DCW or an $OD_{600\ nm}$ of 10, the cultures were induced with the final concentration of 0.8 mgl^{-1} Tween 40®. Previous work carried out in this study using this method, elicited the formation of a large volume of foam

leading to the termination of the process. Therefore, to avoid excess foaming, the solution of Tween was prepared in 98 mL dH_2O with the addition of 2 mL PPG. Addition of the antifoam appeared to suppress some production of foam, however, additional PPG was required thereafter which may have resulted in lower cell growth and productivity. This impact of the antifoam is due to its detrimental impact on the gas-liquid volumetric mass transfer coefficient (kLa) which would dramatically reduce the organisms ability to utilise the available nutrients from the media (Morão *et al.*, 1999).

Comparing the two strains used in this study, *C. glutamicum ΔldhA* empty vector strain (Figure 3-6) exhibited a shorter lag phase than strain ATCC 13869 (Figure 3-7), with mid exponential phase reached within half the process time. No production of L-glutamate was observed before the introduction of Tween into the system with either of the strains. However, after induction, there is a clear indication of glutamate secretion.

Yield of glutamate per gram of biomass was 0.2 and 0.007 g/g for *C. glutamicum ΔldhA* empty vector strain and ATCC 13869 strain respectively, with the final concentration of glutamate at 1.5 gl^{-1} and 0.9 gl^{-1} when glucose was depleted. Compared with Shirai, both strains exhibited lower end product yields however; specific yield for *ΔldhA*-empty vector strain was higher. Glucose utilisation was also higher in both strains, with depletion occurring between 20-28h of the process. This was compared to over 60 h within the aforementioned study. A possible explanation for the increase in glucose consumption in the current system is that the scaled up process allowed for more optimal conditions. Although all specifications were not divulged, a small scale jar fermenter was employed in the previous study. In the present study, a large scale reactor was used that included three Rushton impellers and stainless steel baffles which would improve mixing and mass transfer considerably.

Depletion of glucose occurred relatively soon after induction suggesting that even though system was under glutamate producing conditions there was only a limited amount of glucose left to convert at this point. As there was low end product yield and relatively low DCW created, the production of other by products before this

point was a strong possibility; however at this stage this was not investigated. The depletion of glucose also signalled a deceleration of respiration with the plateauing of biomass production and culminating in the increase of dO_2 within the process. This rise in dO_2 was used as an indication of the process having reached completion.

Tween 40® induction exhibited promise as a viable production method, with its clear ability to elicit L-glutamate secretion upon its introduction in the process. With possible engineering of the organism to divert away from making by products and the introduction of a feeding programme, it could be assumed that production of the desired amino acid could be improved. However, this method was ultimately not applied as controlling foaming issues was hindering the growth of the organism. As more antifoam was required to inhibit foaming, it was noted that respiration was affected with an increased levels of dO_2 observed. Therefore, again as other methods exhibited more promise, Tween 40® induction was rejected.

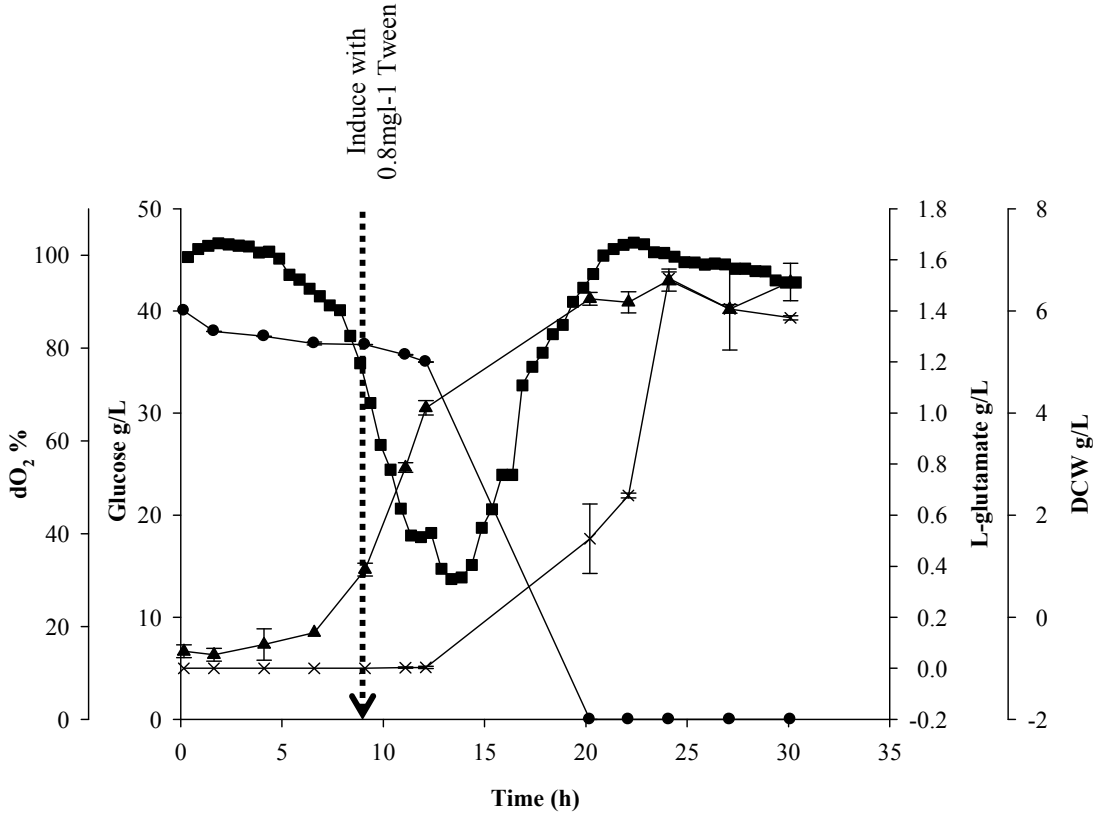


Figure 3-6 Tween 40® L-glutamate induction of *C. glutamicum* ATCC 13032 Δ *ldh*A empty vector

C. glutamicum ATCC 13032 Δ *ldh*A cultures were used to inoculate 10 L of Tween 40® production media to an OD_{600nm} =1. The process was maintained at 30 °C, dO_2>30% with an agitation cascade in a 15 L Bruan BiostatQ bioreactor. Culture was induced with 0.8 mg/mL Tween 40® when an OD_{600nm} = 10 was achieved and process continued until glucose was depleted. Induction time denoted by the dashed arrow. Time profiles of the glucose (●), DCW (▲), dO_2% (■) and L-glutamate accumulation (x) are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

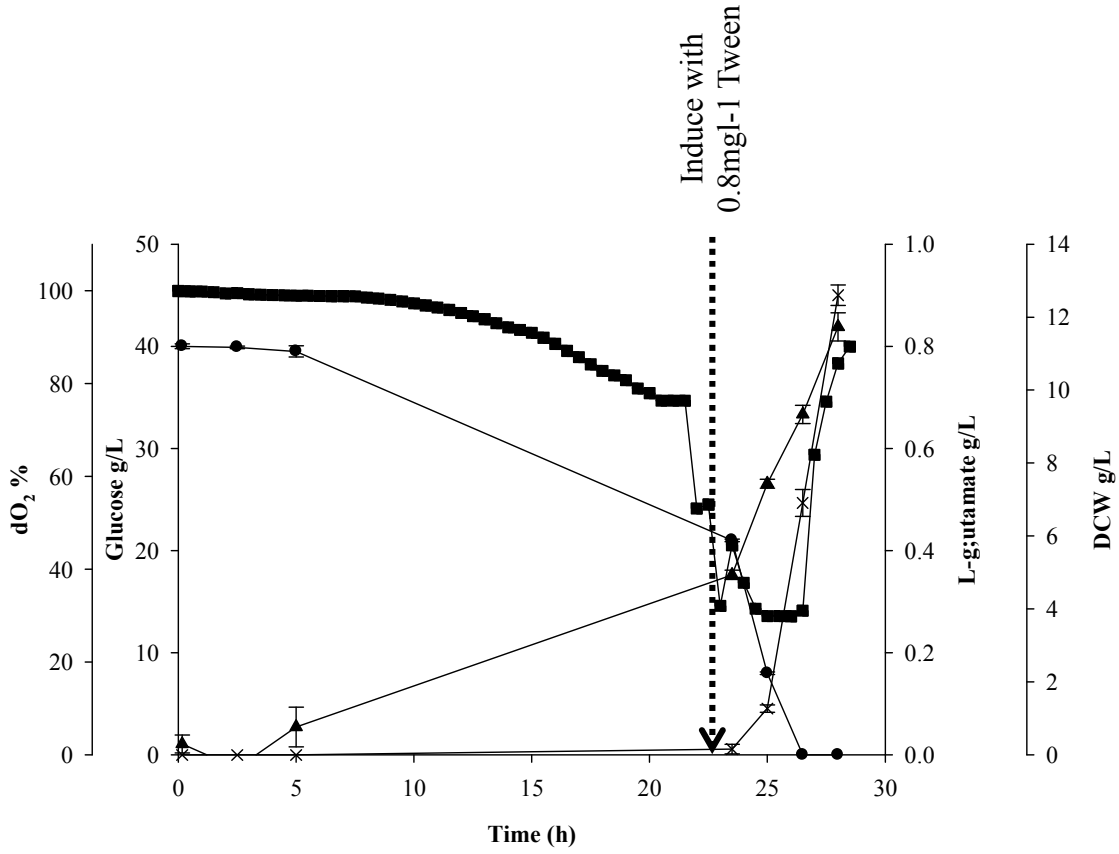


Figure 3-7 Tween 40® L-glutamate induction of *C. glutamicum* ATCC 13869
C. glutamicum ATCC 13869 cultures were used to inoculate 10 L of Tween 40® production media to an OD_{600nm} =1. The process was maintained at 30 °C, dO_2>30% with an agitation cascade in a 15 L Bruan BiostatQ bioreactor. Culture was induced with 0.8 mg/mL Tween 40® when an OD_{600nm} = 10 was achieved and process continued until glucose was depleted. Induction time denoted by the dashed arrow. Time profiles of the glucose (●), DCW (▲), dO_2% (■) and L-glutamate accumulation (x) are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.7 **Ethambutol addition for the induction of L-glutamate in *C. glutamicum***

C. glutamicum, like many *Actinobacteria*, including *Mycobacterium tuberculosis*, contain an abundance of mycolic acids which, together with other cell wall components, make up the outer layer of the cell. Consisting of long fatty acid chains, mycolic acids have been attributed to bacterial resistance to a variety of antibiotics (Nguyen and Thompson, 2006). In a recent study it has also been displayed that mycolic acids may also play a role in glutamate secretion. Hashimoto *et al.* (2006) observed that during glutamate over producing conditions, total mycolic acid content was reduced, and therefore suggested that the acids functioned as a barrier to glutamate production. The study also suggested that even if the treatments themselvess were not directly aimed at the mycolic acids, they were inadvertently decreasing their synthesis. For example, biotin, Tween and cerulenin treatments reduced fatty acid biosynthesis, the building blocks from which mycolic acids are created. Penicillin inhibits peptidoglycan synthesis, reducing the scaffolding on which the acids would attach. Therefore, other induction methods that disrupted the formation of mycolic acid content could also prove useful.

Radmacher *et al.* (2005) described the use of ethambutol as an alternative method for the induction of glutamate production in *C. glutamicum*. Ethambutol was first described in the 1960's as a treatment against *Mycobacterium tuberculosis* however, at the time, its intracellular mechanism of action was not fully understood (Forbes *et al.*, 1962). It is now believed that in the *Mycobacterium* strain, the biostatic drug inhibits arabinosyl transferase, an enzyme that polymerizes arabinose into arabinan which eventually forms arabinoglycan, an essential constituent of the cell wall (Belanger *et al.*, 1996).

3.7.1 **L-glutamate induction by ethambutol in shake flask culture**

To determine the production of L-glutamate by *C. glutamicum* DSMZ 20300 was grown in CGXIII medium with the addition of 30 mgl^{-1} Ethambutol (EMB). EMB was used as a trigger for the secretion L-glutamate because of evidence suggested by Radmacher *et al.* (2005) that foaming of the organism during growth was reduced. It was also suggested that 30 mgl^{-1} was an optimal concentration, as no benefit was achieved by raising the concentration further.

In the shake flask experiment, it was evident that growth was affected slightly initially with a slower rate of growth observed (Figure 3-8). However, after 24h there is no significant difference between the presence of EMB and the control.

In the control culture no significant production of L-glutamate was observed over the 48 hour cultivation. The culture containing 30 mgl^{-1} EMB on the other hand produced approximately 1.7 gl^{-1} (11.6 mMol/L) L-glutamate in the first 24hr. However, after this period, further production does not occur.

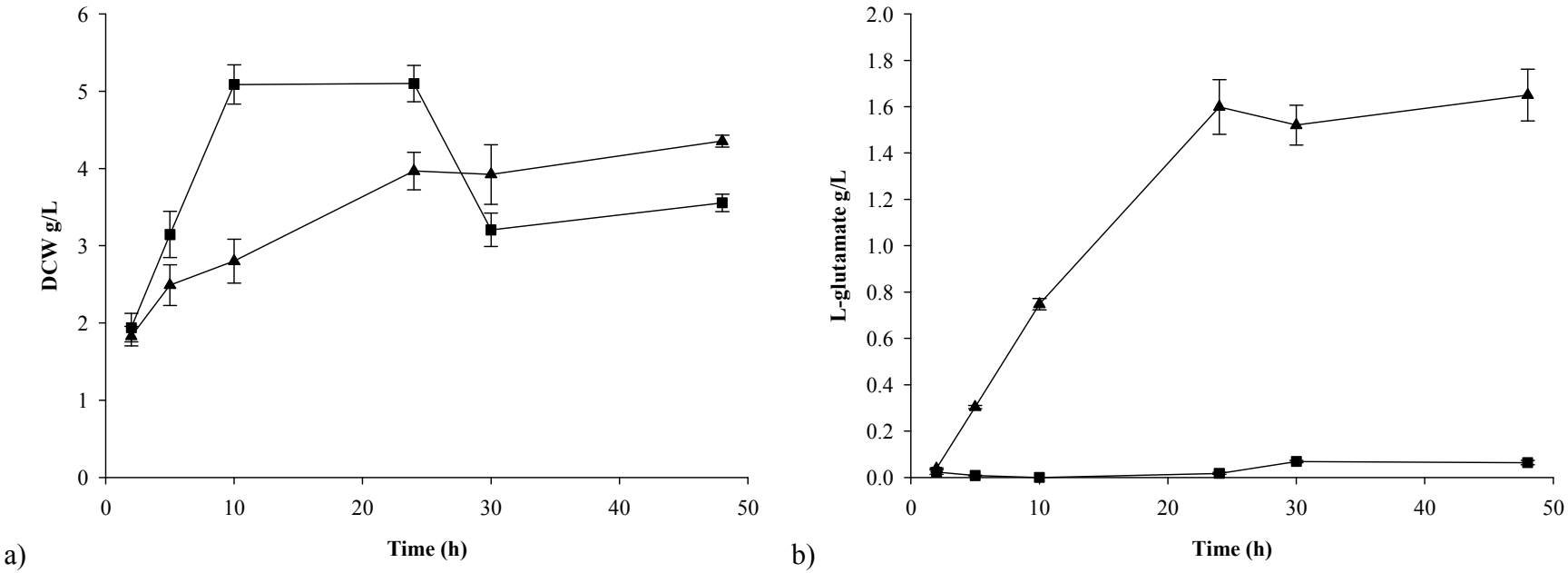


Figure 3-8Analysis of EMB effects on *C. glutamicum* L-glutamate production.

C. glutamicum DSMZ 20300 was pre cultured in yeast extract medium before being used to inoculate shake flasks containing batch medium no EMB (■) or in the presence of 30 mgl^{-1} EMB (▲) using dH2O as a control. Cultures were incubated at 30 °C at 200 rpm on a rotary shaker for 48 hr. Samples were taken regularly aseptically and analysed for a) DCW and b) glutamic acid production. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.7.2 Ethambutol induction of L-glutamate by *C. glutamicum* ATCC 13032 in batch bioreactor process

Initial fermentations were carried out in a 2 L bioreactor. All were unsuccessful in producing appropriate growth. Firstly, foaming issues led to failure after 20 h. Secondly, by not controlling the pH at 7 and letting the pH raise naturally to 8, growth of the organism did not occur even when the process was left to continue for 64 h.

By scaling up the process to a working volume of 10 L in a bioreactor with a maximum volume of 15 L, the foaming issue has been able to be contained and the pH controlled both by acid and base.

Growth was initially slow over the first 24 h with no significant increase observed (Figure 3-9). After 24 h, growth is rapid reaching a maximum of 0.7 $gL^{-1}h^{-1}$. The rate then decreased after 30h to 0.26 $gL^{-1}h^{-1}$ before reaching a plateau around 48 h. The rate at which glucose is consumed increases during the same time frame suggesting that there is a delay in the utilisation of the carbon source and therefore the rate of growth is affected. All the available glucose was consumed after 48 h which could explain the halt in growth.

Unlike in the shake flask experiments L-glutamate was not produced until 29 h reaching a plateau of approximately 1 gl^{-1} at 48 h. This corresponds to both the consumption of glucose and the production of biomass. Due to the absence of glucose at this point in the bioprocess, no further production of the amino acid was observed due to lack of carbon source.

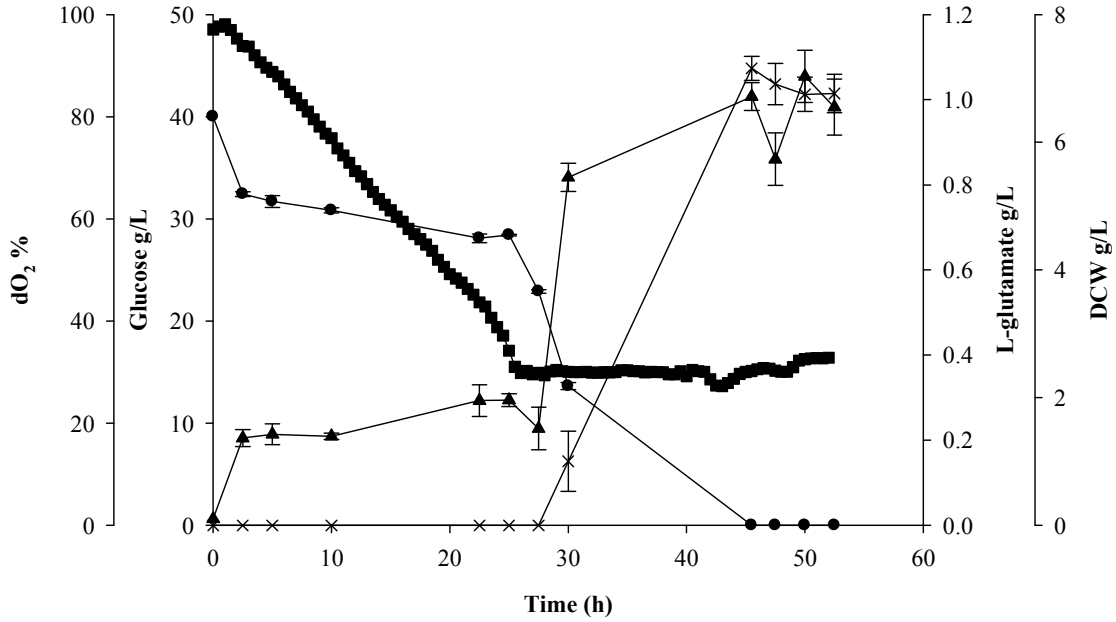


Figure 3-9 Ethambutol induction of *C. glutamicum* ATCC 13032 for the production of L-glutamate in batch culture

C. glutamicum ATCC 13032 was initially cultured overnight in pre-cultivation medium. Once the OD_{600} achieved a state of early log phase (e.g. an optical density of 5), cells were harvested and used to inoculate a bioreactor containing 10 L of batch medium. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.7.3 **Ethambutol induction of L-glutamate by *C. glutamicum* ATCC 13032 *ΔldhA* in batch bioreactor process**

As with *C. glutamicum* ATCC 13032, initial fermentations were carried out in a 2 L Applikon bioreactor which resulted in similar issues. When the process was transferred into the alternative system, growth improved exceeding even that of the wild type strain to an OD of 28 when measured at 600 nm. However, growth was unable to be maintained after 48 h (Figure 3-10).

Unlike the wild type, L-glutamate production was reduced, only reaching a maximum of $0.35gl^{-1}$ after 48h. This was unexpected as it was believed that an increase in biomass would ultimately lead to an increase in productivity. At present there is no exact explanation as to why this occurred. One possibility would be that the concentration of the EMB was not at the correct level. It has been observed that different strains of *C. glutamicum* exhibit unique responses to various triggers of L-glutamate excretion (Schultz *et al.* 2007).

However, when taking into consideration the growth curve as well as production, a similar profile is observed. During the period of exponential growth, approximately 25-35 h, the production of L-glutamate increases. When growth reaches a stationary phase after 40 h, productivity appears to halt. By this time all available glucose was consumed suggesting the organism may be carbon limiting. Therefore, improving growth may help to improve the production of L-glutamate.

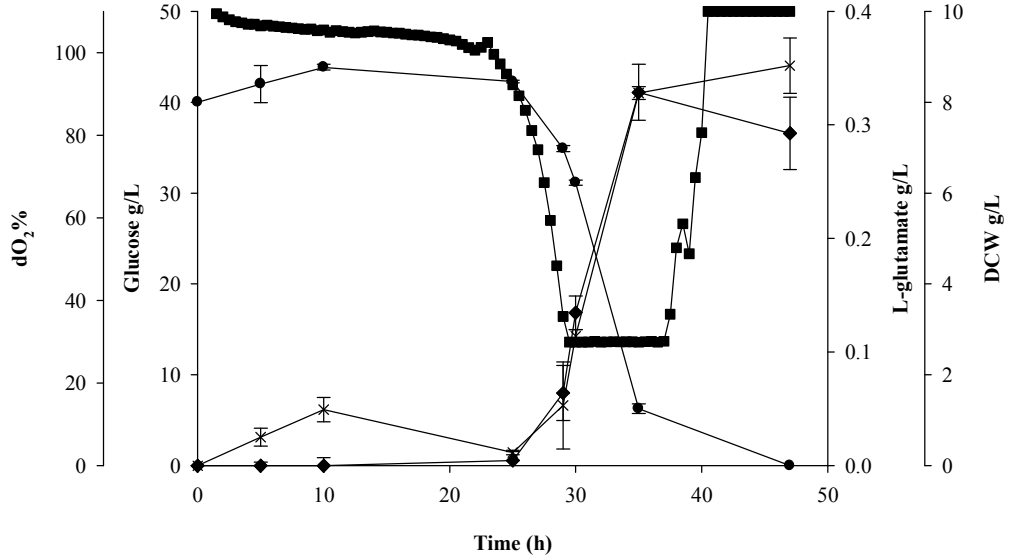


Figure 3-10 *C. glutamicum* 13200 *ΔldhA* 10 L batch fermentation
C. glutamicum ATCC 13200 *ΔldhA* was initially cultured overnight in pre-cultivation medium. Once the OD_{600} achieved a state of early log phase (e.g. an optical density of 5), cells were harvested and used to inoculate a bioreactor containing 10 L of batch medium. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.7.4 **Process Problems : Foaming**

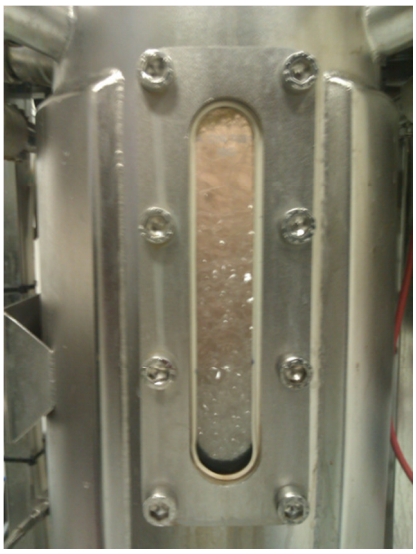
An unexpected issue that arose through the development of this process was the production of an undesirable quantity of foam (Figure 3-11). Whilst moving the process to a larger system, the issue was able to be contained, but the foam would ultimately cause problems if the process was scaled-up to an industrial level. In particularly, the removal of the organism into the foam layer would therefore decrease productivity in the medium, lower oxygen transfer rates and create a possible detrimental effect toward *in situ* instruments. The foaming occurred similarly with both strains and appeared to grow and recede during the fermentation.

After the failure of the initial fermentation, the action of poly-propylene glycol (PPG) was compared to that of a metabolisable antifoam, rape seed oil. The experiments were carried out in shake flasks and therefore, in comparison with cultivation in a bioreactor did not produce the same degree of foam. This is most likely due to aeration of the bioreactor exacerbating the foam. However, it was still noticeable that PPG was more efficient at reducing the level of foam produced. To rule out medium components playing a role, medium only was examined under the process conditions. No foam creation was observed under the normal process condition and this was unchanged when agitation and aeration was increased to beyond these set point.

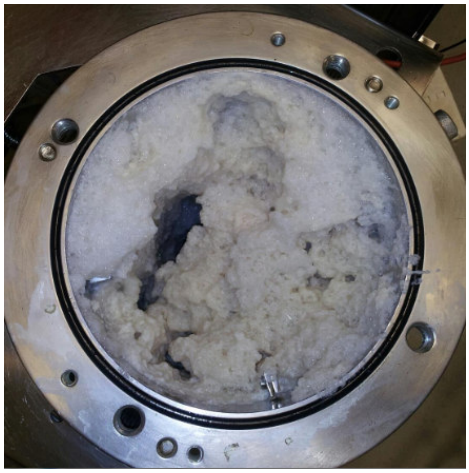
Since the medium alone was not the issue, it was thought that the strains were producing a molecule, possibly a protein exhibiting surfactant like attributes, which was responsible for the stabilisation of the foam (Etoc *et al.*, 2006). Therefore, it was decided that samples of the fermentation broth from different time points, would be analysed by LC-MS, with protein levels examined by a Bradford assay (Section 2.7.2.9). By identifying the molecule, it was hoped that a solution to this phenomenon could be established. However, this endeavour provided no evidence of surfactant related substances and the Bradford assay confirmed, no protein was present in the fermentation samples.

According to Kelle *et al.* (2005) during industrial scale fed batch production of L-lysine by *Corynebacterium*, foaming production is a significant consideration. Cell density of the fermentation broth was considered critical to the creation of foam. However, in this study foam was created at very low cell density suggesting that other factors were more significant. As medium components were ruled out of initiating foam production, the strain itself could be the key issue in this circumstance. In the following section, strain selection was fundamental in developing an operational method to produce L-glutamate to a significant concentration than was previously observed.

a)



b)



c)

Figure 3-11 Foam overproduction by *C. glutamicum* ATCC 13032 in the 10 L bioreactor
a) View from wall window of the 22 L bioreactor during fermentation of *C. glutamicum* ΔIdha. b) Culture medium and inside of reactor after sterilization and c) foam around impeller and sparger of 22 L bioreactor.

3.8 **Biotin limitation for the induction of L-glutamate in *C. glutamicum***

Biotin limitation was the first method that was observed to induce the overproduction of L-glutamate in the wild type strain of *C. glutamicum* during its isolation and characterization (Kinoshita *et al.*, 1957, Shiio *et al.*, 1962b). Shiio *et al.* (1962b) described that when *C. glutamicum* was cultured in medium containing biotin at a concentration of 3 μgl^{-1} or below, L-glutamate excretion would be triggered, but cellular growth would be impeded. By increasing the concentration of biotin to 15 μgl^{-1}, normal growth was observed however, the glutamate production was severely impacted with approximately 8.5 times reduction in end yield titre. For no glutamate to be excreted, the biotin concentration would be required to be in excess of 30 μgl^{-1}. In the presence of no biotin, no growth was observed. Therefore, an important fact was established: biotin concentration in the production medium was required with a concentration low enough to trigger excretion of the desired amino acid but which also allowed for sufficient growth of the organism.

Since the 1960's, this technique has been used throughout the literature in order to investigate the metabolic and structural modifications that occur during L-glutamate overproduction. However, to this date there is still debate over its mechanism of action.

Historically, it was believed that biotin limitation caused the disruption of the membrane integrity leading to a leaky cell wall. This hypothesis was reinforced by Otsuka and Shiio (1968) when they identified a distinct decrease in fatty acid content of the cell wall membrane. More recently, it was noted by Hashimoto *et al.* (2006) that under biotin limitation condition, mycolic acids content decreases. This event was believed to be a direct consequence of the reduction of fatty acid biosynthesis due by the limited availability of biotin.

Biotin appears in *C. glutamicum* as a covalently linked cofactor in the AccBC α-subunit of acetyl-coA carboxylase which is required for fatty acid synthesis (Jäger *et al.*, 1996). Investigation of the gene encoding for the β-subunit of the carboxylase DtsR1 has allowed for a more in depth understanding of the mechanism action

occurring at a molecular level. Disruption of the *dtsR1* gene has been shown to lead to the overproduction of glutamate whereas its amplification reduced production (Kimura *et al.*, 1997) signifying that the biotin-enzyme linked complex including DtsR1 has been thought as the primary target during biotin limitation. It has also been suggested that a metabolic linkage between acetyl-Coa carboxylase and the 2-oxoglutarte dehydrogenase complex (ODHC) exists. When the *dtsR1* gene is disrupted a 30 % reduction in ODHC's activity was observed (Kimura, 2002).

The activity of ODHC itself appears to be essential during the overproduction of L-glutamate. It is responsible for the oxidative decarboxylation of α-ketoglutarate to succinyl coenzyme A and therefore its activity determines the flux of carbon at this branch point. Metabolic flux analysis under biotin limitation identified that the attenuation of the *odhA* gene encoding for OdhA the E1o subunit of the complex, was significant during L-glutamate overproduction (Shimizu *et al.*, 2003, Asakura *et al.*, 2007). It was observed that an *odhA* deletion mutant grown under no induction methods, showed glutamate accumulation was comparable to that of a wild type strain under induction. A regulator for the complex has also been identified as OdhI which in its unphosphorylated form, binds to OdhA protein and inhibits ODHC activity (Niebisch *et al.*, 2006). It has been observed that deletion of *odhI* results in the almost complete abolition of glutamate production under various induction methods (Schultz *et al.*, 2007).

However, the almost simplistic theory of a leaky cell wall model is not sufficient in explaining the entire secretion process. It was observed by Gutmann *et al.* (1992) that under biotin limiting conditions other ions such as H^+, K^+, and Cl^- are not secreted from the cell, therefore suggesting that other elements were involved in the specific excretion of L-glutamate. Recently the discovery of the potential glutamate transporter YggB, encoded by the NCg11221 gene was identified by Nakamura *et al.* (2007) which conveys a more complex mechanism than original envisaged.

In this study we employed the biotin limitation technique described by Eggeling and Reyes (2005) in the Handbook of *Corynebacterium glutamicum* and utilised the medium CGXII. This medium has been used extensively throughout the literature to investigate *C. glutamicum* (Keilhauer *et al.*, 1993, Schultz *et al.*, 2007, Frunzke *et*

al., 2008, Witthof *et al.*, 2013) . It is important to note that unlike other techniques, this method requires a pre-cultivation step in order to deplete the cells of biotin and therefore achieve the desired low concentrations that is necessary to elicit excretion of the amino acid.

3.8.1 Biotin limitation induction of L-glutamic acid in shake flask culture

To assess the extent of L-glutamate excretion under biotin limitation (Section 2.2.2.4), a primary investigation using ATCC 13032 was performed. Biotin concentrations of 0, 1 and 200 $\mu g l^{-1}$ in the production medium were examined and the impact on both DCW and L-glutamate excretion was monitored. In the presence of excess biotin, a DCW of 10 $g l^{-1}$ was achieved after 35 h (Figure 3.10a). This was significantly reduced when biotin was present at only 1 $\mu g l^{-1}$, dropping to only 3 $g l^{-1}$ and to 1.2 $g l^{-1}$ at 0 $\mu g l^{-1}$. The impact on growth is also indicated in the maximum growth rates exhibited by the cultures 0.1, 0.34 and 1.15 h^{-1} by 0, 1 and 200 $\mu g l^{-1}$ respectively. Reduction in overall growth under limited biotin is consistent with original findings by Shiio *et al*. and is unsurprising as it is an essential growth factor for this auxotrophic organism. Unlike the original findings, when no biotin was present, cellular growth was still evident albeit very low. This may have been due to carry over biotin from the previous medium.

As expected in the presence of low concentrations of biotin, glutamate over excretion is observed. A maximum yield of 14.4 $g l^{-1}$ was observed after 48 h of cultivation in the presence of 1 $\mu g l^{-1}$ (Figure 3.10b). This accumulation is similar to that achieved by the wild type strain when it has been examined previously (Shiio *et al.*, 1962b, Hashimoto *et al.*, 2006, Schultz *et al.*, 2007). Unsurprisingly, when biotin is present in excess, little to no glutamate is produced and only slight production is observed when no biotin was included in the medium.

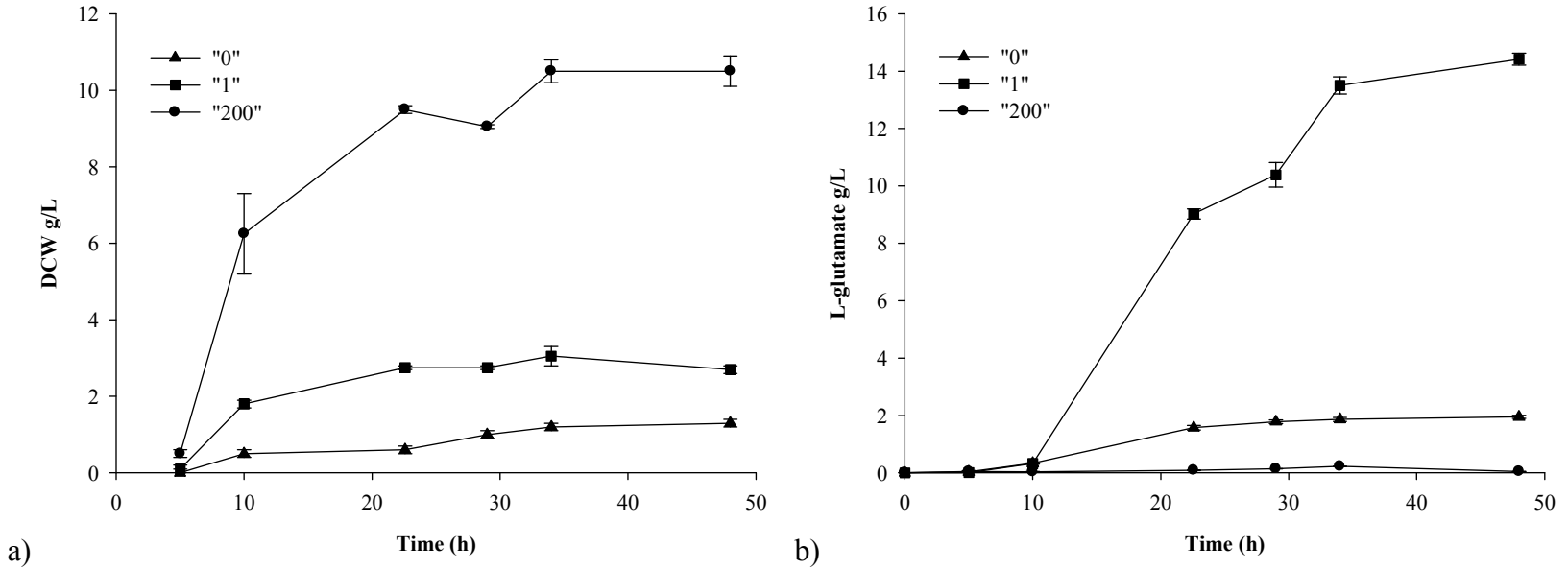


Figure 3-12 Effect of biotin limitation on cellular growth and L-glutamate production

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII biotin limitation media containing 0 (▲), 1 (■) or 200 (●) $\mu g l^{-1}$ of biotin to an OD600nm = 1. Cultures were incubated as previously described. Samples were taken regularly aseptically and analysed for a) DCW and b) glutamic acid production. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

To examine strain stability, *C. glutamicum* ATCC 13032 was cultured on LB agar plates and 3 single colonies, numbered 1-3, were harvested and grown in CGG medium for 16 h. Cultures were then introduced into a biotin depletion medium and finally were cultured in the production medium containing 1 $\mu g l^{-1}$ biotin. Triplicate flask cultures containing the process medium were prepared for each colony to examine reproducibility (Figure 3.11).

Initial growth rates for the colonies over the initial 5 h were observed to be similar with no significant difference between dry cell weight accumulations after 30 h. However, at the end time point of 48 h, dry cell weight accumulation increased to 6.4 and 6.3 $g l^{-1}$ for colonies 1 and 3 but remained constant for colony 2 at approximately 2.5 $g l^{-1}$ (Figure 3.12b). The sudden increase in biomass is unusual and the reason behind this remains unclear. Contamination of the culture was ruled out as no suspect organisms were identified either under microscopy or plate cultivation of the medium.

Crucially, production of L-glutamic acid across the cultures suffered from significant variability. With the exclusion of colony 2, accumulation of L-glutamic acid was observed within the triplicates with large errors obtained. If only the mean values are considered, sub-culturing of the strain appears to lead to unreliable consistency in product accumulation. By 21 h, colony 2 produced around 8 $g l^{-1}$ of L-glutamate, however under the identical conditions strain 1 and 3 had accumulated only 4.5 and 2.2 $g l^{-1}$ respectively (Figure 3.12b). This increase in productivity by colony 2 was still observed at 48 h, with the colony accumulating 25 % more residual L-glutamate compared to the other colonies.

Such variable results obtained from the above procedure were unexpected however, their occurrence may be due to the inconsistency of the colonies themselves. During selection of the colonies, every possible measure was taken to ensure similar sized colonies were chosen. However, the age of the cells within each colony could have been variable and therefore may have inadvertently exhibited different characteristics dependent on viability of the cells. Therefore, to improve reproducibility, it was decided for future procedures, cultures would be directly obtained from the cell bank

that was acquired from Ingenza Ltd. in order to prevent loss of productivity due to subculture.

The yield of L-glutamate obtained by both biotin limitation experiments far exceeded anything observed in the previous techniques attempted in this chapter. Further investigation would be required to determine biotin limitation as a suitable method to take forward. When the method was run in bioreactor with 4 L working volume, foaming challenges similar to those exhibited with ethambutol (Section 3.7.4) were noted. Therefore, based on the hypothesis that the strain could be the ultimate issue, a second wild type strain was examined.

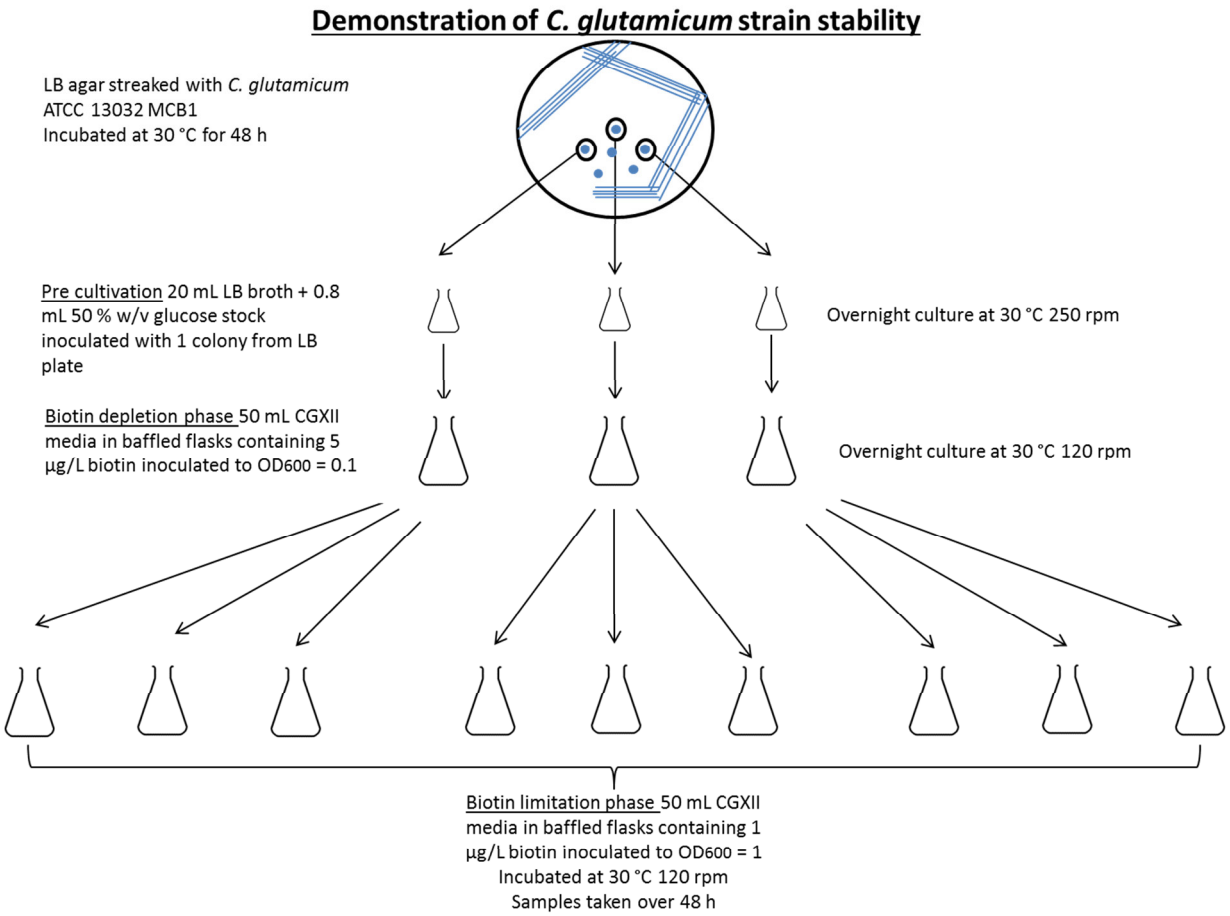


Figure 3-13 *C. glutamicum* strain stability protocol

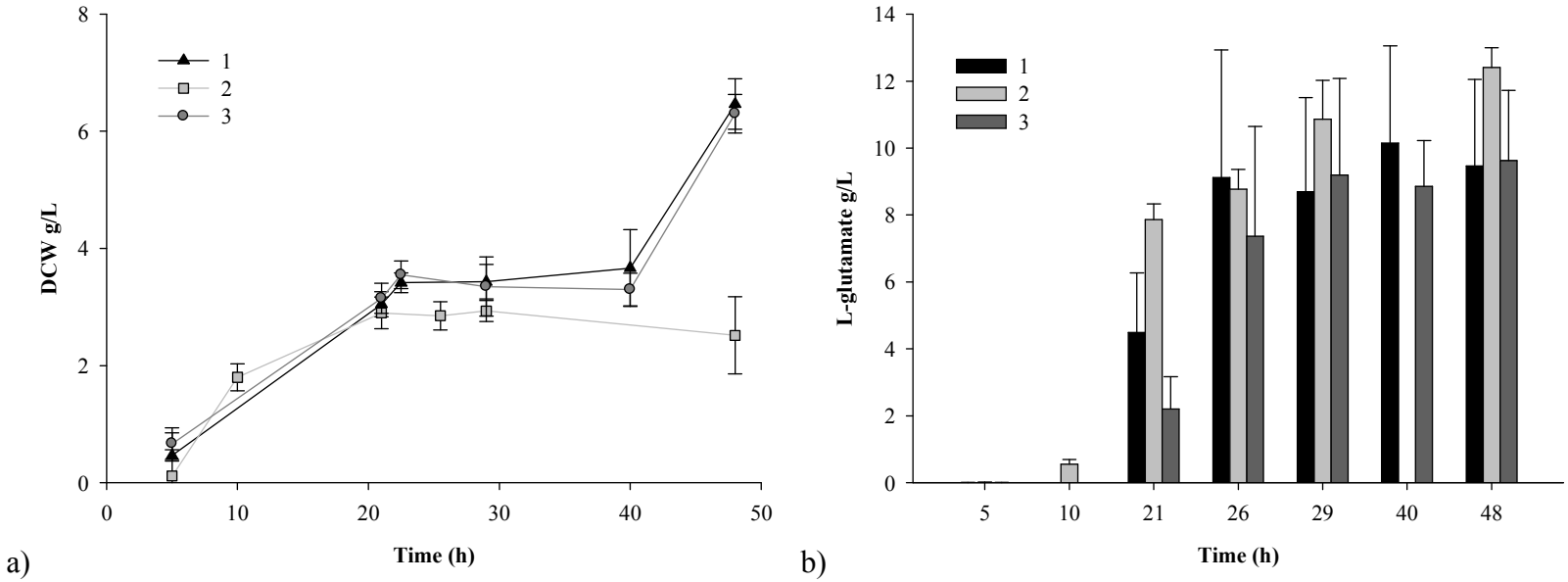


Figure 3-14 Single colony comparison of growth and L-glutamate production

C. glutamicum ATCC 13032 was grown on LB agar plates inoculated at 30 °C. Three separate colonies were selected and used to inoculate a propagation flask. After 16 h of growth the cultures were used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The cultures were then used to inoculate 50 mL of CGXII biotin limitation media containing 1 $\mu g l^{-1}$ of biotin to an OD600nm = 1. Cultures were incubated as previously described. Samples were taken regularly aseptically from cultures 1 (▲), 2 (■) and 3 (●) and analysed for a) DCW and b) glutamic acid production. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

C. glutamicum ATCC 13869, which was previously examined under Tween induction (Section 3.6), was firstly examined in shake flask cultures to determine if a similar accumulation of L-glutamate could be achieved to that observed with ATCC 13032 in the presence of 1 $\mu g l^{-1}$ biotin. Figure 3-15 illustrates that the strains were comparable in both DCW as well as L-glutamate production, with a maximum yield of 14.4 $g l^{-1}$ after 48 h.

In this case, the concentration of glucose and several important TCA cycle organic acids were analysed. Over the 48 h cultivation period, 65 % of the available glucose was consumed with the rate of uptake decreasing from maximum of 1.07 $g L^{-1} h^{-1}$ between 10 and 25 h to 0.12 $g L^{-1} h^{-1}$ by 48 h. The reduction of glucose utilisation occurred at a similar time to the reduction in growth and L-glutamate production, suggesting other limiting factors were influencing cellular activities. Gradual build-up of pyruvic acid (0.86 $g l^{-1}$), α-ketoglutarate (0.87 $g l^{-1}$) and lactic acid (0.42 $g l^{-1}$) was also observed between 30 and 44 h, again suggesting that other limiting factors may be involved. Pyruvic acid and α-ketoglutarate are critical compounds that are precursors to glutamic acid within the TCA cycle (Figure 3-2). This suggests that possible bottlenecks were starting to occur as the bioprocess continued.

Lactic acid is a significant by product in bioprocesses, generally occurring during oxygen limiting conditions which are possible in the shake flask environment. This could be limited in the future by deletion of the *ldh*A gene, responsible for lactate dehydrogenase, which has been performed throughout the literature to reduce by-product production.

Oxygen transfer within the shake flask environment is not optimal and has been shown to have significant impact on growth and L-glutamate accumulation as well as the production of the aforementioned by-products. Comparing two strains of *C. glutamicum*, wild type ATCC 13032 and a lysine production strain DSM 12866, Zimmermann *et al.* (2006) identified when the capacity of oxygen transfer was reduced, cellular respiration was impaired and formation of anaerobic by-products was observed. Oxygen limitation was undoubtedly a significant factor in reducing the overall success of this experiment. Therefore, to obtain a more favourable

environment where dissolved oxygen could be maintained, the procedure was scaled to be operated at a 4 L volume within a bioreactor.

Acidification of the fermentation broth could also cause a decrease in cellular activity. Even with the medium containing the buffering agent MOPS, the pH of the culture medium still fell below 6 after 24 h. Koch-Koerfges *et al.* (2013) reported that the addition of MOPS did reduce the overall effect of pH altering compounds, however, in case of some strains this effect was limited. For *C. glutamicum* wild type strains to maintain an intracellular pH of approximately 7.5, external pHs between 6 and 9 have been shown to be effective (Follmann *et al.*, 2009). However, if the pH shifts above or below these points, cellular respiration is severely inhibited, limiting on both glucose utilisation and productivity.

Scale up of the procedure would not only benefit oxygen transfer but would also allow for the maintenance of the external pH, creating optimum culture conditions.

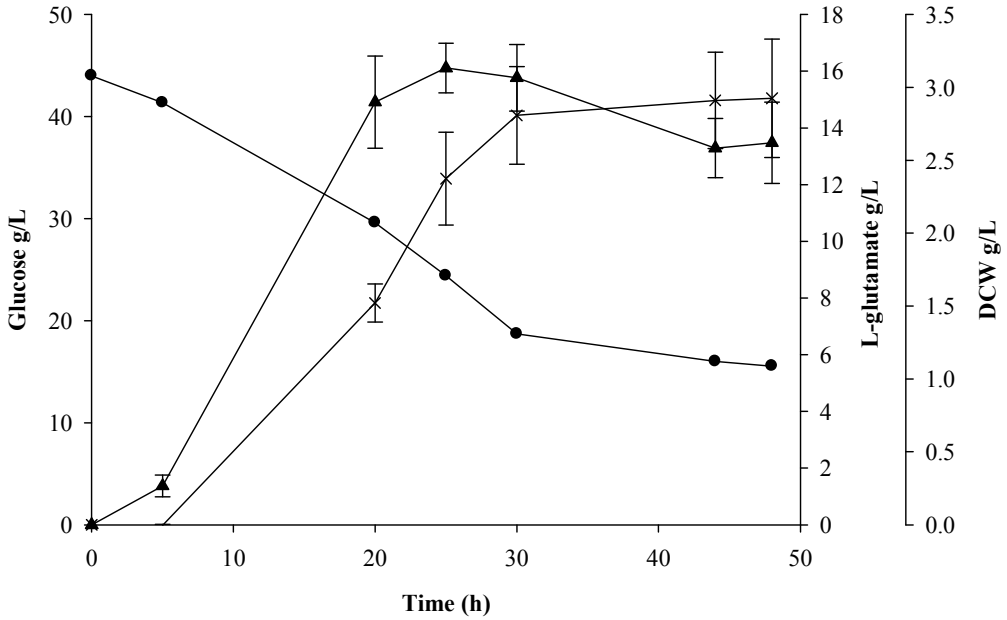


Figure 3-15 Analysis of *C. glutamicum* ATCC 13869 in the presence of 1 μgl^{-1} biotin under shake flask conditions

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. Cultures were incubated as previously described. Samples were taken regularly aseptically and analysed for glucose utilisation (●), DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.8.2 **Analysis of L-glutamic acid production using batch process**

Batch fermentations using *C. glutamicum* ATCC 13869 were performed at a volume of 4L within a Bioflo 110 bioreactor (Section 2.2.4) with a maximum working volume of 10 L. Two Rushton impellers were employed for agitation of the culture. Dissolved oxygen was maintained at or above 30 % using an agitation cascade. pH was also controlled to a set point of 7 ± 0.05 using 2 M H_2SO_4 and 25 % v/v NH_4OH and temperature was maintained at 30 °C with the use of a heating jacket.

To examine the feasibility of a direct scale up of the experiment, the same initial procedure of pre-cultivation and biotin depletion was employed. The latter step was scaled to result in a 10 % v/v inoculum for the bioprocess. This biotin depletion inoculum stage was cultured until an optical density of 10 at 600 nm was achieved and could be used directly to inoculate the batch medium. Cells were once again washed and resuspended in a portion of batch medium. This was to exclude carryover of excess biotin and to exclude other nutrients not consumed during the previous stages, which could alter the starting concentration of nutrient components such as glucose. Processes were deemed to be complete when all available glucose was consumed.

In the case of *C. glutamicum* ATCC 13869 (Figure 3-16), a maximum dry cell weight of 3.6 gl^{-1} was achieved after 24 h of the process. Accumulation was marginally higher than that obtained in the shake flask experiment however; limitation of biotin would curtail any substantial increases. What is more notable is the increase in L-glutamate production and the complete utilisation of glucose after 48 h. A maximum yield of 16.5 gl^{-1} of the amino acid was observed by the time the glucose has been depleted. The rate of its accumulation does decease over time, although this could be due to limited availability of the carbon source towards the end of the bioprocess.

Analysis of other key organic acids suggests that very little by products were produced over the process with the exception of pyruvate towards the end of cultivation. Similar to the analysis in shake flask culture, the presence of pyruvate

($0.52\ gl^{-1}$) suggests that a conversion of the compound may be a limiting factor in the pathway. However, the accumulated concentration was reduced by 40 % when the process was transferred to the bioreactor suggesting improved process control increased productivity. Importantly, anaerobic by-products such as lactate were not obtained, therefore control of the dissolved oxygen above 30 % was sufficient to maintain efficient aerobic conditions.

Maintenance of aerobic conditions and pH at optimal conditions for the organism resulted in an efficient process to overproduce L-glutamate. This basic protocol was then intended for implementation in future processes incorporating engineered strains of ATCC 13689 containing genes coding for enzymes that would continue the pathway from L-glutamate to mesaconate. Conversion to mesaconate would be the first step into creating a competitive process to produce platform chemicals. This transition and implementation of engineered strains into the described process will be discussed in Chapter 4.

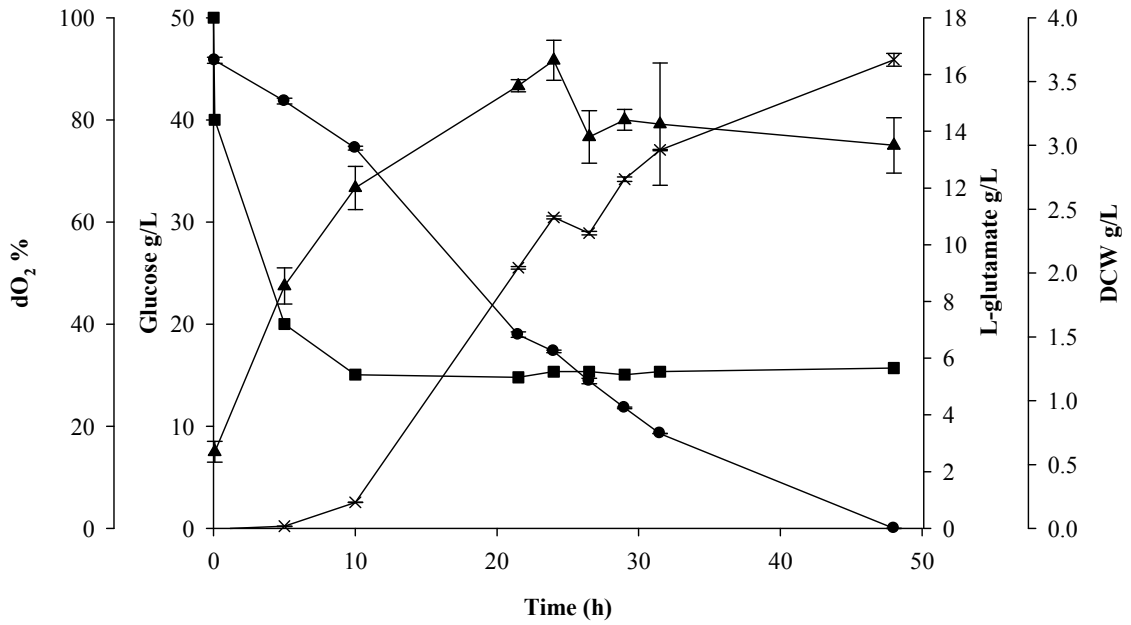


Figure 3-16 Analysis of *C. glutamicum* ATCC 13869 using 1µgl^{-1} Biotin in a 4 L Batch culture

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 µgl^{-1} of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.8.3 *C. glutamicum* Fed batch culture development under biotin limitation

Industrial production of amino acids primarily employs fed batch processes as opposed to batch processes. This method generally results in an improved overall productivity of the process by decreasing production time and end yield titre. To examine if increased productivity of ATCC 13869 could be achieved when glucose was not the limiting factor, a simple glucose feeding process was developed. This was an attempt to build the foundations to develop the continuous production of mesaconate.

During the batch culture, approximately half of the glucose present in the medium is consumed within the initial 20 h of culturing. Therefore, to establish whether a bolus addition of glucose at this point would lead to an increase in production, a simple shake flask experiment was performed. Addition of either 40 gl^{-1} or 20 gl^{-1} of glucose were aseptically transferred to cultures after 15 h of incubation as at this time the cells were still in the exponential phase of growth. Two concentrations of glucose were examined in order to understand if inhibitory effects of excessive sugar would have any negative effect on the organism.

Between the concentrations of bolus feeds, very little difference was observed when DCW, L-glutamate production and glucose consumption were compared (Figure 3-17). However, when examining the results against the batch flask experiment (Figure 3-15) with this strain overall consumption of glucose was reduced. Cultures that experienced no feeding, consumed 29 gl^{-1} glucose whereas only 22 gl^{-1} was consumed following the 40 gl^{-1} and 20 gl^{-1} glucose addition after 48 h. DCW weight was slightly increased compared to the non-fed culture, however, a small decrease in final glutamate titre was observed. These were only minor differences and could be due to experimental errors. More importantly, the addition of the glucose in the shake flask does not make a substantial difference to the productivity. This again relates to the fact the shake flask oxygen restrictive environment may be the crucial step in limiting the potential of the process (Zimmermann *et al.*, 2006).

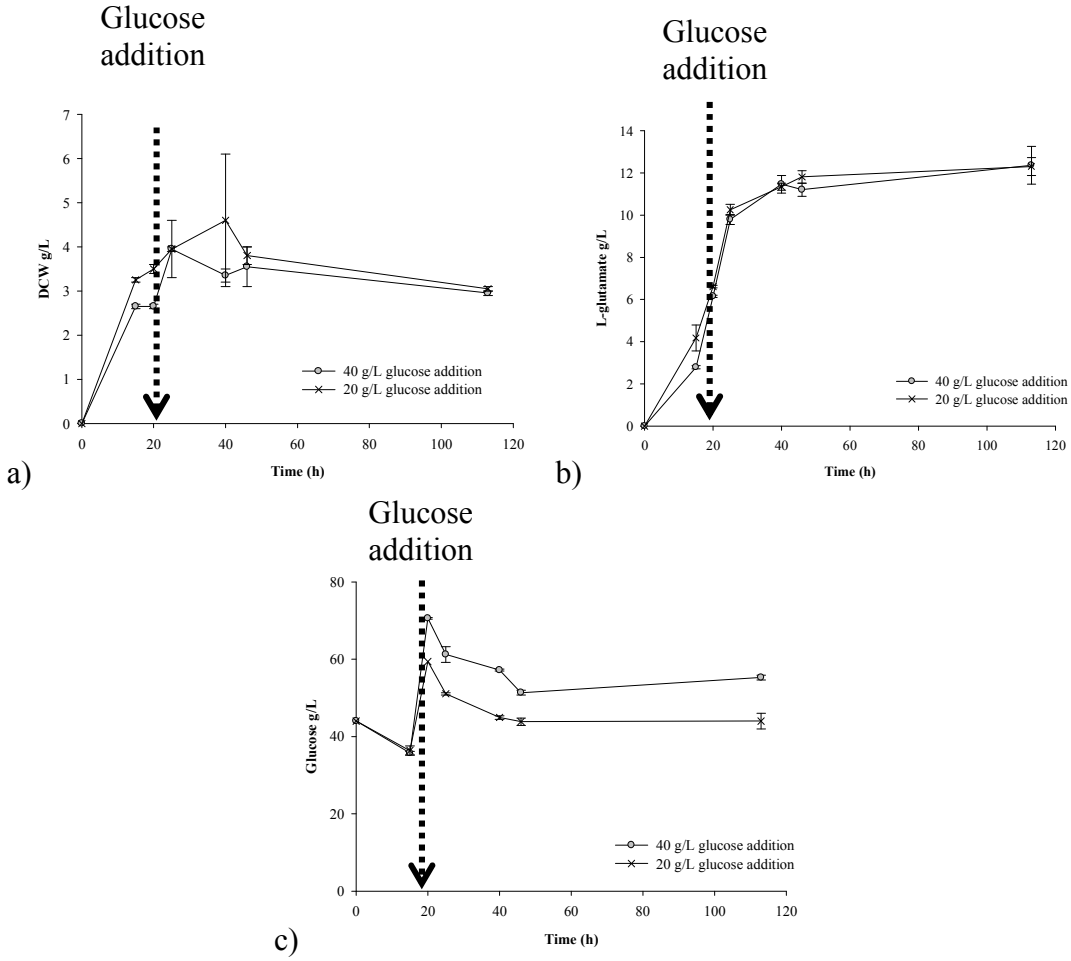


Figure 3-17 Bolase glucose addition during *C. glutamicum* ATCC 13869 shake flask culture

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 3 x 50 mL of CGXII biotin limitation media containing 1 $\mu g l^{-1}$ of biotin to an OD600nm = 1. Cultures were incubated as previously described. After 20 h (denoted with the dashed arrow) an addition of either 40 $g l^{-1}$ (●) or 20 $g l^{-1}$ (x) of glucose was supplemented to the media. Samples were taken regularly aseptically and analysed for DCW (a), glutamic acid production (b) and glucose utilisation (c). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In an attempt to optimise the process, a continuous feed process was designed that introduced a set concentration of glucose to a production culture within a bioreactor environment. In the batch bioreactor process, the glucose utilisation rate over the initial 20 h was 1.4 $gL^{-1}h^{-1}$. Therefore, after 20 h of growth a feed programme was implemented in order to introduce 1.4 $gL^{-1}h^{-1}$ of glucose to the culture for a further 20 h. This gave a final addition of 28 gl^{-1} of extra glucose over the feeding period and therefore a total of 72 gl^{-1} of glucose was available over the course of the process. The process was continued for a further 56 h where respiration was observed to decrease.

Consumption of all available glucose, including that contained in the feed was observed at 48 h (Figure 3-18). At this point glutamate accumulation peaked at 40 gl^{-1}, a 2.5 fold increase from the batch process. This was observed with only a 1.6 times increase in available glucose. A maximum accumulation rate of 1.792 $gL^{-1}h^{-1}$ was recorded which was 4 fold increase compared to the maximum rate observed in the batch culture. Increases in the efficiency of this process compared to the batch can also be observed when examining the yeild (product/DCW) of the cultures with the rate increasing from 5.5 g/g in the batch to 8.2 g/g in the fed batch process.

Examining the production of other organic acids (Table 3-2) reveals a slight build up α-ketoglutarate with its presence at 44 h of 2.28 gl^{-1}. This build up suggests a possible bottleneck in the system where the conversion reaction to L-glutamate may not be efficient, leading to an accumulation of its precursor (Figure 3-2 Table 3-1). Very low concentrations of pyruvate and succinate were also observed, however, their values appear insignificant compared to the secretion of the other organic acids. Accumulation of lactate was also observed, and peaks at a maximum of 1.07 gl^{-1} in the residual medium. Presence of this by product could suggest oxygen limitation was occurring at this point, leading to the formation of anaerobic analytes. However, as the dissolved oxygen was maintained at 30 % this seems unlikely. Interestingly, when the process was examined under oxygen limited conditions where the dissolved oxygen was allowed to fall to 0 %, the accumulation of lactate was much greater, with a maximum of 11.5 gl^{-1} observed at 68 h. Therefore, maintaining the dO_2 % at 30 % resulted in a 10.7 fold decrease in the accumulation of an unwanted anaerobic

by product. An alternative explanation for the formation of lactate, could be due to metabolic leakage. Similar detection of lactate, as well as trehalose and dihydroxyacetone, were detected by Gourdon and Lindley (1999), during temperature induced glutamate production, performed under a glucose feeding strategy. It was hypothesise that this metabolic leakage arose from three key branch points in the glycolysis pathway, which were at; glucose 6-phosphate, pyruvate, and triosephosphates. Tighter control over this pathway, as well as the deletion of critical genes in the pathway towards these by-products, could limit their formation and, in the process, increase carbon efficiency of the glutamate process.

With the depletion of the available glucose and the abundance of organic acids secreted into the medium, it could also be suggested that the organism is re-utilising the by-products it has made, as growth continues for 20 h post glucose depletion. This switch to consumption of product could prove significant when designing industrial processes, as loss of the product would be detrimental. Therefore, a continuous feeding process may be required in order for the organism to continue to grow on the desired substrate.

The efficiency of continuous feeding relies on two key fundamentals; the stability of the production strain and the desired metabolite, in this case glutamate. Unlike lysine and arginine, glutamate is produced by *C. glutamicum* in a growth-independent manner (Ikeda and Takeno, 2013). Therefore, maintaining cells in the growth phase would not be advantageous and could result in reduced productivity. To avoid this prospect, implementation of a cell-recycling technique into a feeding strategy could prove highly effective. This method has been successfully implemented into the production of organic acids such as, succinate and lactate. Using this method, Okino *et al.* (2005) effectively maintained the production rate of the *C. glutamicum* strain for up to 360 h, increasing the overall yield of the process. However, continual use of the cells in this manner could prove problematic when implemented using future engineered strains as their stability and even the purity of the culture could be compromised. Continual sub-culturing may also lead to the loss of functionality of inserted plasmids or even give rise to mutated variants which exhibit a reduction in productivity.

Due to the nature of the future engineered strains and their unknown performance under varying conditions at this stage, only the batch culture process was taken forward to examine the production of mesaconate which is outlined in Chapter 4.

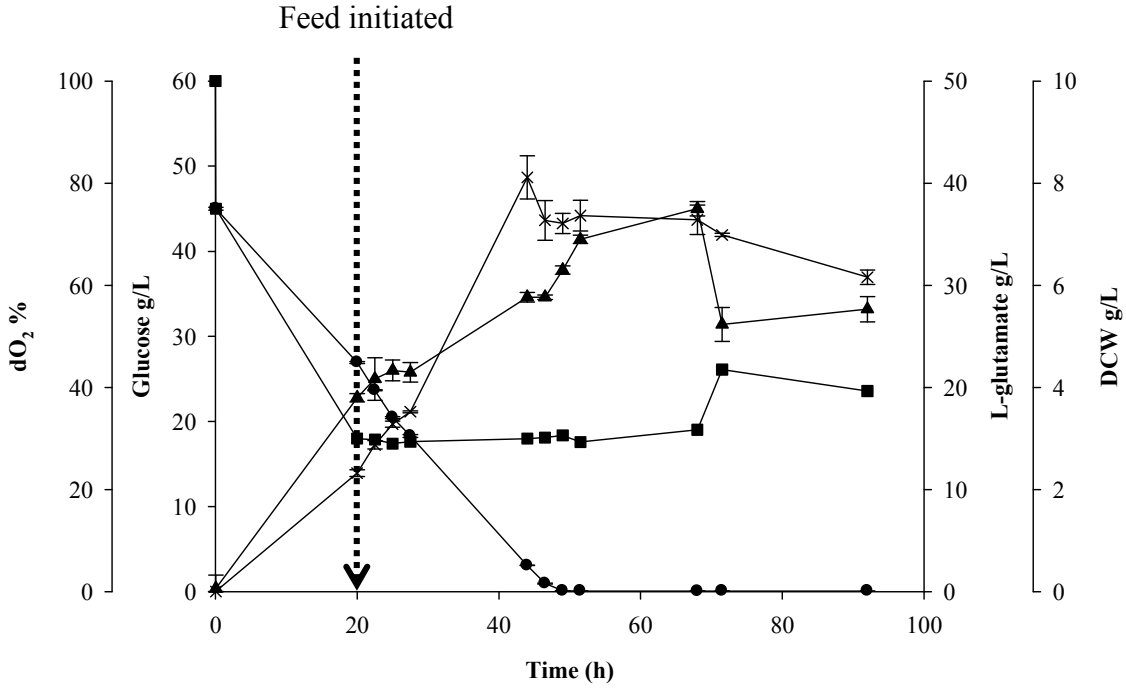


Figure 3-18 *C. glutamicum* ATCC 13869 fed batch fermentation using biotin limitation to produce L-glutamic acid
A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 $\mu g l^{-1}$ of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. After 20 h (denoted with the dashed arrow) a feed was initiated introducing 1.4 $gL^{-1}h^{-1}$ glucose to the process for a further 20 h. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Table 3-2 Residual organic acid analysis of *C. glutamicum* ATCC 13869 during fed batch culture under L-glutamic acid producing conditions.

Time (h)	α-Ketoglutarate gl^{-1}	Pyruvate gl^{-1}	Succinate gl^{-1}	Lactate gl^{-1}	Acetate gl^{-1}
0.08	0 ± 0.0	0.04 ± 0.001	0.02 ± 0.001	0.38 ± 0.02	0.01 ± 0.001
20	0.24 ± 0.05	0.02 ± 0.002	0.03 ± 0.001	0.41 ± 0.03	0 ± 0.0
22.5	0.34 ± 0.03	0.02 ± 0.001	0.02 ± 0.002	0.35 ± 0.05	0 ± 0.0
25	0.46 ± 0.02	0.02 ± 0.003	0.03 ± 0.003	0.44 ± 0.02	0 ± 0.0
27.5	0.55 ± 0.07	0.03 ± 0.001	0.03 ± 0.004	0.36 ± 0.02	0 ± 0.0
44	2.28 ± 0.1	0.37 ± 0.002	0.05 ± 0.007	1.07 ± 0.1	0.03 ± 0.002
46.5	2.56 ± 0.07	0.21 ± 0.003	0.07 ± 0.002	0.79 ± 0.05	0.09 ± 0.002
49	2.29 ± 0.01	0.02 ± 0.001	0.05 ± 0.003	0.39 ± 0.02	0 ± 0.0
51.5	2.28 ± 0.3	0.02 ± 0.001	0.05 ± 0.002	0.45 ± 0.07	0 ± 0.0
68	0 ± 0.0	0 ± 0.0	0.01 ± 0.001	0.45 ± 0.04	0.04 ± 0.001
71.5	0 ± 0.0	0 ± 0.0	0.02 ± 0.002	0.45 ± 0.02	0 ± 0.0
92	0 ± 0.0	0.04 ± 0.001	0.02 ± 0.001	0.45 ± 0.02	0 ± 0.0

3.9 **Chapter 3 Conclusions**

Comparing all of the techniques analysed within this work, it was evident that biotin limitation produced the most efficient method for glutamate production with a specific yield of 8.2 g/g and production rate of 1.792 $gL^{-1}h^{-1}$. This far exceeded any of the other induction methods examined. It was clear from the present study that heat induction was the least efficient method, however, as this was only completed in shake flask culture and conditions may not have been optimal to maintain oxygen transfer. The medium used, as well as the strains used, also may not have been optimal. Tween 40® induction did show promise and an increase in glucose concentration may have resulted in an improved productivity. However, control of the foam created on its addition ultimately rendered this method unsatisfactory at small scale. The addition of ethambutol as an induction method also exhibited potential, however, its ability to reduce the foam created by strain ATCC 13032 and its subsequent mutated strains were not observed in this study as claimed in Radmacher *et al.* (2005).

Therefore, the only method worth taking forward to analyse the future engineered strains was biotin limitation. It was also established that strain ATCC 13869 was the most efficient strain that was examined. Not only was a high productivity achieved during utilising biotin limitation, foaming was kept to a minimum, therefore the addition of additional PPG was not required, thus growth was unaffected.

3.10 **Future work for Chapter 3**

- Once engineering work developed a strain capable of producing a reasonable concentration of mesaconate, revisiting the feeding strategy could prove useful in creating a cost efficient process that would rival that of current production process
- Develop the process further to be more cost efficient by examining potential cheaper substrates than refined glucose. Current developments in use of waste

products from other industries could prove useful in development for industry. However, changing the feed stock may impact on the quality of the product with unexpected by products that may require expensive downstream processing to achieve a pure product

Chapter

4

Mesaconate production development using engineered strains of *C. glutamicum* ATCC 13869

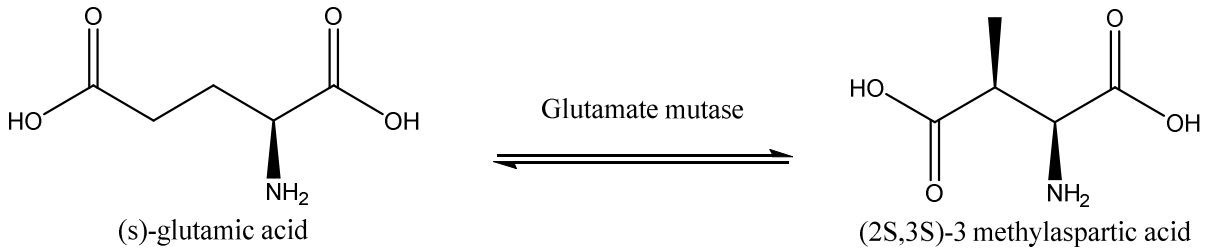
4.1 **Introduction**

Methyl methacrylate is a high value methyl ester with over 3.2 million tons of the compound produced worldwide every year (Choi *et al.*, 2015). The ester is mainly converted to produce polymethyl methacrylate, acrylic acids or co-polymer methyl methacrylate-butadiene-styrene (MBS) used as a modifier for PVC. The principle use for these polymers are in; waterborne coatings (e.g. latex paint), adhesive formulations, plates that keep light spread evenly across liquid crystal displays (LCD) and corrosion casts for anatomical organs such as coronary arteries (Burk *et al.*, 2012).

Commercially, methyl methacrylate is produced by the condensation of acetone and hydrogen cyanide to produce acetone cyanohybrin (ACH). ACH is then hydrolysed in the presence of sulphuric acid to produce sulphate esters of methylacrylamide. An esterification then takes place in the presence of methanol to produce the ester and the by-product ammonium bisulphate. Despite this process having high efficiency it is also accompanied with complications. Firstly, the handling of large quantities of the poisonous organic compound hydrogen cyanide is not ideal and requires stringent control protocols in place to prevent unwanted emission of the compound as a toxic gas (boiling temperature just above room temperature). Secondly, 1.2 tons of the by-product ammonium bisulphate is formed for every ton of MMA produced, disposal of which is highly expensive. For these reasons, as well as the changeable price in oil, development of alternative processes which are more environmentally friendly and economical have attracted a lot of attention in the past decade.

Recently, it has been demonstrated that the organic acid, mesaconate, can be utilised as a precursor in the production of methyl methacrylate. In nature, this compound appears as an intermediate in the degradation of glutamate in facultative and obligate anaerobes. The pathway consists of two enzymatic steps catalysed by glutamate mutase (Glm) and methylaspartate ammonium lyase (MAAL), isolated and characterised firstly in *Clostridium tetanomorphum* (Holloway and Marsh, 1994) and then later in *Clostridium cochlearium*.

Glutamate mutase or methyaspartate mutase is an adenosyl-cobalamin dependent mutase that catalyses the reversible conversion between (S)-glutamate and (2S,3S)-3 methylaspartate with a constant of ~12 in favour of (S)-glutamate (Figure 4-1). This reaction is stereospecific therefore it will result in 100 % of the stereoisomer or the reaction will not occur.



(s)-glutamic acid (2S,3S)-3 methylaspartic acid

Figure 4-1 Conversion of (s)-glutamic acid to (2S,3S)-3 methylasparic acid

Glutamate mutase is found in the *Clostridium* strains mentioned previously, and also within organisms that are capable of growing on glutamate under anaerobic conditions (Buckel and Barker, 1974). The stable protein consists of dimeric large (E) and monomeric small subunit (S) containing the cofactor binding site which includes a cobalt coordination histidine residue (Holloway and Marsh, 1994). In *C. tetanomorphum*, the genes responsible for the ε and σ subunits are mutE and mutS respectively, whereas in *C. cochlearium* they have been denoted as glmE and glmS. Combination of the two subunits allows for the incorporation of coenzyme B12 to form the biochemically active compound. Interestingly, the activity of glutamate mutase is dependent on the concentration of the E and S components with the reaction rate for coenzyme B12 being directly influenced by the alterations in the levels of the subunits (Holloway *et al.*, 1996).

Both strains also contain either an mutL or glmL gene that may code for a molecular chaperone molecule. However, when the genes have been expressed in *E. coli*, the presence of the L gene did not appear to be significant for gene expression and functionality of the enzyme (Zelder *et al.*, 1994). This concept was accepted until recent research by Wang and Zhang (2015), which exhibited contrasting evidence

surrounding the importance of the mutL/glmL gene. Further details of this study will be discussed in Section 4.2.

Inactivation of the glutamate mutase protein has been shown in the presence of oxygen or compounds with similar characteristics to both the substrate and the coenzyme. Substrate analogues include compounds such as α-ketoglutarate, itaconate, fluroglutamate and 2-methyleneglutarate, which confer differing levels of inhibition towards the mutase. In the case of α-ketoglutarate, the compound has been observed to bind to the enzyme resulting in the formation of free radical species, however further transformation is undetected and results in the inactivation of the enzyme (Roymoulik *et al.*, 1999). During the reaction the coenzyme is converted to its inactive state which remains tightly bound to the active site, blocking further activity which results in loss of functionality of the enzyme.

MAAL, is the subsequent enzyme in the mesaconate pathway catalysing the reversible amination-deamination reaction between mesaconate and (2S,3S)-3-methylaspartate (Figure 4-2). As with the glutamate mutase, the enzyme is found in both facultative and obligate anaerobes (Kato and Asano, 1997). It was first isolated in *C.tetanomorphum* with the MAAL genes designated as *bma* and followed on the same gene cluster as the glutamate mutase genes.

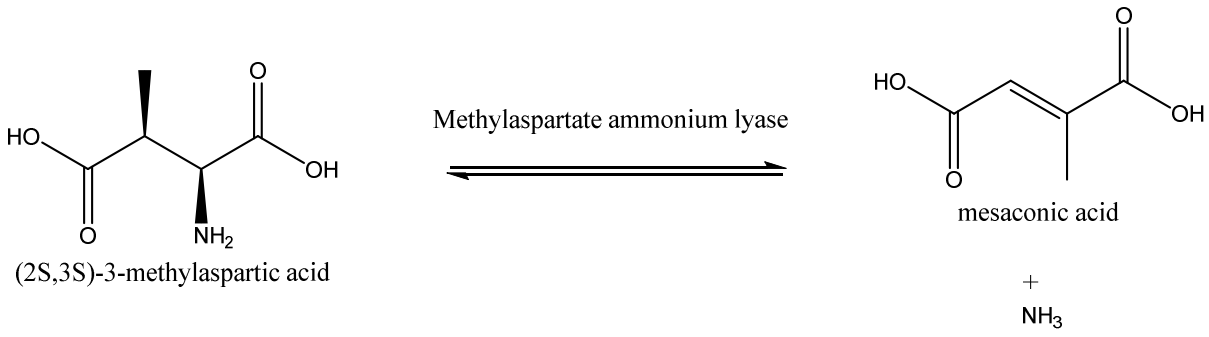


Figure 4-2 Reversible amination of (2S,3S)-3-methylaspartate to mesaconate by MAAL

In this study the forward reaction was obviously preferred to produce mesaconate, however, in recent years utilisation of the reverse reaction has been investigated for use in the synthesis of novel amino acids (Levy *et al.*, 2002, Asuncion *et al.*, 2001, Raj *et al.*, 2012).

4.2 **Novelty**

To the knowledge of the author only one study exists in the literature that discusses the utilisation of Glm and MAAL in order to produce *de novo* synthesis of mesaconate. Metabolic engineering of *E. coli* BW25113 by inserting MAAL, *mutS* and *mutE* genes, Wang and Zhang (2015) successfully produced 1.17 gl^{-1} of mesaconate during glutamate feeding. They also observed the instability and inactivation of the Glm-AdoCbl complex which occurred during the fermentation leading to a reduction in metabolic flux towards mesaconate. The reduction in flux and inactivation of the first enzyme complex in the pathway could explain why only a low concentration of mesaconate was produced. To address these problems, the study went on to investigate the influence of the MutL/GlmL genes had over the process as a whole.

Previously, the purposes of the MutL/GlmL genes were unknown and as they appeared not to affect the expression of the glutamate mutase gene in *E. coli,* they were thought to be unessential as the components were still active without their presence. Even with this insight, Zelder *et al.* (1994) observed the similarities between the amino acid sequence of glmL and that of ATP-ase domains of a wide variety of proteins such as the heat shock proteins HSP70 and DnaK as well as proteins involved with prokaryotic cell cycle FtsA and MreB. Three identical or very similar sequence motifs were present in the genes identified which related to binding sites for specific parts of the ATP molecule. Therefore it was assumed that the GlmL protein would also bind to ATP.

Due to the high similarity between GlmL and MutL, Wang and Zhang (2015) purified the MutL protein from *E. coli* and examined its activity *in vitro*. They

observed that the presence of the purified protein led to the reactivation of the inactive mutase enzyme in the presence of both coenzyme B12 and ATP which reinforces the theory, at least in part, that the L protein contains an ATP-ase sequence motif. When MutL was introduced to the strain, mesaconate accumulation increased to 2.65 gl^{-1}. This improvement was a significant finding as it proved that the presence of MutL could influence the reactivation of the Glm-complex and further its activity life span. However, it was still noted that the production of mesaconate was slow due to the higher binding affinity of the pseudoenzyme B12 compared to that of the coenzyme.

To address the issues of inactivity of the coenzyme the current study explored the possibility of exploiting *E. coli*'s natural ability to covert vitamin B12 into the active coenzyme. Genes involved in the regeneration of the Coenzyme B12 were examined and it was found that adenosylation was the key bottleneck. Enhancement of these genes increase mesaconate production to 5.9 gl^{-1} which was a 74.3 % rise compared to the original engineered strain.

Interestingly, on investigation of the stability of the MutE gene, it was found that by replacing this with the GlmE protein found in *C. cochlearium* the flux towards mesaconate would again be improved with an end yield titre of 7.81 gl^{-1}. This finding appears to reinforce the theory that E subunit from *C. cochlearium* is more oxygen stable than its similar counterpart in *C. tetanomorphum* even though their sequence and structure exhibit high levels of similarities.

The improvement of the yield due to the presence of more stable glutamate mutase proteins appear to be vital to increase the production of mesaconate. Therefore, in this study we examine the use of glutamate mutase genes identified in other organisms in an attempt to improve stability of the enzyme complex and success of the process. Process conditions were also examined to determine the optimum operating conditions that would produce the most efficient reaction.

Similar to the study by Wang and Zhang (2015), genetically engineering an organism to produce *de novo* mesaconate directly from glucose was the main concept. It was proposed that glucose would be consumed and through the TCA cycle glutamate

would be produced from the branch point at α-ketoglutarate. Glutamate mutase and methylaspartate ammonium lyase genes would then be inserted to carry on the pathway to produce mesaconate (Figure 4-3). Unlike the previous study, a different approach was adopted in that instead of engineering the genes into an *E. coli* strain, they would instead be inserted into a natural glutamate overproducing organism. As previously stated in this study, *C. glutamicum* is the industrial workhorse in the production of L-glutamate. Therefore, it was assumed that an increase in glutamate production efficiency would in turn lead to an improvement in downstream mesaconate conversion. In Chapter 3, *C. glutamicum* ATCC 13869 was identified was as the most efficient strain available from Ingenza Ltd. accumulating the highest yield of L-glutamate under biotin limitation. Therefore it was chosen as the parent strain to produce the subsequent engineered mutants.

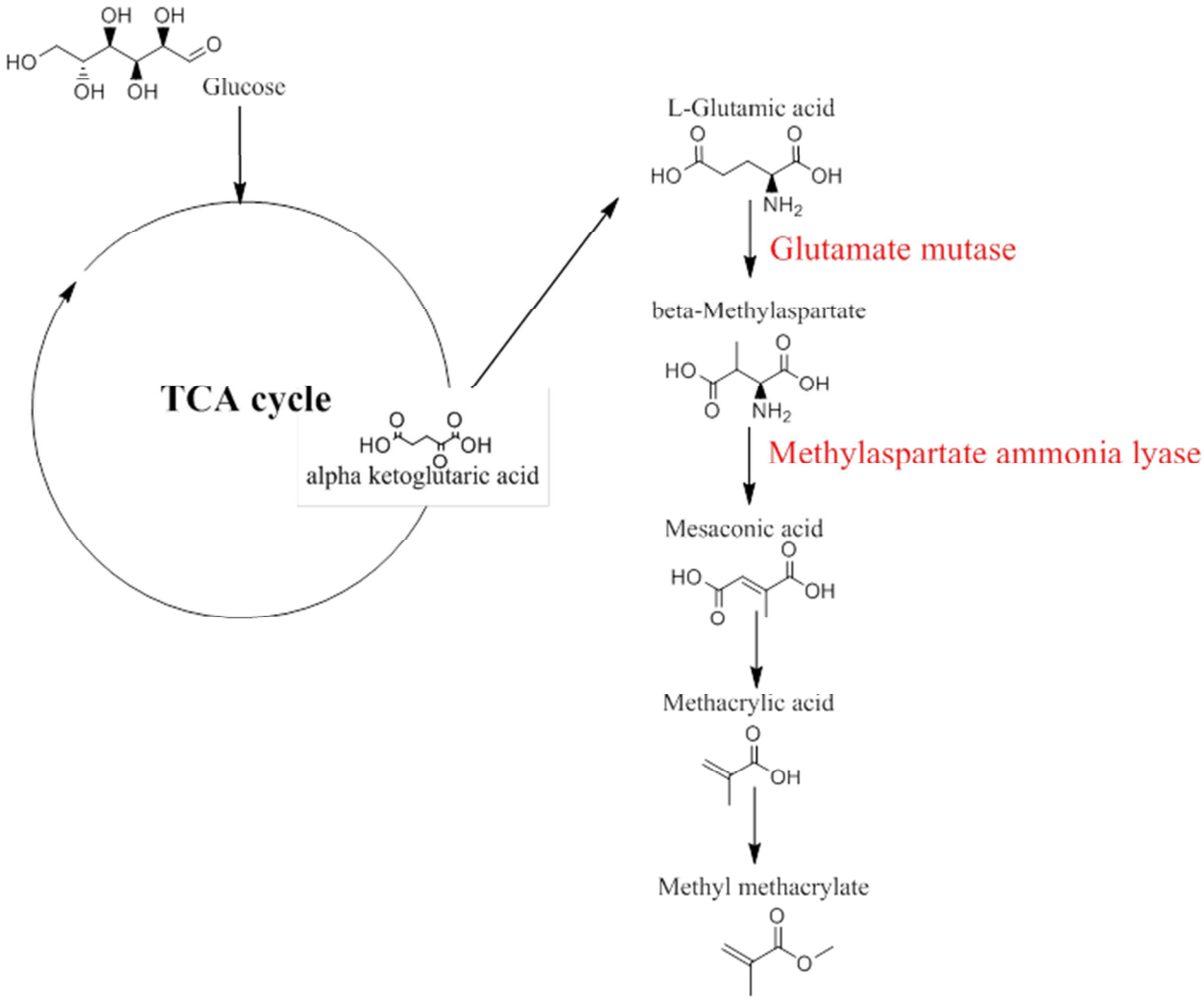


Figure 4-3 Proposed schematic for Methyl methacrylate production utilising the mesaconate pathway.

4.3 **Strain Construction**

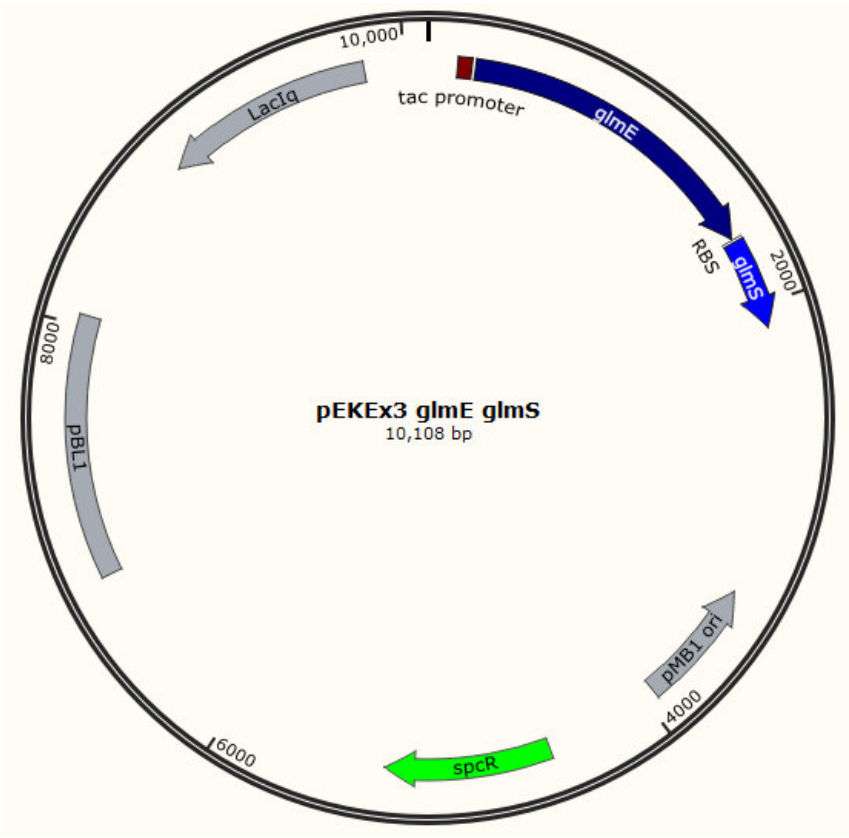
All engineering of the *C. glutamicum* ATCC 13869 was carried out in house at Ingenza Ltd. with the decisions on specific gene insertions being dependent upon company demands. These modifications are detailed in Table 4-1 and plasmid maps are depicted in Figure 4-4, Figure 4-5 and Figure 4-6. Unlike the genes used in previous studies (Wang and Zhang, 2015) , the glutamate mutase genes were taken from *Desulfosporosinus meridiei* for strains 1 and 2 and from *Actinoplanes friuliensis* for strain 3. Previous work carried out by Ingenza Ltd. using a variety of glutamate mutase genes from various origins identified the genes from *D. meridiei* gave the greatest efficiency of converting L-glutamate to β-methylaspartate. Therefore it was used in the first instance for genetic modification. By examining glutamate mutase genes from other organisms it was intended to identify a more stable protein that would with-stand oxygen inactivation, a vital characteristic in the proposed bioprocess. In all instances, methylaspartate ammonium lyase genes were obtained from *C. tetanomorphum* and are denoted as tMAAL. Antibiotic resistance was also incorporated onto the vector for strain selection and denoted in the table.

Two types of promoters were employed in the construction of these strains. For strains 1 and 2, the P*tac* promoter was utilised whereas strain 3 contained the P*tuf* (translational elongation factor) promoter. The major difference between the two types of promoter is their mechanism of transcriptional regulation. P*tuf* allows the genes to be expressed continuously and is known as constitutive expression. This means while the organism is growing, expression of the genes should increase simultaneously. Alternatively, P*tac* is an inducible promoter and is so called as it is a mixture of the *trp* and l*ac* promoters (de Boer *et al.*, 1983). It is the most commonly used inducible promoter for metabolic engineering in a wide variety of bacteria. In the presence of the lacI repressor molecule, encoded by $lacI^q$, the *tac* promoter is tightly regulated inhibiting transcription. Addition of IPTG inactivates lacI, relieving repression and therefore induces expression.

Although P*tac* exhibits desirable attributes such as high expression levels and precision of induction time, at industrial scale the expense of the IPTG required would be great. Therefore, a constitutively expressed system would be more desirable to develop into a large scale process as the running costs would be greatly reduced.

Table 4-1Engineered strains of *C. glutamicum* ATCC 13869

Strain no.	**Plasmid vector**	**Genes inserted**	**Antibiotics resistance**	**Expression**	**Ingenza ID**	**Figure No.**
1	pMB1 pEKEx3 pCG1 pVWEx1	glmS glmE tMAAL spec kan	spectinomycin kanamycin	Inducible 1 mM IPTG	IL.5.0.2(G2).1.(F1F2)	4.4
2	pMB1 pEKEx3 pMB1 pVWEx1	glmS glmE glmL tMAAL spec kan	spectinomycin kanamycin	Inducible 1 mM IPTG	IL.5.0.1(F1.F2-F3).2(G2)	4.5
3	pCG1 pIL-1	glmA glmB tMAAL kan	kanamycin	Constitutive	IL.5.0.1(F16.F17.G2).0	4.6



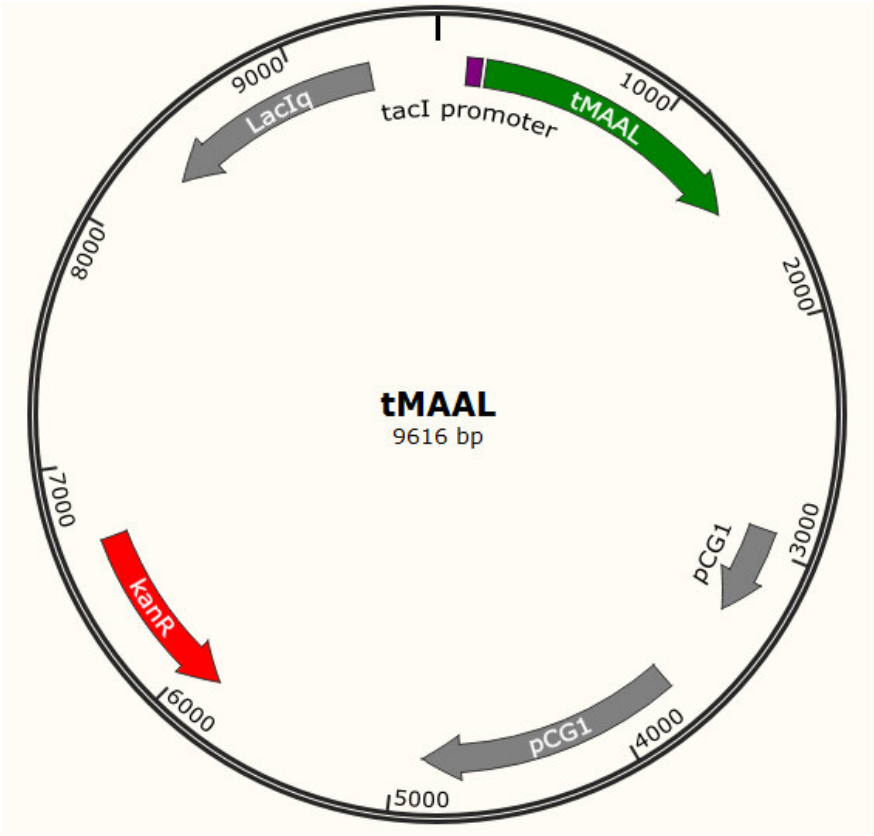


Figure 4-4 Strain 1 plasmid maps for fusion protein *glmES* and *tMAAL*

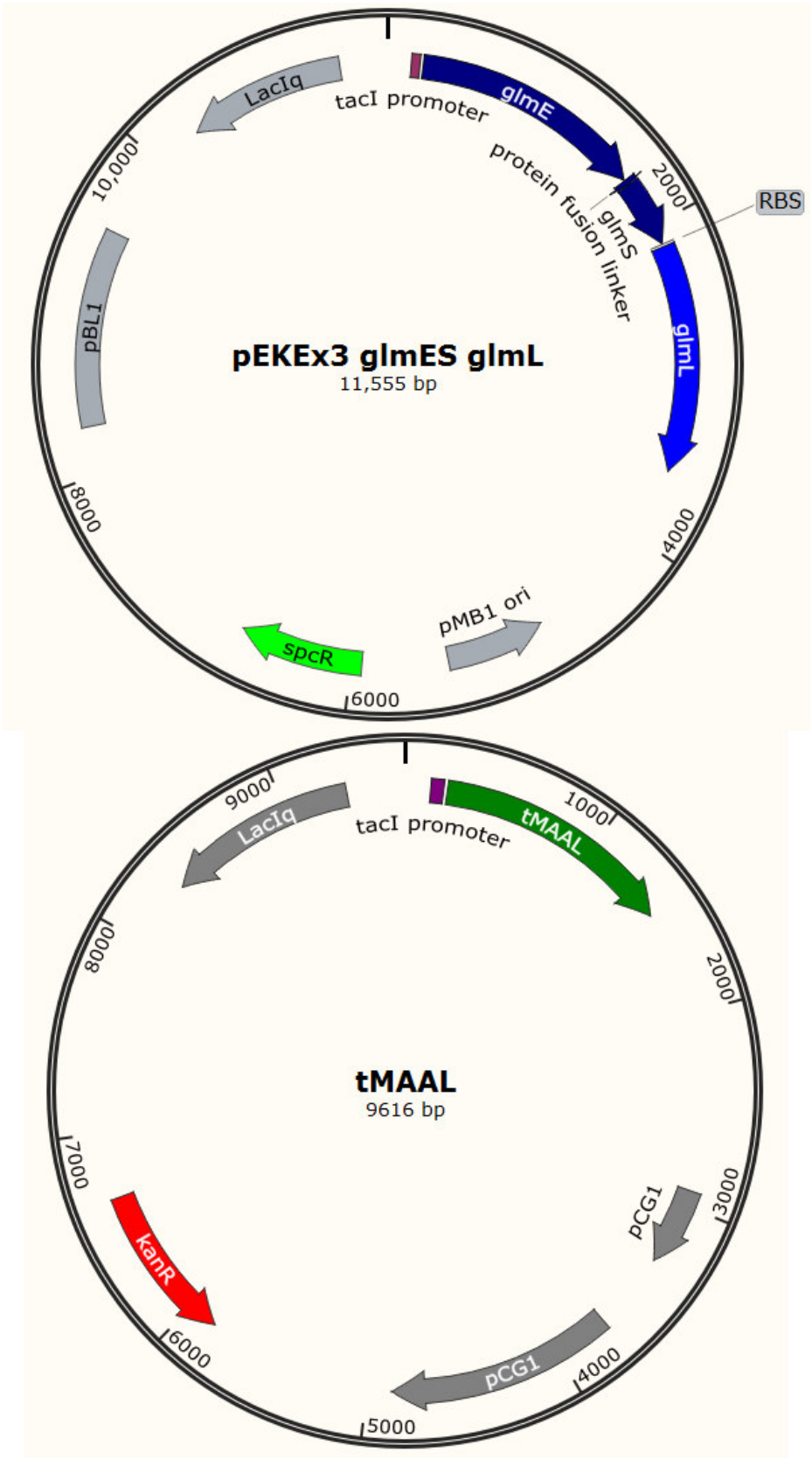


Figure 4-5 Strain 2 plasmid maps for fusion protein *glmES*/*glmL* and *tMAAL*

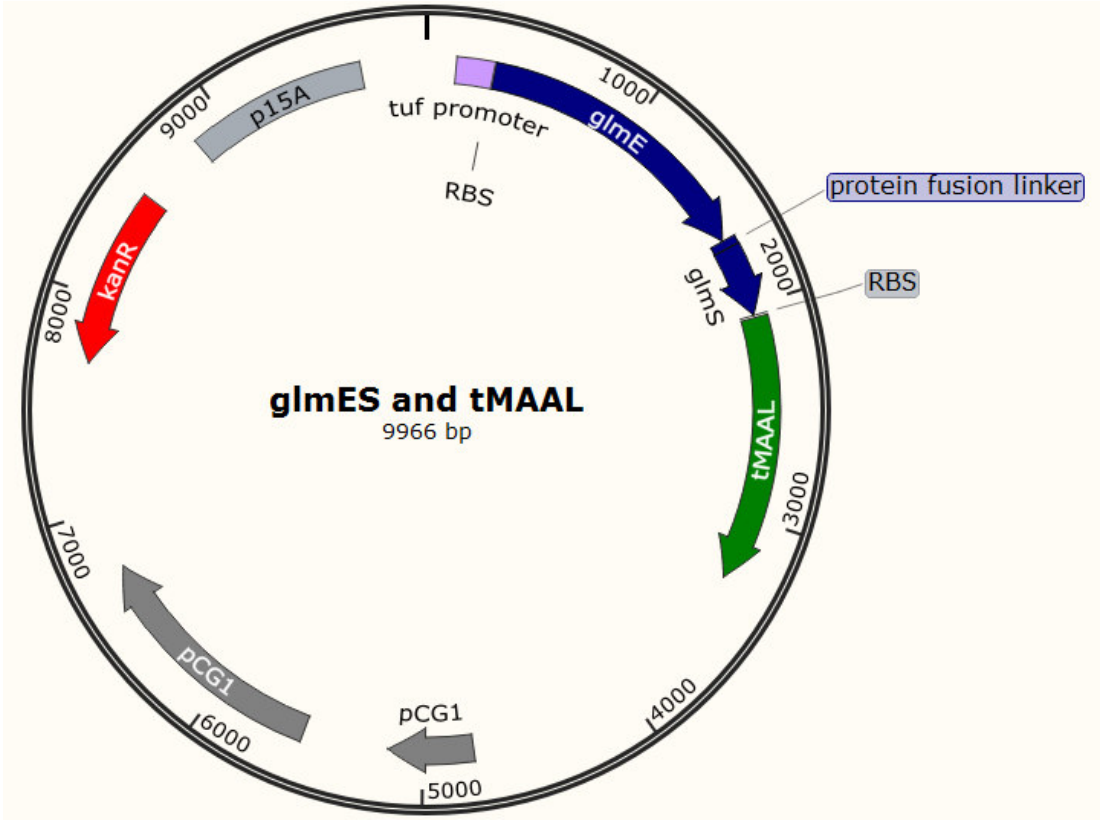


Figure 4-6 Strain 3 plasmid map containing *glmES* and *tMAAL*

4.4 **Process Overview**

Biotin limitation at 1 $\mu g l^{-1}$ biotin produced the greatest accumulation of L-glutamate using the wild type *C. glutamicum* ATCC 13689 (Section 3.8.2), when using the standard batch medium for this process (Section 2.2.2.4). As a result, it was used throughout this investigation with the addition of the cofactors cobalt chloride and 60 μM coenzyme B12. Addition of these chemicals to the medium was intended to provide the glutamate mutase enzyme with increased levels of essential cofactors leading to a more efficient reaction. Neither of the chemicals hindered the growth or the production of the parental strain. All experiments were compared to the standard batch culture using the unmodified ATCC 13869 under biotin limitation to observe changes in growth, L-glutamate and mesaconate production.

To obtain an overview of the feasibility of the various strains constructed to produce mesaconate, several process conditions were examined (Table 4-2). Firstly, the strains were examined under normal batch conditions (Process 1) where no induction methods were employed for strains 1 and 2. As strain 3 included constitutively expressed genes and a glutamate mutase protein that was believed to be oxygen stable, this strain was only examined using process 1. For Strains 1 and 2, two aerobic induction processes were analysed. Induction with 1mM IPTG was examined at 0 h (Process 2) and 25 h (Process 3). A final process (Process 4) was then developed with an initial aerobic growth phase and upon induction at 25 h the process was switched to a microaerobic environment. It was intended that by maintaining a low concentration of oxygen, the glutamate mutase enzyme would not become inactive and would convert the glutamate being produced in the process to β-methylaspartate, the intermediate in the glutamate degradation pathway towards mesaconate (Figure 4-3).

Table 4-2 Process development overview

Process no.	***C. glutamicum* ATCC 13869 engineered strain No.**	**Induction**	**Method**
1	1, 2, 3*	No	Standard batch conditions**
2	1, 2	Yes	IPTG at 0h of batch
3	1, 2	Yes	IPTG at 25 h of batch
4	1, 2	Yes	ITPG at 25 h of batch immediate switch to micoraerobic environment

*Strain 3 was constitutively expressed and contained a more oxygen stable glutamate mutase therefore was only examined under standard batch conditions

**Standard batch culture for biotin limitation induction of L-glutamate production used in Section 3.8.2

4.5 **Aims**

The present work aims to identify whether the constructs developed by Ingenza Ltd. can produce mesaconate with the induction method and oxygen levels examined. Production of glutamate and cellular growth would also be compared to the previous growth exhibited by ATCC 13869 during biotin limitation batch conditions (Section 3.8.2). As well as the regular sample analysis performed on all previous bioprocesses, samples from Process 4 of both strains 1 and 2 and Process 1 of strain 3 were prepared in order to analyse protein expression. This information would give an insight into the viability of the strains for their potential commercialisation.

4.6 **Cultivation of Strain 1 under different process conditions**

Strain 1 containing glutamate mutase genes from *D. meridiei* and methylaspartate ammonium lyase genes from *C. tetanomorphum* was the first of the engineered strains examined (Figure 4-4). The genes for the two proteins reside on two separate plasmids that conferred resistance to kanamycin and spectinomycin respectively.

To obtain an understanding on how the new strain would perform compared to the parental strain (Section 3.8.2), the strain was examined under biotin limitation (Section 2.2.2.4). All processes were performed at 4 L in a Bioflo 110 bioreactor system (Section 2.2.4).

Similar to the parental strain, strain 1 utilised all glucose within 48h (Figure 4-7). Biomass accumulation decreased slightly, with only 2.66 gl^{-1} DCW accumulating after 24 h compared to 3.6 gl^{-1} achieved by the control at the same time point. A maximum of 3.6 gl^{-1} was eventually achieved by the engineered strain after an additional 24 h period. Glutamate accumulation also reached a maximum of 15.99 gl^{-1} at 48 h which was 96% of the yield that the parental strain exhibited. Yield of glutamate per gram of biomass was therefore 4.4 g/g of glutamate to DCW for strain 1 compared to 4.58 g/g for the parental strain. The yield appears to have remained fairly constant, however, the rate of accumulation has decreased marginally.

At 31 h, approximately 30 % of the glucose was still present in the medium, however, only a small portion of this available carbon is converted to either biomass, glutamate or other by-products. Terminal location of the carbon could be explained, by analysing the respiratory coefficient (RQ) of the bioprocess. RQ was determined using the carbon evolution rate (CER) and oxygen uptake rate (OUR), which was calculated using the exhaust gas from the bioprocess. At this point, the RQ was equal to 0.8, which was approaching the theoretical maximum RQ value of 1. This theoretical value of RQ, represented a TCA cycle that was fully functioning and where all glucose was converted to CO_2 (Xiao *et al.*, 2006). Therefore, exhibiting an RQ close to the theoretical maximum, would suggest that a large amount of carbon was being lost to CO_2. Therefore, future improvement of the flux towards glutamate

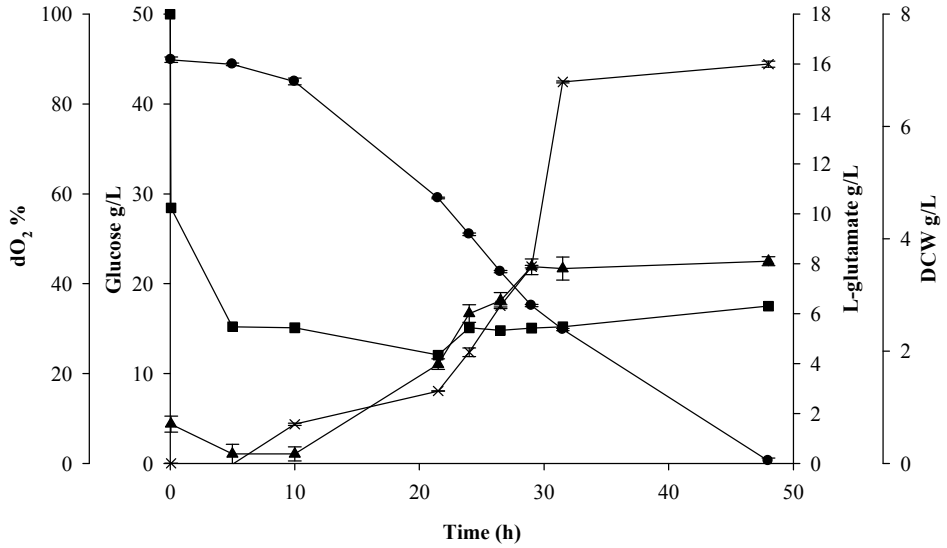


Figure 4-7 Strain 1 analysed using Process 1

A propagation flask of Strain 1 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

would be required to improve efficiency of the process to meet industrial requirements.

Different protocols for induction of the strain were examined (Table 4-2). Initially, the strain was exposed to 1 mM IPTG from time zero in the batch process (Process 2). IPTG concentration was based on previous work carried in house at Ingenza Ltd. as well as concentration used in the literature (Carpinelli *et al.*, 2006). The starting OD_{600}nm of the culture was approximately 1, in all cases, i.e. similar to cellular levels used in previous studies investigating gene overexpression in *C. glutamicum* (Kortmann *et al.*, 2015). Examination from time zero also enabled the determination of negative effects the IPTG would exhibit on the functionality of the organism. Induction from this early point was also used by Wang and Zhang (2015) who induced their culture overnight to successfully express both Glm and MAAL proteins. This early induction elicited protein production that ultimately led to the accumulation of mesaconate from the utilisation of batch glucose.

In the current study, the growth was very similar to that of the un-induced process (Figure 4-7), with a maximum of 3.6 gl^{-1} of DCW achieved and all glucose consumed within 48h (Figure 4-8). End point glutamate accumulation was 16.8 gl^{-1} which was a 0.8 gl^{-1} increase on the un-induced process, however, the rate of accumulation decreased. At 31.5h only 9.6 gl^{-1} of residual glutamate was observed in the medium compared to 15.99 gl^{-1} accumulated by the un-induced process. Interestingly, low levels of pyruvate were also observed. Pyruvate was not, however, observed as a by-product in the un-induced process. This could again indicate a bottleneck that, if improved, could increase the flux of glucose towards the desired product.

Slight differences in growth and amino acid production were observed between the Process 1 and 2, however, this could be due to experimental error. It was expected that by inducing with IPTG, the enzymes would actively convert the L-glutamate produced into mesaconate. Analysing for residual mesaconate, or its intermediate β-methylaspartate, indicated that these compounds were not present in the fermentation broth. It was postulated that allowing the culture to grow for a period of time before induction would increase the likelihood of functional enzymes being produced.

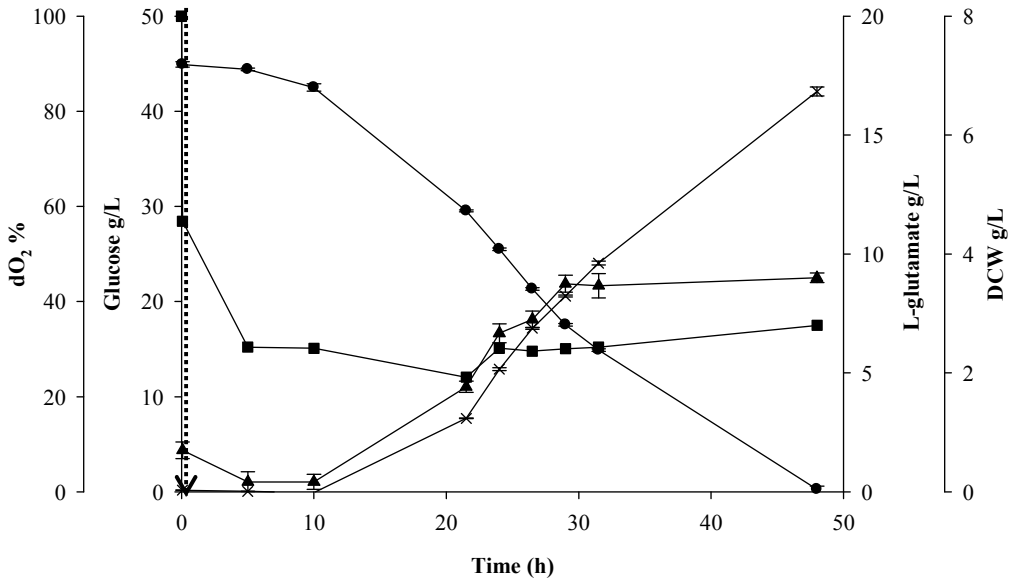


Figure 4-8 Strain 1 analysed using process 2

A propagation flask of Strain 1 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. Induction of the strain was achieved by 1mM IPTG at 0h of the batch process (denoted by dashed arrow). The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

To examine this hypothesis, the culture was grown for 25 h before being induced with 1 mM IPTG (Table 4-2, Process 4). This time point was chosen for induction as the culture in both processes 1 and 2 were still in the growth phase; L-glutamate production had commenced and over 50 % of the initial glucose was still available.

With the process prepared following the same protocol as previously, it was expected that similar growth and glutamate accumulation would be observed up until the point of induction. The bioreactor was seeded with the same concentration of cells and all parameters were maintained at the same level. However, growth exceeded that of that observed in Process 1 and 2 as well as the parental strain, reaching a maximum of 8.4 gl^{-1} after 24 h (Figure 4-9). Only 4 gl^{-1} of L-glutamate was achieved after 29 h. Interestingly, substantial production of alanine was also observed in this particular process, with 2.5 gl^{-1} at 48 h suggesting that the carbon flux was being diverted to the production of other metabolic routes.

Reduction of productivity for the desired amino raised concerns about strain stability, however, as glutamate was present it was still assumed that upon induction, mesaconate might be produced. On analysis of the culture broth, this was not the case as no evidence of the desired product or the intermediate was found.

The process outcome differed from that of the control, however, enzymatic conversion of L-glutamate to mesaconate still remained unsuccessful. Therefore, increasing cell density before induction did not necessarily improve enzyme expression in the strain. As this was an aerobic process, there was a possibility that the glutamate mutase protein was being inactivated due to the high levels of oxygen present in the medium. Therefore, process 4 was developed to examine if reducing oxygen concentration after induction would lead to the production of functional enzymes (Table 4-2).

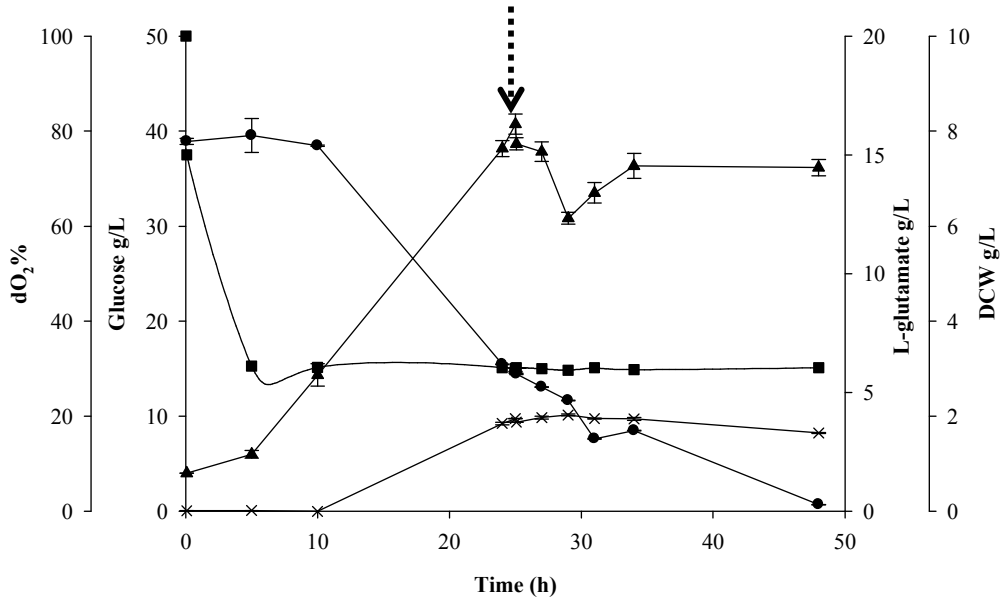


Figure 4-9 Strain 1 analysed using Process 3

A propagation flask of Strain 1 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. Induction of the strain was achieved by 1mM IPTG at 25 h of the batch process (denoted by dashed arrow). The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Process 4 involved preparation of the initial bioprocess in the same manner as that of Process 3. Upon induction at 25h, the air supply to the reactor was closed and all outlets were clamped to maintain a sealed unit. Agitation was also reduced to 50 rpm to maintain mixing. As expected, when the air supply was disconnected, the dO_2 decreased to zero rapidly (Figure 4-10) and no further accumulation of biomass or glutamate was observed after this point. Glucose, however, continued to be utilised therefore, cellular metabolism was not completely inhibited by the limited availability of oxygen within the reactor. However, lactate accumulation was inevitable as it is the major by product that is accumulated under oxygen limiting conditions (Inui *et al.*, 2004b, Okino *et al.*, 2008b). By 48 h, 21 gl^{-1} of lactic acid was present in the medium which accounted for the total utilisation of the carbon source. Deletion of the lactate dehydrogenase gene could inhibit the production of this unwanted product.

Again, no mesaconate production was observed. To examine if expression of the inserted genes were taking place, pre and post induction samples were analysed by RNA-seq by Dr Alison MacFadyen (University of Strathclyde). Interestingly, it was discovered that only the methylaspartate ammonium lyase gene was being overexpressed after induction. No expression of glutamate mutase was observed at any point, indicating that the strain was not functioning correctly.

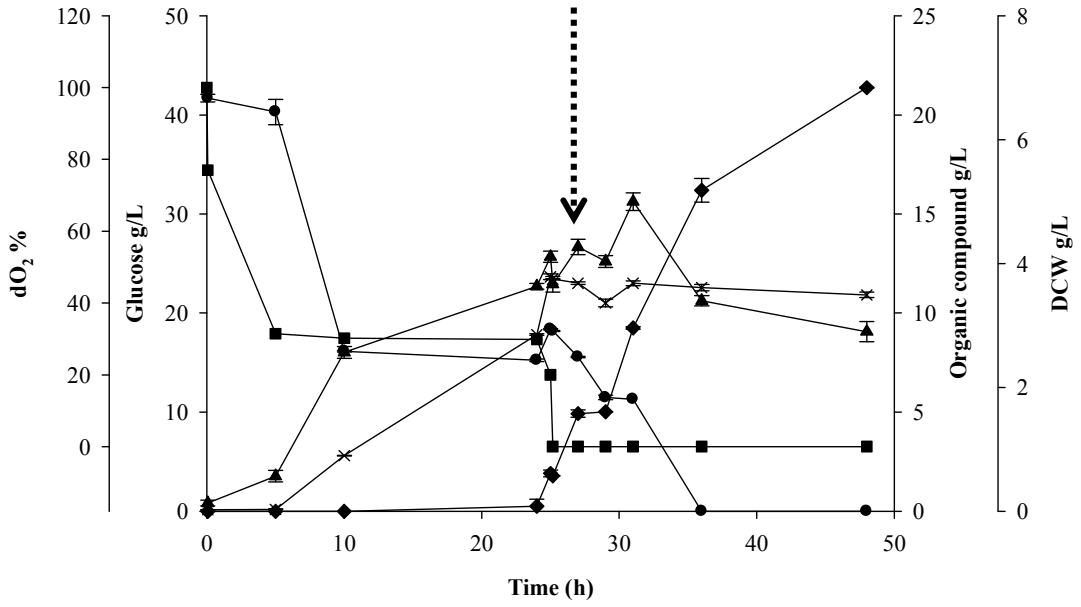


Figure 4-10 Strain 1 analysis using Process 4

A propagation flask of Strain 1 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. At 25 h the culture was induced with 1mM IPTG of the batch process (denoted by dashed arrow). Upon induction the air supply was cut, the vessel sealed and agitation was lowered to 50 rpm. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲), glutamic acid production (x) and lactic acid (♦). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

To validate that the strain was not functional, the strain was cultured in the rich CGG medium for 25 h and induced with 1 mM IPTG. In this case, samples were then examined by qPCR instead of RNA-seq as a more cost effective method which would elicit results as efficiently. Strain 2 was also examined using this method to understand the efficiency of both strains simultaneously. Figure 4-11 represents the qPCR copy numbers that were present for *glmS*, *glmE*, *tMAAL* and *glmL* (for strain 2 only). As expected from the RNA-seq results, no expression of any of the glutamate mutase genes was observed from strain 1. This confirmed that the strain was incapable of producing the enzyme and resulted in the process being unsuccessful. On the other hand, it is clear that upon induction of strain 2, the glutamate mutase genes are overexpressed. Therefore, this strain exhibited the potential to be a more successful candidate for production of mesaconate.

Interestingly, was observed in both pre and post induction samples for both of the strains suggesting that the plasmid was leaky. However, an increase was still observed suggesting induction was still required to obtain efficient expression.

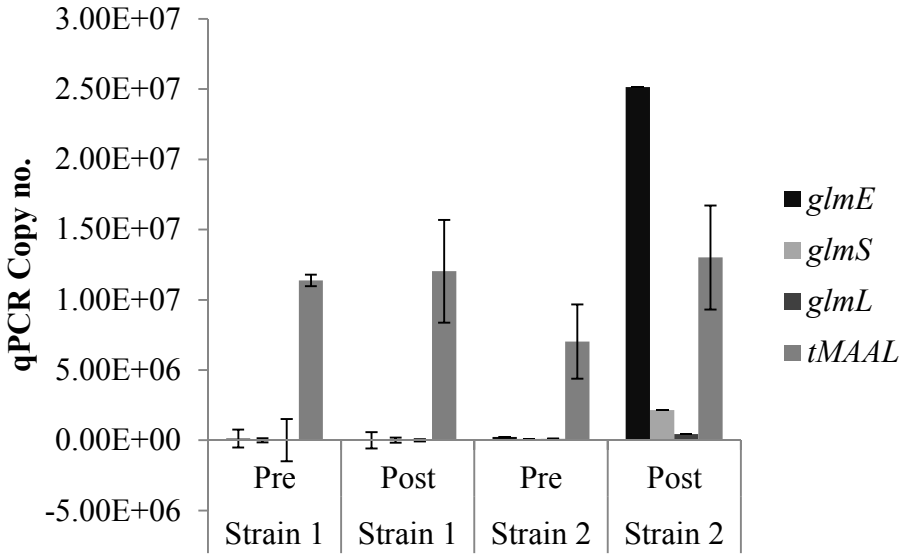


Figure 4-11 qPCR results of Strain 1 and 2

Samples were acquired pre-induction and 10 minutes after induction with 1 mM IPTG. RNA protect was added to the sample at ratio of 1:2 sample to protectant. Samples were incubated at room temperature for 5 minutes before being pelleted to remove the supernatant. Dried pellets were stored at –80 °C until cells were disrupted an analysed by qPCR.

4.7 **Cultivation of Strain 2 under different process conditions**

With the results from the qPCR suggesting that all of the required genes were expressed, it was anticipated that the strain would be able to convert the L-glutamate produced in the process to mesaconate. For comparison, the same 4 process conditions were examined that were used to analyse strain1 (Table 4-2).

Unlike Strain 1 and the parental strain, Strain 2 exhibited a prolonged growth phase during the biotin depleted inoculum stage and required 1.5 times longer to achieve an optical density of 10. Similar growth profiles were observed during repetition of this stage. However, an increased lag phase was not observed when the strain was investigated during an un-induced batch process (Figure 4-12).

DCW comparable with the parental strain was achieved after 24 h. A maximum yield of 4.2 gl^{-1} of DCW was attained after 48 h which was an increase over strain 1 and the parental strain of 14%. However, productivity was reduced slightly with a maximum yield of only 14.3 gl^{-1} giving a yield of L-glutamate produced per gram of biomass of 3.4 g/g. This was 14 % and 11 % reduction in product yield compared with that of the parental and strain 1 respectively.

Unlike the parental strain and strain 1, the batch carbon source was not fully utilised within 48h, with 7.3 gl^{-1} residual glucose remaining in the batch medium. It could be hypothesised that incomplete utilisation of the carbon source could have resulted in the lower yield at 48 h. Similar to the aforementioned strains, very little by-product production was observed which was considered a positive characteristic of this strain. Since no induction method was employed, no production of mesaconate or its intermediate β-methylaspartate was observed.

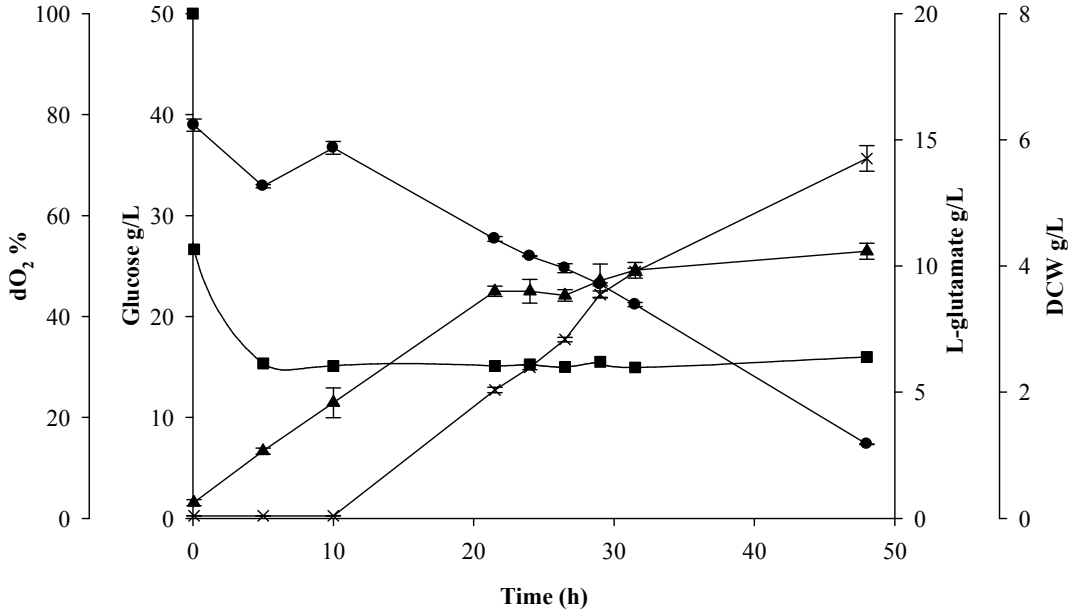


Figure 4-12 Strain 2 analysed using Process 1

A propagation flask of Strain 2 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Analysis of Process 2 (Figure 4-13), showed that the growth of strain 2 was slower in comparison to that observed when no IPTG was present. However, a comparable DCW was reached by 48 h suggesting that the initial growth phase may be effected by the presence of IPTG in the initial medium. At 24 h a DCW of 2.6 gl^{-1} was achieved whereas at 48 h 4.4 gl^{-1} was obtained. In stark contrast with the previous and parental strains, a titre of 6 gl^{-1} of L-glutamate was achieved after 48 h which was only 36 % of the yield accumulated by the parental strain. Further reduction in utilisation of the carbon source was also observed, with 21.5 gl^{-1} of residual glucose remaining at the end time point.

As with previous attempts, this strain, under these conditions, failed to produce any mesaconate. Poor conversion to the desired chemical may be due to reduced production of L-glutamate. On repetition of this process no improvement in glutamate production could be achieved and mesaconate was still not produced.

In comparison with the previous process examining Strain 2, Process 3 had a similar utilisation of glucose to that observed in process 2, with 22 gl^{-1} of the carbon source remaining after 48 h (Figure 4-14). In contrast, growth was in line with process 1, with a maximum DCW weight of 4 gl^{-1} achieved at 25 h. However, at 48h the accumulation of glutamate was only 60 % of the un-induced process. Yield of glutamate produce per gram of biomass was therefore reduced from 3.4 g/g to 2.5 g/g glutamate to biomass with the addition of IPTG at 25 h. Nevertheless, this was still an improvement from when IPTG was introduced at the beginning of the batch process where the yield was only 1.4 g/g (glutamate/biomass).

Unsuccessful production of mesaconate by Strain 2 was also observed during process 3. However, as this was still an aerobic process, there remained a possibility that the glutamate mutase protein was being inhibited as dissolved oxygen was still maintained at 30 % within the bioreactor.

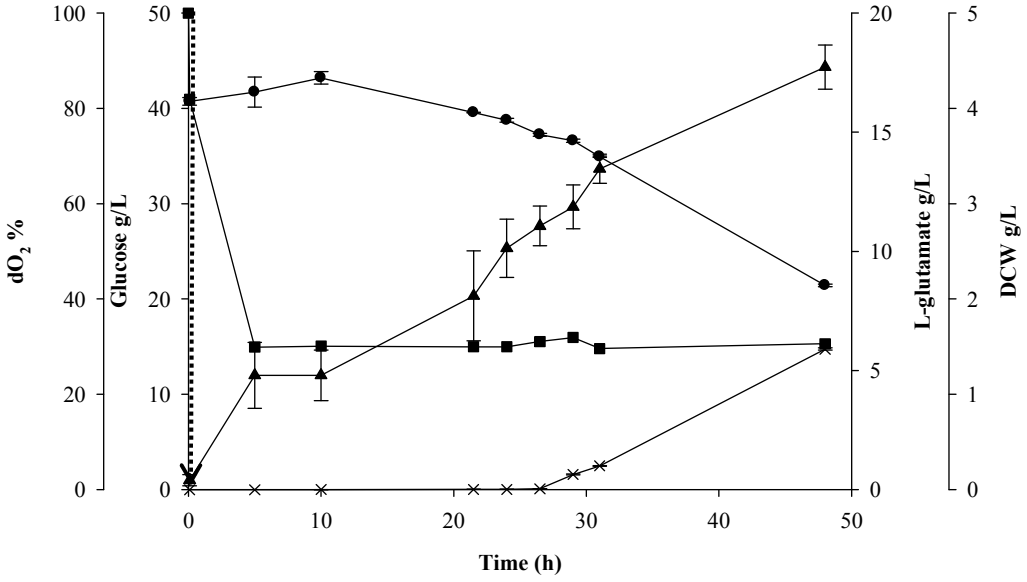


Figure 4-13 Strain 2 analysed using Process 2

A propagation flask of Strain 2 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. Induction of the strain was achieved by 1mM IPTG at 0h of the batch process (denoted by dashed arrow). The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

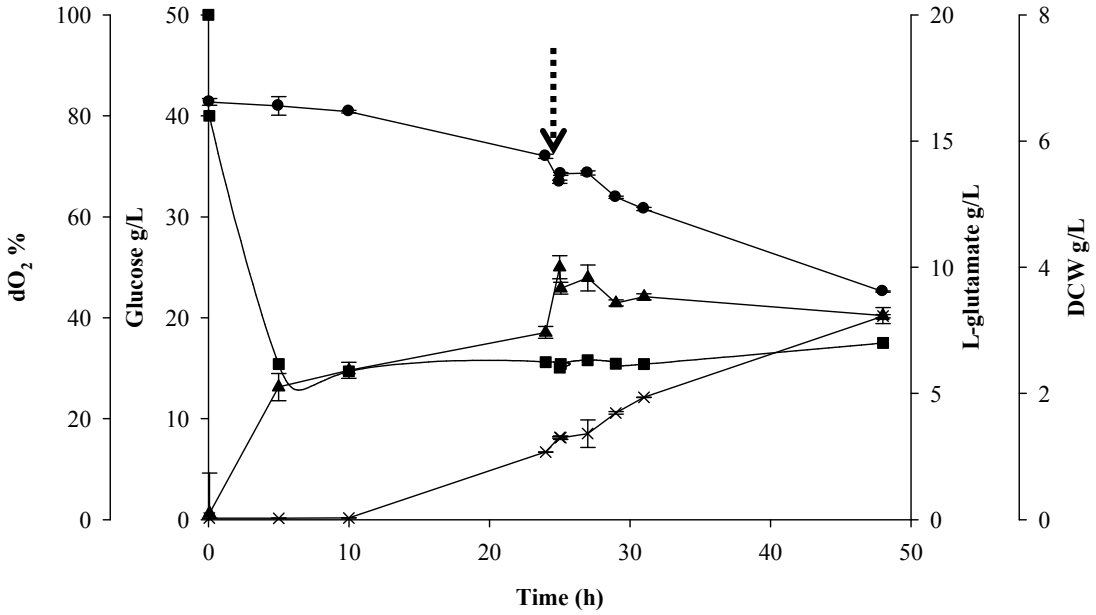


Figure 4-14 Strain 2 analysed using Process 3

A propagation flask of Strain 2 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. Induction of the strain was achieved by 1mM IPTG at 25 h of the batch process (denoted by dashed arrow). The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid production (x). All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

From the flask culture qPCR results (Figure 4-11), it was established that Strain 2 could express all of the genes inserted to produce mesaconate. Therefore, to ascertain if the oxygen availability in the vessel was detrimental to the success of the process, the strain was examined using process 4. As with Strain 1, Strain 2 was grown for 25 h under aerobic conditions before the culture was induced with 1 mM IPTG and the aeration switched off.

Similar to the other bioprocesses involving this strain, a maximum DCW of 3.5 gl^{-1} was achieved at 24 h (Figure 4-15). However, glutamate production was considerably lower than what had been observed previously with the strain. An initiation of glutamate accumulation was detected before induction and the cessation of the air supply. Thereafter, glutamate production appears to plateau. This characteristic was also observed when strain 1 was deprived of oxygen in Process 4. Lack of oxygen could be causing the organism to adapt to utilising an anaerobic metabolism of the available carbon source. Continued utilisation of the residual glucose was observed after the air supply was discontinued, and as was observed with strain 1, lactic acid accumulation was noted.

To examine whether the genes were being expressed in this process, samples were acquired before, 10 minutes and 4 h after induction and examined by qPCR to determine copy numbers of the genes (Figure 4-16) (work carried out by Dr Alison MacFadyen, University of Strathclyde). Before induction, low levels of all of gene transcripts were present in the culture samples with *tMAAL* appeared to exhibited the greatest expression. Presence of the transcripts copies suggests that the expression system is leaky which is comparable with the previous qPCR performed in the flask culture (Figure 4-11). However, after 10 minutes there was a significant increase in expression of *glmS*, *glmE* and *tMAAL* suggesting IPTG induction was successful in promoting the production of these genes. 4 h after induction, expression of *glmE* and *glmS* were reduced in comparison to 10 minutes after induction whereas *tMAAL* continued to increase. In contrast, *glmL* overexpression was not observed either at 10 minutes or 4 h post induction. Low expression of this gene was consistent with the results obtained previously in the shake flask qPCR analysis. The absence of *glmL* may not be significant for the initial production of mesaconate as its function remains

debatable within the literature (Zelder *et al.*, 1994, Wang and Zhang, 2015). Therefore, with the presence of the other genes conversion of glutamate to mesaconate should have been successful

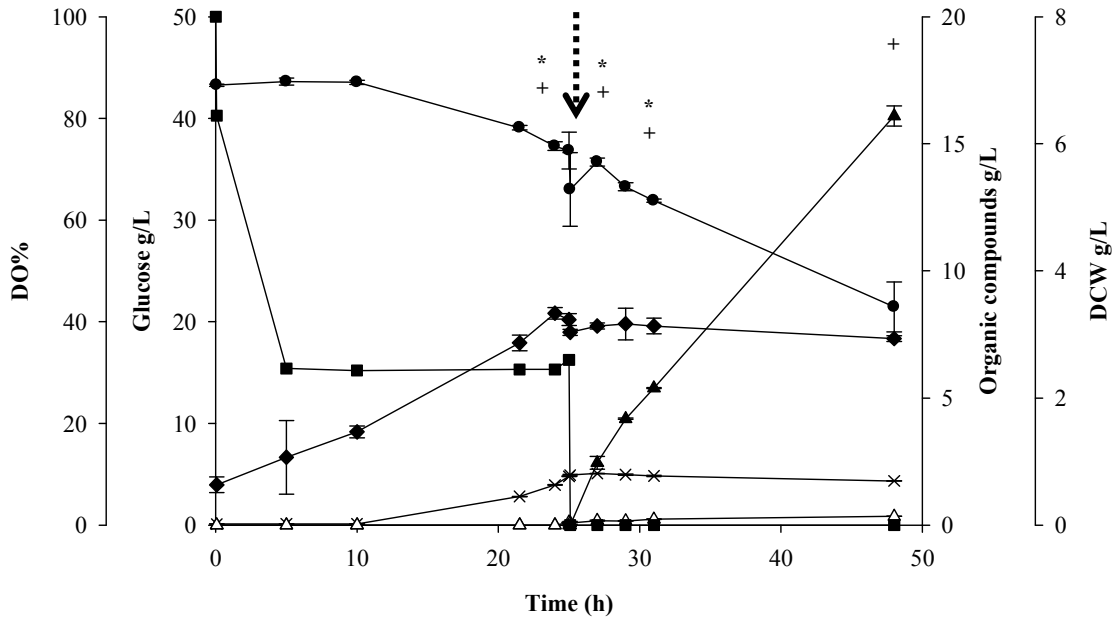


Figure 4-15 Strain 2 analysis using Process 4

A propagation flask of Strain 2 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. At 25 h the culture was induced with 1mM IPTG of the batch process (denoted by dashed arrow). Upon induction the air supply was cut, the vessel sealed and agitation was lowered to 50 rpm. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲), glutamic acid (x), lactic acid (♦) and mesaconate production (Δ). All analysis methods were carried out in triplicate. Samples were also obtained for qPCR (*) and LC-MS (+) analysis. Error bars were calculated from the standard deviation of the sample population.

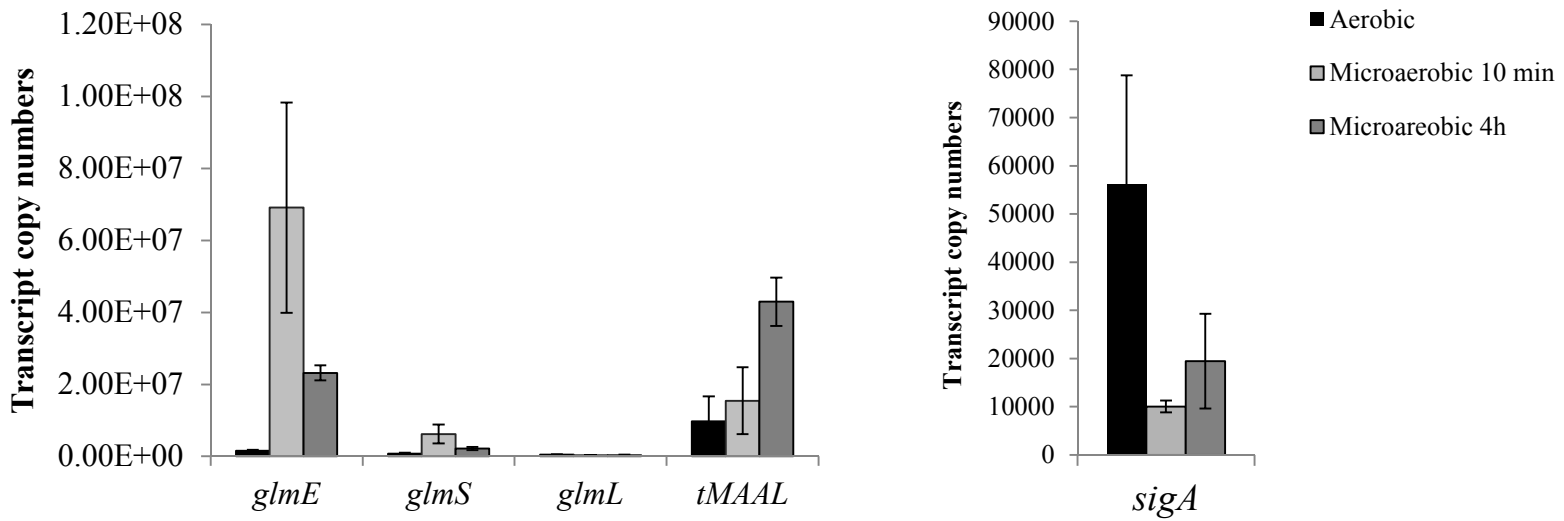


Figure 4-16 qPCR analysis of Strain 2 gene expression during Process 4

Aerobic values represent samples acquired pre-induction, micro-aerobic 10 mins represent expression 10 mins after induction with 1mM IPTG and aeration cessation and micro-aerobic 4 h represent samples obtain 4 h post induction. RNA protect was added to the sample at ratio of 1:2 sample to protectant. Samples were incubated at room temperature for 5 minutes before being pelleted to remove the supernatant. Dried pellets were stored at –80 °C until cells were disrupted an analysed by qPCR by Dr Alison MacFadyen. Transcript copy numbers were then evaluated with the average value and standard deviation of technical replicates being depicted graphically a) *glmE, glmS, glmL* and *tMAAL* and b) *sigA*.

HPLC analysis of the samples revealed a possible chromatography feature that exhibited a similar, but not exact, retention time to the mesaconate standard. The feature was present in samples before induction, however, gene transcripts were present in the samples at low levels at this point (Figure 4-16). It could be hypothesised that production of mesaconate could still be a possibility.

To analyse this sample further, the fraction where the peak appeared was collected. As the size of the sample was small, the fraction was collected 10 times with multiple HPLC injections. The mobile phase was then evaporated and the precipitate dissolved in deuterium oxide and evaluated by proton NMR spectroscopy (WestCHEM NMR Facility at University of Strathclyde). For comparison, a pure sample of mesaconate was used as a standard (Figure 4-17). No correlating signals could be ascertained between the standard and the HPLC sample suggesting that the feature was not in fact mesaconate (Figure 4-18).

Although the sample did not appear to be mesaconate, examining the NMR trace further, it was possible to postulate structural features that may be present in the sample by comparing the signals to known proton shift characteristics.

Five prominent signals were present in the sample. The doublet peak at 1.3 ppm indicates the possible presence of a secondary aliphatic group (R_2CH_2) with adjacent protons, the singlet signal at 1.8 ppm could indicate the presence of an allylic group ($C=C-CH_3$). Another singlet signal at 2.5 ppm could represent either an acid (HC-COOR) or a carbonyl compound (HC-C=O). And the presence of a peak at 4.75 could indicate the presence of a vinylic group (C=C-H). Variations in the chemical shift values may also result from the presence of heteroatoms within the molecule which could have resulted in the quartet peak visible at 4.2. However this hypothesis requires further scrutiny.

Full structural identification of the suspected peak was not possible and would require further C^{13} analysis to gain a definitive structure. However, it was suspected that due to the combination of potential structures present, the sample contained a mixture of compounds rather than one chemical.

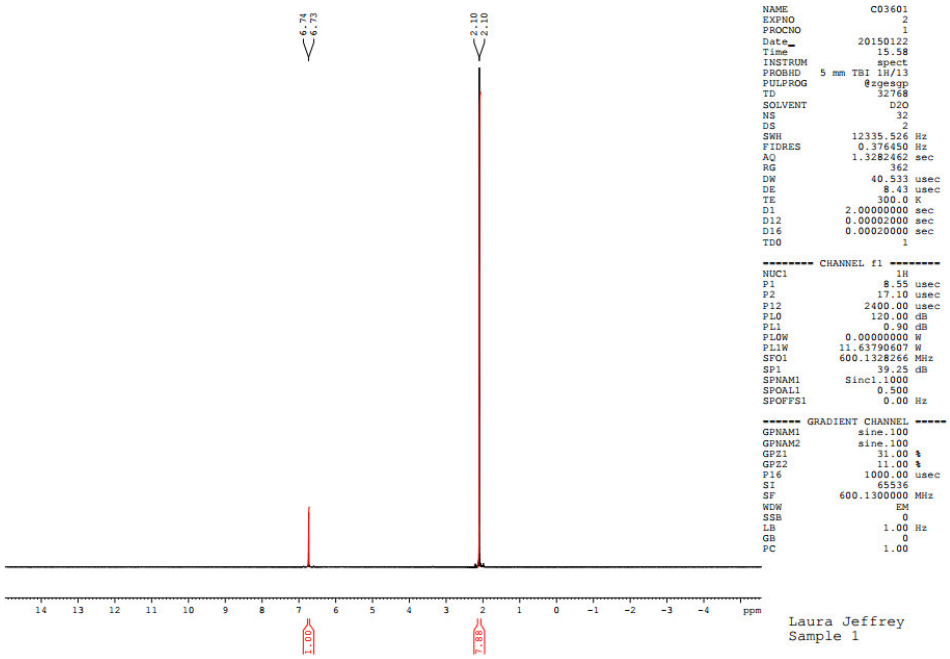


Figure 4-17 NMR trace of 10mM Mesaconate standard

10 mM of Mesaconate was prepared in 750 µL of deuterium oxide and evaluated by proton NMR analysis.

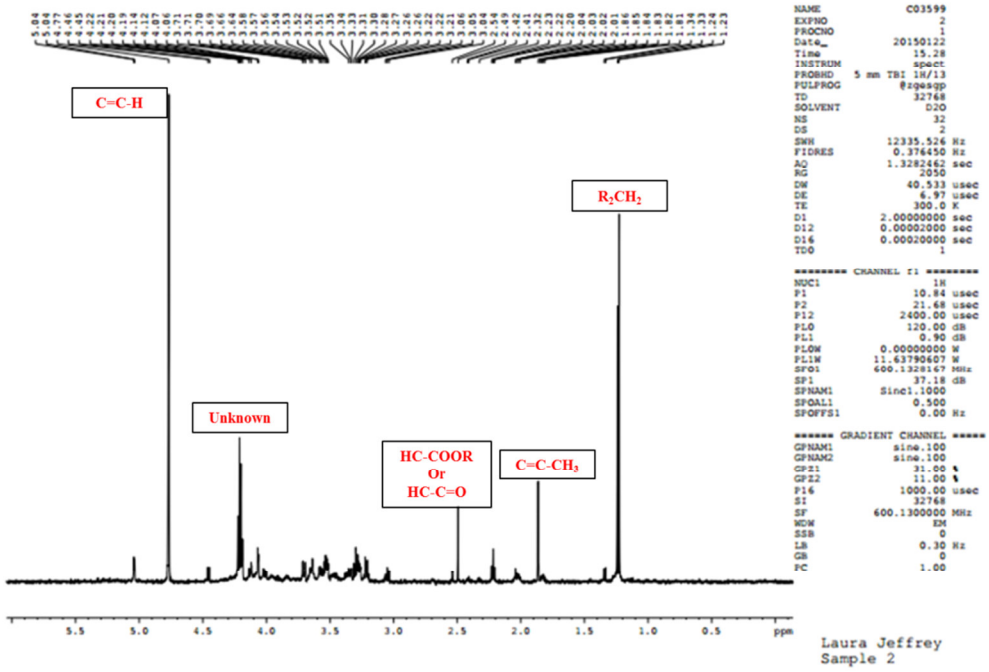


Figure 4-18 NMR trace of suspect peak analysed for its potential to be mesaconate Samples were obtained during organic acid analysis using a Gilson HPLC with a flow rate of 1 mL/min, 0.005N H_2SO_4 mobile phase and a column temperature of 70 °C. Fractions that appeared to correspond with mesaconate standards were obtained using a fraction collector. Liquid from the samples were then evaporated a precipitate resuspended in deuterium oxide and examined using proton NMR analysis. Potential structures present in the sample are indicated.

Contrasting results were obtained when samples were examined using LC-MS. Samples were obtained pre-induction, 10 mins after induction with 1mM IPTG and aeration cessation, 4 h and 24h post induction. Cells were harvested, with both the separated cells and supernatant frozen under liquid nitrogen before being analysed by Dr Alison MacFadyen (University of Strathclyde). Analysis of the mesaconate standard presented a retention time of 15.0 min and a molecular mass of 129.0196 (Figure 4-19). A correlating peak was observed in the sample that exhibited the exact molecular weight of the standard and only differed from the retention time by 0.2 min. Compared to the standard, the signals were relatively small and corresponded to a very low concentration. However, as it appears to exhibit the same molecular weight as the standard it is very promising. As these values are particularly low it could be hypothesised that they were being concealed in the HPLC chromatography due to possible limitations in the detection level.

Currently, this is the most promising evidence that the strain is producing mesaconate albeit at a very low level. The low level that has been produced here is in stark contrast with what has been produced in the literature with Wang and Zhang (2015) attaining a maximum yield of 6.96 gl^{-1} directly from glucose. Therefore, further work is still required in order to produce an efficient process using *C. glutamicum*.

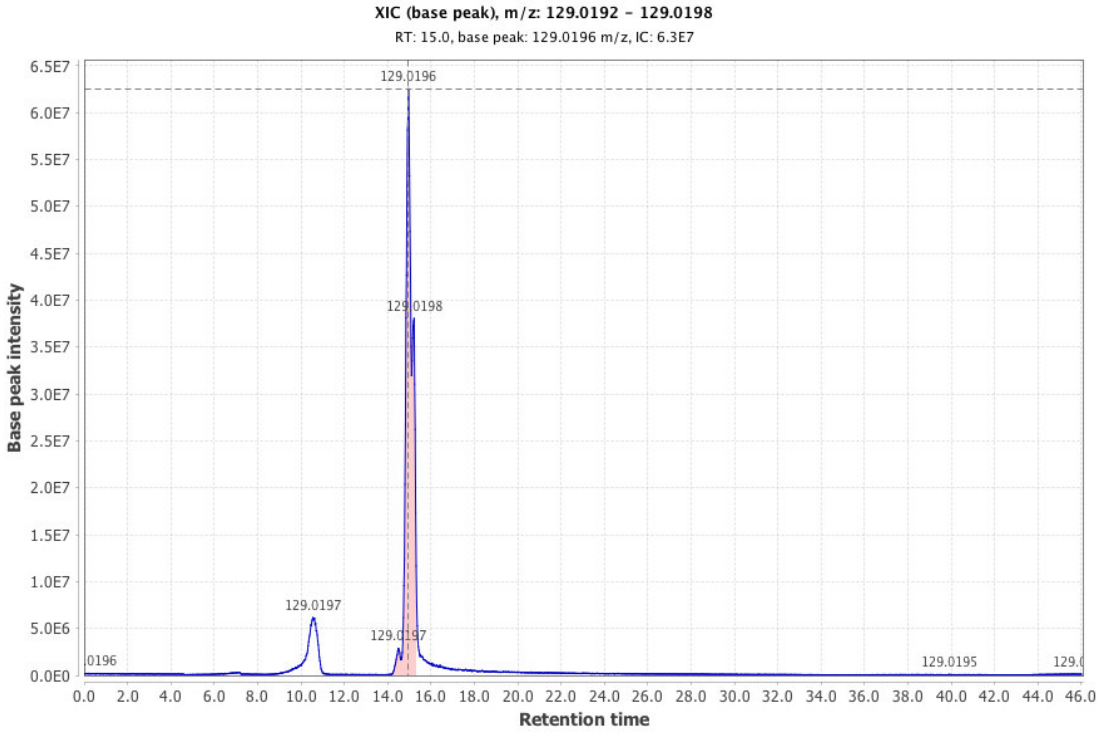


Figure 4-19 Mesaconate standard analysed using LC-MS

A 2 mg/mL standard of mesaconate was prepared in HPLC grade MeOH and processed using LCMS analysis. Samples were separated on HILIC column using mobile phase of acetonitrile and ammonium carbonate. Retention time of the analyte was noted at 15.0 min with a suspected mass of 129.0192 g. Analysis of the chromatographs was carried out by Dr Alison MacFadyen using MZmine software (v.2.17).

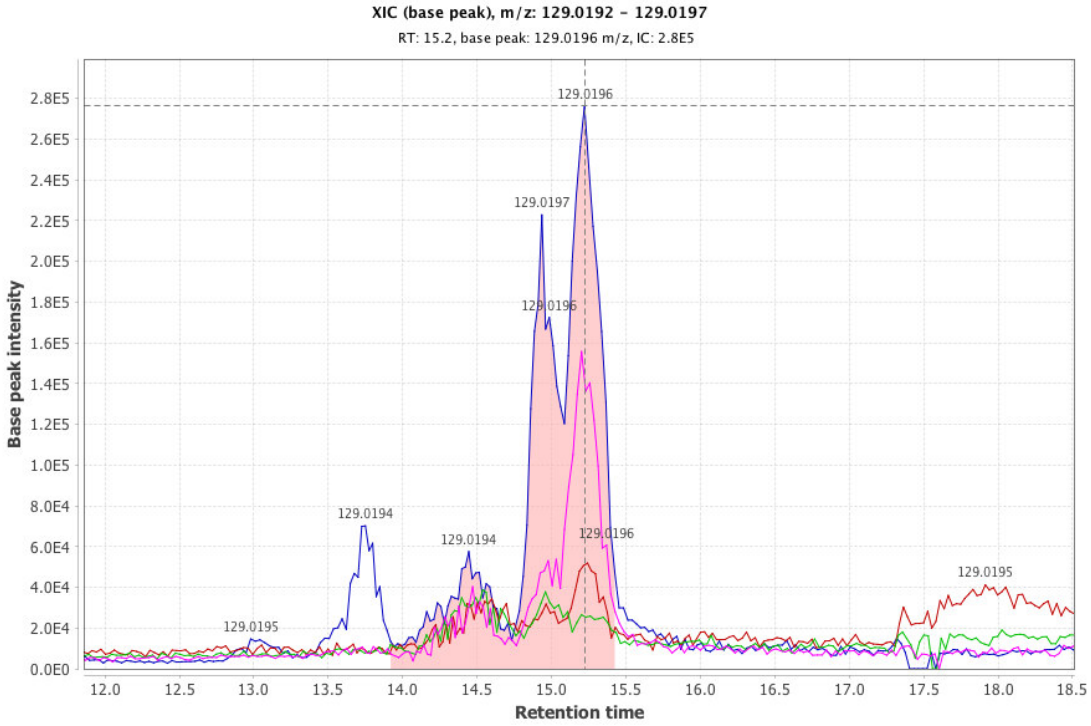


Figure 4-20 LC-MS analysis of samples obtained from Strain 2 under Process 4 conditions

Samples from the fermentation were obtained pre-induction (red), 10 minutes after induction (green), 4 h after induction (pink) and 24 h after induction (blue), and frozen immediately on liquid nitrogen. Cultures were then freeze dried and resuspended in HPLC grade MeOH before processing by LCMS analysis. Samples were separated on HILIC column using mobile phase of acetonitrile and ammonium carbonate Retention time of the analyte was noted at 15.2 min with a suspected mass of 129.0192 g. Analysis of the chromatographs was carried out by Dr Alison MacFadyen using MZmine software (v.2.17).

4.8 **Cultivation of Strain 3 under different process conditions**

Switching to anaerobic conditions in the middle of the process may not be desirable as this could lead to cessation of cellular growth (Inui *et al.*, 2004b, Yamamoto *et al.*, 2011). Deprivation of oxygen could also lead to a change in metabolism with the organism relying on the reductive instead of the oxidative arm of the TCA cycle. As yet, there is no evidence that glutamate can be over produced under anaerobic conditions. This could explain the low levels of glutamate produced by Strain 2 when a micro-aerobic environment was created (Figure 4-15). To continue production of glutamate in an aerobic manner that allowed for the conversion to mesaconate, an oxygen stable glutamate mutase gene was required.

This requirement led to the development of Strain 3 by Ingenza Ltd (Figure 4-6) which contained a more oxygen stable glutamate mutase protein. Unlike the previous engineered strains, Strain 3 contained constitutively expressed genes, therefore, the strain was only examined as a batch process identical to that of the un-induced experiments of Strains 1 and 2 (Figure 4-7 and Figure 4-12). Plasmid construction was also altered with genes for both Glm and tMAAL residing on the same plasmid.

Strain 3 exhibited a significantly higher growth rate, with a maximum of 0.42 h^{-1} achieved over the first 10 h which was approximately double of the parental strain (Figure 4-21). L-glutamate accumulation reached 14.35 gl^{-1} at 27 h which was an increase from the parental strain at this specific time point. However, the maximum yield was only 86 % of that which the parental strain achieved over 48 h. This value was comparable to strain 2. Glucose was consumed in its entirety by 27 h which coincided with a reduction of cellular growth and glutamate production terminating. Therefore, it could be assumed that with the introduction of more glucose through a feeding strategy, growth and production could be improved.

Again, qPCR was used to analyse samples to ascertain expression of the genes inserted (Figure 4-22). As the genes were constitutively induced, they were expected to be expressed by the organism early in the process. At 5 h, expression of both the fused Glm and tMAAL genes was observed, however, the copy numbers obtained

are significantly lower than those obtained using Strain 2. Reduction in expression could be responsible for the inefficient production of mesaconate. Samples obtained from a later point during the process revealed a decrease in overall expression of both sets of genes which is consistent with the organism going into the death phase.

Due to time constraints, this strain could only be analysed once and therefore requires further work to establish the true characteristics. The rapid growth of the organism caused significant foaming of the culture which had not been thus far observed using the previous engineered or parental strains. This unfavourable characteristic will require further examination to help understand its activation in order to be able to limit its detrimental impact in future replicates of this process.

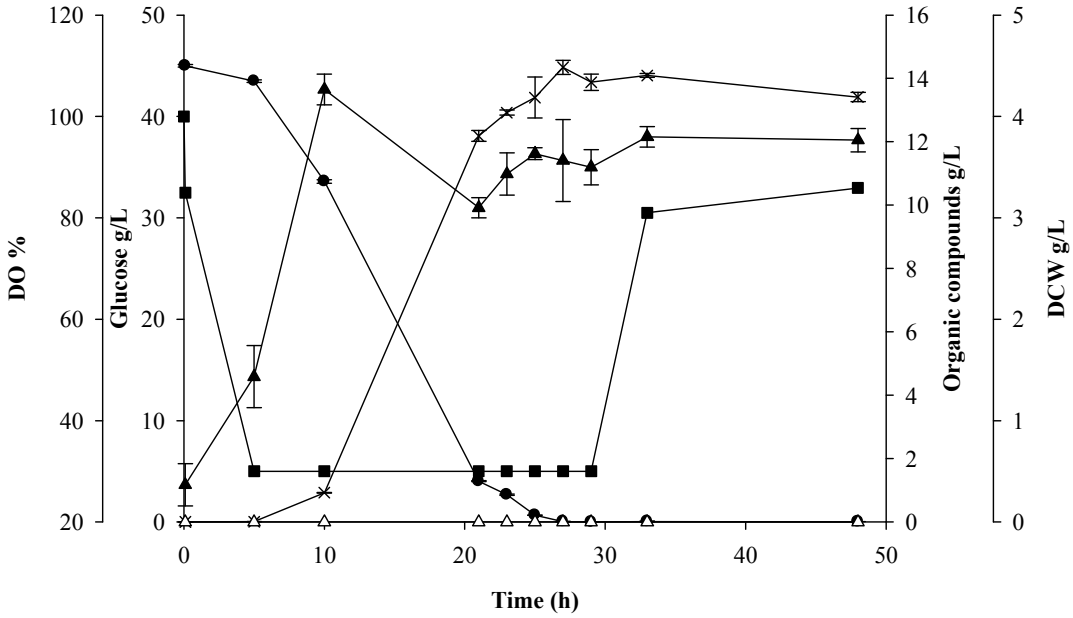


Figure 4-21 Strain 3 analysis using Process 1

A propagation flask of Strain 3 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a Bioflo 110 bioreactor containing 4 L of CGXII biotin limitation media containing 1 μgl^{-1} of biotin to an OD600nm = 1. The process was monitored and control loops set to maintain the dO_2 above 30% (■), the pH at 7, air flow rate at 1 vvm and vessel temperature at 30 °C. Samples were taken regularly aseptically and analysed for glucose consumption (●) and DCW (▲) and glutamic acid (x) and mesaconate (Δ) production. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

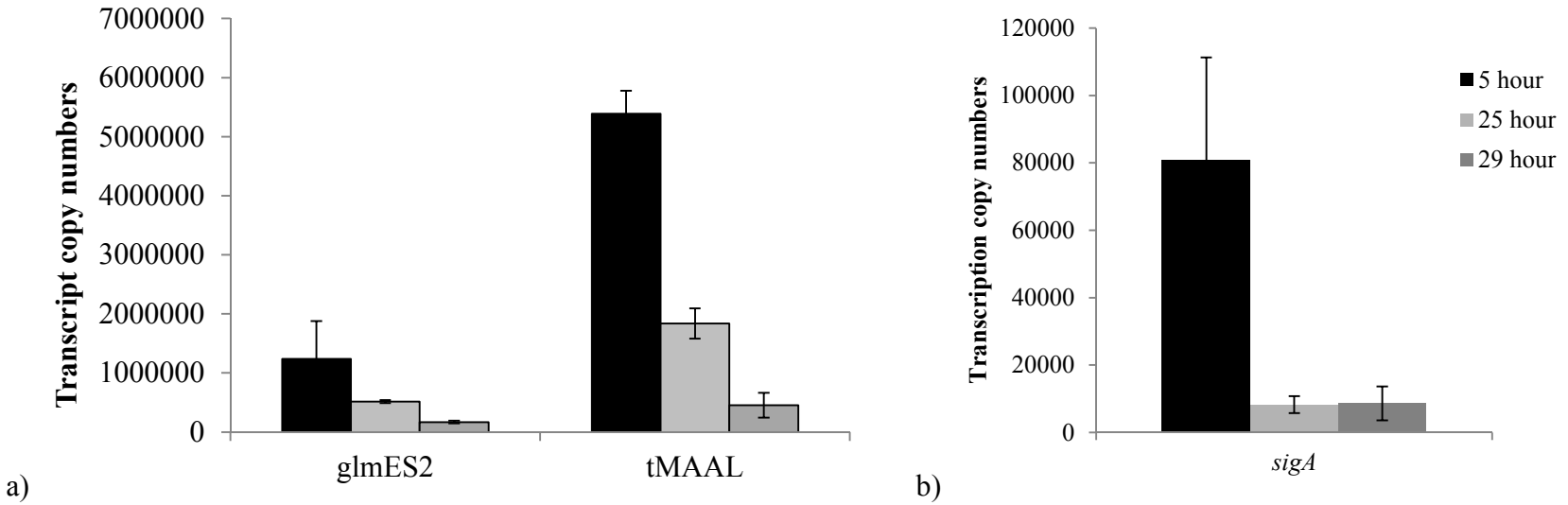


Figure 4-22 qPCR analysis of Strain 3 gene expression duing Process 1

Samples were acquired 5, 25 and 29 h after batch culture was initiated. RNA protect was added to the sample at ratio of 1:2 sample to protectant. Samples were incubated at room temperature for 5 minutes before being pelleted to remove the supernatant. Dried pellets were stored at –80 °C until cells were disrupted an analysed by qPCR by Dr Alison MacFadyen. Transcript copy numbers were then evaluated with the average value and standard deviation of technical replicates being depicted graphically a) *glmES2* and *tMAAL* and b) *sigA*.

4.9 **Chapter 4 Conclusions**

Results obtained during qPCR analysis permitted an insight into the level of expression exhibited by each of the strains examined here. Although the plasmid containing the glutamate mutase genes were inserted into Strain 1 and verified by sequence analysis, overexpression could not be detected either in Process 4 or in flask culture. Therefore, further examination of this strain would not be worthwhile.

In contrast, Strain 2 exhibited over expression of *glmS*, *glmE* and *tMAAL* and displayed promising LC-MS results that indicated low levels of mesaconate production. However, the strain displayed variability during the examination with fluctuating growth rates and L-glutamate productivity. Therefore further work would be required to obtain reproducible results. Interestingly overexpression *glmL* did not appear to occur upon induction with IPTG. Presence of this subunit may play a more significant role in the successful activity of the enzyme. The functionality of this subunit remains ambiguous, however, its presence in the original *Clostridium* strains would suggest that its existence is significant. Therefore, improved expression of this gene may improve production also.

Strain 3 was the first iteration of the genetically engineered strains to contain the glutamate mutase gene that exhibited a higher tolerance to oxygen levels. However, only low level expression was observed from this strain. By transforming this gene into an inducible construct this may improve productivity.

This project is in the early stages of development and requires a great deal of further examination before the process will be suitable for the industrial production of mesaconate. However, overexpression of both glutamate mutase and methylaspartate ammonium lyase in this *C. glutamicum* strain is, to the knowledge of the author, the first of its kind. It is important that the continuation of the strain to over produce glutamate was maintained to be in line with the proposed process. However, it could be construed that putting the organism under additional stress of overproducing led to poor accumulation of mesaconate. It also cannot be confirmed as yet that the

glutamate produced by the strains can be converted to mesaconate, therefore, further work is required to understand the optimum process conditions.

4.10 **Future work for Chapter 4**

- Although strain 3 exhibited expression of both genes, qPCR results showed reduction in copy numbers of the genes compared to the previous inducible strains. Production of an inducible strain containing the oxygen stable glutamate mutase gene would allow for enhanced control over the expression of the proteins. At the time of writing, this strain was currently under development by Ingenza Ltd.
- Examination of the expression of the genes under non-glutamate producing conditions. Although in *E. coli*, Wang and Zhang (2015) displayed successful *de novo* conversion of glucose to mesaconate without inducing the overproduction of glutamate. Therefore, to understand if mesaconate production was being hindered due to cellular stress, Strain 2 could be examined under a surplus of biotin. By understanding if cellular stress affected the activity of the proteins would assist in the future development of the process.
- If a successful microaerobic process were to be developed, then production of glutamate would have to be maintained under the oxygen limited conditions. Process development for the production of L-glutamates precursor α-ketoglutarate under this environment is discussed in more detail in Chapter 5.
- Regeneration of coenzyme B12 will be vital for the prolonged production of mesaconate as the internal concentration of the cofactor is essential for the efficiency of the glutamate mutase enzyme. Again, this has been successfully proven by Wang and Zhang (2015) by overexpressing genes that were essential for the regeneration process. A similar approach could also be employed in the *C. glutamicum* strain to improve the activity of the enzyme.
- Once successful production is established under a batch culture, a fed strategy could be implemented to increase end production yield.

Chapter

5

Production of organic compounds under oxygen limited conditions

5.1 Chapter 5 Background

Aerobic cultivation of *C. glutamicum* has been well documented in the literature and has been adopted, for a number of years, by industry for the production of a variety of compounds destined for the food, pharmaceutical and chemical sectors. However, anaerobic production of desirable compounds by this organism has only recently received more focus from industry. For industry, the major advantage of anaerobic over aerobic processes ultimately comes down to cost. Anaerobic bioreactors require lower specifications than their aerobic counterparts leading to a reduction in construction and, as well as, running costs. Such processes generally still require an aerobic growth phase to achieve high cell densities, which are then harvested and used to populate the anaerobic vessel. The use of a high cell density in itself facilitates the high end product yield. Moreover, as no growth is required in this second stage, microbial energy is primarily used for production creating a more cost efficient process. Also, unlike oxygen limitation during growth, low by-product accumulation has been observed in micro-aerobic bioconversions as carbon flux is directed at the desired compounds and therefore supports high end yields (Inui *et al.*, 2004b).

Anaerobic production by anaerobes and facultative anaerobes has already been used within industry to produce a variety of organic acids such as acetic, lactic, succinic, citric, propinonic and butyric acid (Okino *et al.*, 2005). As with glutamic acid, which has been discussed in previous chapters (Chapter 3), production of the above compounds, in particular lactic and succinic acid, have received increasing amounts of interest as they can be used as an alternative building block chemical for historically petroleum based materials such as the production of biodegradable polymers (Hofvendahla and Hahn-Hägerdala, 2000).

Traditionally, lactic and succinic acid have been produced by anaerobes such as members of the lactic acid bacterial genre, examples of which are from the *Lactococcus spp*, *Enterococcus ssp,* and *Anaerobiosirillum succiniciproducens* (Guettler and Jain, 1996) respectively. Although these organisms are capable of producing their respective compounds at high levels, they rely on a complex medium

containing material such as whey and corn steep liquor. These make the purification and recovery of the desired compound more difficult. At large scale, this increases the cost of downstream processing, which would ultimately affect the products worth and its use as a more cost efficient alternative raw material. Progress has been made with respect to engineered strains that can produce both lactic and succinic acid using both minimal medium and renewable raw materials (Durnin *et al.*, 2009). Mazumdar *et al.* (2013b) utilised metabolically engineered *E. coli* strains to produce lactic acid from glycerol which exhibited 93 % of the theoretical yield and 97 % chemical purity.

Continued research to obtain an optimised organism that can be utilised to produce high levels of desirable organic acids, with a process that is competitive with existing commercial methods, has led to further investigation of the potential use of the industrial workhorse *C. glutamicum*. As this organism has a long historical use within other sectors of industry, adaption into this particular area could be easily implemented as regulatory guidelines for standard operating procedures already exist for bacterial cultivation.

It is generally agreed that under oxygen deprived conditions the growth of *C. glutamicum* is restricted (Inui *et al.*, 2004b, Fukui *et al.*, 2011, Yamamoto *et al.*, 2011). However, secretion of various organic acids by *C. glutamicum* ATCC 17965 was described by Dominguez *et al.* (1993) where they observed lactate, succinate and acetate production under oxygen deprived conditions. As a consequence of these findings, further research has allowed for a fuller understanding of the underlying mechanisms permitting *C. glutamicum* to retain its metabolic activity. Significantly, the central enzymes that are crucial in the anaerobic metabolism have been identified and are discussed in the next section. Previous discussion about the importance of these genes in carbon metabolism has also been covered in Section 3.1.2.

By disrupting the *ppc* gene, Inui *et al.* (2004b) identified phosphoenolpyruvate carboxylase (PEPC) as a key enzyme in the production of succinic and lactic acid. The *ppc* mutant gave rise to lower production of both the organic acids, decreased glucose utilisation and reduced NAD^+/NADH ratio. As this enzyme is responsible

the synthesis of oxaloacetate, from phosphoenolpyruvate, its disruption would limit the generation of succinate precursors.

In contrast, pyruvate carboxylase (PC), which catalyses the production of oxaloacetate by its conversion from pyruvate, appeared not to be dominant during oxygen deprivation. Over expression of the *ppc* gene did not increase the production of succinic acid suggesting that this was not a bottleneck of the process. Addition of sodium bicarbonate, which is fixed by both PEPC and PC, did lead to an increased production of succinate by 3.6 fold, glucose consumption by 2.5 fold and lactate production by 2.3 fold. Interestingly, a disruption of both the *ldh*A (lactate dehydrogenase) and the *ppc* genes result in total inhibition of the glycolytic pathway as NADH regeneration is disabled. To demonstrate the organic acids were being produced by the reductive arm *only* of the TCA cycle, a fumerase mutant was created which was incapable of producing any succinate. In addition, no CO_2, a key indicator of the forward pathway produced during the conversion of isocitrate to α-ketoglutarate and α-ketoglutarate to succinyl-CoA, was detected throughout the experiment.

Increasing expression of these genes and their impact on enzymatic activity under oxygen deprived conditions are not exclusive to *C. glutamicum*. Yamamoto *et al.* (2011) examined 17 strains closely related to *C. glutamicum*, of which 8 were capable of producing organic acids under oxygen deprived conditions. As with the previously discussed study (Inui *et al.*, 2004b) PEPC and LDH activity appear to be crucial in the production of both succinic and lactic acid with the differing levels of enzyme activity relating to the specific organism's ability to produce the certain organic acids. Unlike *C. glutamicum* R and ATCC 13032 which both exhibited high levels of LDH and PEPC activities, *Corynebacterium halotolerans* levels were reduced and resulted in decreased production of both lactic and succinic acid. In contrast, *Corynebacterium efficiens* harbours all of the genes required to produce organic acids anaerobically but no production of succinate was observed. However, this strain possess low levels of fumerase activity which reiterates the theory that succinate is produced through the reductive arm of the TCA cycle under oxygen deprived conditions (Figure 5-1).

With the production of organic acids using *C. glutamicum* becoming ever more prominent, several new engineered strains have been developed that, as well as displaying high yields also exhibited a reduction in by-product formation. Litsanov *et al.* (2012) developed a series of engineered strains that firstly lacked essential genes required in all known pathways for the production of acetate and lactate (BOL-1 strain). This strain was capable of producing succinate in a 1:1 molar ratio to glucose carbon source. The addition of chromosomal integration of the PC gene (BOL-2 strain) did not increase the production of succinate which is in agreement with the earlier study by Dominguez *et al.* (1993). However, significant changes in both glucose consumption and succinate accumulation resulted in observed increases of 80 and 85% respectively. Mutant BOL-3 was then subsequently engineered to obtain a strain containing formate dehydrogenase gene (*fdh*) that allowed for the formation of additional reducing cofactors from formate contained in the medium. Increased cofactor generation from this conversion was sought as this would increase the availability of NADH required for the conversion of oxaloacetate to malate in the reductive TCA cycle. With the addition of a single copy of the *fdh* gene, an increase in succinate production was observed from 1.05 Mol/Mol (succinate/glucose) when using glucose as the sole carbon source to 1.26 Mol/Mol (succinate/glucose) when a mix of glucose and formate were used. Undesirably, these values were obtained from only the first three hours of the process as the organism appeared to enter a stationary phase. To overcome this undesirable phase, overexpression of *gapA*, responsible for glyceraldehyde 3-phosphate dehydrogenase enabled the yield to be increased further to 1.67 Mol/Mol (succinate/glucose), one of the highest yields reported for anaerobic production of succinate.

5.2 **Novelty**

C. glutamicum synthesis of succinate under micro-aerobic/anaerobic conditions has been thoroughly demonstrated in the literature and has the potential to be implemented by industry in the near future. However, continuation of the reductive arm of the TCA cycle towards α-ketoglutarate has yet to be thoroughly investigated

(Figure 5-1). Successful production of this key organic acid in this manner could give rise to the potential of developing a bioprocess that could be more cost effective due to efficient production of high end titres and low by-product formation.

As discussed previously in Chapters 3 and 4, the proposed pathway towards methyl methacrylate requires the efficient production of L-glutamate. In order to achieve this, the TCA cycle must function optimally to synthesise α-ketoglutarate. Due to its proven ability to produce succinate in an efficient manner under anaerobic conditions, the Bol-3/pAN6-*gap* strain was obtained and used as an initial starting point for future strain iterations (Table 5.1). By introducing the ketoglutarate synthase gene within the stain, it was proposed that under optimal conditions the titre of succinate exhibited by Bol-3/pAN6-*gap* would be converted to α-ketoglutarate (Figure 5.1). Future development of the process and the strain would then be required to produce the desired L-glutamate, the initial building block in the proposed pathway for methyl methacrylate. From there, it was proposed that a series of enzymes would be engineered into the strain to produce the intermediate mesaconate, aerobic production of which was discussed in Chapter 4. Further downstream processing would then be required for the conversion of mesaconate to methacrylic acid by a decarboxylation before finally undergoing esterification to produce methyl methacrylate.

Although not the main focus of the final process, *E. coli* was also considered as a candidate for the production of organic acids through the reductive arm of the TCA cycle. This versatile Gram-negative bacterium has an extensive history as an industrial workhouse for a variety of products including organic acids (Wendisch *et al.*, 2006) and recombinant proteins (Voulgaris *et al.*, 2011). Recent results have also demonstrated the organism's ability to produce mesaconate, a key intermediate in the process, aerobically (Wang and Zhang, 2015). *E. coli* also has a large molecular toolbox which assists in the efficient genetic manipulation of the organism. Here, Ingenza Ltd. utilised the *E. coli* cell line BW25113, to introduce similar genes to those that were introduced to the above *C. glutamicum* strain (Table 5.1)

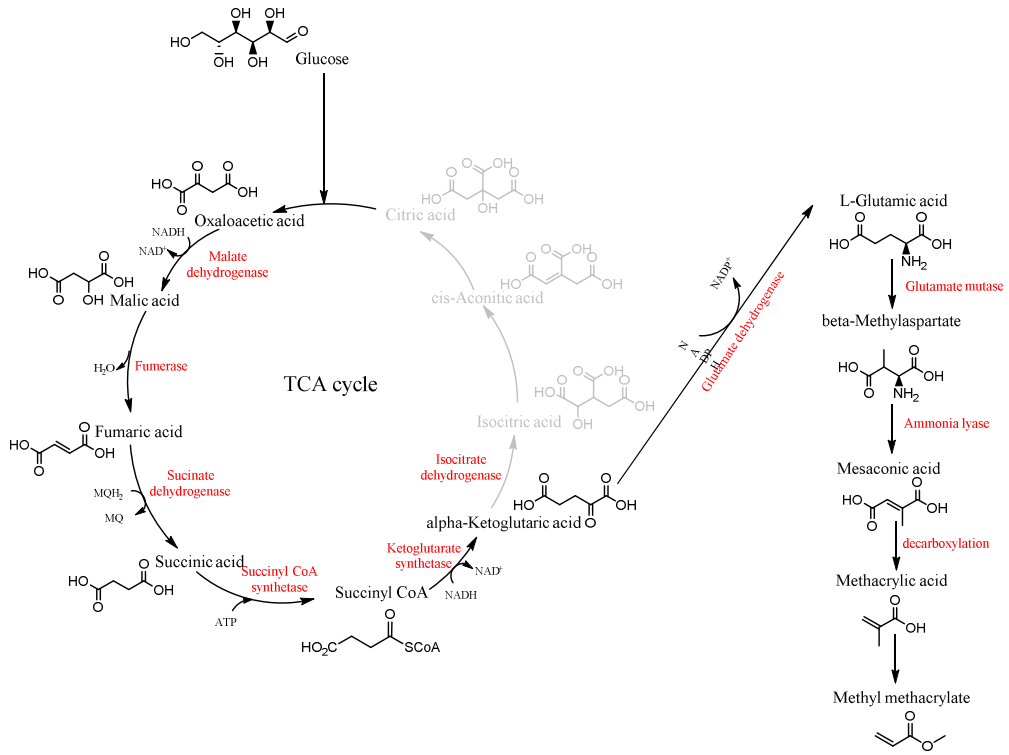


Figure 5-1 Proposed biological/chemical production of methyl methacrylate from glucose

Using modified strains of *C. glutamicum*, glucose is utilised by the cell through the glycolysis pathway. Under anaerobic conditions it was proposed that the carbon would then flux through the reductive branch of the TCA cycle with α-ketoglutarate being the terminal compound. The conversion of succinyl CoA to α-ketoglutarate would be catalysed by ketoglutarate synthase. Glutamate dehydrogenase would then catalyse the conversion between α-ketoglutarate and L-glutamate in the presence of ammonia and NADPH. From L-glutamate, a series of chemical reactions using glutamate mutase and ammonia lyase would produce mesaconic acid. Decarboxylation of mesaconic acid would produce methacrylic acid with its esterification creating methyl methacrylate. Molecules in grey depict the remainder of the TCA cycle.

5.3 **Process Overview**

To examine the possibility of enhancing the production of α-ketoglutarate under oxygen deprived conditions, several engineered strains of *C. glutamicum* and *E. coli* were designed in house by Ingenza Ltd. and are outlined in (Table 5-1). These strains contained important genes crucial for the enzymes required for the improvement of the reductive arm of the TCA cycle towards α-ketoglutarate. In particular, incorporation of the ketoglutarate synthase (KGS) required for the conversion of succinyl CoA to α-ketoglutarate was expected to drive the carbon flow further around the TCA cycle than has previously been reported.

Similar to the literature discussed in Section 5.1, which analysed the production of organic acids under oxygen deprived conditions, a biotransformation process was developed (Section 2.4.2). Primarily, the strain was cultured aerobically to obtain a high cell density. During this growth phase, depending on the strain in question, inserted genes were either constitutively expressed or induced by IPTG. Cells were then harvested and either immediately used, stored at 4 °C or -20°C. Examining different storage conditions were crucial for future development of the industrial process as it gave an insight into the length of at which the cells could maintain their enzymatic activity over a period of time.

Harvested cells were then resuspended in biotransformation medium at a cell density of 4 g/150 mL. The standard medium consisted of glucose, sodium chloride and sodium bicarbonate with the addition of sodium formate, ammonium formate, iron sulphate or L-cysteine in certain cases in an attempt to improve process conditions. The latter two compounds were added as they are required for the formation of the KGS, as it contains at least four Fe-S clusters in the holoenzyme structure. These changes in process conditions were heavily influenced by industrial demands at the time of investigations.

Bioconversions were generally carried out in round bottomed flasks which were constantly heated to 30 °C when examining *C. glutamicum* or 37 °C when examining

Table 5-1 Strain construction

Strain	Organism	Genotype	Plasmid	Gene	Source
IL.1.6.6.(K).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pAN6	GapDH	(Litsanov *et al.*, 2012)
IL.1.6.5.(A).0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pIL-3 (pEKEx3 Ptuf)	Ketoglutarate synthase	Ingenza Ltd.
IL.1.6.1.0	*C. glutamicum* ATCC 13032	*ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pyc^{P4585} ΔldhA*	pEKEx3	Empty vector	Ingenza Ltd.
IL.1.7.0.0	*C. glutamicum* ATCC 13032	ΔcatΔpqo::PtuF_FDH Δpta-ack::PtuF_pycP4585 *ΔldhA* ΔsucA	pEKEx3	ΔSucA	Ingenza Ltd.
IL.2.1.1.0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Empty vector	Ingenza Ltd.
IL.2.1.1.(A).0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Ketoglutarate synthase	Ingenza Ltd.
IL.2.1.1.(ABC).0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Ketoglutarate synthase Pyruvate synthase Ferredoxin	Ingenza Ltd.
IL.2.1.1.(ADE).0	*E. coli* BW25113	*ΔldhA*	pEKEx3	Ketoglutarate synthase Flavoprotein reductase Flavoprotein	Ingenza Ltd.

All genetic manipulation were carried out by Ingenza Ltd. to company standard operating procedures

E. coli unless otherwise stated. This was achieved using a thermocoupled heating block and was stirred with a magnetic stirrer bar at 250 rpm. Samples were periodically obtained and examined for organic acid production by HPLC analysis. In certain cases, a methyl viologen (MV) assay was performed on cell free extracts (CFE) of the strains by Ingenza Ltd. The assay saw the conversion of α-ketoglutarate to succinyl CoA .catalysed by KGS enzyme present in the CFE. This was coupled with the reduction of methyl viologen with the transfer of an electron generating a UV active methyl viologen cation radical species. Detection of the radical species was made by the spectrophotometrically at 578 nm and was a sign of successful KGS activity.

Further modifications to this protocol were made to examine the process under a nitrogen blanket to create an anaerobic environment. These are detailed, where employed, in the relevant sections below.

5.4 **Aims**

The aim of this part of the research programme was to develop an anaerobic process for the successful overproduction of α-ketoglutarate, with the prospect of producing L-glutamate in the future. To determine critical enzymatic bottlenecks of the reductive TCA cycle, an enzymatic assay was developed to examine the activity of succinyl CoA synthetase. An isocitrate dehydrogenase assay was also developed in order to discount the activity of the forward pathway of the TCA cycle.

5.5 **Biotransformation of *C. glutamicum* strain 1 IL.1.6.6.(k).0**

IL.1.6.6.(K).0 was created by Litsanov *et al.* (2012) and was entitled Bol-3/pAN6 *gap*. This strain lacks multiple genes necessary for the synthesis of acetate and lactate. These genes include coA transferase (*cat*), pyruvate:quinone oxidoreductase (*pqo*), phosphotransacetylase and acetate kinase (*pta-ack*) and lactate dehydrogenase

(*ldhA*). It also has two chromosomal gene integrations of the pyruvate decarboxylase gene, pyc^{P4585}, increasing flux through glycolysis and an NAD^+ coupled formate dehydrogenase, *fdh*, providing additional reducing power for the cell. A further modification to improve flux in glycolysis was made by plasmid borne expression of glyceraldhyde-3-phosphate dehydrogenase gene, g*apDH*.

As stated in the process overview (Section 5.3) the constitutively expressed strain was aerobically cultivated in CGXII media for 16 h to produce a high biomass. The culture was then harvested and either used immediately (Fresh), or stored at 4 or -21 °C before use (Section 2.4.1). For the process, the pelleted cultures were resuspended in the biotransformation standard medium with or without the addition of ammonium or sodium formate (Section 0) and transferred to a round bottom flask. Process temperature was maintained at a constant temperature of 30 °C and stirred continuously. Samples were obtained periodically to access the accumulation of residual organic acid and utilisation of glucose and ammonium formate. Conditions and the correlating production of organic acids are outlined in Table 5-2.

The strain was predominately created for over production of succinic acid under anaerobic conditions. This goal was achieved with the accumulation of 14.6 gl^{-1} succinate after 21 h. In this study, equivalent levels of succinic acid yields were observed in the absence of ammonium formate when the sample was fresh. The rate of accumulation was lowered as the sample was stored over a period of time. Faster deterioration was observed if the sample was stored at 4 °C rather than -20 °C. The other main compounds produced in this study were pyruvic acid and α-ketoglutarate. Compared to previously reported results, in the absence of formate fresh cells accumulated almost double the titre of pyruvic acid whereas α-ketoglutarate was halved. As with succinate, the accumulation rate was reduced when the cells were stored.

With the presence of *fdh* gene, the organism was able to utilise formic acid resulting in increased levels of intracellular NADH (Litsanov et al. 2012). This in turn would allow the potential for increased productivity of anaerobically accumulated organic acids, in particular succinic acid. Comparing experiments with and without formate presents no significant increase in the main products observed. This suggests that

other factors are impeding further production. In the presence of formate, glucose consumption rate is increased, therefore reducing the initial concentration of formate may help to alleviate possible inhibitions.

The shift to ammonium formate from sodium formate increased ammonium ion concentration in the cell. Ammonia is a key requirement for the synthesis of L-glutamate from α-ketoglutarate. However, very little production of L-glutamate was seen throughout the experiments even in the presence of ammonia. A factor that was not considered at the time was the requirement for NADPH as a cofactor in the reaction. Anaerobically, NADPH is not produced, resulting in very little conversion. Therefore, increased levels of NADPH would be essential.

Table 5-2 Summary of Biotransformation by Strain IL.1.6.6.(K).0

Biotransformation No.	Medium	Induction	temp (°C)	Cultivation (h)	storage (°C)	Biotrans medium	Succinate gl^{-1}	Pyruvate gl^{-1}	αkg gl^{-1}
1	CGXII+kan200	C	30	16	4	Standard	11.8	3	1
2	CGXII+kan200	C	30	16	4	Standard	12.0	3.8	0.7
3	CGXII+kan200	C	30	16	4	+HCOONa	13.8	1.2	0.2
4	CGXII+kan200	C	30	16	Fresh	Standard	10.0	3.0	1.1
5	CGXII+kan200	C	30	16	4	standard	9.8	1.3	0.8
6	CGXII+kan200	C	30	16	-20	+NH4HCO2	11.6	1.4	1.5
7	CGXII+kan200	C	30	16	-20	+NH4HCO2	14.3	1.0	0.0

This table summarises the data gathered from biotransformations using strain IL.1.6.6.(K).0. Numbers 1-4 denote experimental evidence gathered prior to start of project by Ingenza Ltd. To obtain a high cell density for the biotransformation, the strain was initially grown in CGXII medium containing kanamycin for 16 h before the culture was harvested. Several condition parameters were examined including storage of cells before use, addition of either sodium formate (HCOONa) or ammonium formate (NH_4HCO_2) to the biotransformation standard medium. The biotransformation was maintained at a constant temperature of 30 °C with samples acquired periodically and Samples were heat shocked at 95 °C for 5 mins and centrifuged at 13500 rpm to pellet the cells. Supernatant obtained from the samples were examined for residual organic acid content. Values for Succinate, pyruvate and α-ketoglutarate (αkg) are conveyed in the table.

5.6 **Biotransformation *C. glutamicum* strain IL.1.6.5.(A).0**

IL.1.6.5.(A).0 strain, was again based on the Bol-3 strain, with the addition of the α ketoglutarate synthetase (KGS) gene from *Chlorobium tepidum*. This enzyme was required to catalyse the conversion of succinly CoA to α-ketoglutarate. To understand how the expression of this gene would improve the flux of carbon through to alpha-ketoglutarate, varying growth and process conditions were examined and are outlined in Table 5-3. The organism was initially grown aerobically in either CGXII or CGG medium (Section 2.4.1). As this was a constitutively expressed strain, cultures were grown for varying times to understand the optimal growth for efficient expression. Cells were then harvested and either used immediately in the bioconversion or stored at 4 or -20 °C to access strain viability on storage. Pelleted cells were resuspended in the biotransformation medium (Section 2.4.2) with or without ammonium formate. Addition of iron sulphate, L-cysteine was also investigated in attempt to improve the enzyme activity as the coupounds are both required in structure formation of KGS. Organic acid production was monitored during a biotransformation and maximum yields of significant compounds are presented in Table 5-3.

Initial preparation of the harvested cells appeared to have significant impact on the biotransformation product composition, with the main analytes being succinate, pyruvate and alpha-ketoglutarate. The type of growth medium directly affected the activity of the KGS, with a methyl violegen assay exhibiting a negative result when the organism was grown in a minimal medium (GGXII). When the organism was grown in a rich medium (CGG), KGS activity was observed, resulting in this medium being favoured for future experiments. However, expressed activity in the MV assay did not always correlate with increased yield of α-ketoglutarate in the biotransformation.

The addition of L-cysteine into the growth medium increased the production of pyruvate in the presence of formate significantly compared to when grown in its

Table 5-3 Summary of Biotransformation by Strain IL.1.6.5(A).0

Biotransformation No.	Medium	FeSo4	L-cys	Induction	Growth phase temp (°C)	Cultivation (h)	storage (°C)	Biotrans medium	Succinate gl^{-1}	Pyruvate gl^{-1}	αkg gl^{-1}	MV assay*
1	CGXII+spec200	N	N	C	30	16.5	Fresh	Standard	14.1	3.1	1.1	N/A
2	CGXII+spec200	Y	N	C	30	16.5	Fresh	+ NH_4HCO_2	14.8	1.1	0.2	N/A
3	CGXII+spec200	N	N	C	30	16.5	Fresh	+ NH_4HCO_2	20.2	1.0	0.2	N/A
4	CGG+spec200	N	N	C	30	16.5	Fresh	+ NH_4HCO_2	9.9	1.9	0.2	++
5	CGG+spec200	Y	Y	C	30	6	-20	+ NH_4HCO_2	9.5	5.2	1.3	+
6	CGG+spec200	Y	Y	C	30	22.5	-20	+ NH_4HCO_2	4.3	0.7	0.0	+
7	CGG+spec200	Y	Y	C	37	6	-20	+ NH_4HCO_2	7.3	10.6	1.2	+
8	CGG+spec200	Y	Y	C	37	22.5	-20	+ NH_4HCO_2	8.3	12.1	0.9	N/A
9	CGG+spec200	Y	N	C	30	24	-20	+ NH_4HCO_2	8.6	0.0	2.9	-
10	CGG+spec200	N	N	C	30	24	-20	+ NH_4HCO_2	7.7	0.0	2.1	-

This table summarises the data gathered from biotransformations using strain IL.1.6.5(A).0. To obtain a high cell density for the biotransformation, the strain was initially grown in CGXII or CGG medium containing spectinomycin for between 16-24 h before the culture was harvested. Several condition parameters were examined including storage of cells before use, addition of ammonium formate (NH_4HCO_2), iron sulphate ($FeSO_4$) or L-cysteine (L-cys) to the biotransformation standard medium. Gluconate was also used a replacement carbon source for biotransformation 14 and 15.The process was maintained at a constant temperature of 30 °C with samples acquired periodically. Samples were heat shocked at 95 °C for 5 mins and centrifuged at 13500 rpm to pellet the cells Supernatant obtained from the samples were examined for residual organic acid content. Values for Succinate, pyruvate and α-ketoglutarate (αkg) are conveyed in the table. *Work carried out by Ingenza Ltd.. N/A work not available at time of writing.

Table 5-3 Summary of Biotransformation by Strain IL.1.6.5(A).0 continued

Biotransformation No.	Medium	FeSo4	L-cys	Induction	Growth phase temp (°C)	Cultivation (h)	storage (°C)	Biotrans medium	Succinate gl^{-1}	Pyruvate gl^{-1}	αkg gl^{-1}	MV assay *
11	CGG+spec200	Y	N	C	30	24	-20	+ NH_4HCO_2	8.4	3.1	0.2	+
12	CGG+spec200	Y	N	C	30	24	Fresh	+ NH_4HCO_2	15.6	2.9	0.2	N/A
13	CGG+spec200	Y	N	C	30	24	Fresh	gluconate + NH_4HCO_2	0.9	0.0	0.1	N/A
14	CGG+spec200	Y	N	C	30	24	Fresh	glucose/gluconate NH_4HCO_2	8.8	0.0	0.0	N/A
15	CGG+spec200	Y	N	C	30	24	Fresh	Standard	13.8	9.2	0.1	N/A

Continuation of the table summarises the data gathered from biotransformations using strain IL.1.6.5(A).0. To obtain a high cell density for the biotransformation, the strain was initially grown in CGXII or CGG medium containing spectinomycin for between 16-24 h before the culture was harvested. Several condition parameters were examined including storage of cells before use, addition of ammonium formate (NH_4HCO_2), iron sulphate ($FeSO_4$) or L-cysteine (L-cys) to the biotransformation standard medium. Gluconate was also used a replacement carbon source for biotransformation 14 and 15.The process was maintained at a constant temperature of 30 °C with samples acquired periodically. Samples were heat shocked at 95 °C for 5 mins and centrifuged at 13500 rpm to pellet the cells Supernatant obtained from the samples were examined for residual organic acid content. Values for Succinate, pyruvate and α-ketoglutarate (αkg) are conveyed in the table. *Work carried out by Ingenza Ltd.. N/A work not available at time of writing.

absence. The same increase was not observed for α-ketoglutarate. Therefore, the use of cysteine was eliminated from the growth medium.

Temperature during the growth phase may also effected the organic acid production during the biotransformation, with significantly increased levels of pyruvic acid observed when cells were grown initially at 37 °C compared to 30 °C. This increase in productivity was observed with cells harvested at 6 h and 22.5 h cultivation suggesting that temperature was more of an influence over cellular characteristics rather that cultivation time. Similar increases of either succinate or α-ketoglutarate were not detected. It could be assumed that the increased temperature during the growth phase could affect the ability of the enzyme involved with glycolysis and the TCA cycle, which could then confer a disruption in productivity during the biotransformation. In this case, glucose conversion to pyruvate appears to be up-regulated.

Overall, very little α-ketoglutarate was observed, with 0.1 gl^{-1} commonly occurring. This increased to 2.9 and 2.1 gl^{-1}, in the presence (Biotransformation No. 9) and absence (Biotransformation No. 10) of iron sulphate respectively in the growth medium. The cell pellets used in this experiment were frozen on harvesting, which, compared with pellets stored at 4 °C, had higher productivity. Pyruvate accumulation was also abolished. However, the methyl viologen assay for these pellets exhibited no activity for KGS. This suggests that another pathway is being used by the cell to produce α-ketoglutarate. The oxidative arm of the TCA cycle is normally thought to be inactive under anaerobic conditions, however, hypothetically the cycle could be active if sufficient cofactors were present. This theory has yet to be proven within this piece of work and would require further validation.

The ultimate end goal of the biotransformation was to produce L-glutamate which requires an NADPH driven step. Aerobically, the NADPH is generated during the production of α-ketoglutarate from isocitrate. However, during anaerobic production with glucose being the sole carbon source there is a deficiency of this cofactor. In an attempt to increase the levels of NADPH present in the cell, glucose was substituted for gluconate in the biotransformation. Gluconate is utilised by the cell through the pentose phosphate pathway yielding 2 molecules of NADPH which could then be

used to produce L-glutamate. However, during the biotransformation no utilisation of gluconate was observed and very little production of organic or amino acids was detected. Prior to the biotransformation, the biomass had been developed in CCG medium containing glucose as the carbon source. One argument would be that the organism needs to be cultured using gluconate as the sole carbon source. Presence of gluconate during the growth phase is important in regulating the genes required for its utilization (Frunzke *et al.*, 2008). Therefore, if the genes are not expressed, no utilisation will occur. A second argument would be that a negative feedback loop was being created with the production of excessive NADPH. NADPH is a potent inhibitor of 6-phosphogluconate dehydrogenase, an enzyme required for the conversion of 6-phosphogluconate to ribulose 5-phosphate during the pentose phosphate pathway (PPP) (Moritz *et al.*, 2000). Therefore, if this enzyme was inhibited, then utilisation of the gluconate through the PPP would halt, with further product synthesis being obstructed.

The reproducibility of this strain is still in an experimental stage due to the instability of the KGS gene. By scaling up of the biotransformation volume to 500 mL and 1L (Section 2.4.3), analysis of the strain could be examined under more controlled anaerobic condition. At 500 mL scale, an inert atmosphere, using a nitrogen balloon, was created. At 1L scale, the biotransformation was prepared in a Dasgip bioreactor, with the medium maintained under a nitrogen blanket. Production of organic acids from both of these systems was low, suggesting a microareobic environment may be better suited to for the future process. Microaerobic conditions may increase the utilisation of the carbon source and, in turn, increase the efficiency of the process. This has been successfully demonstrated in *E. coli*, where glycerol utilisation was improved under microaerobic compared to anaerobic conditions (Durnin *et al.*, 2009). In this case, the microaerobic environment was established by flushing the head space of the bioreactor with air at a flow rate of 0.01 L/min.

5.7 **Biotransformation of *C. glutamicum* strain IL.1.6.1.0**

IL.1.6.1.0 was a strain based on the Bol3 construct. Subsequent additions to this strain were made using a pEKEx3 plasmid containing; A= ketoglutarate synthase gene, ABC = ketoglutarate synthase, pyruvate synthase and ferredoxin genes or ADE = ketogluarate synthase, flavoprotein reductase, flavoprotein (flavodoxin) genes. Addition of pyruvate synthase and ferredoxin was hypothesised to increase the conversion of pyruvate to acetyl CoA and therefore increase the flux of carbon into the TCA cycle. With inclusion of the flavoproteins genes, it was hypothesised that additional cofactors would be available crucial for the reductive arm of the pathway.

Similar to previous experiments in this chapter, the organism was cultured aerobically to create a high cell density for the biotransformation. During this growth phase, genes were induced by the addition of IPTG to the medium for 3 or 22 h before harvesting and storage at -20 °C. Pelleted cells were resuspended in biotransformation medium (Section 0) containing ammonium formate and transferred to a round bottom flask. Process temperature was maintained at a constant temperature of 30 °C and stirred continuously. Samples were obtained periodically to access the accumulation of residual organic acid and utilisation of glucose and ammonium formate.

No significant difference in organic acid composition was observed between the parental strain and the subsequent mutants when the same induction length was implemented. (Table 5.4) However, increasing induction time appears to have an effect by lowering the natural production of organic acids and increasing the rate of glucose consumption. It could be hypothesised that culturing for different periods of time could lead to the creation of different levels of key enzymes and therefore end productivity would be completely different.

Further work is required to determine the significance of this strain. However a methyl viologen test exhibited no KGS activity therefore an understanding of this enzymes optimum condition has to be gathered.

Table 5-4 Summary of Biotransformation by Strain IL.1.6.1.0

Strain	Medium	Induction	temp (°C)	Induction length (h)	storage (°C)	Biotrans medium	Succinate gl^{-1}	Pyruvate gl^{-1}	αkg gl^{-1}
IL.1.6.1.0	CGG+spec200	IPTG	30	3	-20	+ NH_4HCO_2	8.47	7.91	1.31
IL.1.6.1.0	CGG+spec200	IPTG	30	22	-20	+ NH_4HCO_2	5.57	3.34	0.08
IL.1.6.1.(A).0	CGG+spec200	IPTG	30	3	-20	+ NH_4HCO_2	8.1	9.06	1.3
IL.1.6.1.(A).0	CGG+spec200	IPTG	30	22	-20	+ NH_4HCO_2	6.77	3.91	0.07
IL.1.6.1.(ABC).0	CGG+spec200	IPTG	30	3	-20	+ NH_4HCO_2	9.15	9.46	1.62
IL.1.6.1.(ABC).0	CGG+spec200	IPTG	30	22	-20	+ NH_4HCO_2	5.82	4.41	0.1
IL.1.6.1.(ADE).0	CGG+spec200	IPTG	30	3	-20	+ NH_4HCO_2	8.23	9.02	1.28
IL.1.6.1.(ADE).0	CGG+spec200	IPTG	30	22	-20	+ NH_4HCO_2	4.98	3.42	0.1

This table summarises the data gathered from biotransformations using strain IL.1.6.5(A).0. To obtain a high cell density for the biotransformation, the strain was initially grown in CGG medium containing spectinomycin with induction of the incorporated genes with IPTG for either 3 or 22 h before the culture was harvested. Several condition parameters were examined including storage of cells before use, addition of ammonium formate (NH_4HCO_2), to the biotransformation standard medium. The process was maintained at a constant temperature of 30 °C with samples acquired periodically over the process. Samples were heat shocked at 95 °C for 5 mins and centrifuged at 13500 rpm to pellet the cells. Supernatant obtained from the samples were examined for residual organic acid content. Values for Succinate, pyruvate and α-ketoglutarate (αkg) are conveyed in the table.

5.8 **Biotransformation of *C. glutamicum* strain IL.1.7.0.0**

Il.1.7.0.0 based on the Bol-3 construct with the additional deletion of the *sucA* gene. This gene is required for the production of 2-oxoglutarate dehydrogenase, the E1 subunit, of the 2-oxoglutarate dehydrogenase complex (ODHC) (Pettit *et al.*, 1973, Spencer and Guest, 1982). With the disruption of this subunit it was hoped that that any reverse conversion (oxidative pathway) of α-ketoglutarate to succinyl coA would not occur. With the high yield of succinic acid in previous experiments in this chapter, it could be hypothesised that there may be a feedback loop redirecting any accumulated α-ketoglutarate back towards succinate as this would be more energetically favourable. However, the likelihood of this eventuality was unknown.

Again, the cells were initially cultured aerobically in CGG media containing IPTG to induce the incorporated genes. The culture was then harvested and resuspended in biotransformation medium containing ammonium formate and transferred to a round bottom flask. Process temperature was maintained at a constant temperature of 30 °C and stirred continuously. Samples were obtained periodically to access the accumulation of residual organic acid and utilisation of glucose and ammonium formate.

From this analysis, the deletion of *sucA* effects the productivity of the organism overall (Figure 5.2). The expected increase in α-ketoglutarate was not observed with only 0.14 gl^{-1} being accumulated over 48h. Succinate production and glucose utilisation was also reduced in comparison with previously described strains. Reduced productivity could be a direct detrimental effect of the exclusion of *sucA* from the organism, suggesting its influence is further reaching than just the activity of ODHC.

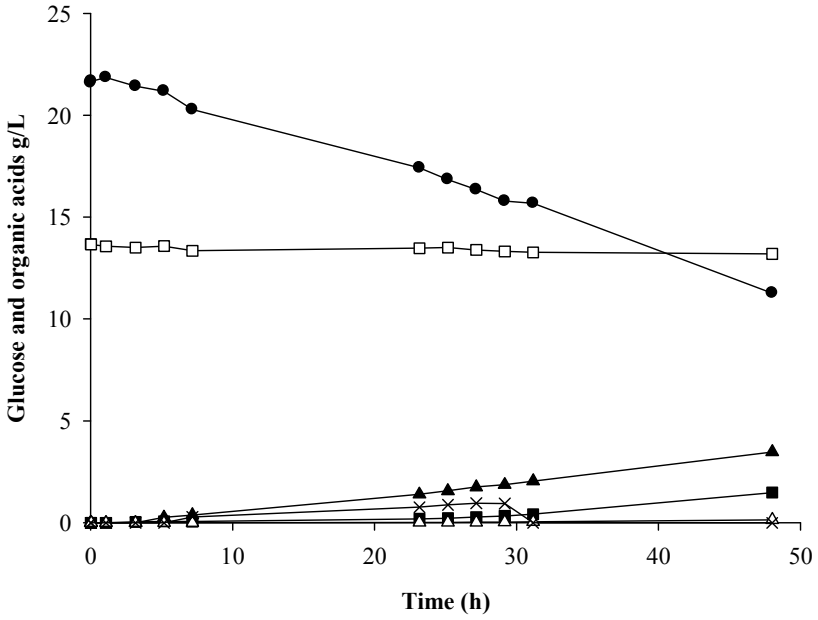


Figure 5-2 IL.1.7.0.0 Biotransformation product composition and glucose consumption

Biotransformation of IL.1.7.0.0 was carried out as standard. 1 mL samples were acquired periodically after which they were heat treated at 95 °C for 5 minutes. The sample was then pelleted and stored at 4 °C until analysed using the organic acid HPLC method. As this was a singular experiment and only single injections of each sample was analysed by HPLC. Time profiles of glucose (●), succinic acid (▲), pyruvic acid (■), alpha ketoglutarate (Δ)acetic acid (x) ammonium formate (□).

5.9 **Biotransformation of *E. coli* strain IL.2.1.1.0**

IL.1.2.1.1 was a modified strain of *Escherichia coli* BW2113, lacking the lactate dehydrogenase (*ldhA*) gene and the insertion of a pEKEx3 plasmid. Subsequent additions to this strain were made by the insertion of the plasmid containing; A= ketoglutarate synthase gene, ABC = ketoglutarate synthase, pyruvate synthase and ferredoxin genes or ADE = ketogluarate synthase, flavoprotein reductase, flavoprotein (flavodoxin) genes. The strains were grown aerobically in LB medium to obtain the biomass for the biotransformation (Section 2.4.1). During this growth phase, incorporated genes were induced by the addition of IPTG. Cultures were then harvested and stored at -20 °C before their use. Pelleted cells were resuspended in biotransformation media with or without ammonium formate. The process was maintained at 37 °C and continually mixed. Organic acid production was monitored during a biotransformation and maximum yields of significant compounds are presented in Table 5-5.

To understand how varying anaerobic conditions affected the organism productivity, three different systems were implemented (Table 5.5). Initially, the system setup was per previous experiments, with the vessel openings sealed using supa-seals. With this set up, no oxygen was introduced to the system, however, a limited volume was available in the head space of the vessel. In this instance, the main product observed across all strains was acetate with very little production of other organic acids. When the same experiment was repeated it was carried out under a nitrogen atmosphere to eliminate oxygen from the medium and the surrounding environment. Ammonium formate was also excluded from the batch medium as the current strain was yet to be optimised to produce L-glutamate.

Under these conditions, an increase in succinate was observed with the quantity of acetate reducing. Strain IL.2.1.1.(ABC).0 exhibited significantly higher concentrations of succinate compared with the other *E. coli* strains. It was also comparable to levels observed by IL.1.6.6.(K).0 (Section 5.5). However, the favoured product α-ketoglutarate was only observed at low concentrations, the

Table 5-5 Summary of Biotransformation by Strain IL.2.1.1.0

Strain	Medium	Induction	temp (°C)	storage (°C)	Biotrans medium	Anaerobic condition	Succinate gl^{-1}	Pyruvate gl^{-1}	αkg gl^{-1}	Acetate gl^{-1}	MV assay*
IL.2.1.1.0	LB+spec100	IPTG	37	-20	+ NH_4HCO_2	Standard	0.08	0.07	0.09	7.1	-
IL.2.1.1.(A).0	LB+spec100	IPTG	37	-20	+ NH_4HCO_2	Standard	0	0.04	0.07	3.04	+++
IL.2.1.1.(ABC).0	LB+spec100	IPTG	37	-20	+ NH_4HCO_2	Standard	0.32	0.07	0.04	5.3	+++
IL.2.1.1.(ADE).0	LB+spec100	IPTG	37	-20	+ NH_4HCO_2	Standard	0	0.06	0.106	4.09	+++
IL.2.1.1.0	LB+spec100	IPTG	37	-20	Standard	UNB	1.63	0.008	0.002	2.085	-
IL.2.1.1.(A).0	LB+spec100	IPTG	37	-20	Standard	UNB	2.82	0.012	0	0.52	+++
IL.2.1.1.(ABC).0	LB+spec100	IPTG	37	-20	Standard	UNB	12.5	0.0056	0.0009	0.927	+++
IL.2.1.1.(ADE).0	LB+spec100	IPTG	37	-20	Standard	UNB	4.5	0.04	0.0004	0.6	+++
IL.2.1.1.0	LB+spec100	IPTG	37	-20	Standard	Flacon tube	0.66	1.06	0.07	3.82	N/A
IL.2.1.1.(A).0	LB+spec100	IPTG	37	-20	Standard	Flacon tube	0	0	0	0	N/A
IL.2.1.1.(ABC).0	LB+spec100	IPTG	37	-20	Standard	Flacon tube	0	0	0	0	N/A
IL.2.1.1.(ADE).0	LB+spec100	IPTG	37	-20	Standard	Flacon tube	0	0	0	0	N/A

This table summarises the data gathered from biotransformations using iterations of stain IL.2.1.1.0. To obtain a high cell density for the biotransformation, the strain was initially grown in LB medium containing spectnomycin with induction of the incorporated genes with IPTG before the culture was harvested. Biotransformation were carried out in round bottom flask with (UNB) or without nitrogen (Standard) or prepared in a sealed Falcon tube. Despite the vessel used the process was maintained at a constant temperature of 30 °C with samples acquired periodically. Samples were heat shocked at 95 °C for 5 mins and centrifuged at 13500 rpm to pellet the cells Supernatant obtained from the samples were examined for residual organic acid content. Values for Succinate, pyruvate, α-ketoglutarate (αkg) and acetate are conveyed in the table. *Work carried out by Ingenza Ltd.. N/A work not available at time of writing.

highest of which was 90 mM. It should be noted that during the biotransformation, the pH increased to approximately 10 which is not optimal conditions for *E. coli*.

In a final attempt to create an atmosphere that allowed for the harnessing of the reductive arm of the TCA cycle, a third set of experiments were prepared in Falcon tubes with a sealed screw top lids. This method was unsuccessful as very little productivity of any kind was observed. This may be attributed to the change of mixing method. In this particular experiment, mixing was via the use of orbital shaker to accommodate the Falcon tubes. This method may not be as efficient as an agitation method as the stirrer bar, leading to a less homogenous biotransformation.

As with all of the biotransformations discussed within this chapter, they act as a preliminary screening investigation used as an indication only to meet the company's immediate objectives. Further replicates would have to be carried out to draw definitive conclusions.

5.10 **Development of Enzymatic assays**

5.10.1 **Succinyl CoA synthetase assay method development**

Due to the lack of α-ketoglutarate production, it was hypothesised that the enzyme responsible for the conversion of succinic acid to the intermediate compound succinyl CoA (Figure 5-1) may be a limiting factor. Succinyl CoA synthetase catalyses the reversible reaction, therefore, to test for its activity, an assay was developed to examine future strains activity of this enzyme. The objective was to obtain a control assay that could be implemented when screening future strains for their activity of this particular enzyme. Success of the assay was analysed by both HPLC and spectrophotometrically (Section 2.6.1).

The results from the HPLC analysis clearly demonstrate the conversion of sodium succinate to succinyl CoA in the positive reaction mixture with a signal for the latter compound appearing at a retention time of 8.5 minutes (Figure 5.3). Addition of

magnesium chloride does not seem to have an effect on the reaction, as when it is not present there is no significant decrease in succinyl CoA production compared to the positive reaction (Figure 5.4). As expected, when the key components, ATP, coenzyme A, succinic acid or succinyl CoA synthetase are removed from the mixture no succinyl CoA is produced (Figure 5.5-8). Interestingly, when the Tris-HCl buffer is replace with deionized water, and thus altering the pH of the final reaction, no succinyl CoA is produced. This suggests that the enzyme may have a pH dependency for activity (Figure 5.9).

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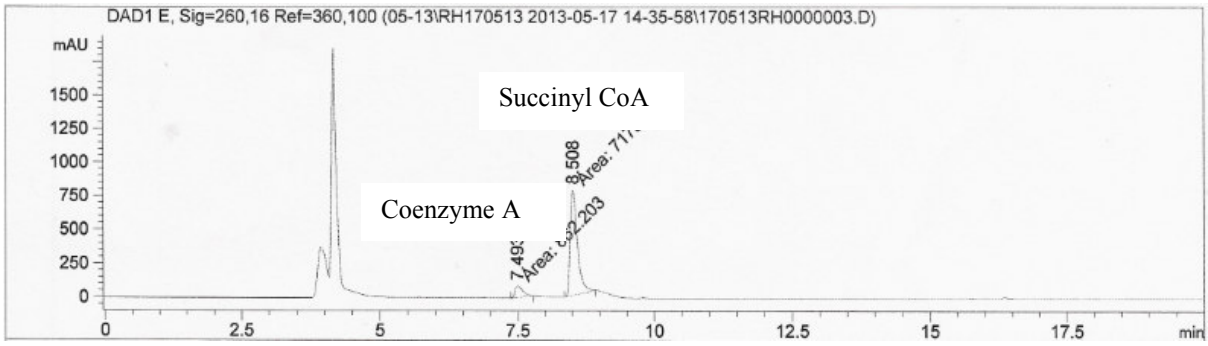

Figure 5-3 HPLC chromatograph of positive succinyl CoA synthetase reaction
Reaction mix containing succinate, ATP, Coenzyme A, magnesium chloride and buffer was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis. Conenzyme A was detected at 7.4 mins retention time and succinyl CoA at 8.5 mins retention time.

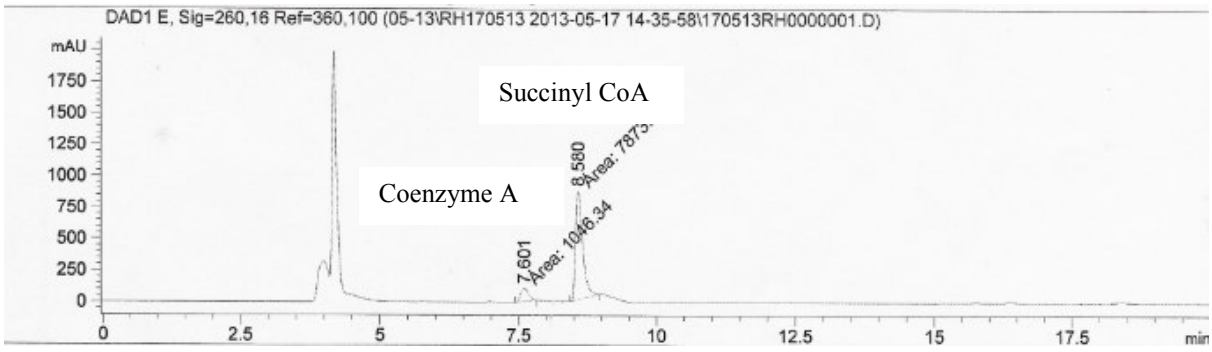

Figure 5-4 HPLC succinyl CoA synthetase assay with no magnesium chloride Reaction mix containing succinate, ATP, Coenzyme A and buffer was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis. Conenzyme A was detected at 7.6 mins retention time and succinyl CoA at 8.5 mins retention time.

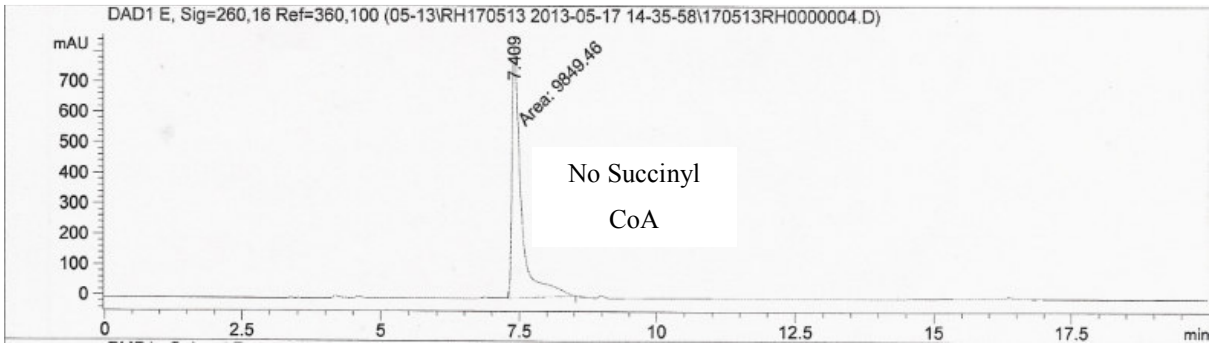

Figure 5-5 HPLC succinyl CoA synthetase assay with no coenzyme A

Reaction mix containing succinate, ATP, Coenzyme A, magnesium chloride and buffer was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis.

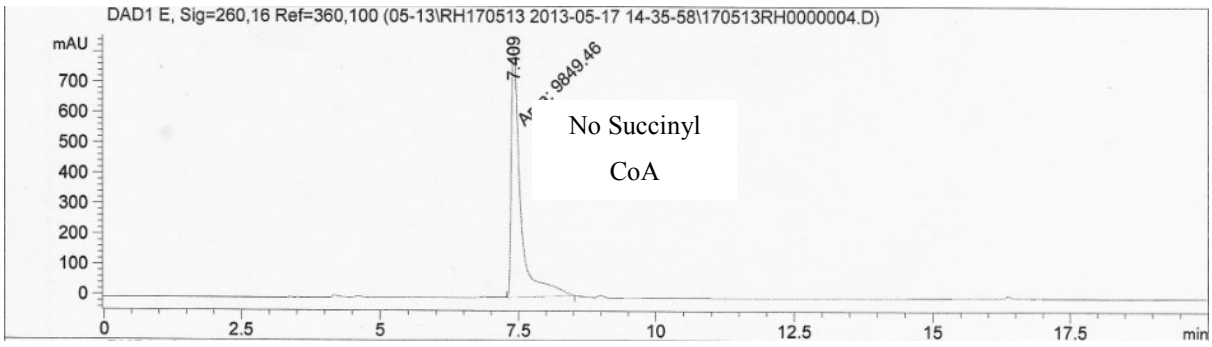

Figure 5-6 HPLC succinyl CoA synthetase assay with no ATP

Reaction mix containing succinate, Coenzyme A, magnesium chloride and buffer was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis.

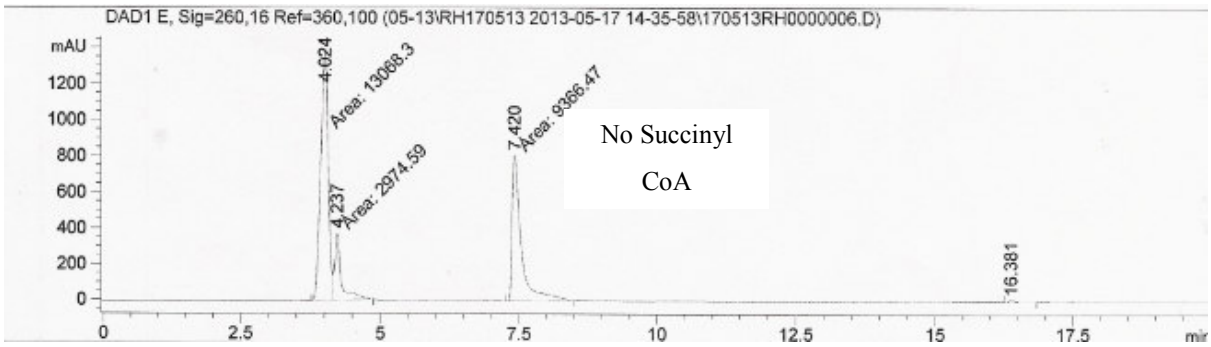

Figure 5-7 HPLC succinyl CoA synthetase assay with no sodium succinate

Reaction mix containing ATP, Coenzyme A, magnesium chloride and buffer was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis.

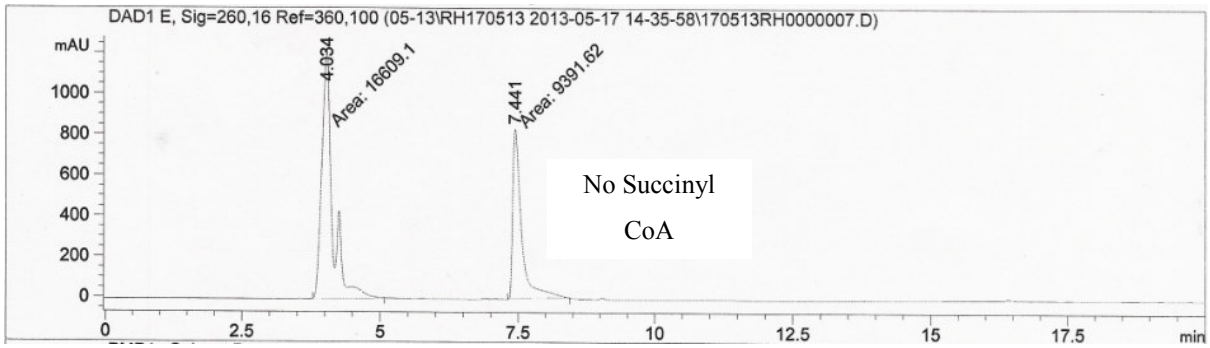

Figure 5-8 HPLC succinyl CoA synthetase assay No Succinyl CoA Synthetase Reaction mix containing succinate, ATP, magnesium chloride and buffer was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis.

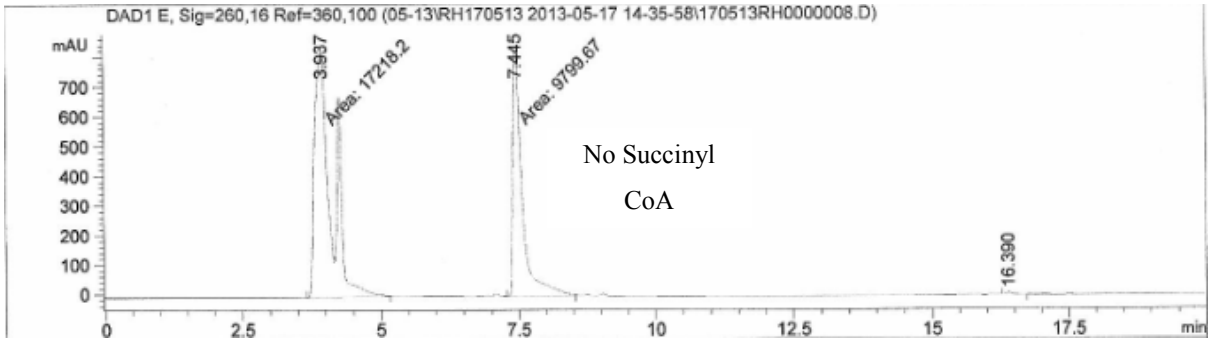

Figure 5-9 HPLC succinyl CoA synthetase assay with no Tris.HCl buffer Reaction mix containing succinate, ATP, Coenzyme A, magnesium chloride and water was prepared and incubated at room temperature for 5 minutes. The reaction was then quenched and stored at -20 ° C until examined by HPLC analysis.

The results from the spectrophotometric assay demonstrated that only the positive reaction exhibited an increase in absorption at 230 nm. When any of the required components of the assay are omitted, namely, succinate, Coenzyme A, ATP or enzyme, the absorbance increase fails to occur (Figure 10). This observation was consistent with the HPLC data that displayed only the positive reaction containing succinyl CoA in the reaction mix and where any required component of the assay is omitted, succinyl CoA is not detected.

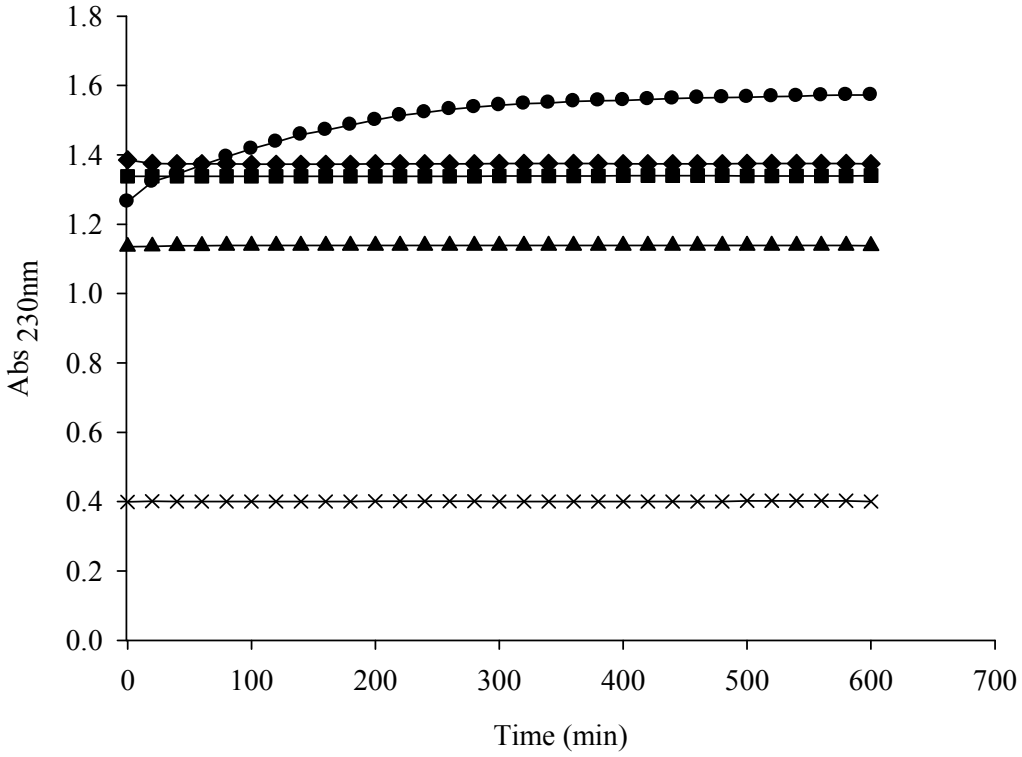

Figure 5-10 Spectrophotometric succinyl CoA synthetase assay
A positive reaction mixture containing all of the relevant constituents (succinate, ATP, Coenzyme A, magnesium chloride and buffer) was prepared and subsequently its absorbance at 230 nm was measured for 10 minutes. Negative reactions were also prepared, where one key substitute was omitted; no succinate (■), no ATP (x), No coenzyme A (▲) no succinyl CoA synthetase (♦).

It was hoped with the success of both the HPLC and spectrophotometric experiments, the assays could be implemented using cell free extract of the mutant *C. glutamicum* strains. This would allow determination of the activity of the succinyl CoA synthetase present in the cell. If activity was found to be low, then this may help to explain the insufficient conversion of high levels of succinic acid to α-ketoglutarate.

5.10.2 **Isocitrate dehydrogenase assay method development**

As mentioned in Section 5.6, it has yet to be determined in this study if the oxidative arm of the TCA cycle was active. Therefore, the activity of the isocitrate dehydrogenase, which catalyses the conversion of isocitrate to α-ketoglutarate, was identified as a key target to investigate. Activity of this enzyme under anaerobic conditions was also favourable as it produces the vital cofactor NADPH which would later be required for the conversion of α-ketoglutarate to L-glutamate. As with the succinyl CoA synthetase assay, it was anticipated that with a successful assay in place this procedure could act as a control when comparing future strains for their activity of this enzyme.

The method employed was adapted from the Sigma Aldrich protocol SPISOC01.001 and contained some alterations (Section 2.6.2). Unlike the protocol the buffer was changed from Glycylglycine to Tris-HCl as it was assume it was only required for controlling the pH. The final volume was also scaled appropriately to accommodate standard quartz cuvette dimensions. Another initial adaption was to run the assay at room temperature as a thermostated spectrophotometer was unavailable. This preliminary examination of the isocitrate dehydrogenase assay proved unsuccessful. Therefore, in an attempt to develop the process into a viable assay several alterations were attempted.

To address the possibility of temperature being the limiting factor, the assay was prepared in the absence of the enzyme. The sample was then incubated at 37 °C for five minutes until the absorbance at 340 nm was stable. An aliquot of the enzyme

was then added with the initial A_{340nm} being measured before the sample was returned to the orbital incubator. The absorbance was then measure at 60 second intervals. An initial increase was observed after the addition of the enzyme, however, this was also detected in the negative control indicating the enzyme itself absorbed at this particular wavelength which may interfere with future results. All further assessments were carried out at both room temperature and 37 °C to determine if multiple factors required addressing.

To determine if the enzyme loading was insufficient, the dosage added to the assay was increased by a factor of 5. After the initial change in absorbance due to addition of the enzyme, no subsequent increase was observed either at room temperature or incubating at 37 °C.

On closer inspection of the protocol, it was discovered that an error between the stated reaction mixture and the specified final concentration of the assay components differed. Therefore, in the 2 mL reaction mixture, the final concentrations were altered to: Tris-HCl pH7.4, 67 mM; DL-Isocitrate acid trisodium salt, 0.44 mM; NADP+, 1 mM; manganese chloride, 0.6 mM; 0.06 IDH. However, as before, after the initial increase in Abs $_{340nm}$, no subsequent change was observed either at room temperature or by incubating at 37 °C.

These unsuccessful alterations suggested this assay was unreliable with the given analytes, therefore, a different approach was required. An assay described by Alp *et al.* (1976) was used with the final concentrations in the reaction mixtures being; Tris-HCl pH 7.4 buffer, 70 mM; NADP, 0.5 mM; magnesium chloride, 8 mM; DL-isocitrate trisodium salt, 3 mM; 0.06 units/mL IDH. The reaction was performed at 25 °C as stated by the protocol. Once again, no increase in absorbance was observed after the enzyme was added.

Lack of success in the above experiment led to the assumption that one or more of the key elements were likely to be limiting the assay. Firstly, reliability of the $NADP^+$ was brought into question as previous treatment and storage of the chemical could not be verified. To confirm this theory, fresh chemicals would be required and the assay repeated. Secondly, a constant temperature of 37 °C may need to be

maintained in order for the enzyme to be active. With the unavailability of a thermostated spectrophotometer, an alternative procedure may need to be developed in order to achieve a positive assay. Finally, a HPLC protocol could be completed in tandem with the assay. This would allow low level analysis to investigate if any isocitrate present was being converted to α-ketoglutarate.

5.11 **Chapter 5 Conclusions**

Early success with the IL.1.6.6.(K).0 (Bol-3/pAN6 *gap*) (Section 5.4) strain exhibited promise that an anaerobic process using *C. glutamicum* strains could be developed. Comparable values of succinic acid were achieved, leading to the assumption that with the right manipulations in place, the flow of reaction would continue to form an excess titre of α-ketoglutarate. With this in mind, strain IL.1.6.5.(A).0 was created, in the hope that with the inclusion of the ketoglutarate synthase gene conversion would occur. This was not the case, with the gene appearing to be very unstable in its expression. For example, during a biochemical assay, enzyme activity could be detected, however, during biotransformation using cells from the equivalent batch, no increase in α-ketoglutarate titre was observed (Table 5.3). The lack of improved α-ketoglutarate production was not limited to the above strain. Modifications made to IL.1.6.1.0, failed to increase the yield compared to that reached by the empty vector strain (Table 5.4). Attempts to create an α-ketoglutarate producing strain of *E. coli* were also unsuccessful even though the organism containing an active form of the ketoglutarate synthase gene (Table 5.5). For all the described strains, inconsistency was prevalent, suggesting an inherent instability in the construct. It is noteworthy that the results from strain IL.1.6.5.(A).0, which exhibited the most significance (Table 5.3) did not confer ketoglutarate synthase activity. This emphasises the need to reanalyse the strains to determine if they require further development to ensure consistent expression of the desire genes.

For the reasons explained above, it can be assumed that other factors in the biotransformation itself could also play a major role in determining the production of α-ketoglutarate under anaerobic conditions.

It should be noted at this time, that no conclusive evidence has been presented that verifies the α-ketoglutarate present at the end of any of the biotransformation has in fact been created through only the reductive branch of the TCA cycle. To understand if the oxidative branch is operational during anaerobic conditions, it was hoped that with a fully functioning isocitrate dehydrogenase assay (Section 5.9.2), cell free extract (CFE) could be analysed for activity. For this assessment of activity to be valid, the assay would be prepared under a nitrogen blanket to eliminate oxygen in the immediate environment.

Resolving the bottlenecks are vital in the development of any efficient bioprocess. Therefore, examining the metabolic controls of the production of succinate and pyruvate could be beneficial. It appears in this case that the carbon flow is effectively being held back at these significant points. A major bottleneck may be the inefficiency of the succinyl CoA synthetase enzyme to covert excess succinic acid to succinyl CoA. To analyse the activity of this enzyme in CFE, use of the developed SCS assay (Section 5.9.1) could be implemented.

5.12 **Future work for Chapter 5**

Key aspects of this work have to be improved before an efficient anaerobic process can be developed.

- The inconsistency of *C. glutamicum* strain IL.1.6.5.(A).0 proved that gene expression in the model was unstable. As expression was observed in biochemical analysis but increase product accumulation was not observed in the biotransformation, the reaction mixture could be examined to identify if a more suitable combination was available. The biotransformation medium used throughout, is based on the composition used for succinate production

(Litsanov *et al.*, 2012), therefore may not be optimal for α-ketoglutarate synthesis.

- Co-factor regeneration is also a key issue that requires addressing. Understanding the rate at which formate dehydrogenase reduces NAD^+ to NADH, may assist in determining if production is inhibited due to the lack of NADH available. Also, developing gluconate utilisation during the biotransformation could prove useful in supplying essential NADPH required for L-glutamate production. Metabolomic analysis of CFE could provide an insight into co-factor concentrations inside the cell and identify insufficiencies.
- Understanding if all the steps in the cycle towards α-ketoglutarate are performing efficiently could identify key bottlenecks. Using the assays developed in this investigation (Section 5.9), determining if efficient conversion from succinate to succinyl CoA could be analysed using CFE. Also determining if the oxidative branch of the TCA was operation could be examined.
- Once a strain has been acquired that exhibits consistent increased production of α-ketoglutarate, L-glutamate producing conditions could be incorporated into the biotransformation. This would create a direct anaerobic route to the production of L-glutamate, the desired amino acid. A scale up development process could then occur to produce L-glutamate anaerobically at large volumes. From this point, the synthetic pathway to methacrylic acid could be introduced (Figure 5.1).

Chapter 6

Toxicity Examination of potential compound candidates

6.1 **Process Overview**

Before the intended process could be implemented into an industrial setting as an alternative pathway to produce methyl methacrylate and similar compounds, the effects of these desired chemicals and their intermediates have to be fully understood (

Figure 6-1). To achieve an insight into the potential problems faced by an industrial process, the chemicals mentioned below were tested for toxicity against the following industrially relevant microorganisms, *C. glutamicum* ATCC 13869, *E. coli* BW25113 *ΔpflB ΔldhA* and *A .niger* A60. As the priority of the project shifted from a fungal to a bacterial bioprocess, the conditions were modified to be incorporated into Ingenza Ltd.'s priorities and as a result different approaches were developed for each different organism.

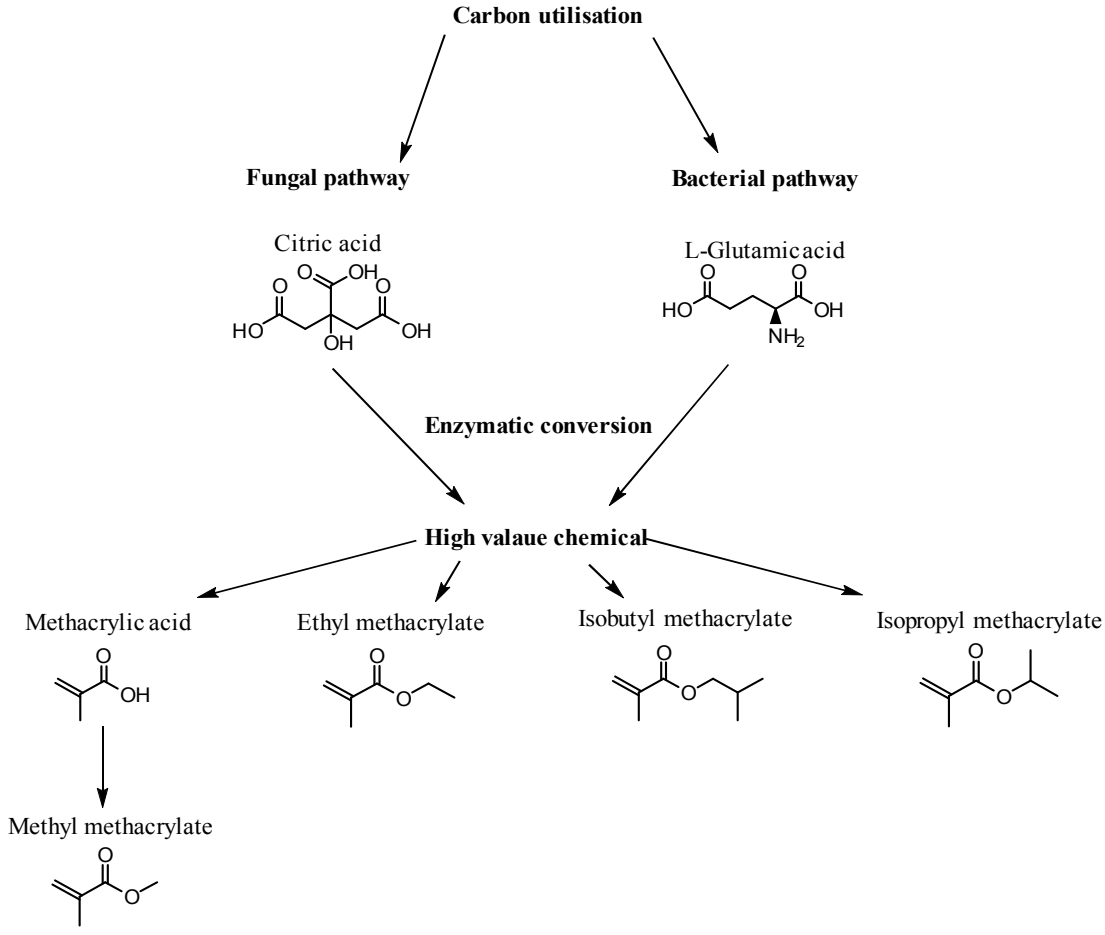

Figure 6-1 Proposed biochemical process pathway towards the production of high value chemicals.

6.1.1 *Aspergillus niger* A60

Aspergillus niger is an important industrial producer of a variety of organic compounds, in particular citric acid. Therefore, the organism was considered in the early stages of this project as a potential candidate to be used in the biological synthesis of methyl methacrylate. Similar to L-glutamate, citric acid can be used as a chemical building block in the conversion process. To analyse suitability of *A. niger* in terms of resistance to the toxic effects of methyl methacrylate and its immediate precursor, methacrylic acid, strain A60 was examined using the concentrations outlined in Table 6-1.The organism was grown both on solid and in liquid medium, in triplicate, to obtain a full understanding of growth, morphological and production variations when the desired chemicals were present. When it came to investigating in liquid culture two approaches were examined. Firstly, the organism was cultured in medium containing the chemical and secondly, cultured for 24h before introduction of the chemical. As well as growth, citric acid accumulation was monitored as its production would be the critical in the formation of the downstream chemicals.

Table 6-1 Concentrations of chemicals examined for *A. niger*

	gl^{-1}	gl^{-1}	gl^{-1}	gl^{-1}
Methyl methacrylate (MMA)	0	0.5	1	2.5
Methacrylic acid (MAA)	0	0.5	1	2.5

6.1.2 *C. glutamicum* ATCC 13689 & *E. coli* BW25113 *ΔpflB ΔldhA*

A high throughput system was developed for the microbial examination as growth inhibition was perceived to be of high importance. Using this, two cell densities were investigated to determine if cell loading would be a significant factor in determining the future efficiency of the process. Cell loadings of 0.1 gl^{-1} and 2.5 gl^{-1} were examined for both microbial strains. As this process ultimately would be performed under conditions that produced excess amino acids for conversion to mesaconate,

production medium was chosen to examine the effects of each chemical. Previously, biotin limitation process medium elicited the greatest L-glutamate concentration (Chapter 3), therefore it was selected for examination of *C. glutamicum*. On the other hand, for *E. coli*, a production medium previously used by Ingenza Ltd. was utilized (Voulgaris *et al.*, 2011)

Cultures were loaded onto a 96 well plate with each concentration of compound detailed in Table 6-2 and assessed in triplicate. Cells were also cultured in medium only to act as a control to assess normal growth under the conditions. Growth was continuously monitored over a 24 hour period using a shaking thermocouple plate reader. As the chemicals being examined were all commercially obtained, they all contained the stabilizing agent 4-methoxyphenol to prevented radical polymerization of the monomer chemical. To ensure the presence of this compound did not cause undesirable effects to the organism and therefore influencing the results, 4-methoxyphenol was also examined at concentrations that spanned that of which were present in the chemicals. Triton-x at a concentration of 0.1 % v/v was used as a positive control.

Table 6-2 Concentrations of chemicals examined for *C. glutamicum* and *E. coli*

	gl^{-1}	gl^{-1}	gl^{-1}	gl^{-1}
Methyl methacrylate (MMA)	0	2.5	5	7.5
Ethyl methacrylate (EMA)	0	0.43	0.9	1.35
Isopropyl methacrylate (iPMA)	0	0.225	0.4	0.675
Isobutyl methacrylate (iBMA)	0	0.09	0.18	0.27
Methanol (MeOH)	0	3	6	9
Methacrylic acid (MAA)	0	0.5	1	2.5

	ppm	ppm	ppm	ppm
4-methoxyphenol (Mex)	0	15	30	45

Equipped with the preliminary growth examination interaction, the investigation was scaled up to flask scale which allowed not only a better understanding of cell growth but also enabled productivity to be monitored. Data obtained in this section were obtained and provided by Cameron Howard and David MacNicol at the University of Strathclyde, and examined the effects of the above chemicals on *C. glutamicum* ATCC 13689 at a cell loading of 0.1 gl^{-1}. Repeat of results of interest were then performed by the author.

6.2 **Novelty**

The proposed pathway towards methyl methacrylate using respiratory organic acids is a relatively novel concept and has only been demonstrated partly by Wang and Zhang (2015) to produce the intermediate mesaconate. However, the influences of the desired chemicals and potential substrates on the microorganisms themselves are relatively unknown, with the exception of methanol. Methanol has been identified as a possible feedstock of the industrial process as it can be obtained from renewable sources such as glycerol and synthesis gas produced from biogas (Schrader *et al.*, 2009). Unlike the methacrylates examined here, methanol toxicity towards many microorganisms is well documented in the literature (Salakkam and Webb, 2015). However, metabolic analysis of *C. glutamicum* ATCC 13032 by Witthof *et al.* (2013) demonstrated that the wild type strain could in fact oxidise methanol to produce CO_2 through an endogenous pathway. Therefore, this could lend itself to be a potential candidate as a sustainable raw material if used in a feed style manner whilst maintaining the levels of methanol under a toxic level.

Although no research of the toxicity of the other pre-mentioned chemicals on the potential microbial candidates has been published, considerable research has been performed to investigate acrylates (including methyl methacrylate) toxicity in relation to their uses in the production of medical and dental products. Focus of these studies considered the toxicity to mammalian cell lines or animal models with many exhibiting varying levels of toxicity or even dose like responses dependent on the type of acrylate used (Yoshii, 1997). Growth of HeLa cells was examined in the

presence of thirty-nine acrylates and methacrylates by Yoshii (1997), several of which were examined in the current study. Using the half maximal inhibitory concentration (IC_{50}) it was observed that the acrylates examined were more toxic to growth than that of the methacrylates with the IC_{50} of methyl methacrylate of 89.89 mMol/L. This was double that of ethyl methacrylate which had an IC_{50} value of 29.26 mMol/L and isobutyl methacrylate at 2.94 mMol/L, all of which are examined in this study. Structure of the monomer also appeared to be influential with the addition of a hydroxyl group considerably increasing the cytotoxicity of the chemical. If similar toxicity effects were observed using the proposed organism then the conversion efficiency between the intermediate methacrylic acid and methyl methacrylate could prove crucial in future development of the process to maintain harmful intermediates below toxic levels.

In the cases of animal model investigations, Bereznowski (1995) exposed rats to 8 mMol/kg of methyl methacrylate using intravenous injections to the stomach cavity. No histopathological changes were observed to the major organs or tissues of the animal and specific enzyme activity such as aspartate aminotransferase were maintained. Break down of MMA was observed with the appearance of methacrylic acid in the serum 5 minutes after its introduction, peaking in concentration between 10 and 15 minutes. However, after 1 hour only low levels of the intermediate chemical could be detected, suggesting that it could be hydrolysed within the blood. It was suggested that this breakdown of MMA was due to non-specific carboxylesterases, which are commonly found in mammalian livers. This lipolytic enzyme that catalyses the cleavage of ester bonds is also widely distributed in nature and has been identified in a variety of organisms including thermophilic bacterium (Kakugawa *et al.*, 2007). This could be put forward as a hypothesis as to how the organisms examined here would metabolise MMA. Such metabolism would be detrimental for the biochemical production of methyl methacrylate.

In contrast to the above research, other animal model studies have found severe detrimental effects caused by the presence of methacrylates and acrylic acids. Singh *et al.* (1972) observed gross deformities and foetal abnormalities when pregnant mice

were injected with the compounds. However, as with Yoshii, dose dependent trends were observed with acrylic acid exhibiting the greatest level of toxicity compared to that of the methacrylate esters.

6.3 **Aims**

Although the toxicity of several of the methacrylate esters summarised in the process outline (Section 6.1) have been documented in mammalian studies, there is not a comparable large body of research focusing on the effects these compounds have on microorganisms. Therefore, the presented work aims to identify undesirable effects resulting from the presence of these potential end compounds and substrates (methanol) on both growth and productivity prospective.

6.4 **Effect of Methacrylic acid on *Aspergillus niger* A60**

With both solid and liquid medium, the initial presence of the intermediate methacrylic acid led to severe inhibition. Concentrations as low as $0.5gl^{-1}$ inhibited sporulation on the solid medium, however, lawn growth was still observed (Figure 6-2). Increasing this concentration to 1 gl^{-1} MAA resulted in complete inhibition of growth. This phenomenon was further verified when *Aspergillus niger* A60 was examined in liquid culture where no indication of growth was observed whatsoever (Figure 6-3a). In contrast to the cultures containing MAA, the control cultures in this case achieved, on average, a maximum dry cell weight (DCW) of 10.4 gl^{-1} after 168h which was considered to be typical for this strain under the conditions (Papagianni and Mattey, 2006).

To confirm utilisation of glucose was inhibited, filtrate collected during DCW determination was analysed by HPLC using the developed organic acid method (Section 2.8.3). Organic acid production was also measured using the same method. As expected, there was no utilisation of glucose and no product formation throughout

the samples containing MAA at any concentration. Glucose consumption was observed in the control consistent with growth and increasing biomass (Figure 6-3b). However, not all the glucose present in the medium was utilised suggesting that under flask culture conditions, growth and productivity was not optimum. This was confirmed by the low yield of citrate observed, which at 168 h, was a maximum of only 26.1gl^{-1} (Figure 6-3c). However, examining the values in more detail indicated that prolonging the culture period could have further increased the end point yield. Examining the yield of citric acid over the second half of the process, the accumulation rate increases from 0.16 $gL^{-1}h^{-1}$ between 96 and 144 h to 0.3 $gL^{-1}h^{-1}$ between 144 and 168 h. If this rate were to continue or increase glucose would have continued to be utilised and converted into citrate.

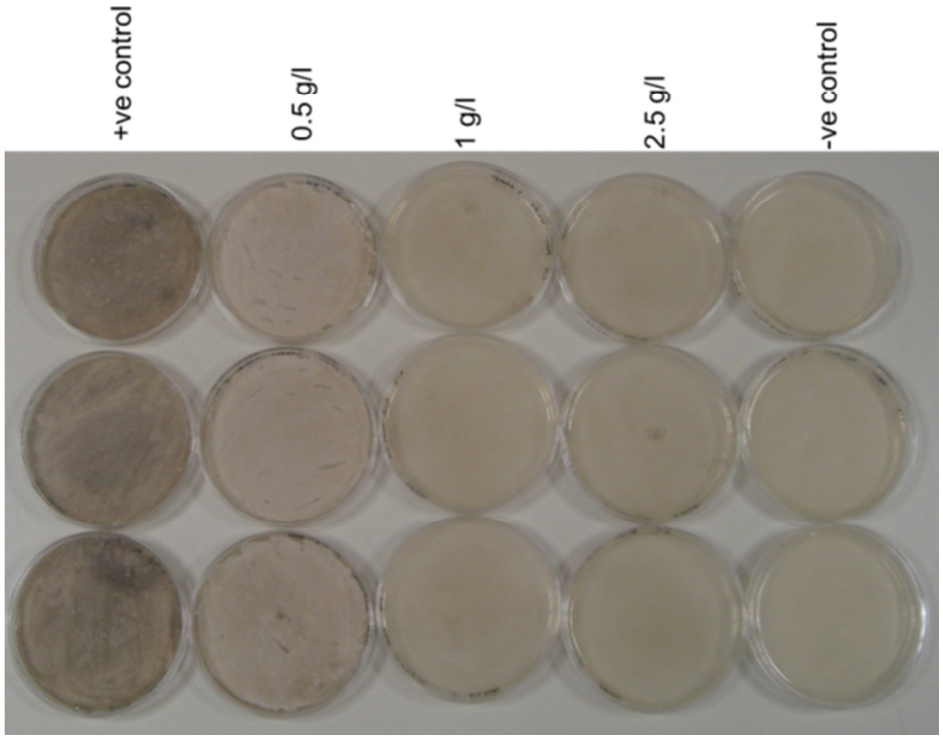

Figure 6-2 Effect of Methacrylic acid on growth and sporulation of A60 on solid medium.
To 75ml of molten PDA concentrations ranging from 0-2.5gl^{-1} of Methacrylic acid was added. The agar was then split between 3 plates. When set 1.08 x 10^7 *A. niger* A60 spores were aseptically transferred to the plate. The plates were then incubated at 30°C in the dark for 168h. Examination of the plates was carried out every 24 h analysing colony growth and colour, spore colour and agar pigmentation.

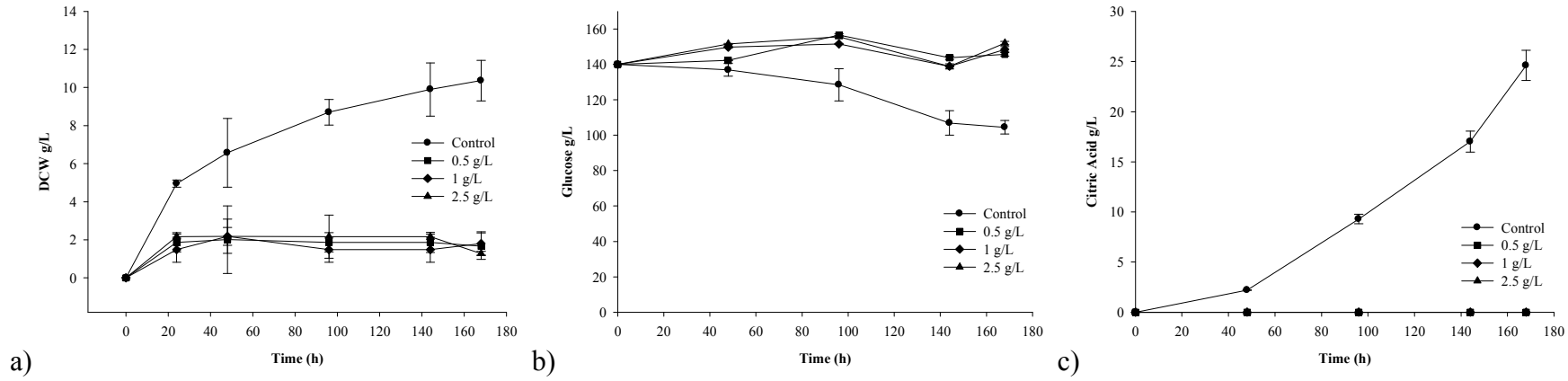

Figure 6-3 Effect of Methacrylic acid on biomass of A60 in liquid medium.

To 200ml of liquid medium concentrations ranging from 0 (●), 0.5 (■), 1 (♦) or 2.5 (▲) gl^{-1} of Methacrylic acid were added after sterilisation. The medium was mixed thoroughly before 1.08 x $10^{\wedge 7}$/mL *A. niger* A60 spores were aseptically transferred. The flasks were then incubated in a rotary shaker at 180rpm at 30ºC. 2 x 5ml samples were taken every 48 h and analysed for a) DCW b) glucose utilisation and c) citric acid production. Error bars are represented by the SD (n=3).

6.5 **Effect of Methyl methacrylate on *Aspergillus niger* A60**

Unlike its intermediate, methyl methacrylate appears to have less influence over *A. niger*'s growth. On solid medium, growth and sporulation was unhindered by the presence of the monomer and was consistent to that displayed by the control (Figure 6-4). This was also the case when MMA was increased to a concentration of 10 gl^{-1} in the agar medium (plate observation identical to that observed in Figure 6-4).

When examined in liquid culture, this apparent trend in comparable growth with the control was observed once again. No significant differences in the DCW (Figure 6-5a) or glucose (Figure 6-5b) consumption were noted, signifying that the presence of MMA may not be as detrimental as its precursor. However, as the concentration increased, there did appear to be a significant delay in citrate production (Figure 6-5c). This delay in product formation appeared to have a direct correlation with increasing concentration of MMA. At 96 h, citrate accumulation was observed in the control and when 0.5 gl^{-1} of MMA was present in the medium. An additional 48 h was required when 1 gl^{-1} of MMA was present and a further 48h with 2.5 gl^{-1} of MMA present, for accumulation of citrate to be observed. This association could prove problematic if an efficient *in-situ* conversion process was to be put into practice. Product inhibition of a process is a common problem in industry and steps to reduce this would have to be investigated.

As with the result obtained using MAA, glucose consumption was not complete. However, by allowing the culture to continue past 168h, an increase in the end yield of citric acid was observed with a maximum accumulation rate of 0.4 $gL^{-1}h^{-1}$ between 192 and 240 h. Prolonging culturing further would undoubtedly continue the conversion of glucose to the desired product.

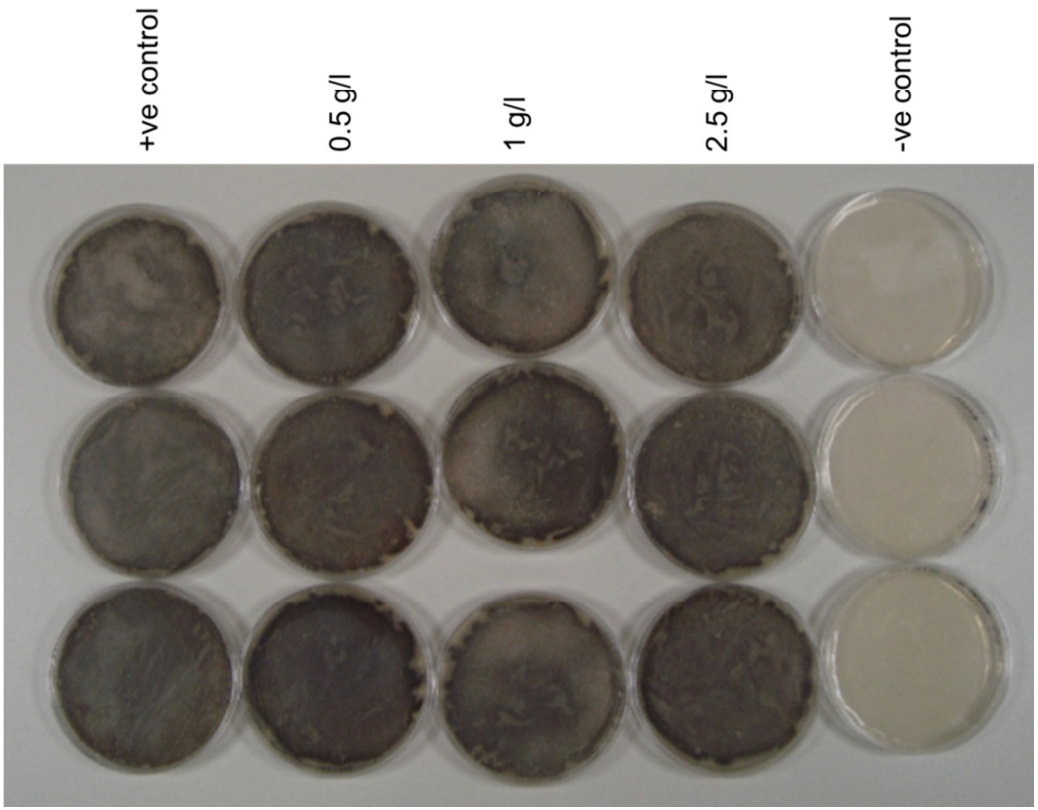

Figure 6-4 Effect of Methyl methacrylate on growth and sporulation of A60 on solid medium.
To 75ml of molten PDA concentrations ranging from 0-2.5gl^{-1} of Methyl methacrylate was added. The agar was then split between 3 plates. When set 1.08 x 10$^{\wedge 7}$ *A. niger* A60 spores were aseptically transferred to each plate. The plates were then incubated at 30°C in the dark for 7 days. Examination of the plates was carried out every 24 h analysing colony growth and colour, spore colour and agar pigmentation.

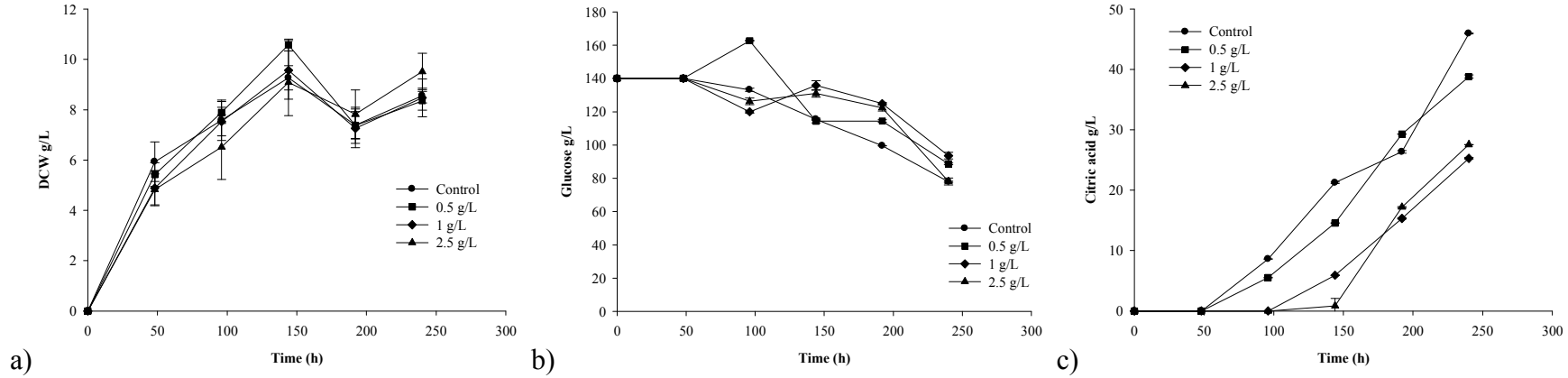

Figure 6-5 Effect of Methyl methacrylate on biomass of A60 in liquid medium

To 200ml of liquid medium concentrations ranging from 0 (●), 0.5 (■), 1 (♦) or 2.5 (▲) gl^{-1} of methyl methacrylate were added after sterilisation. The medium was mixed thoroughly before 1.08 x $10^{\wedge 7}$/mL *A. niger* A60 spores were aseptically transferred. The flasks were then incubated in a rotary shaker at 180rpm at 30ºC. 2 x 5ml samples were taken every 48 h was analysed for a) DCW b)glucose utilisation and c) citric acid production Error bars are represented by the SD (n=3).

6.6 **Effect of MAA and MMA addition after 48 hour growth of A60**

To determine whether similar effects would be replicated if the organism was in a growth phase, cultures were grown for 48 h before addition of either MAA or MMA. This determination would allow for the understanding at which point the inhibition was occurring, whether it be in germination of the spores or in exponential growth. As with the previous study, MMA appeared to have no effect on DCW being comparable to that of the control. On the other hand, presence of MAA appeared to inhibit further growth and cause aggregation of the pellets (Figure 6-6a).

The complete inhibition by MAA was also supported by the halt in the production of acid after the intermediate was added (Figure 6-6d). Interestingly, although end point acid yield is comparable to that of the control, the addition of MMA appears to hinder the production rate of acid. This revelation is in keeping with the results observed previously (Figure 6-5c). Both the complete inhibition observed by MAA and the acid production delays instigated by MMA could signify a decrease in an *in-situ* process's efficiency as end product accumulation would be decreased.

There were no significant differences in pH levels between the samples containing MAA or MMA compared with the control. However, slight decrease of the pH from 2.5 to 2 could be enough to create the ideal conditions for citric acid production (Figure 6-6b) (Roukas & Harvey 1988).

Increased toxicity of MAA compared to MMA may be due the presence of a hydroxyl group within the compound's structure. It was observed by Yoshii (1997), that compounds containing the functional group exhibited a lower IC_{50} value and therefore higher toxicity towards HeLa cells compared to that of monomethacrylates such as methyl methacrylate. The results presented in this work correspond to these findings and exhibit a similar dose dependent toxicity. Presence of the hydroxyl group in the compound may result in the acidification of the media, as the labile proton on this functional group is easily displaced (Hart *et al.*, 2007). Similar evidence of growth disruption by a weak acid has also been observed by Plumridge *et al.* (2004), noting inhibition of mycelial growth in the presence of sorbic acid.

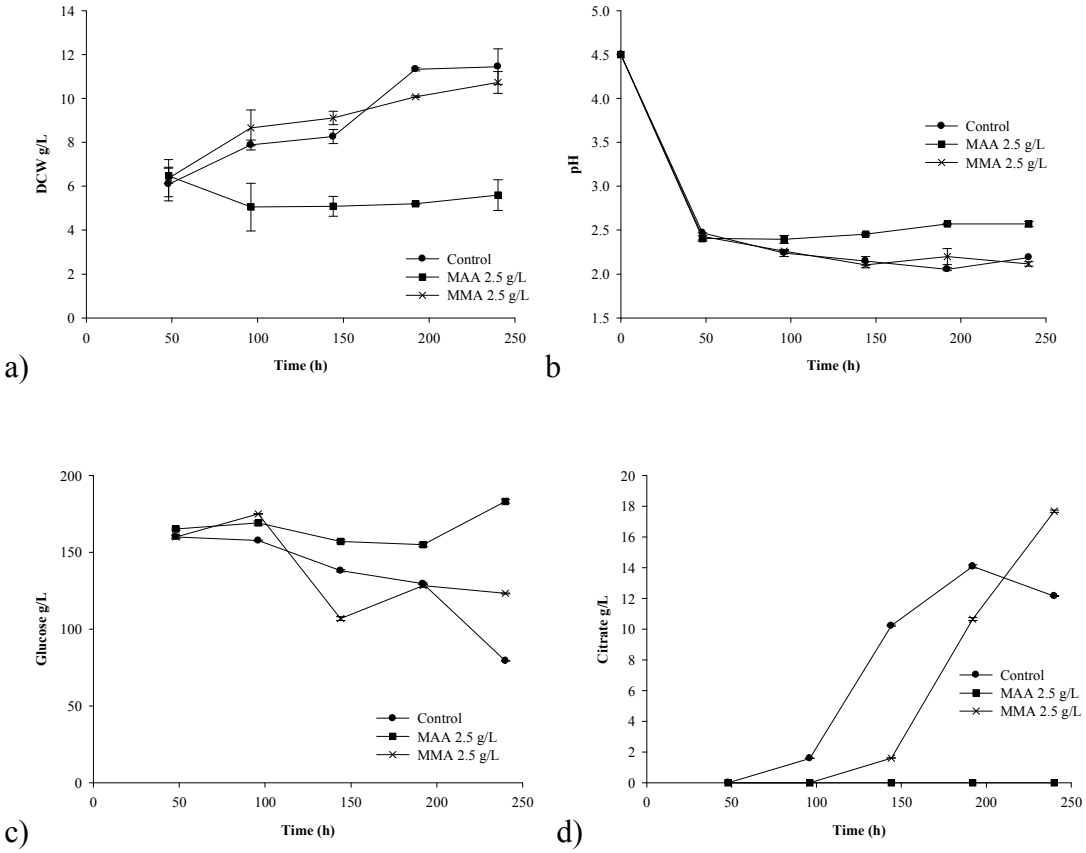

Figure 6-6 Effect of MAA or MMA addition after 48 h growth of *A. niger*
To 200ml liquid medium (glucose carbon source) 1.08 x $10^{\wedge 7}$/ml *A. niger* A60 spores were aseptically transferred. The flasks were incubated in a rotary shaker at 180rpm at 30ºC. After 48 h 2.5gl^{-1} of either methacrylic acid (■) or methyl methacrylate (x) was added. 2.5gl^{-1} of water (●) was used as control. Samples were acquired every 48hr to examine a) DCW, b) pH c) glucose utilisation and d) citrate production. Error bars are represented by the SD (n=3).

6.7 High throughput examination of *C. glutamicum* and *E. coli* growth

Influences caused by the presence of the identified chemicals on *C. glutamicum* and *E. coli* were initially measured by examining the growth profiles of the organisms over a 24 h period. This investigation was performed at small scale, examining 200 µL cultures using 96 well plates allowing the cultures growth to be monitored closely. In this experiment, the highest concentrations of the compounds of interest were selected as these values were close to the maximum concentrations soluble in water.

At a low cell loading (DCW 0.1 gl^{-1}) of *C. glutamicum* (Figure 6-7-Figure 6-9) the effect of the chemicals was relatively negligible. In particular, over the initial 5 h of growth, the optical denisities were in good correlation with the control. After 5 h, cell densities appear to plateau with only slight variation observed over 10-24 h in the presence of methanol and 4-methoxyphenol. Methanol presence at 9 gl^{-1} did appear to exhibit a small decrease in growth after 7 h, however, at this small a scale values obtained could be circumstantial. As this experiment was designed to act as a rapid screening process, subtle differences would be better assessed on a larger scale. Interestingly, the presence of 4-methoxyphenol in the medium led to a slight increase in cell density compared with the control after 9 h of culturing. It is unlikely, but there is a possibility that the 4-methoxyphenol was being consumed as a carbon source, however, this is unlikely as glucose would still be readily available at this point within the medium. Growth of the cultures in the presence of compounds was in stark contrast to the cultures treated with Triton-x, which did not increase in cell density whatsoever over the full 24 h.

Similar patterns were observed when the cell density was increased to 2.5 gl^{-1}. Taking into consideration the deviation between the triplicate measurements, no discernible differences were observed in the presence of all of the chemicals examined with the exception of methanol. After 10 h, methanol appeared to have a gradual negative effect on cell growth. Again, at this scale true influences were difficult to understand (Appendex Figure 9-1).

As with *C. glutamicum*, *E. coli* appeared to be unaffected by the presence of the chemicals during the initial stages of growth, at both 0.1 gl^{-1} and 2.5 gl^{-1} cell loadings. After 5 h of growth, variance between the cultures was observed in particular at low cell loadings when MMA and iBMA were present. However, when examined under statistical scrutiny, no significance differences could be concluded (Appendix Figure 9-2 and Figure 9-3).

However, the nature of the plate culture is not optimal for growth as limitations due to other factors, such as oxygen deprivation, could be effecting growth and therefore obscuring the true effects of the chemicals.

C. glutamicum is credited as having a high oxygen demand, in particular during the initial growth phase (Zimmermann *et al.*, 2006). Therefore, the conditions within the 96 well plate would quickly become oxygen limited and growth would be hindered. However, for productivity this is not necessarily detrimental. Yamamoto *et al.* (2011) suggested that, although growth would be ultimately inhibited, metabolic acitivity continued under oxygen deprived conditions. With an oxygen limited state plausible under these conditions, the organism would naturally convert to producing anaerobic fermentation products such as lactate. Although not optimal for growth, the creation of an acidic environment may not be critical for the organism it has been shown to be tolerant of a range of pH between 6-9.5 (Barriuso-Iglesias *et al.*, 2006, Follmann *et al.*, 2009).

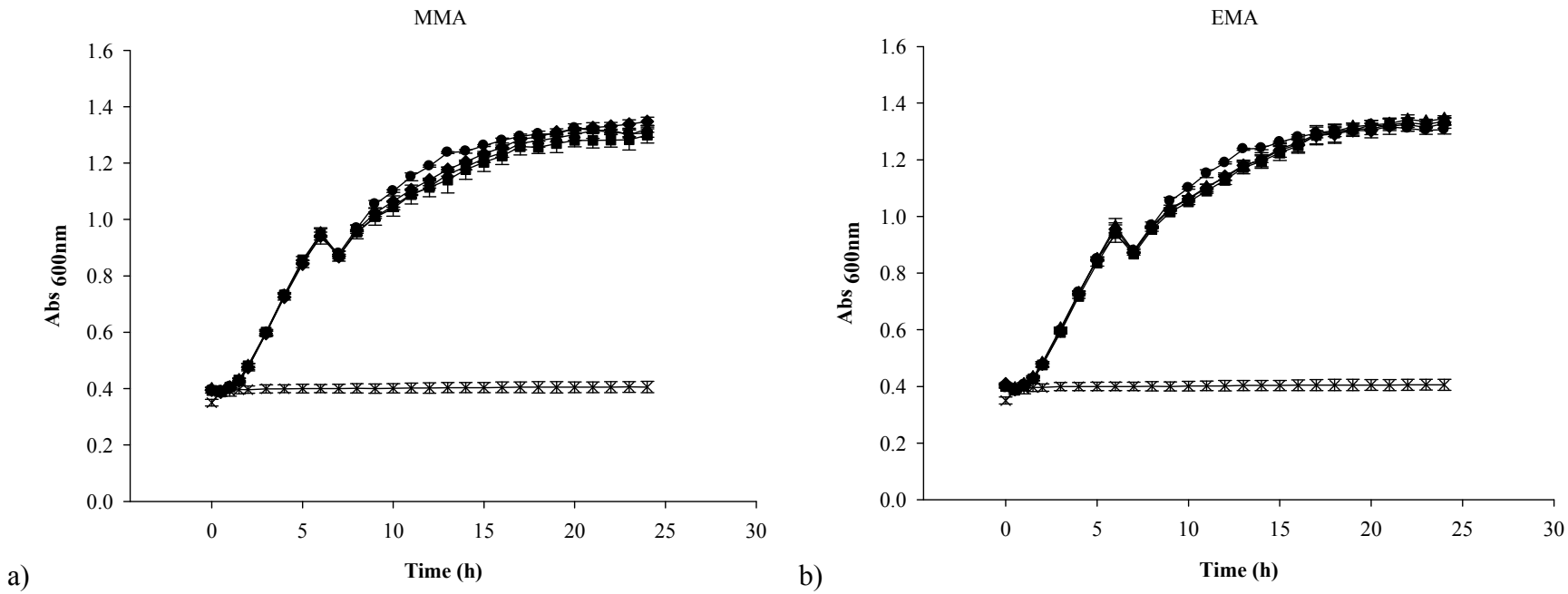

Figure 6-7 Effects of Methyl and Ethyl methacrylate on *C. glutamicum* growth

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of CGXII media with 1 $\mu g l^{-1}$ of biotin and either a) 0 (●), 2.5 (■), 5 (♦) or 7.5 (▲), $g l^{-1}$ of MMA or b) 0 (●), 0.43 (■), 0.9 (♦) or 1.35 (▲) $g l^{-1}$ EMA, to an OD600nm = 1. 0.1 % v/v Triton-x (x) was used as a control. Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

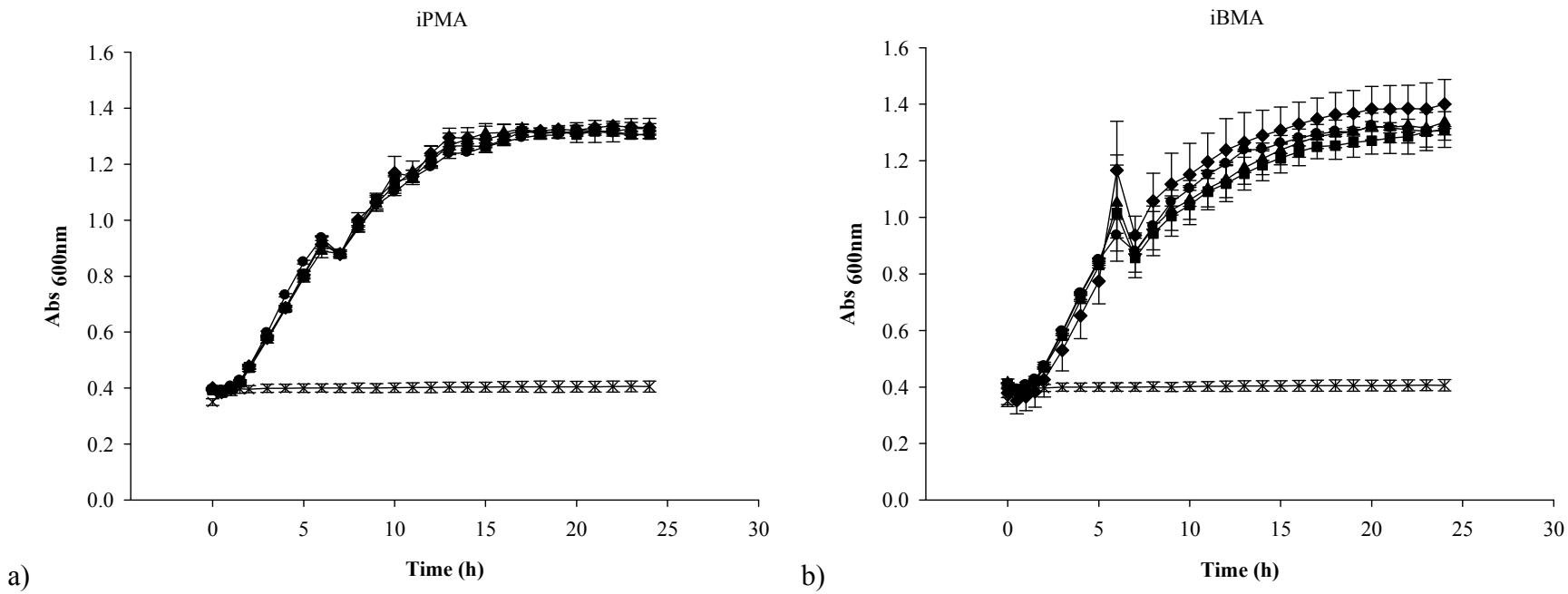

Figure 6-8 Effects of Isopropyl and Isobutyl methyl methacrylate on *C. glutamicum* growth

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of CGXII media with 1 μgl^{-1} of biotin and either a) 0 (●), 0.225 (■), 0.4 (♦) or 0.675 (▲), gl^{-1} of iPMA or b) 0 (●), 0.09 (■), 0.18 (♦) or 0.27 (▲) gl^{-1} of iBMA, to an OD600nm = 1. 0.1 % v/v Triton-x (x) was used as a control. Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

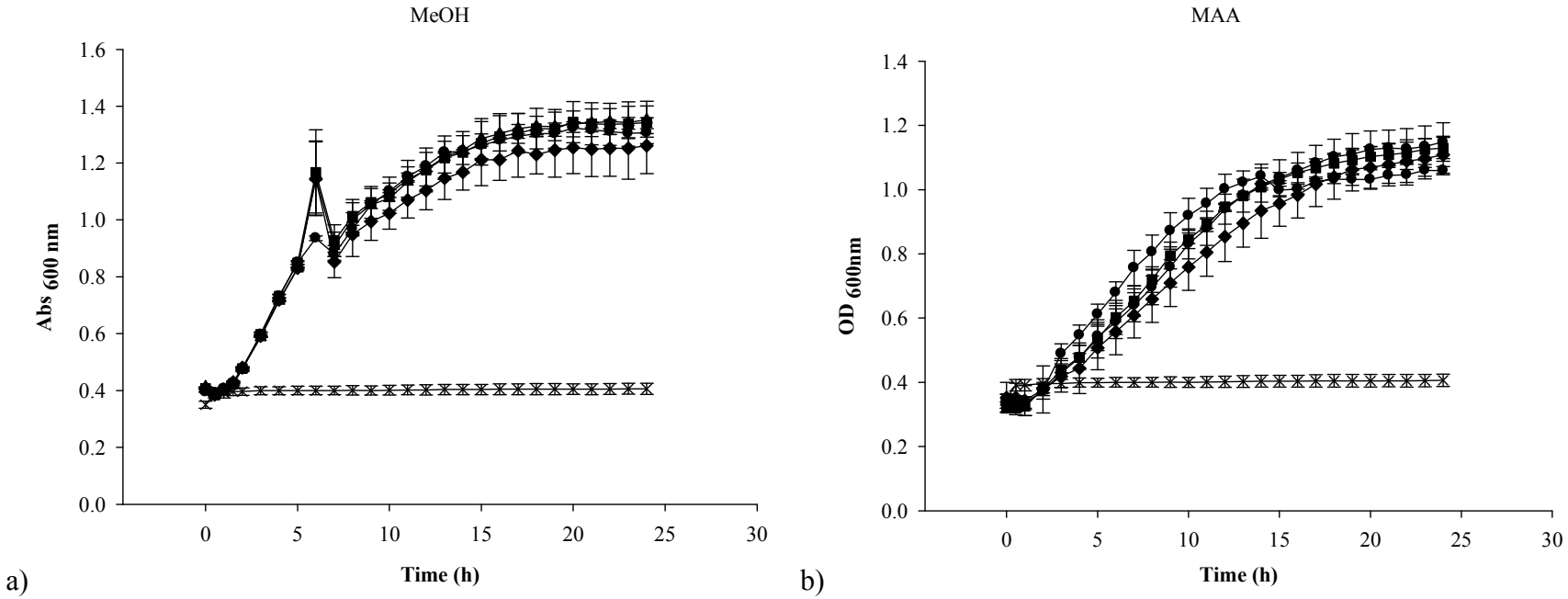

Figure 6-9 Effects of MeOH and Methacrylic acid on *C. glutamicum* growth

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of CGXII media with 1 μgl^{-1} of biotin and either a) 0 (●), 3(■), 6 (♦) or 9 (▲), gl^{-1} of MeOH or b) 0 (●), 0.5 (■), 1 (♦) or 2.5 (▲) gl^{-1} of MAA, to an OD600nm = 1. 0.1 % v/v Triton-x (x) was used as a control. Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

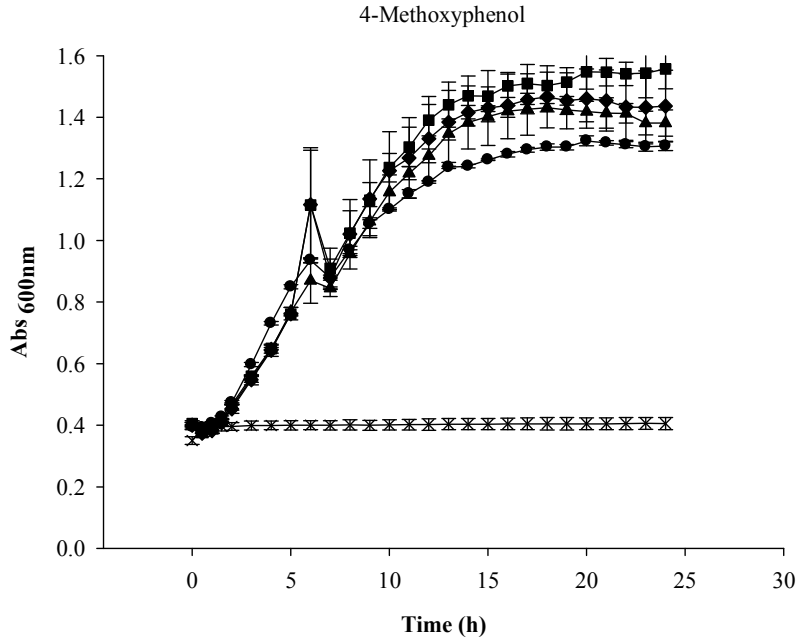

Figure 6-10 Effects of 4-Methoxyphenol on *C. glutamicum* growth

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of CGXII media with 1 μgl^{-1} of biotin and either 0 (●), 15 (■), 30 (♦) or 45 (▲) ppm of Mex, to an OD600nm = 1. 0.1 % v/v Triton-x (x) was used as a control. Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

6.8 **Shake flask examination of *C. glutamicum* growth and productivity**

As the results from the high throughput method only gave a small insight into influences of the chemicals on growth, the experiment was scaled-up to examine the organisms in flask culture. Scaling also addressed the detrimental effects of an oxygen limited environment on productivity, if any. The pH of the culture could also be monitored. Being the favoured organism for the industrial process, *C. glutamicum* only was examined in this case. This section of work was performed as part of two Honours projects within the University of Strathclyde and were designed and supervised by the author. Results of interest were then repeated by the author. As with previous results, values obtained were analysed by One-Way ANOVs and significance scrutinised by a Tukey multiple comparison test, a mean comparison method at the 95 % confidence interval. Compounds that exhibited a significant detrimental effect compared with their relative control are discussed in further detail.

Unlike the high throughput method, several differences were observed between the cultures containing the chemicals. Table 6-3 and Table 6-4 outline a condensed overview of the influences the presence of these compounds in the medium exhibited on a variety of cellular processes, including maximum growth rate, yield as well as subsequent differences in pH observed throughout the process. Examining the pH of the culture medium gave an immediate indication if the organism was in a state of acid production, however, this was not specific for L-glutamate accumulation.

Table 6-3 Effect of examined chemicals on pH during *C. glutamicum* culture

		pH		
	Concentration gl^{-1} or ppm	**0h**	**24h**	**48h**
Control	0	7.18	5.27	5.23
Methanol	3	7.17	8.05	7.85
	6	7.15	8.18	7.88
	9	7.13	8.19	7.96
iBMA	0.09	7.15	5.64	5.23
	0.18	7.32	7.45	5.59
	0.27	7.08	6.66	7.36
EMA	0.45	7.04	6.46	4.75
	0.9	7.05	5.57	6.66
	1.35	7.02	5.67	4.9
iPMA	0.225	7.09	5.42	5.95
	0.45	7.1	5.19	4.99
	0.675	7.09	5.18	4.99
MMA	2.5	7.27	7.26	5.48
	5	7.25	7.48	5.74
	7.5	7.24	7.77	6.72
MAA	0.5	7.40	7.28	5.25
	1	7.36	7.55	5.41
	2.5	7.15	7.68	6.15
Mex	15	7.07	5.42	6.17
	30	7.09	5.49	5.04
	45	7.08	5.39	5

Table 6-4 Effect of examined chemicals on growth and productivity of *C. glutamicum*

	Concentration gl^{-1} or ppm	Max specific growth rate h^{-1}	Max product titre gl^{-1}	Yield coefficient of product/biomass gg^{-1}	Specific glucose uptake rate $gl^{-1}\cdot h$
Control	0	0.34	14.36	3.8	0.94
Methanol	3	0.08	8.35	4.32	0.51
	6	0.07	3.34	1.52	0.36
	9	0.06	3.54	2.10	0.34
iBMA	0.09	0.37	11.23	1.66	1.03
	0.18	0.10	10.88	2.9	0.89
	0.27	0.46	7.92	0.89	0.85
EMA	0.45	0.26	9.54	1.61	0.98
	0.9	0.24	12.46	2.06	0.82
	1.35	0.22	12.43	2.22	0.70
iPMA	0.225	0.22	8.80	1.91	1.61
	0.45	0.23	10.85	1.96	1.66
	0.675	0.25	10.70	2.13	1.70
MMA	2.5	0.11	10.34	3.8	0.91
	5	0.07	9.26	3.33	0.70
	7.5	0.06	9.75	4.2	1.09
MAA	0.5	0.22	4.35	0.97	0.86
	1	0.21	2.48	0.47	0.81
	2.5	0.21	0.32	0.06	1.0
Mex	15	0.18	10.45	2.02	1.66
	30	0.22	10.85	1.87	1.71
	45	0.22	8.80	1.4	1.64

The approximate starting pH of all cultures was between 7-7.3, which was optimal for *C. glutamicum* growth. With the exception of methanol and methyl methacrylate, the trends of the pH over the 48 h of cultivation were similar to that observed with the control, falling below pH 6 in most cases (Table 6-3). This was a strong indication of acid production. In the case of MeOH, MMA and MAA, an increase in pH was observed at 24 h across all of the concentrations of both compounds examined. By 48 h, the pH for all MMA and MAA concentrations had decreased below 7, with the lowest concentration exhibiting a value close to that of the control. Cultures containing methanol also decrease from the value at 24 h, however, were still significantly higher than that of the starting pH and the control at 48 h. For both compounds this suggested that although acid production may be occurring, accumulation may be delayed compared to the control.

Examining the cultures containing MeOH further, it was observed that growth was significantly inhibited by the presence of the alcohol at all concentrations compared to the control (Figure 6-11a). A maximum DCW of 6 gl^{-1} was achieved by the control after 18 h. At the same time point, all of the cultures containing MeOH had only achieved DCW of approximately 1.4 gl^{-1}, with that value decreasing to 1.1 gl^{-1} at 9 gl^{-1} MeOH. As expected, glucose consumption was not complete over the 48 h period, with the rate decreasing to a third of that of the control (Figure 6-11b).

A recent study investigating metabolism of methanol by *C. glutamicum* ATTCC 13032 by Witthof *et al.* (2013) observed inhibition of the growth rate of the wild-type strain when methanol concentrations were increased in the medium. A maximum of 0.28 M (9 gl^{-1}) was examined in the present study which was considerably lower than the 3 M concentration where complete inhibition was observed by Witthof *et al.* (2013). Values obtained here follow a similar trend to that observed by Witthof *et al.* (2013) with increasing levels of MeOH reducing growth rate, however, the rates achieved here were considerably lower. For comparison with 0.28 M, a growth rate of 0.33 h^{-1} was achieved by Witthof *et al.* (2013) whereas only a rate of 0.07 h^{-1} was achieved here for the same concentration. Both studies utilise the same CGXII minimal medium, however, it is unclear if biotin limitation was employed in the aforementioned study as it was here. As previously observed in this

work, biotin is a key requirement in cellular growth, therefore, its limitation will influence the growth rate of the organism. This may explain why lower growth rates were observed in this case.

A significant decrease in glutamate accumulation was observed in the presence of MeOH (Figure 6-11c). A maximum of 13.9 gl^{-1} of L-glutamate was observed at 42 h by the control culture. Maximum yields by the cultures contain MeOH were as follows; 8.35 gl^{-1} (3 gl^{-1}), 3.34 gl^{-1} (6 gl^{-1}), 3.54 gl^{-1} (9 gl^{-1}) of L-glutamate. Although it appears that MeOH inhibits productivity the yield obtained at 3 gl^{-1} of MeOH was double that of the control. This demonstrates possible adaption of the organism to maintain productivity within the presence of the alcohol and still excrete the desired chemical.

Using methyl methacrylate, growth rates were also decreased in the presence of increasing concentrations of the compound (Figure 6-12a). Examining the mean variation of the DCW weight values, a significant decrease in accumulation was observed when 7.5 gl^{-1} MMA was present in the medium compared to that of the control and in the presence of 2.5 gl^{-1} MMA at 24 h. Further significance decrease was also observed at 30 h when compared to the control, however, this is nullified by 48 h. This suggests that increasing the levels of MMA in the medium could be detrimental for initial growth although its presence would not be completely inhibitory. No significance differences were observed between the control and the two lower concentrations of MMA examined, which was similar to the results obtained when *A. niger* (Figure 6-5) was examined with this compound. These results would suggest that MMA, at low levels, can be tolerated across multiple organisms.

The significant decrease observed in the DCW correlate with the decrease in glucose utilisation (Figure 6-12b) when MMA concentrations are increased. This supports the argument that the presence of the compound was detrimental to growth. However, this significance, like that observed with the DCW was nullified by 48 h. Again, this supports the idea that although the compound is unfavourable, the organism is able to recover to normal metabolic levels.

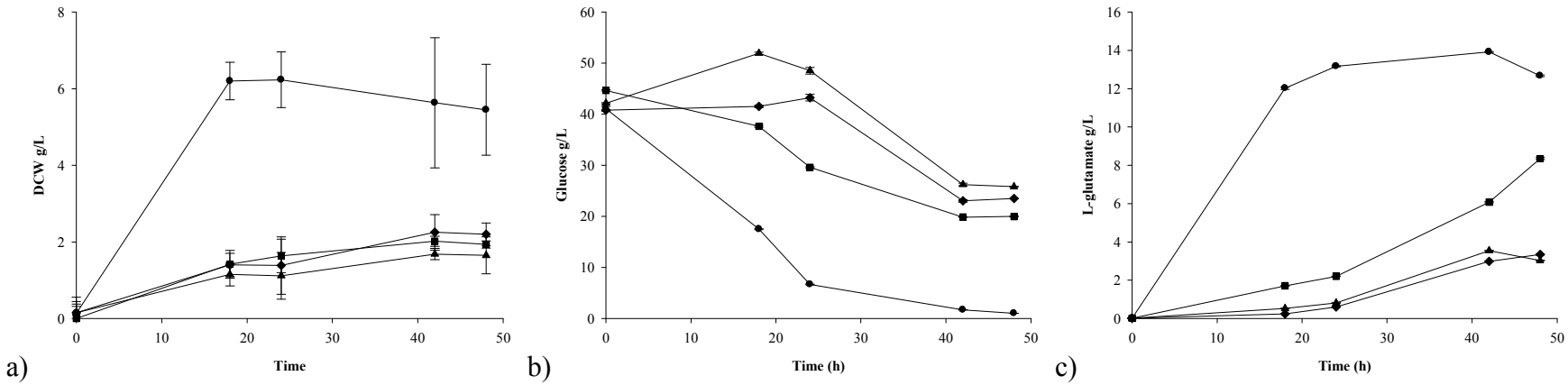

Figure 6-11 Effect of MeOH on *C. glutamicum* during shake flask culture

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 3(■), 6 (♦) or 9 (▲), gl^{-1} of MeOH to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW, b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Examination of L-glutamate production indicates that increasing the levels of MMA in the medium can contribute to a lag in the production of the amino acid (Figure 6-12c). No significance in production was observed between the control and 2.5 gl^{-1} MMA at any point of the experiment. When the concentration was increased to 5 and 7.5 gl^{-1} delayed accumulation was evident with a significant decrease observed at both 24 and 30 h compared with the control. However by 48 h the end yields of all cultures are relatively similar.

Although the cell type and compound concentrations examined in this experiment were different to those examined in previous papers (Yoshii, 1997), a dose dependent influence of MMA was still observed. Increasing concentrations of the compound did exhibit adverse effects on both growth and productivity. After further process time, significance of these effects decreased suggesting that although a delay in cellular activities was observed the organism was still capable of growing and producing the desired product after what appeared to be a period of adaption.

Unlike during fungal cultivation (Section 6.4), the presence of methacrylic acid had no significant detrimental effects on growth under any of the concentrations examined (Figure 6-13a). Glucose consumption also appeared to be un-effected by the presence of the compound with only slight significance at 24 h between 2.5 gl^{-1} MMA and the control (Figure 6-13b). Significance became obsolete by the end of cultivation at 48 h with the final residual glucose level being similar to that of the control. Both of these features suggest that the presence of MAA was not detrimental to *C. glutamicum* when considering growth alone. This was in stark contrast to *A. niger* where growth appeared to be completely inhibited by its presence suggesting individual characteristics of the organisms may play a role in the protecting the cell from damage.

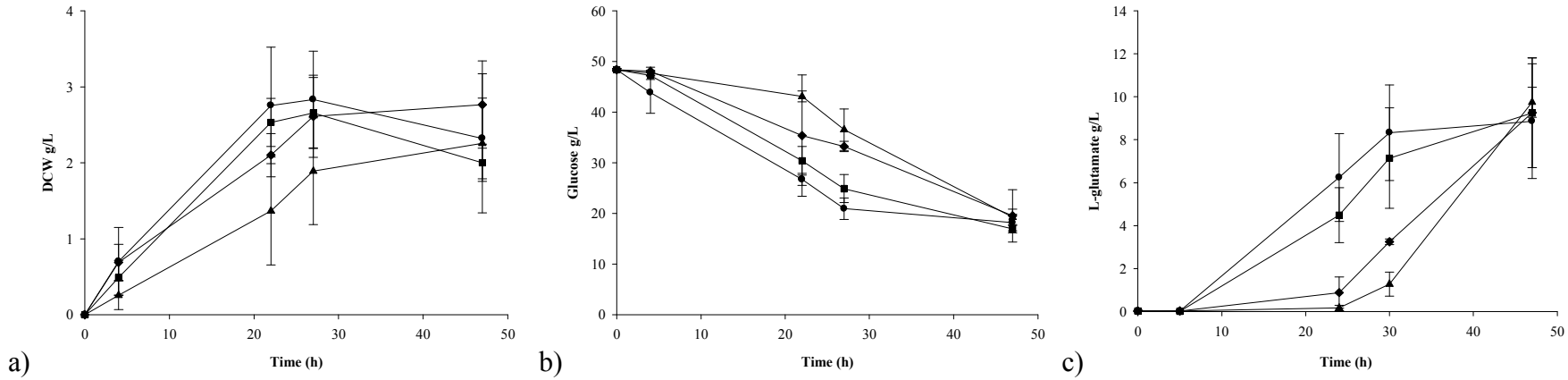

Figure 6-12 Effect of MMA on *C. glutamicum* under shake flask cultivation

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD_{600nm} =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 2.5 (■), 5 (♦) or 7.5 (▲), gl^{-1} of MMA to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW, b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Although presence of MAA appears not to hinder growth, an increase in DCW at 48h was observed. As end residual glucose levels were similar to that of the control, it could be postulated that the organism was potentially utilising MAA as a carbon source. While utilisation of MAA *C. glutamicum* has not been discussed previously, degradation of MAA has been observed to occur in rat serum due to the presence of lypolitic enzymes (Bereznowski, 1995). Presence of similar enzymes in *C. glutamicum* could, in theory, degrade the compound and utilise the carbon for growth. This is only theoretical hypothesis, as the end concentration of the compound was not measured and requires further investigation.

While growth appeared un-effected by MAA, L-glutamate accumulation was severely impacted (Figure 6-13c). As with the presence of MMA, a dose dependent inhibition on production was observed. Compared to the control, which yielded 8.6 gl^{-1} by 48 h, the presence of 0.5 gl^{-1} MAA halved the potential yield of L-glutamate to 4.4 gl^{-1}. By increasing the concentration of MAA to 1 gl^{-1}, the yield of L-glutamate was reduced even further to 2.5 gl^{-1} and almost complete inhibition of amino acid production was observed at 2.5 gl^{-1} MAA.

Amino acid production inhibition by methacrylic acid could prove problematic if an *in situ* production process were to be implemented, if the presence of MAA was prolonged. By insuring conversion to the final product was efficient, detrimental effects of the compound could be prevented.

No significant inhibition of growth, utilisation of glucose or production of L-glutamate was observed by the other compounds examined. This suggests that their inclusion in future process development would not be detrimental to the organism's activity. (Appendix Figure 9-4 - Figure 9-7)

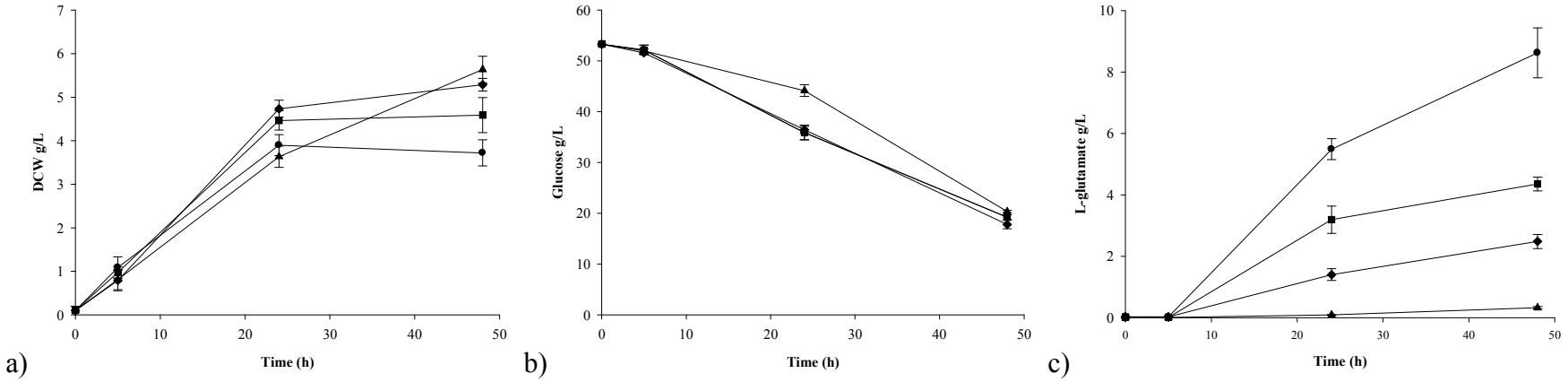

Figure 6-13 Effect of MAA on *C. glutamicum* during shake flask culture

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 0.5 (■), 1 (♦) or 1.5 (▲), gl^{-1} of MAA to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW, b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

6.9 **Chapter 6 Conclusions**

Examination of all the compounds indicated varying levels of toxicity with the organisms assessed. The high throughput method used to understand the initial growth of *C. glutamicum* and *E. coli* was unable to detect the detrimental effects observed when the experiment was increased in scale to shake flask volume. However, only methanol, methyl methacrylate and methacrylic acid exhibited significant effects that lowered growth, glucose consumption and amino acid accumulation. Further analysis of toxicity would be required before a process was implemented to ensure efficiency.

Influences of methacrylic acid appeared to differ depending on the organism examined. In *A. niger*, this compound exhibited the greatest inhibition of all the chemicals with the effects observed both from initial exposure and introduction after 48 h. In its presence, low growth and complete inhibition of productivity was observed at all concentrations examined. In *C. glutamicum*, growth was not hindered however, production of L-glutamate was significantly inhibited. Further investigations of MAA's influences on bacterial cultures, in particular high cell density cultures, are still required to obtain a full understanding of its potential impacts on future process development. As this compound is an intermediate chemical in the pathway towards methyl methacrylate, a high conversion rate would be required in order to ensure detrimental effects were maintained at a low level to reduce impact on the production pathway. Insuring a fast conversion rate may also reduce the potential for the organism to utilise the compound as a carbon source and therefore reducing productivity.

In the presence of MMA, a delayed effect was observed in both *A. niger* and *C. glutamicum*. However, these effects appeared to be limited as the organism appeared to recover and end yields of both DCW and production compound were comparable with the control. This suggests that at a higher cell density the effects of the compounds are either neutralised or the organisms have adapted to their new environment.

According to Witthof *et al.* (2013), methanol could be a potential future raw material for this process. However, due to the severe inhibitory effect observed with this compound in this study further analysis of limits of toxicity would be required in order to determine maximum inhibitory concentrations. If this alternative carbon source was to be used, future design of the process could be critical in creating an efficient process. A strategy wherein glucose is used in the batch medium to initiate growth of the organism, followed by a continuous feed of methanol could be employed. This would maintain the level of methanol below the inhibitory concentration but utilisation of the compound would still be observed.

Overall, the presence of these methacrylates and methanol exhibit various effects on the organisms examined. However, as only the parental strains were examined, the results obtained can only give an insight into the possible interactions the compounds would have on the genetically engineered strains that would be employed in the final process. Therefore, similar procedures would be required in order to establish if the same effects were relevant and if they would impact on the efficiency of the proposed process.

6.10 **Future work for Chapter 6**

- Examine at shake flask scale the impact of compounds at higher starting cell concentration. In reality most of the chemicals assessed in this study are end products and therefore would by present when the cell density was higher.

- Identify maximum inhibitory levels of desired compounds to determine how future production processes will have to be designed in order to improve/maintain required productivity levels. Product inhibition would result in a process that was not cost efficient. Designing a chemostat fermentation that maintained the level of product below that of the inhibitory concentration could improve continuous production of the desired compound.

- End concentrations of compounds were not examined here, however, to ensure that the organism are not consuming these as a carbon source, this should be investigated. Utilisation of the desired product would lead to a reduction in the efficiency of the process. Again, by designing a chemostat process would allow for the removal of production broth containing the desired compound which could then be purified with downstream processing.

Chapter

7

Summary of conclusions

This thesis contains the initial investigation into the viability of using *Corynebacterium glutamicum* as a microbial factory for the production of mesaconate, an important building block for the production of polymeric material. By utilising *C. glutamicum*'s natural affinity to overproduce L-glutamate, it was intended to incorporate the glutamate degradation pathway into the organism to produce mesaconate through a *de novo* pathway with glucose as its initial carbon source. Several conclusions could be drawn from the project as a whole and are discussed below.

Firstly, to determine the most efficient method to aerobically overproduce the initial building block compound, L-glutamate, several induction conditions were analysed. While a variety of process conditions were examined, the classical induction method of biotin limitation elicited the highest yield of L-glutamate by a substantial margin and was therefore chosen to examine the engineered strains in Chapter 4. However, future work may be required to refine the method to allow the use of different feedstocks from sustainable resources.

Secondly, initial strain selection also appeared to be significant, with *C. glutamicum* ATCC 13869 obtaining higher yields of L-glutamate compared to that of ATCC 13032 within the bioreactor environment. This was due, in large, to the latter strains continual overproduction of foam during the process, ultimately leading to lower cellular growth and productivity. Therefore, to ensure the most efficient process, ATCC 13869 was used as the parental strain for the construction of the engineered strains examined in Chapter 4.

Although mesaconate production by the engineered strains remains inconclusive, overexpression of both the glutamate mutase and ammonium lyase genes were observed in both strains 2 and 3 examined in Chapter 4. To the knowledge of the author, this is the first time the enzymes required for the glutamate degradation pathway has been expressed in *C. glutamicum* ATCC 13869. As the process remains in the early stages of development, expression of these genes for the intended pathway, in the intended organism, has given the project a valuable launch pad for continued advancement of the process. By examining the use of glutamate mutase

enzymes that are less sensitive to oxygen degradation, improvement in mesaconate production could be achieved.

While a large majority of this study was focused on mesaconate production through an oxidative pathway, Chapter 5 examined the possibility of using the reductive arm of the TCA cycle to produce the desired compounds. Although successful production of succinate was observed, increased accumulation of α-ketoglutarate could not be achieved and requires further work to enable a sufficient process to be developed.

Encouragingly, the primary organism for the proposed process, *C. glutamicum*, appeared unaffected at low cell densities by the presence of several of the potential terminal compounds (Chapter 6). However, future consideration must be paid to the potential use of methanol as a sustainable carbon source, as its presence in high concentrations caused significant detrimental effects on growth and L-glutamate accumulation. A dose dependent inhibitory effect on productivity was also observed in the presence of methacrylic acid and methyl methacrylate. Therefore, to minimise these potential detrimental effects, efficient conversion between the two compounds should be paramount. Further, continual removal of the final product, in this case methyl methacrylate could be incorporated into the final design of the process to maintain low levels of the compound, therefore reducing its effect.

This project is in the early stages of development and requires a great deal of further examination to make the process attractive for industry. Detailed future work for each aspect of this thesis is presented at the end of each chapter. However, based on the overall conclusions reached here, the priority of the future work can be made

Chapter 4

1. The extent of oxygen's detrimental effect on the glutamate mutase enzyme needs to be established in order to assist future development of this process

2. Continued development of an inducible strain containing a more oxygen resistant glutamate mutase enzyme would greatly improve the feasibility of the process.
3. Investigation of potential cofactor inhibition of the glutamate mutase enzyme would also aid in developing an efficient process. Incorporating a cofactor regeneration system may be crucial in maintaining the activity of the enzyme and therefore subsequently sustaining the production of mesaconate.

Chapter 5

1. As with the aerobic process, understanding the cofactor needs of the process is vital in developing an efficient process.

Chapter 6

1. Although most of the compounds examined in Chapter 6 did not appear to effect *C. glutamicum* at low cell densities, methacrylic acid and methyl methacrylate, lowered the end yield of glutamate. As it would be expected that these chemicals would be produced at the end of the production process, examination at high cell densities would show if these characteristics remained when exposed to an established culture.
2. Determination of product uptake is also necessary, as product loss in this way would not be cost efficient.

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Chapter 9 Appendix

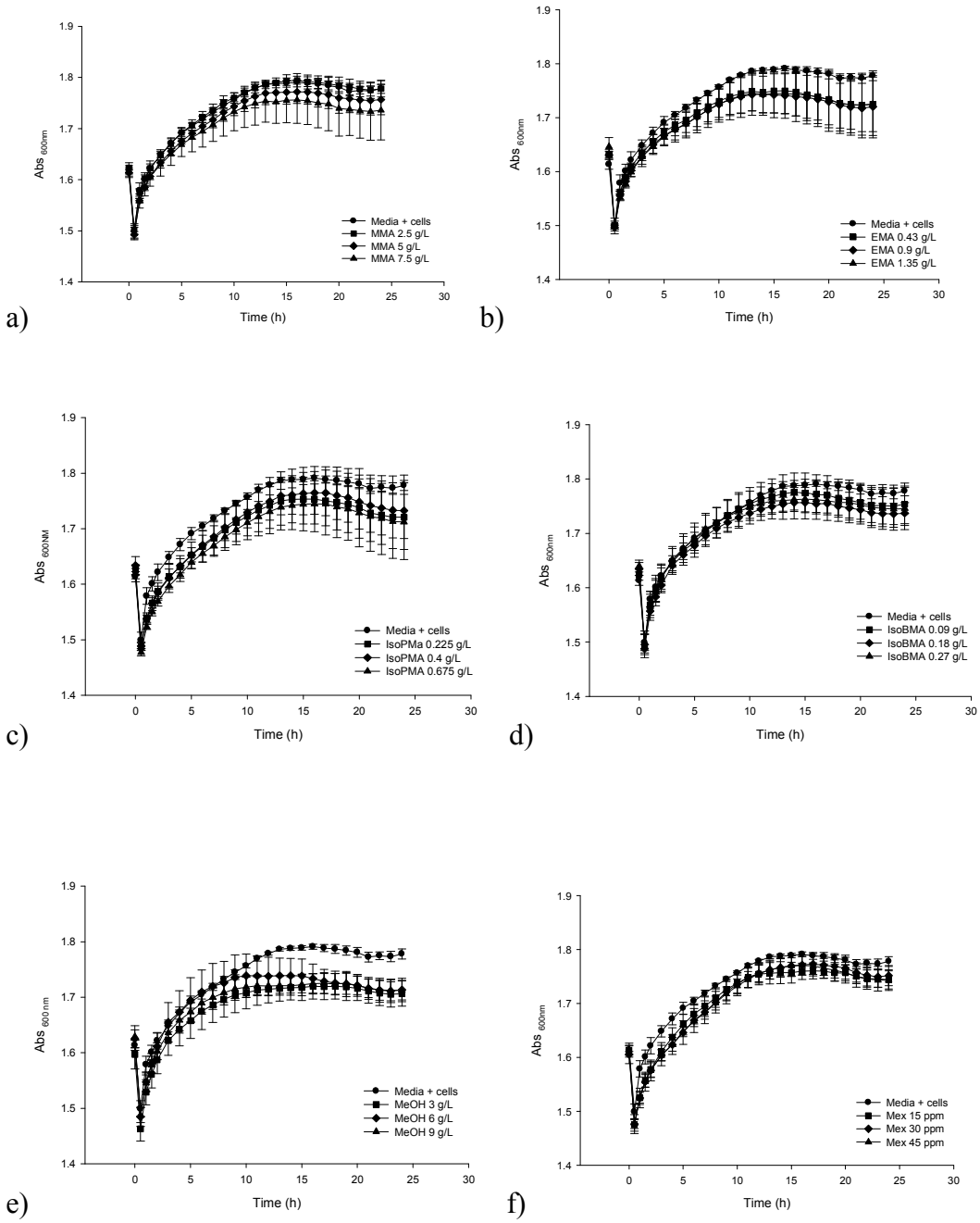

Figure 9-1 *C. glutamicum* 2.5 gl-1 starting cell density

A propagation flask of *C. glutamicum* ATCC 13032 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of CGXII media with 1 µgl-1 of biotin and a) MMA, b) EMA, c) iPMA, d) iBMA, e)MeOH, f) 4-methoxyphenol to an OD600nm = 10. 0.1 % v/v Triton-x (x) was used as a control. Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

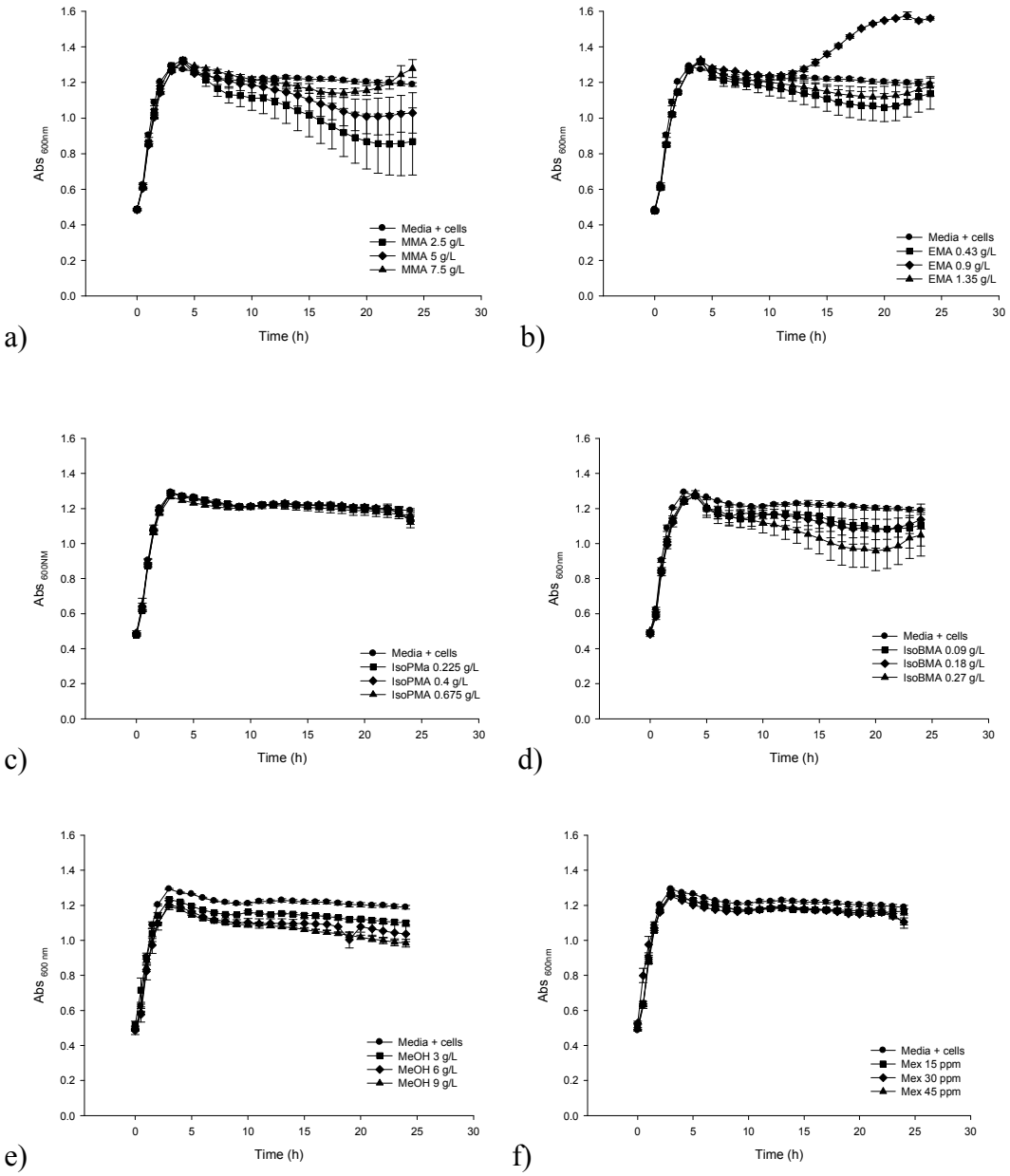

Figure 9-2 *E. coli* 0.1 gl^{-1} starting cell density

E. coli BW25113 ΔpflB *ΔldhA* was used to inoculate 50 mL of Lund media and incubated at 37 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of Lund media and a) MMA, b) EMA, c) iPMA, d) iBMA, e)MeOH, f) 4-methoxyphenol to an OD600nm = 0.5 Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

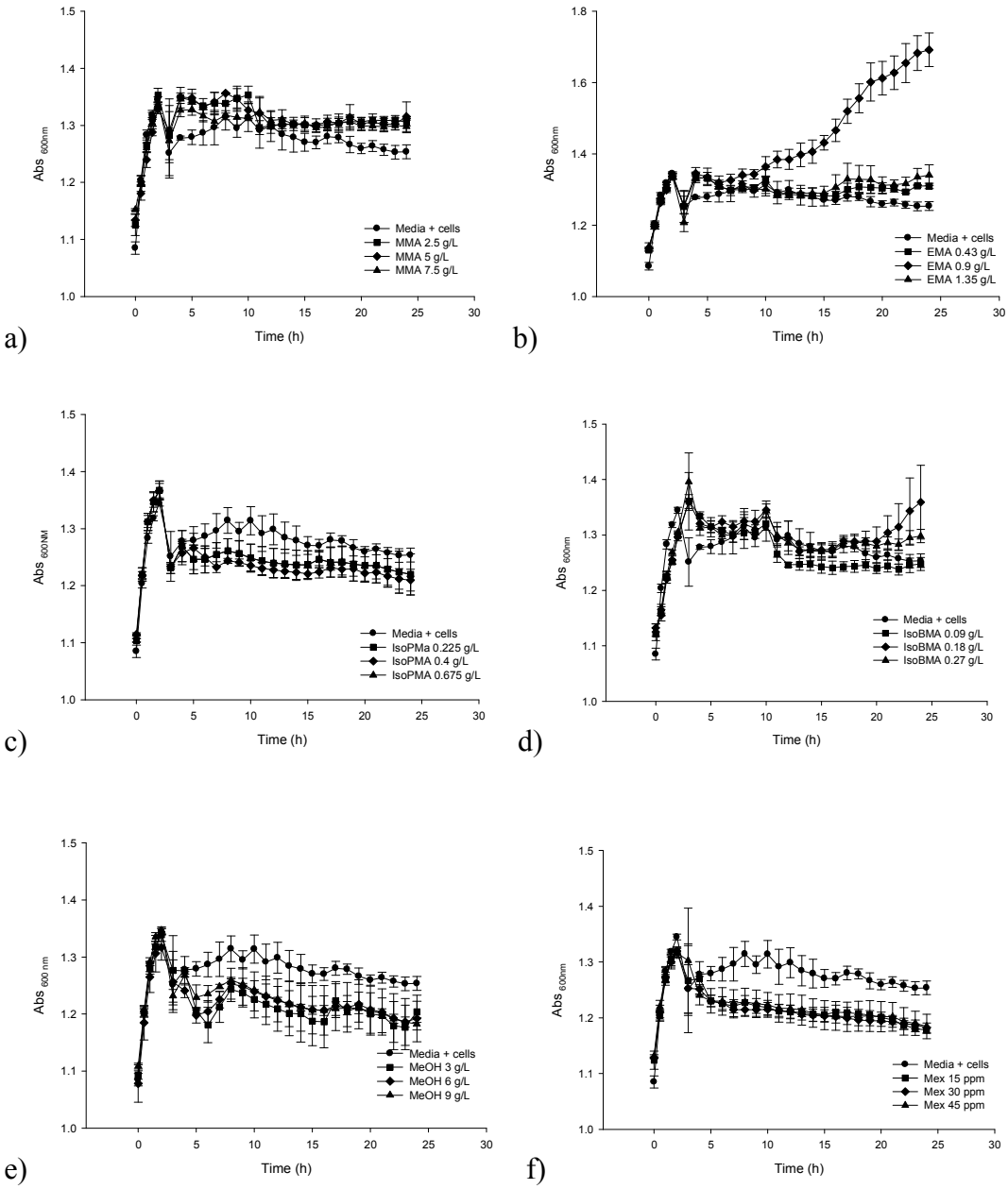

Figure 9-3 *E. coli* 2.5 gl^{-1} starting cell density

E. coli BW25113 ΔpflB *ΔldhA* was used to inoculate 50 mL of Lund media and incubated at 37 °C 250 rpm for 16h. The culture was then used to inoculate a 96 welled containing 200 µL of Lund media and a) MMA, b) EMA, c) iPMA, d) iBMA, e)MeOH, f) 4-methoxyphenol to an OD600nm = 10 Cultures were analysed at 30 °C in a temperature controlled plate reader. Time profiles of the OD_{600nm} are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

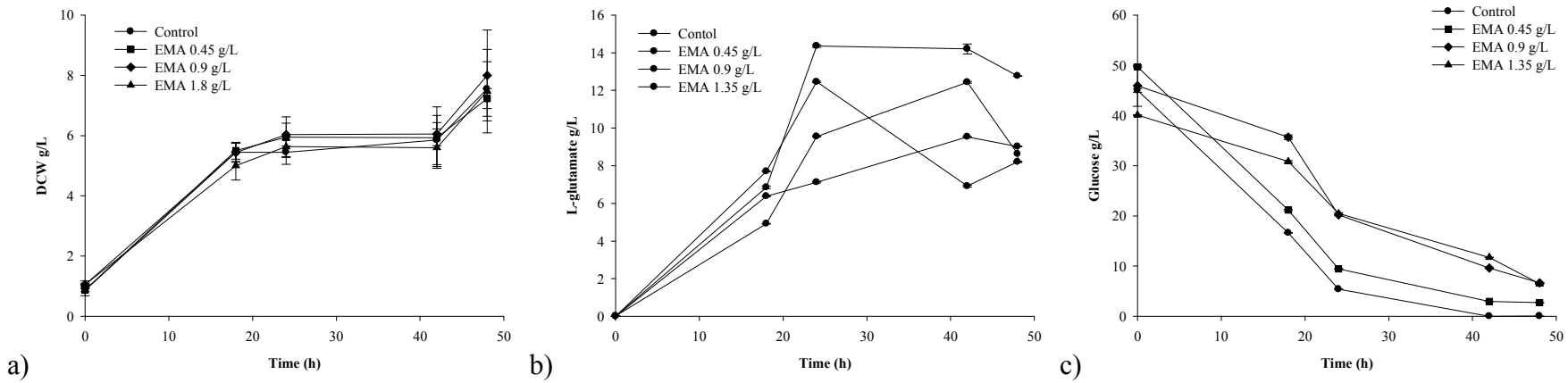

Figure 9-4 Effect of Ethyl methacrylate on *C. glutamicum* growth and productivity

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 0.45 (■), 0.9 (♦) or 1.8 (▲), gl^{-1} of EMA to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

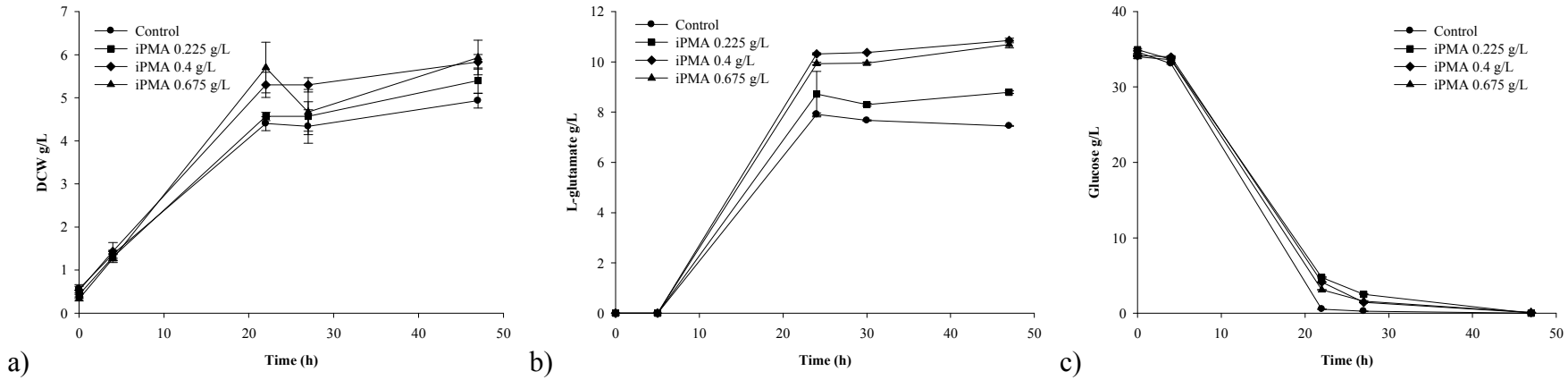

Figure 9-5 Effect of Isopropyl methacrylate on *C. glutamicum* growth and productivity

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 0.225 (■), 0.4 (♦) or 0.675 (▲), gl^{-1} of iPMA to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

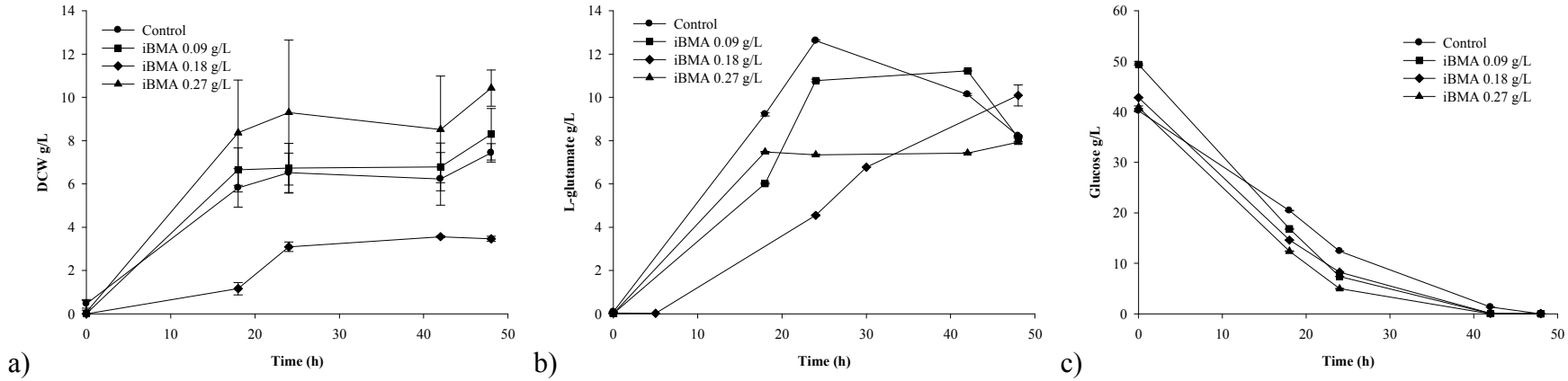

Figure 9-6 Effect of Isobutyl methacrylate on *C. glutamicum* growth and productivity

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 0.09 (■), 0.18 (♦) or 0.27 (▲), gl^{-1} of iBMA to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

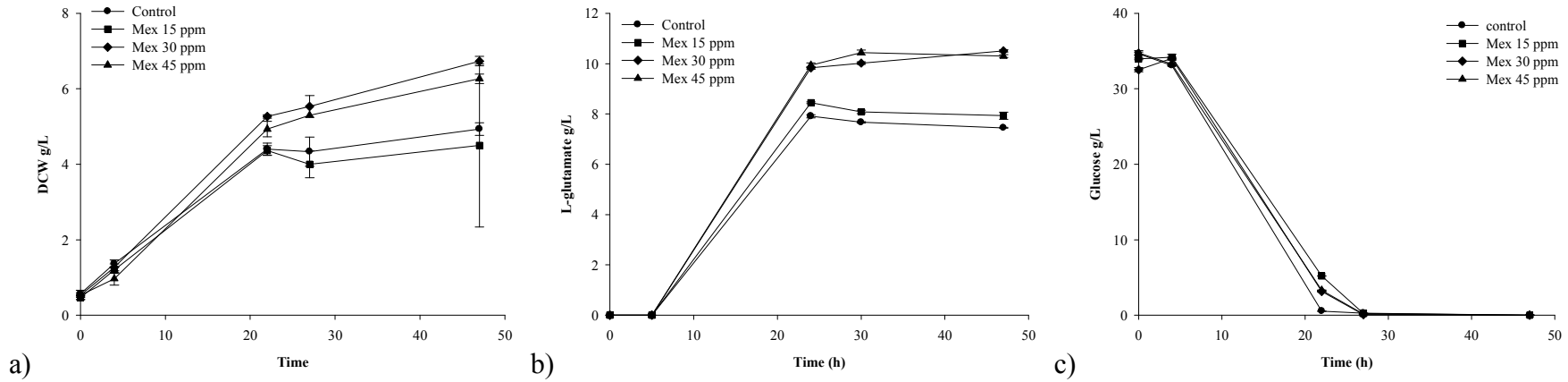

Figure 9-7 Effect of 4-Methoxyphenol on *C. glutamicum* growth and productivity

A propagation flask of *C. glutamicum* ATCC 13869 was used to inoculate the CGXII biotin depletion media to an OD600nm =0.1 and incubated at 30 °C 250 rpm for 16h. The culture was then used to inoculate 50 mL of CGXII media with 1 μgl^{-1} of biotin containing 0 (●), 15 (■), 30 (♦) or 45 (▲) ppm of 4-methoxyphenol to an OD600nm = 1. Cultures were incubated as previously described. Time profiles of a) DCW b) glucose utilisation and c) glutamate accumulation are displayed. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.