Development of bioprocesses for the production of biocatalysts used in the synthesis of amino acids and amines

Ph.D. Thesis

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---------BE---------
WHO YOU ARE
AND SAY
WHAT YOU FEEL,
BECAUSE
THOSE WHO MIND
DON'T MATTER
---------AND---------
THOSE WHO MATTER
DON'T MIND

Dr. Seuss

US author & illustrator (1904-1991)

Acknowledgements

Supervisors. Colleagues. Friends. Family. Everyone Else.

They say the people come in to our lives for a reason. I encountered a lot of people during my time as a PhD student and often that reason was not so clear to me; some of these encounters have been welcomed, others not so much. There are some people in the world who, given the opportunity, would chose to go back in time, and change their lives; to erase certain people from their past. This sounds great, but I *would* be lying to myself by choosing to acknowledge only the positive. I'm embracing the negative too. I'm choosing now, after having left my student days behind me, to accept that ALL of these encounters have helped shape who I am now. I am choosing to thank you for making me laugh and cry; for making me so full of rage and for helping me to recover. You made me smile and you made me miserable. You made me want to quit and you encouraged me to keep going. You made me trusting and mistrusting of everyone I'll ever encounter in the future. There will be times in the not too distant future when I will meet some of you again, either by choice or through the randomness of life; sometimes I will walk on by, and others times I will stop to talk. I put my heart and soul in to my PhD work and it nearly broke me, and it's only right that I be completely honest with you as this chapter of my life comes to a final close.

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Contents

Abstract

Research in to enzyme catalysis has enormously enriched the success of biological organic synthesis; particularly in the last decade, remarkable progress in biological catalysis has taken place leading to the application of enzymes to a wider extent in industrial processes. The benefits of establishing enzymes as industrial catalysts are founded on the need to develop a 'clean' and 'pure' technology which carry out isomer and region selective reactions; generate or transform pure isomers compared with racemic mixtures and produce pure compounds in comparison with mixtures of by-products often arising from conventional chemical synthesis. With the onset of molecular biology and genetics, the manufacturing of high value proteins in recombinant host cells has become commonplace; nevertheless, there needs to be a better understanding of the optimum conditions for the production of every recombinant protein. In this study, the over expression of an industrially important amine oxidase – D-Amino Acid Oxidase (DAAO) – was attempted using two different expression systems: *Escherichia coli* and *Pichia pastoris* in order to establish the optimum conditions for industrial production. Amine oxidases exhibit stereoselectivity; ergo they can theoretically be used for the deracemisation of non-optically pure mixtures of amines leading to optically pure amines of high value. Furthermore, DAAO is involved in the production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin c (CPC), which can be used in the synthesis of novel cephalosporin antibiotic derivatives. In order to accelerate development of these new antibiotics DAAO must be readily available. Overexpression of DAAO in *E. coli* was studied under varying experimental conditions in order to ascertain the best conditions for enzymatic activity whilst simultaneously investigating the variations between both defined and complex media to understand suitability for scale-up. Although *E. coli* is still regarded as the 'go to' organisms for initial laboratory scale up the methylotrophic yeast *P. pastoris* is considered an industrial workhorse for the production of recombinant proteins and thus the expression of DAAO was also investigated to ascertain how enzyme activities are affected by this expression system. Furthermore, the supplementation of media with different nutrient sources of varying complexity was investigated, as was the implementation of co-feeding in order to improve carbon flux during recombinant protein production. Finally, the

oxygen availability was investigated in order to gain important understandings on its effects on microbial physiology and DAAO activity.

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Abbreviations

7-ACA	7-Aminocephalosporanic Acid
AOX	Alcohol Oxidase
ATP	Adenosine Triphosphate
CAT	Catalase
DAAO	D-Amino Acid Oxidase
DHA	Dihydroxy Acetone
DHAS	Dihydroxy Acetone Synthase
DO_2	Dissolved Oxygen
FAD	Flavine Adenine Dinucleotide
FOX	Ferrous Oxidation-Xylenol Orange Assay
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GSH	Glutathione
H_2O	Water
H_2O_2	Hydrogen Peroxide
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth
Lpp	Lipoprotein
LTA	Low Specificity Threonine Aldolase
MAO	Monoamine Oxidase
O_2	Oxygen
O_2-	Superoxide Anion
OH	Hydroxyl Radical
OmpA	Outside Membrane Protein
ORT	Operator Repressor Titration
pDNA	Plasmid Deoxyribonucleic Acid
pFLD1	Formaldehyde Dehydrogenase
ppGpp	Guanosine Pentaphosphate
RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
SCP	Single Cell Protein
SHMT	Serine Hydroxymethyl Transferase
SOD	Superoxide Dismutase
SUMO	Small Ubiquitin-Like Modifier

Chapter One

Literature Review & Aims

1 Literature Review & Aims

1.1 *The Physiology of Microbial Expression Systems*

1.1.1 *Enzyme Production*

Research in the area of heterologous (recombinant) protein production using microorganisms is prodigious; numerous expression systems exist which allow for the overexpression of many industrially and medically important proteins (Chen, 2012) such as xylanases for biobleaching and animal feeds; proteases such as Alcalase and Esparase for detergents; lipases for the dairy industry and cellulases for agriculture.Proteins are the elementary units of life, engineered by all living things. Ranging from biocatalysts to the constituents of the cytoskeletal system; from having key roles in immunological responses, cellular signalling pathways and cellular metabolism to being important players in the biopharmaceutical, industrial and agricultural industries, there is no doubting how intrinsic proteins are to life today (Demain and Vaishnav, 2009). Due to the ease with which enzymes in particular can be produced by microbial expression systems the use of industrial enzymes is estimated to make almost $8 billion dollars by 2018 (Joshi and Satyanarayana, 2015).

With the advancement of recombinant DNA technology, and protein engineering, enzymes can be engineered to the demands of the end-user: the native characteristics of an enzyme need no longer be adhered to. Synthetic biology is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms. Site directed mutagenesis is a powerful tool that changes a single amino acid sequence, yet can output physiological transformations that can positively effect changes in pH optimization, thermostability and substrate specificity amongst others (Demain and Vaishnav, 2009). Directed evolution has also been employed to increase the best characteristics of the expression system in producing the desired enzyme (Tobin *et al*., 2000, Voulgaris *et al*., 2011). Theoretically enzyme properties can be modified by rational design. Unfortunately, rational design is prevented in practice by the intricacies of protein function. Directed evolution mimics natural evolution by generating a protein with novel activity through screening or selection of a desired function(s) from a vast array of protein variants (Tao and Cornish, 2002). The review

paper ‘Directed enzyme evolution’ by Farinas *et al*. (2001) discusses in detail the development of therapeutic enzymes using the aforementioned method. In 1998, therapeutic enzymology was estimated to be a $1.5 billion dollar industry with an annual growth rate of 2-15% depending on the industry in which the enzyme(s) is associated with (van Beilen and Li, 2002).

1.1.2 *Systems for Recombinant Protein Production*

The preliminary stage in the production of recombinant protein production is the isolation and duplication of the desired DNA; the protein is then produced in large volumes in the preferred expression system. Expression of proteins can occur in cell cultures of bacteria as diverse as *Escherichia coli* and *Pseudomonas fluorescens* (Retallack *et al*., 2012*)*; yeasts including *Pichia pastoris* and *Saccharomyces cerivisiae* (Li *et al*., 2008); molds (Singh and Satyanarayana, 2011); plants (Xu *et al*., 2011) and viruses and insects or insect cells using the baculovirus expression system (Yamaji and Konishi, 2013, Chen, 2012). The expression systems that will be discussed in this review are *Escherichia coli* and *Pichia pastoris.*

1.2 *Escherichia coli*

1.2.1 *Background*

The Gram negative bacterium *Escherichia coli* is a driving force in the field of expression systems (Gomes et al.). Possessing the most well characterised genome of any microorganism it helped to revolutionise the biotechnology industry (Lecointre *et al*., 1998, Hugenholtz, 2002). *E. coli* was used for the production of the world’s first recombinant DNA pharmaceutical in 1982: human insulin (Swartz, 2001). Thanks to advances in the biotechnology and fermentation industries, human insulin could be produced economically, efficiently and in sufficient quantities to help treat millions of diabetes mellitus patients worldwide. As pioneering as *E. coli* once was, advances in eukaryotic and mammalian cell culture systems have seen the popularity of *E. coli* dwindle since the mid nineteen-nineties. However, as an initial platform for laboratory investigations and as a comparative tool for other expression systems, *E. coli* is still the prokaryotic expression system of choice (Chen, 2012). Table 1-1 highlights some of the advantages and disadvantages of *E. coli* as an expression system.

Table 1-1. Advantages and Disadvantaged of E. coli as an expression system.

Advantages	Disadvantages
Rapid Expression	Proteins with Disulphide Bonds difficult to express
High Yields	Produce Unglycosylated Proteins
Ease of Culture and Genome Modifications	Proteins Produced with Endotoxins
Inexpensive	Acetate formation resulting in cell toxicity
Mass Production Fast and Cost Effective	Proteins produced as inclusion bodies; are inactive; require folding

For many years *E. coli* was thought to be incapable of assembling glycosylated proteins. Subsequent investigations in to the Gram negative bacterium *Campylobacter jejuni* identified an N-linked glycosylation system that could theoretically be transferred in to *E. coli* expression systems (Wacker *et al*., 2002). More recent studies in 2011 by Ihssen *et al*. (2010) have further demonstrated the glycosylation capabilities of *E. coli*. Unfortunately, glycosylation is often imperfect, with single figure percentage success rates for complete glycosylation (Chen, 2012). In general however, eukaryotic protein expression is favoured in eukaryotic organisms for post translational modifications.

Interestingly, the procedures used to create the genetically altered organism can be problematic. Antibiotic resistance plasmids are used to exert selective pressure on the *E. coli* organisms. The general principal of antibiotic resistance plasmids involves the insertion of an antibiotic resistance gene in to the plasmid along with the gene for the protein to be expressed. In nature bacteria have various enzymes that cut up the DNA of their natural enemies, such as bacteriophages (bacterial viruses). Researchers have taken advantage of these so-called restriction enzymes to splice DNA for use in engineering bacteria. Hundreds of restriction enzymes have been isolated and each will cut a DNA strand at a specific sequence of nucleotides. Some restriction enzymes generate blunt ends, cutting across both strands of DNA. Others generate a staggered cut, producing "sticky ends." These ends anneal by hydrogen

bonding to similar ends on another DNA segment cut with the same restriction enzyme.

Cloning a gene involves identifying a gene of interest in an organism, isolating DNA from that organism, and then using a restriction enzyme to snip the gene from the DNA strand. The gene containing segment can then be spliced into a plasmid cut by the same restriction enzyme. The bacteria take up the plasmid and are allowed to replicate.

Ordinarily, bacterial cells do not readily take up plasmids. Researchers can use various tricks, however, to get cells more ready to do so. One common method holds the cells on ice in a solution of calcium chloride. The cells are then briefly heat shocked so the plasmid can cross the plasma membrane. An alternate method, electro corporation_uses a short electrical pulse to open pores in the plasma membrane, allowing the plasmid to pass through.

Marker genes, such as genes for antibiotic resistance, are often engineered into plasmids. These marker genes enable researchers to know which bacteria have the plasmids. The antibiotic is added to the media used to grow the bacteria. Cells that do not contain the plasmid will fail to reproduce. In addition to marker genes, plasmids typically contain one or more genes of interest. For example, a protein not otherwise expressed by the recipient cell might be produced only when the plasmid is present. Individual colonies of bacteria, each derived from a single cell, can be evaluated for the expression of such novel gene products.

If the *E. coli* has successfully taken up the plasmid, and they are then subsequently cultured on an agar plate containing the antibiotic, then only the cells harbouring the plasmid will begin to replicate. Therefore it is safe to assume that the *E. coli* growing is fully capable of expressing the desired protein (Chen, 2012). The major drawback of this system is that it aids the development of antibiotic resistance and the antibiotic itself needs to be removed from the final product and thus requires expensive downstream processing (Vidal *et al*., 2008). Fortunately, several novel methods of plasmid selection or construction exist and will be discussed later in section **1.2.5.**

1.2.2 *Cellular Response to Recombinant Protein Production*

Industrial production of therapeutic proteins is a billion dollar industry (Lecina *et al*., 2013). It is a fundamental fact that in order improve the quality of produced recombinant proteins, there needs to be a greater understanding of not only the genetics of the expression system used, but also of the production process itself, as well as a greater perspective of the structures and functions of the recombinant proteins produced; in many respects, *E. coli* is the catalyst facilitating this (Papaneophytou and Kontopidis, 2014, Feng *et al*., 2014).

1.2.2.1 *Physiological Overview*

All bacteria require energy for growth and metabolism. To maintain, propagate and ensure complete functionality of a plasmid would require an increase in energy that is subsequently diverted from normal cellular function to the harboured plasmid; this is referred to as "*metabolic burden*" (Carneiro *et al*., 2013). When a bacterium is genetically engineered to produce heterologous proteins, metabolic burden is further increased when said proteins are induced via a promoter system: target genes are cloned in the host bacterium, usually resulting their high level expression and, therefore, metabolic burden (Özkan *et al*., 2005). Quantitative physiology incorporating metabolic flux analysis is a progressive technique being used by many researchers to further advance the understanding of metabolic burden and how this bottleneck can theoretically be improved with the optimisation of the host system and bioprocess conditions (Heyland *et al*., 2011). Furthermore, the advent of systems biology (Bose, 2013) has allowed for a more statistical approach to metabolic burden and the maximisation of recombinant protein production in *E.* coli (Papaneophytou and Kontopidis, 2014, Royle and Kontoravdi, 2013). Concurrent to the degradation of formed heterologous proteins, synthesis rates can be in excess of the accumulation rates and as a consequence of this, biomass formation can be lost as a result of the elevated energy demand of the heterologous protein produced, usually as a consequence of augmented respiration for the regeneration of adenosine triphosphate (ATP); often the amassing of product can be imperceptible or extremely low. *E. coli* is known to have major difficulty in excreting soluble protein products to the external medium in a bioreactor vessel (Chen, 2012).

E. coli often produces proteins as inclusion bodies. The formation of inclusion bodies is multifactorial; no one single step in the protein expression process can be

attributed as the sole problem (Ventura and Villaverde, 2006). Overexpression of a particular gene product can have disastrous consequences when it comes to protein formation, such as miss-folding, and solubility (Hsu *et al*., 2014). Temperature, growth and stress responses, amongst others, all factor in to why *E. coli* grapples to secret proteins as effectively as eukaryotic and mammalian expression systems (Moll and Engelberg-Kulka, 2012). However, inclusion bodies are generally a source of pure protein and as such should not be looked upon as purely inconveniences. Several recent studies have aimed to improve the remuneration of proteins from inclusion bodies, such as sequential tentacle grafting and charge modification for enhancing the charge density of mono-sized beads for facilitated protein refolding and purification from inclusion bodies (Dong *et al*., 2014); the targeted expression, purification, and cleavage of fusion proteins from inclusion bodies (Hwang *et al*., 2014) and the expression of soluble and active interferon consensus in SUMO fusion expression systems in *E. coli* (Peciak *et al*., 2014). Previously a multitude of lipoproteins derived from Gram negative bacteria have been utilised in the creation of fusion proteins affiliated with the outer membrane (Cornelis, 2000). The rudimentary lipoprotein based system is the miscegenation of Braun's lipoprotein (Lpp)-OmpA protein composed of the signal peptide and eight amino acids of the major Braun's lipoprotein of *E. coli* and amino acids of the OmpA outer membrane protein (Cornelis, 2000, Francisco *et al*., 1992). A signal peptide (sometimes referred to as signal sequence, targeting signal, localization signal, localization sequence, transit peptide, leader sequence or leader peptide) is a short (5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway. OmpA has extensively been used as a model protein for understanding the folding and membrane insertion of β-barrel membrane proteins; comprised of a 171-residue N-terminal 8-stranded β-barrel transmembrane domain immured within the outer membrane and a 154-residue C-terminal periplasmic domain, it is one of the most bountiful structural proteins within *E. coli's* outer membrane, necessary for counterbalancing the effects of aggregate mating during conjugation; as bacteriophage receptors (Nair *et al*., 2009) and low permeability porins which facilitate the slow diffusion of minute solutes (Piggot *et al*., 2013, Wang *et al*., 2013).

1.2.3 *Stress Responses*

Heterologous protein production in *E. coli* can trigger various stress responses giving rise to high levels of stress proteins which, in the company of increased heterologous proteins, culminates in increased respiration rates and subsequently, maintenance energy requirements: this signifies that heightened energy demands during the synthesis of these proteins is the root cause of metabolic burden. Depending on the process conditions, whether induction occurs or not, such stresses can include, but are not limited to, cold shock response (Vidovic *et al*., 2011), pH stress response (Fritz *et al*., 2009), hyperosmotic response (Oh *et al*., 1998), heat shock response (Vostiar *et al*., 2003) SOS response (Shah *et al*., 2013) oxidative stress response (Smirnova *et al*., 2009) and the stringent response (Edwards *et al*., 2011).

In vivo protein folding has a somewhat mutually beneficent relationship with molecular chaperons helping to aid proper isomerisation and cellular targeting of other polypeptides by temporarily interacting with folding intermediates and through the use of foldases, which hasten rate-limiting steps in the folding process. The best defined of *E. coli*'s molecular chaperones are the ATP-dependent DnaK-DnaJ-GrpE and GroEL-Gro-ES systems (Zahn *et al*., 2013, Calloni *et al*., 2012, Hartl and Martin, 1995, Castanie-Cornet *et al*., 2014). There has been a comprehensive demonstration that co-overproduction of the DnaK-DnaJ or GroEL-Gro-ES chaperones can considerably raise the levels of soluble aggregation-prone proteins (Mayer and Bukau, 2005).

However, this is associated with the nature of the overproduced protein (Vostiar *et al*., 2003). Amassing of misfolded proteins in *E.* coli can cause induction of heat shock response which is controlled by the sigma factor σ^{32} with regulation dependent on the DnaK chaperone discussed above; thus exemplifying the intimate relationship between non-growth related products resulting in an increase in maintenance requirements during heterologous protein production. DnaK, by attaching to aggregation liable proteins, is detached from σ^{32} causing σ^{32} to have elevated stability and activity. A temperature rise results in the synthesis rates of σ^{32} being increased by translation of mRNA and thus numerous genes regulated by σ^{32} including *dnaK* are briefly expressed at heightened levels subsequent to an upshift in temperature (Roy *et al*., 2012). The principal function of heat shock proteins is the

maintenance of protein quality; chaperoning the folding of proteins, they simultaneously prevent the aggregation and re-solubilisation of aggregated proteins; additionally proteins incapable of folding in to their correct configuration are degraded (Roy *et al*., 2012). Provocation of a heat-shock-like response after the initiation of recombinant protein synthesis has been well documented for many years in a number of alternative recombinant expression systems.

DnaK and GroEL are the most plentiful heat shock chaperones and display increased transcription, synthesis rates and accumulation levels during heterologous protein production in *E. coli*. The stringent response in *E. coli* is when cells that have been induced afterward suffer from an abrupt deficiency in aminoacylated tRNA which in turn leads to an instantaneous interruption of the synthesis of functional RNA's including tRNA and rRNA which ultimately affects recombinant protein production (Hoffmann and Rinas, 2001, Hoffmann and Rinas, 2004, Hoffmann *et al*., 1999).

The SOS response in *E. coli* is activated when chemicals or conditions which damage DNA and/or interfere with its replication are exposed to the cells (Hamilton *et al*., 2013, Bellio *et al*., 2014, Belov *et al*., 2013). Governed by the induction of an intricate cascade of enzymes providing repair to damaged DNA along with an enhanced mutational frequency, the SOS response is unfortunately crucial for the maintenance of healthy cells. Through the utilisation of *lac-Z-based* SOS-reporter gene fusions, it has been demonstrated that the SOS response is generated by temperature-induced heterologous protein production but not if the exact same product is produced in an IPTG-inducible expression system *even* when the temperatures are elevated (Hoffmann and Rinas, 2004). Moreover, several experimental studies have produced results indicative of SOS induction being dependent upon the construct employed and complies only with heterologous protein production, not homologous *E. coli* proteins or indeed when a particular strain carrying the insert negative expression vector was exposed to the optimum conditions for protein induction (Lee *et al*., 2002).

The above global regulatory stress response systems can also have an abstruse effect upon cells by inducing or increasing protease activity and there is further evidence that heightened levels of ppGpp (the nucleotide guanosine 5'-diphosphate

3’-diphosphate) elicited by the reduction of amino acids or the astringent energy/carbon limitation, gives rise to the stringent response (Maciag-Dorszynska *et al*., 2013). An inverse correlation between intracellular concentration of ppGpp, growths rates and the quantity of steadfast RNA transcripts exists.

1.2.4 *Metabolic Burden*

As discussed previously, the overexpression of heterologous proteins in *E. coli* is oftentimes accompanied by unrelenting metabolic changes in the host cell with growth retardation indicative of this; fundamentally attributed to the aberration of monomers and the diversion of energy toward the recombinant protein (Özkan *et al*., 2005).

Despite the commodious knowledge of the molecular genetics of *E. coli*, not all genes can be expressed as easily as others in *E. coli.* The reason for this is multifactorial: gene sequences are unique and structurally dissimilar, albeit somewhat inconspicuously so; mRNA has fluctuations in both stability and translational efficiency; protein folding is not always straightforward; secreted heterologous proteins can be easily degraded by host cell proteases and codon usage amongst the foreign gene and native *E. coli* differs greatly. For several years *E. coli* was thought to be incapable of assembling glycosylated proteins. Subsequent investigations in to the Gram negative bacterium *Campylobacter jejuni* identified an N-linked glycosylation system that could theoretically be transferred in to *E. coli* expression systems (Ihssen *et al*., 2010, Wacker *et al*., 2002). More recent studies by, for example have further demonstrated the glycosylation capabilities of *E. coli*. Srichaisupakit *et al*. (2014) successfully constructed the first *E. col*i system to provide synthesis of an N-glycoprotein with a homogeneous N-glycan structure, which might be applicable as a platform for the production of other homogeneous N-glycoproteins. Unfortunately, glycosylation is often imperfect, with single figure percentage success rates for complete glycosylation. In general however, eukaryotic protein expression is favoured in eukaryotic organisms for post translational modifications.

Interestingly, the procedures used to create a genetically altered organism can be problematic. Antibiotic resistance is used to exert selective pressures upon the *E.*

coli. The general principle of antibiotic resistance plasmids involves the insertion of an antibiotic resistance gene in to the plasmid along with the gene for the protein to be expressed. If the *E. coli* has successfully taken up the plasmid, and then are subsequently cultured on an agar plate containing the antibiotic of choice, whether it is kanamycin amongst others, then only the cells harbouring the plasmid will begin to replicate. Therefore it is safe to assume that the *E. coli* culture growing is fully capable of expressing the desired protein (Chen, 2012). The major drawback of this system is that it aids the development of antibiotic resistance and the antibiotic itself needs to be removed from the final product; consequently downstream processing can become very expensive (Vidal *et al*., 2008).

1.2.5 *Plasmid Maintenance and Antibiotic Free Plasmid Systems*

Antibiotics and antibiotic resistance genes are commonplace in the selection and maintenance of recombinant plasmids in hosts such as *E. coli*. Though a strong and effective selection tool, their use has been deemed unsatisfactory by regulatory authorities in the biotechnology industry so much so that there is worldwide scientific and regulatory investigations taking place (Oliveira and Mairhofer, 2013). The spread of bacterial genes that give resistance to antibiotics presents an environmental hazard, which is worsened by the release of antibiotic from the fermentation facility also (Overton, 2014). The optimal expression vector used in bacteria for industrial cultivation should be highly stable in the growing culture; it should have no negative effect on growth rates of the host cell and it should not create any environmental problems, like the potential spreading of antibiotic resistance genes to natural bacterial populations.

Plasmids existing naturally are passed to the two daughter cells, since different stabilization systems are in place which all give an elevated level of stability. Such examples include (i) Site specific recombination systems such as the *cer* system of the ColEl-plasimid (Sasagawa *et al*., 2011). Multimeric forms of the plasmid are resolved in to monomers to ensure an even spread of the plasmid in the daughter cells (ii) Partitioning systems, such as the sop^{+} locus of the F-plasmid, which distributes evenly the plasmid between daughter cells (iii) Addiction systems, like the *parB* (*hok/sok)* locus of the R1-plasmid of the *ccd*-locus of the F-plasmid. In this instance, all the daughter cells lacking a plasmid are killed by way of a system of

stable toxins and unstable antitoxins, both of which are expressed via the plasmid (Hägg *et al*., 2004).

An alternative maintenance strategy is the operator-repressor titration system (ORT) reported by Cranenburgh *et al*. (2001) which makes use of *E. coli* strains possessing essential genes for host survival under the control of the lac operator/promoter system. This system permits the growth of the modified host strain only when the lac promoter is induced by the addition of β-Galactose or IPTG or when the cell is transformed with a multicopy plasmid containing the short *lac* operator sequence. This system has demonstrated the maintenance of plasmid stability during high cell density cultivation and subsequent expression of a heterologous protein (Durany *et al*., 2005).

Regardless, the majority of these stabilization systems have yet to be investigated under bioprocess conditions for the production of recombinant proteins, nor have they been tested to transform conventional production systems that are already in use in order to avoid antibiotics and antibiotic resistance genes during high cell density cultivations, and further compared in terms of genetic stability and process productivities (Vidal *et al*., 2008).

The instability of a plasmid in a bacterial culture is mainly caused by factors leading to an uneven distribution of plasmids to the daughter cells during cell division. Faster growth of bacteria which no longer have to deal with the metabolic burden of carrying a plasmid will consequently result in an overgrowth of plasmid-less segregates in the culture. Since basic replicon plasmids do not carry any gene system for an ordered distribution to the daughter cells, plasmid loss takes place. The loss of plasmids presents a significant industrial problem in regards the large scale cultivation of host bacteria carrying basic replicon plasmids, which are generally used for the expression of cloned genes. Several approaches have been used in an attempt to hinder the segregation and instability of cloning plasmids.

1.2.6 *Plasmid Selection and pDNA Vaccines*

As discussed previously, the demand for pDNA for vaccines and gene therapy has, over the last decade, aggrandized as a consequence of the continued improvements in molecular biology and genetics (Mairhofer *et al*., 2010). To permit selective retention in *E. coli* plasmid vectors, which are adopted for the production of

biopharmaceuticals, as a rule code for the aminoglycoside phosphotransferase (*nptII)* conciliating resistance to kanamycin, an aminoglycoside bactericidal antibiotic isolated from the bacterium *Streptomyces kanamyceticus (*Shao *et al.,* 2013*).* Currently this is the only one which appeases the standards of regulatory bodies. Using selection markers that grant resistance to antibiotics in vaccine plasmids may allow for the transformation of the patients micro flora and spread resistance genes. Furthermore, in recombinant protein production for therapeutic applications, the antibiotic must be removed from the final product. An additional problem can come in to sight from the potential loss of selective pressure as a consequence of the deterioration of the antibiotic; ampicillin can deteriorate through β-lactamase degradation in under 30 minutes in a high cell density culture (Vidal *et al*., 2008, Glenting *et al*., 2002).

Considerable efforts have been made to replace antibiotics as selective markers for plasmid stability in bacteria; notably, several systems which use auxotrophic markers based on complementation of a mutation or deletion in host chromosomes have been engineered to provide stable plasmid selection based on auxotrophies in biosynthetic pathways for the production of recombinant proteins in *Lactococcus lactis (*Glenting *et al., 2002)*; for the production of amino acids in *Corynebacterium* or *Brevibacterium;* or for engineering a live bacterial viral delivery system for an array of antigens (Spreng and Viret, 2005) in *E. coli*, *Salmonella* and *Vibrio cholera* strains (Ryan *et al*., 2000).

Mairhofer *et al*. (2010) discuss how pDNA quality and consummation are negatively affected by antibiotic selection markers and non-essential bacterial sequences. Furthermore, they discuss how pDNA can have the potential to evoke *in vivo* inflammatory responses as well as impede the efficiency of transfection as a result of increased size. Overburdening of the host machinery during bioprocesses developed for the production of pDNA is commonplace especially with the immense over-replication of pDNA effected by runaway replication (Olsson *et al*., 2012) amongst others. The determinant of metabolic burden on host cells is the expression of the antibiotic resistance gene. Theoretically, these sequences need to be eliminated from the backbone of the vector because they are ultimately non-essential for the final attributes of the product as well as abating the effectiveness of the final pDNA product. It is possible, as illustrated by Mairhofer (Mairhofer *et al*., 2010,

Mairhofer and Grabherr, 2008), that the maintenance of antibiotic free plasmid systems is achievable by using the origin of replication (ori)-encoded RNAI in lieu of the antibiotic resistance marker. Vidal *et al*. (2008) utilised an amino acid auxotrophy complementation system in *E. coli* as substitute for antibiotic selection markers. The formation of glycine is achived in *E. coli* by serine hydroxymethyl transferase (SHMT) when glucose is the sole carbon source. Encoded by the *glyA* gene, this enzyme has two main functions (i) Threonine aldolase activity which catalyses the reversible permutation of glycine and L-threonine as well as acetaldehyde and (ii) serine hydroxymethyl transferase activity which catalyses the reversible permutation of serine and glycine. Furthermore, a thermostable low specificity threonine aldolase (LTA) encoded by the *ltaE* gene has been discovered in *E. coli* and is responsible for the relatively poor growth rates observed for *E. coli* GS245, a mutant with the inability to synthesise glycine due to an inactivated *glyA* gene (Vidal *et al*., 2008, Iurescia *et al*., 1996). Post segregation killing is a method of plasmid stabilisation which involves the expression of a gene product that to a cell lacking a plasmid upon division would be toxic (Peubez *et al*., 2010). The bacterial toxin-antitoxin (TA) system is a constituent of bacteria that has the potential to play a key role in cell perpetuation under stress conditions including nutrient limitation; antibiotic treatment; temperature fluctuations as well as oxidative stress regardless though they are not necessitous for normal cellular growth (Park *et al*., 2013). The *hok/sok*_system of post segregation killing is an example of the TA system. *E. coli* possesses numerous type I TA systems such as SymR–SymE (Park *et al*., 2013). Sberro *et al*. (2013) have an excellent review focussed on the discovery of Functional Toxin/Antitoxin Systems in Bacteria by Shotgun Cloning.

Repressor titration is another example of a novel antibiotic free selection system. If the *lac* operator (or *tet* operator) is present on the plasmid and there is an antibiotic gene which is controlled by the appropriate operator on the chromosome, the subsequent titration of the repressor by the operator causes the chromosomal gene to be expressed and thus antibiotic resistance. Several therapeutic plasmids have been created using this particular construction. An alternative system has utilised the *dapD* gene but under the control of the lac operator/promoter. These systems are advantageous because of their small size and the removal of antibiotic resistance, in the case of *dapD* though unfortunately fermentations which give a high yield of

products under these maintenance systems have yet to be reported (Luke *et al*., 2009).

1.3 *Pichia pastoris*

1.3.1 *Background*

The industrially important methylotrophic yeast *Pichia pastoris* (9.8 Mbp genome) was developed in the early 1970's as a source of single cell proteins (SCP). Because methanol can be synthesised from natural gas (methane), historically, *P. pastoris* was exploited industrially to convert bounteous methanol supplies to a source of protein which was to be employed as an animal feed. Both methods and media were developed by Phillips Petroleum Company throughout the seventies which could theoretically yield dry cell weights of in excess of 130g/L. Concurrent to this was the Oil Crisis of the 1970's which unfortunately resulted in the price of methane rising exponentially whilst, rather ironically, the cost of alternative animal feeds (such as soy beans) fell dramatically thus rendering the production of SCP from *P. pastoris* as economically unviable. Seemingly unfazed by this turn of events, Phillips Petroleum Company continued their research in to the *AOX1* promoter of *P. pastoris* during the 1980's and by the turn of the 1990's they had concluded that combining the previously developed methods and media with expression of products under the control of the extremely powerful and tightly regulated *AOX1* promoter, made *P. pastoris* an exciting concept for the industrial production of recombinant proteins (Cregg *et al*., 2000). *P. pastoris* has become a reference mark for the expression of recombinant proteins in eukaryotes (Potvin *et al*., 2012, Sasagawa *et al*., 2011, Tortajada *et al*., 2012).

It's versatility as a system for recombinant protein expression has led to *P. pastoris* becoming one of the most extensively studied yeasts (Potvin *et al*., 2012). Thanks in part to the decision by Phillips Petroleum Company to release the *P. pastoris* expression system to research laboratories in 1993 (Cregg *et al*., 2000) its excellent ability to generate high-cell density cultures utilising inexpensive substrates including glycerol and methanol, coupled with its capacity to introduce post-translational modifications as well as high protein yields which can be secreted to the extracellular milieu, and the abundance of commercial vectors and hosts.

1.3.2 *Methanol Metabolism*

A unique methanol utilisation pathway is shared by all yeasts possessing the ability to utilise methanol as a carbon source; expression of which is controlled and regulated at a transcriptional level. The methanol inducible pAOX1 must be credited for *P. pastoris'* success as an expression system in the field of recombinant protein production. Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA) first isolated the aforementioned promoter whilst working in conjunction with Phillips Petroleum Company to develop *P. pastoris* as a recombinant gene expression system in the 1980's (Cregg *et al*., 2000, Potvin *et al*., 2012).

Figure 1-1 illustrates the unique methanol utilisation pathway. Peroxisomes are essential whilst *P. pastoris* is growing on methanol because contained within them are 3 enzymes key for methanol metabolism, alcohol oxidase (AOX), catalase (CAT) and dihydroxyacetone synthase (DHA) (Jahic *et al*., 2002, Jahic *et al*., 2003). As can be seen in Figure 1 methanol is oxidised to formaldehyde, generating hydrogen peroxide through the actions of the enzyme AOX. This hydrogen peroxide (H_2O_2) is broken down to oxygen (O_2) and water (H_2O) through the actions of catalase. Formaldehyde is either oxidised by two dehydrogenase reactions (dissimilation pathway) or assimilated in the cell metabolism by condensation with xyulose-5-phosphate (assimilation pathway). The condensation is facilitated by dihydroxyacetone synthase which catalyses the formation of dihydroxyacetone and glyceraldehyde-3-phosphate from formaldehyde and xyulose-5-P. These C_3 compounds are further assimilated within the cytosol. Fructose-1-6-biphosphate is formed by an aldose reaction of dihydroxyacetone and glyceraldehyde 3-phosphate. Xyulose-5-phosphate is regenerated by the xyulose monophosphate cycle involving transaldoase, transketolase, pentose isomerase and epimerase reactions. According to Jahic *et al*. (2003) only one third of the glyceraldehyde-3-phosphate generated is used for biomass and the generation of energy. In the dissimilation pathway, formaldehyde spontaneously reacts with glutathione to S-hydroxymethylglutathione after diffusing from the peroxisomes. This is further oxidised in two consecutive reactions to carbon dioxide by the action of a glutathione (GSH) and NAD^+ dependent formaldehyde dehydrogenase and an NAD^+ dependent formate dehydrogenase, both of which are located in the cytosol.

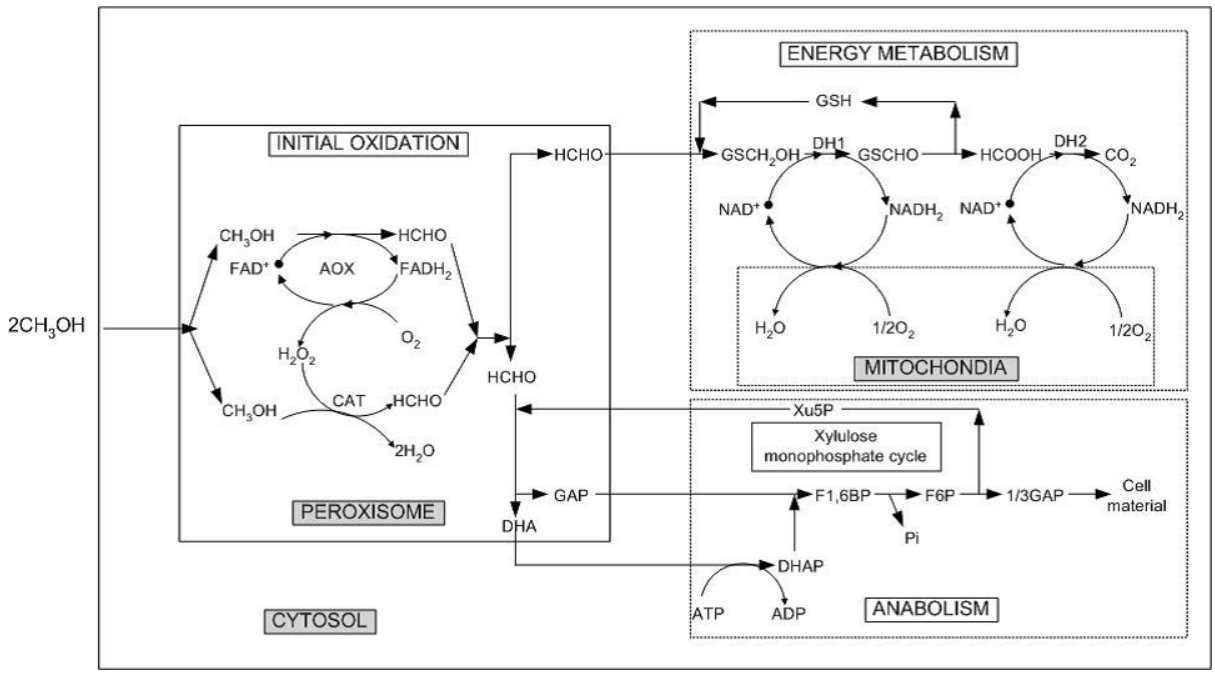

Figure 1-1. Methanol metabolism in P. pastoris. AOX: alcohol oxidase; CAT: catalase; DH1: formaldehyde dehydrogenase; DH2: formate dehydrogenase; GSCH2OH S-hydroxymethylglutathione; GSCHO, S-formylglutathione;GAP: glyceroldehyde-3-phosphate; DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose-1,6-bisphosphate; F6P: fructose-6-phosphate; Xu5P: xylulose-5-phosphate. Adapted from Charoenrat et al. (2005).

Glucose and ethanol strongly repress the methanol pathway genes in all methylotrophic yeasts whilst the strength of repression of glycerol is dependent upon the yeast species, with *P. pastoris* showing being strongly repressed. Ultimately methanol results in induction (Yurimoto *et al*., 2011).

1.3.3 *Methanol Utilisation Phenotypes: AOX Promoters*

The success of *P. pastoris* as an expression system can be attributed to its methanol-inducible pAOX1 promoter (Potvin *et al*., 2012); the enzymes needed for the metabolism of methanol are only available when the cells are cultured on methanol (Macauley-Patrick *et al*., 2005). However, as powerful as this system is, methanol presents a severe danger in terms of flammability, especially when considering the amounts required for industrial scale fermentations, where huge amounts need to be stored in the production facilities (Potvin *et al*., 2012). AOX1 accounts for up to 95% of the total expressed alcohol oxidase due to the relative tenacity of its promoter (Macauley-Patrick *et al*., 2005). Because of this reason,

pAOX1 systems are highly sought after, though significant progress has been made with regards to pAOX2 systems also (Sasagawa *et al*., 2011, Kuwae *et al*., 2005).

P. pastoris possesses three methanol utilisation phenotypes; both AOX1 and AOX2 are operational in the Mut^+ strains which grow at the wild type rate on methanol, but which require copious amounts of it during cultivation. The responsiveness of Mut^+ strains to large concentrations of methanol makes such systems more challenging to control and scale-up and AOX expression may result in competition for cellular machinery with heterologous protein production (Potvin *et al*., 2012). This consequently reduces the yield of the wanted product.

Mut^S strains have their AOX1 gene deleted, thus the growth of this strain is limited by the expression of AOX2. In certain circumstances greater productivities were acquired in these Mut^S strains compared with the wild type strains (Cos *et al*., 2005, Cos *et al*., 2006). Because of reduced growth rates, challenges fundamentally associated with high density cultivations of accelerated growth strains, for example oxygen limitation, are lessened. Reduced sensitivity to methanol also allows for a more straightforward scale up. Mut^- strains are lacking in both AOX genes and thus are incapable of growing on methanol (Chiruvolu *et al*., 1997).

P. pastoris offsets deficiency of the alcohol oxidase of pAOX1 which has a poor affinity for oxygen (Gurkan and Ellar, *2005*), by producing larger quantities of the AOX1 enzyme by up regulating the *AOX1* promoter to increase expression of the *AOX1* gene. Subsequently, the AOX1 enzyme can account for approximately 30% of the total cellular protein when methanol is utilised as the sole carbon source (Gellissen, 2000).

1.3.4 *Alternative Promoters*

The promoter for the glutathione-dependent enzyme formaldehyde dehydrogenase (pFLD1) is an attractive alternative to pAOX1 as it allows the regulation of protein expression through induction with either methanol (carbon source) or methylamine (nitrogen source) (Shen *et al*., 1998). A methanol-free fed-batch cultivation system was developed for the methylamine-regulated production of a *Rhizopus oryzae* lipase under the control of pFLD1, with resulting productivity comparable to those of pAOX1 systems (Cos *et al*., 2005). Gelatin and GFP, under the control of pAOX1 and pFLD respectively, were successfully simultaneously

expressed in *P. pastoris.* As both are methanol inducible but only pFLD1 is methylamine inducible, such systems will prove useful for the simultaneous expression of co-dependent proteins or the modulation of expression of co-dependent proteins to determine their effects on others (Duan *et al*., 2009). Dihydroxyacetone synthase (DHAS) is involved in the methanol assimilation pathway, and its promoter (pDHAS) is also inducible by methanol. Due to its strong promoter DHAS can account for up to 20% of total protein content (TPC) in cells grown on methanol (Gellissen, 2000).

Likewise, inductive promoters have been isolated that do not require methanol for induction. PEX8 for example is a peroxisome membrane protein, inducible by both methanol and oleic acid. Due to low expression however these promoters, though providing an alternative to methanol-inducible systems, are rarely used (Liu *et al*., 1995).

In 1997 Waterham *et al*., isolated the constitutive promoter (pGAP) of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Potvin *et al*., 2010). Subsequently this has been utilised in the expression of many recombinant proteins in cells with either glycerol or glucose as carbon sources for growth thus forgoing the safety concerns associated methanol (Cos *et al*., 2006). Biomass and protein synthesis occur simultaneously in GAP systems and are correlated directly with pGAP-regulated gene dosage, whilst in pAOX1 systems protein synthesis is limited during the induction phase by the availability of methanol (Potvin *et al*., 2012, Vassileva *et al*., 2001b, Vassileva *et al*., 2001a). Unlike pAOX1 systems whose fermentation parameters must be strictly controlled during the induction phase, the same requirements do not apply to GAP systems (Boer *et al*., 2000) and induction phases can last anywhere to 30 days after commencement of induction. Furthermore, when cells are grown on glycerol, methanol or glucose, expression of pGAP-genes is very high.

1.3.5 *Fed Batch Cultivation of P. pastoris and Process Control*

As with all expression systems, the optimum conditions for media, feed profiles, pH and temperature etc. essential for the production of a recombinant protein are dependent upon the strain of the microorganism and the nature of said recombinant protein. Usually, fed-batch is adopted over continuous cultivation because these

systems are able to achieve high cell densities whilst simultaneously being much simpler to control. Glycerol batch and fed-batch phases followed by a methanol induction phase are the standard phases for the cultivation of pAOX1-regulated *P. pastoris*.

1.4 *D Amino Acid Oxidase (DAAO)*

1.4.1 *An Introduction to DAAO*

Research in to enzyme catalysis has enormously enriched the success of biological organic synthesis; particularly in the last decade, remarkable progress in biological catalysis has taken place leading to the application of enzymes to a greater extent in industrial processes (Pilone and Pollegioni, 2002). The benefits of establishing enzymes as industrial catalysts are founded on the need to develop a 'clean' and 'pure' technology which can carry out isomer-and regio-selective reactions; generate or transform pure isomers compared with racemic mixtures, and produce pure compounds in comparison with mixtures of by-products (Pilone and Pollegioni, 2002). D amino acid oxidase (DAAO) is a flavoenzyme which catalyses the oxidation of D-amino acids into the corresponding α-keto acids (Pilone, 2000). DAAO was only the second flavoenzyme to be discovered. DAAO activity was first identified in tissue specimens by Krebs (1935) and FAD was consequently identified as its enzyme cofactor (Pilone, 2000).

All DAAOs distinguished thus far possess noncovalently bound FAD as their prosthetic group. Due to the fact that they have been widely studied they are looked upon as model flavooxidase catalysts. Within the last decade, the interest in DAAOs has escalated due to their boundless presence in eukaryotes and the disputes surrounding their reaction mechanism(s) (Harris *et al*., 1999, Molla *et al*., 2000, Pilone, 2000, Pilone and Pollegioni, 2002, Pollegioni *et al*., 2007a, Pollegioni *et al*., 2008, Pollegioni *et al*., 2007b, Umhau *et al*., 2000)

DAAO catalyzes the following reaction:

$$RCHNH_2COOH + E\text{–}FAD \rightarrow RC$$

$$= NHCOOH + E\text{-}FADH_2 \quad (1a)$$

$$E\text{-}FADH_2 + O_2 \rightarrow E\text{-}FAD + H_2O_2 \quad (1b)$$

$$RC= NHCOOH + H_2O \rightarrow RCOCOH + NH_3 \quad (1c)$$

The reductive half-reaction, the only enzymatic step of the whole scheme, dehydrogenates the amino acid to the corresponding imino acid, coupled with the reduction of FAD **(1a)**. FAD reoxidizes extemporaneously in the presence of molecular oxygen, which subsequently, is reduced to hydrogen peroxide **(1b)**. The imino acid then hydrolyses to form α-keto acid and ammonia **(1c).** The stereospecifity of the reaction is complete and is restricted to the D-isomers when both D- and L-amino acids are supplied as substrates (Pilone, 2000, Pilone and Pollegioni, 2002).

In spite of the expansive dispersal of DAAO in eukaryotic organisms and mammals, only three enzymes have been obtained as a homogenous preparation, specifically pkDAAO from Pig kidney and additionally two from the yeasts *Rhodotorula gracilis* (*Rg*DAAO) and *Trigonopsis variablis* (*Tv*DAAO).

The expression of these DAAO proteins in *E. coli* has been reported. It is intriguing that the expressed pkDAAO could reach approximately 49% of the total soluble protein. Unfortunately, the low specific activity renders the recombinant enzyme as being unsuitable for industrial use (Hwang *et al*., 2000)

1.4.2 *DAAO as an Industrial Biocatalyst*

The redox reaction of DAAO can be employed in numerous processes of enzyme-based technology, like the production of α-keto acids and of glutaryl-7-ACA, the resolution of racemic mixtures of amino acids and the analytical determination of D-amino acids. DAAO of yeast origin is an optimal biocatalyst for biotechnological and industrial work since it possesses a high turnover, tight binding of the coenzyme FAD, and a broad substrate specificity. Unfortunately, DAAO in its free form is restricted from use due in part to its thermal instability and inactivation by denaturing agents.

D-amino acids achieve different roles in nature and have been found in different bioactive peptides. DAAO, as well as glutaryl-7-amino-cephalosporanic acid acylase (GL-7-ACA acylase) are industrially important enzymes used in the development of 7-aminocephalosporanic acid (7-ACA) from cephalosporin C (CPC) in a two-step process. In step one cephalosporin C is converted to 7-β-(5-carboxy-5-oxopentanamido) cephalosporanic acids (keto_AD-7-ACA) by DAAO, which spontaneously reacts with the hydrogen peroxide produced in the reaction to produce 7-β-(4-carboxybutanamido)-cephalosporanic acid (GL-7-ACA). In the second step, GL-7-ACA is further hydrolysed to 7-ACA by a GL-7-ACA acylase (Harris *et al*., 1999, Molla *et al*., 2000, Pilone, 2000, Pilone and Pollegioni, 2002, Pollegioni *et al*., 2007a, Pollegioni *et al*., 2008, Pollegioni *et al*., 2007b, Umhau *et al*., 2000, Luo *et al*., 2004, Yu *et al*., 2002).

Additionally DAAO can also be used for production of α-keto acids and pure L-amino acids (Fischer *et al*., 1996, Gabler *et al*., 2000) and therefore many efforts have been made to obtain DAAO in large amounts. Recombinant expression is a promising approach to acquire DAAO to meet its large demand in industry. Protein engineering has facilitated a redesign of the DAAO substrate specificity, oxygen affinity, cofactor binding, stability and oligomeric state. D-amino acids complete different roles in nature and have been shown to be part of bioactive peptides (e.g. transmitters, neuromodulators, neurohormones, and antimicrobials) (Harris *et al*., 1999, Molla *et al*., 2000, Pilone, 2000, Pilone and Pollegioni, 2002, Pollegioni *et al*., 2007a, Pollegioni *et al*., 2008, Pollegioni *et al*., 2007b, Umhau *et al*., 2000).

DAAO has many different physiological roles, making it an example of functional enzyme promiscuity. It serves as a catabolic enzyme for using D-amino acids as a source of carbon, nitrogen and energy (in yeasts); it is involved in thiamine (in *Bacillus subtilis*) or eye pigment (in insects) biosynthesis; and it helps to eliminate D-amino acids in the kidney and the neuromodulator D-serine in the brain. As a result of the absolute stereoselectivity (L-amino acids are neither substrates nor inhibitors) and broad substrate specificity, DAAO has been investigated for use as an industrial biocatalyst Recently significant efforts have been made in the mass production of wild type (WT) DAAO mainly from (*Trigonopsis variablis, Rhodotorula gracilis, Candida boidinii* and *Arthrobacter protophormiae)* at a reasonable cost, by fermenting the original organism or as a recombinant protein (Harris *et al*., 1999,

Molla *et al*., 2000, Pilone, 2000, Pilone and Pollegioni, 2002, Pollegioni *et al*., 2007a, Pollegioni *et al*., 2008, Pollegioni *et al*., 2007b, Umhau *et al*., 2000).

Most recently, *R. gracilis* and *T. variabilis* DAAOs have been expressed in *P. pastoris* cells using a fed batch culture, and reaching a productivity of 350 and 220 kU/l of fermentation broth respectively (Abad *et al*., 2011). Nevertheless, light has only recently been shed on the extraordinary flexibility of this enzyme; in large part this is due to many detailed investigations in to structure-function relations of yeast DAAO (i.e. elucidating the 3D structure (Umhau *et al*., 2000) and carrying out site directed mutagenesis (Harris *et al*., 1999) and by identifying novel DAAOs from various microorganisms (Gabler *et al*., 2000).

A significant application of DAAO is the development of optically pure L-amino acids by resolution of D, L-racemic mixtures. Enantiomerically pure, synthetic α-amino acids or unnatural amino acids are of increasing interest in the fine chemical and pharmaceutical industries, particularly for drug discovery (Pollegioni and Molla, 2011). The most interesting methods employ an enantioselective oxidase as the biocatalyst and a non-selective chemical reducing agent or transition metal catalyst (Fotheringham *et al*., 2006).

Pollegioni and Molla (2011) have shown that complete recovery of the single L-enantiomer of 2-napthyl-alanine from racemic mixtures has been obtained by combining *Rg*DAAO with L-aspartate amino transferase and catalase. Through using this multi-enzymatic system, the α-keto acid that is produced by M213G (a variant) *Rg*DAAO from D-2-napthyl-alanine can be converted in to the L-isomer with a 98% yield (in terms of product recovery) and a 99.5% enantiomeric excess. Similarly, D-methionine has been fully converted to the L-isomer in a four enzyme system (Findrik and Vasić-Rački, 2007).

1.4.3 *General Properties of DAAO*

Though a large a number of patents have been filed on the industrial application of DAAO as a biocatalyst, only a minute amount of DAAO proteins have been isolated in a homogenous form and characterised in detail. DAAO isolated from the kidney of a pig was the first to be obtained as a homogenous flavoprotein; 14 years passed before a second purified DAAO, from the yeast *R. gracilis* became available and a further six years before a second microbial DAAO was isolated from the yeast

T. variabilis All react rapidly with oxygen in the reduced form to give hydrogen peroxide and the oxidized flavin, and all stabilize the red anionic flavin radical via one electron reduction, the flavin *N(5)*-sulphite adduct, and the benzoquinoid anionic form of 6- and 8- substituted hydroxyl- and mercaptoflavins (Pilone, 2000, Pollegioni *et al*., 2007a, Pollegioni *et al*., 2008).

1.4.4 *Molecular Biology of DAAO and Structural Properties*

The DAAO gene is present in a single copy in the mammalian genome. The complementary DNA (cDNA) of the complete pig kidney enzyme has been cloned and sequenced. An open reading frame of 1041bp, comprising the coding region, encodes 347 amino acids of the enzyme, indicating the that proteolytic posttranslational processing is not present (Pilone, 2000).

The active pkDAAO holoenzyme is a monomer of 347 amino acids containing one molecule of non-covalently bound FAD with a molecular mass of 39.6kDa. The dissociation constant of the FAD-apoenzyme complex is 2.2×10^{-7}M. pkDAAO exists in solution as a mixture of monomer-dimer and even higher molecular aggregates in equilibrium, depending on the enzyme concentrations and its forms (holoenzyme, apoprotein, or holoenzyme-benzoate complex. *Rg*DAAO is a homodimeric holoenzyme of 80kDa, each subunit of 368 amino acids containing a non-covalently bound FAD molecule (K_d is 2.0×10^{-8}M). The dimer is fairly stable and is not reliant upon the concentration of the enzyme. Significant discrepancies within the literature about the molecular mass of DAAO from *T, variabilis* exist (Pilone and Pollegioni, 2002).

1.4.5 *Spectral and Kinetic Properties*

All characterised DAAOs show the expected absorbance spectrum for FAD-containing enzymes in the oxidized state, with two maxima in the visible region (at ≈ 455 and 380nm) and one in the UV region (at ≈ 280nm). Moreover, fluorescence emission, which is related to the presence of the flavin fluorophor can also be observed. Benzoate represents a well-studied example of a number of carboxylic acids which are competitive inhibitors of DAAO and which bind at the enzyme-active

site, yielding typical absorbance spectrum perturbations (Pilone and Pollegioni, 2002).

pkDAAO, *Tv*DAAO, and *Rg*DAAO have all been studied for their redox properties. For the free enzyme forms, a two single-electron transfer was established (with a midpoint redox potential, E_m, at pH 7.5 of -109, -91, and -62 mV for pk-, *Rg*-, and *Tv*DAAO, respectively). The binding of benzoate mimics the presence of the substrate and thus modulates these properties, facilitating a two-electron transfer (E_m at pH7.5, of -166, -98, and -107mV for pk-, *Rg*-, and *Tv*DAAO, respectively) Pig kidney and yeast DAAO possess wide substrate specificity. Excluding the acidic amino acids like D-glutamate and D-aspartate, DAAOs can deaminate all the other D-amino acids by oxidation, though with a different efficiency (expressed as the $Vmax/K_m$ ratio) (Molla *et al*., 2000, Pilone, 2000, Pilone and Pollegioni, 2002, Pollegioni *et al*., 2007a, Pollegioni *et al*., 2008, Pollegioni *et al*., 2007b, Umhau *et al*., 2000).

1.4.6 *Reaction Mechanisms and Site Directed Mutagenesis*

Numerous hypotheses exist with regards to the molecular mechanism used by DAAO to catalyse the dehydrogenation of D-amino acids (*Pilone*, 2000). The knowledge that DAAO elicits the removal of chloride from β-chloro-D-alanine via a catalytic reaction indicates that there is a carbanion mechanism reliant upon the active site base for removing the α-hydrogen. In comparison, results obtained through experimentation with pkDAAO manipulated to incorporate the artificial flavin 5-deazaFAD were indicative of a hydride transfer mechanism (to the flavin N5, or C5 in 5-deazaFAD). During 1996, two groups separately determined the crystal structure of pkDAAO; though the interpretations of the mechanism of the data contrast greatly (Mattevi *et al*., 1996, Miura *et al*., 1997).

Mattevi's group presented the crystal structure as a complex with benzoate at a maximum of 2.6 Á thus supporting the direct transfer of the substrate α-hydrogen to the flavin in the reductive half reaction: no side chains are present at the active site of pkDAAO in a position to act as a general base and consequently this rules out the possibility of a carbanion reaction mechanism. Miura *et al* (1997) and his group carried out research using a recombinant form of pkDAAO. The authors hypothesised an electron-proton-electron transfer mechanism that gives the anionic

form of the reduced flavin and the imino acid with cationic imino nitrogen from the molecular modelling of the pkDAAO-D-Leucine complex (Miura *et al*., 1997).

The crystal structures of various forms of DAAO are presented in Figure 1-2.

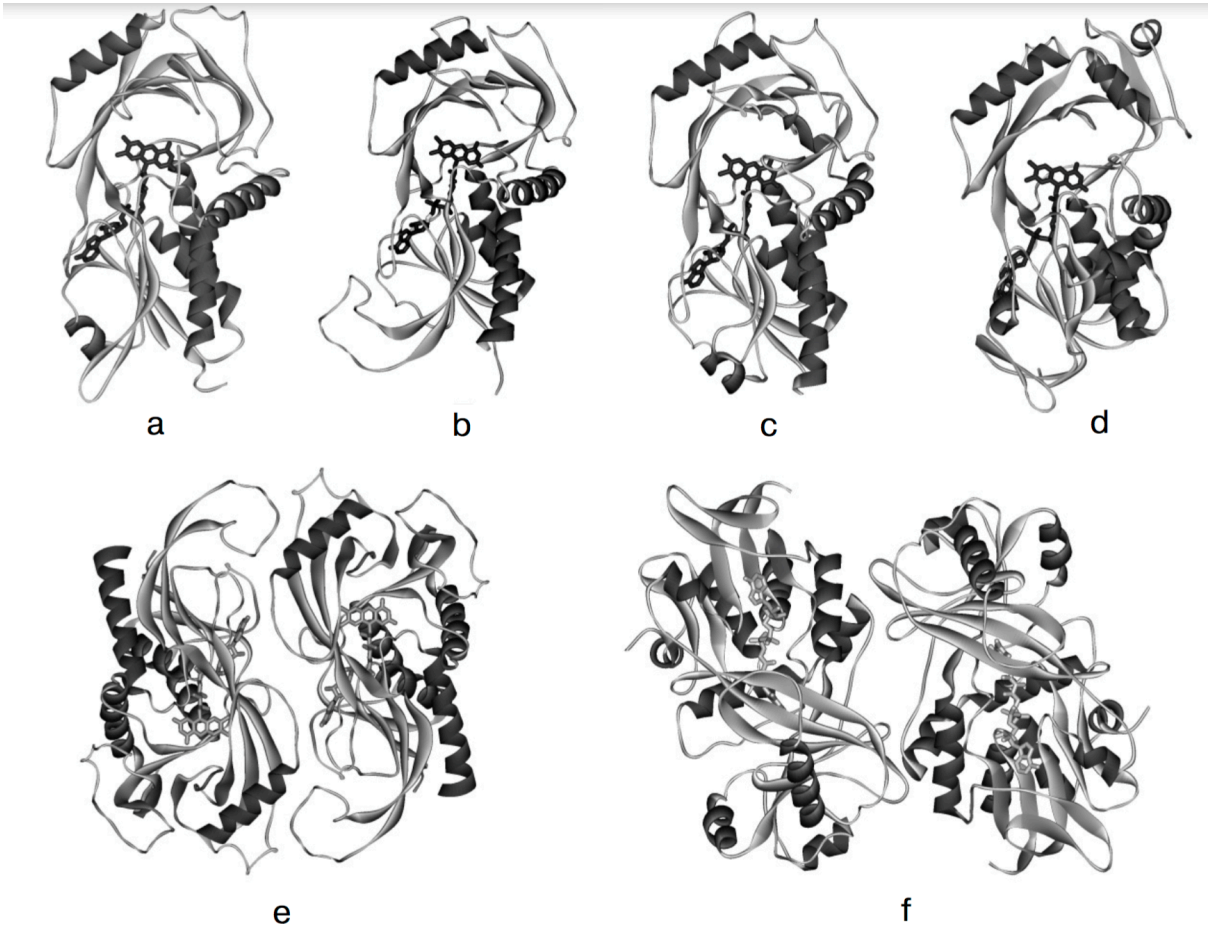

Figure 1-2. Spatial structures of DAAO subunits from pig kidney (a), yeast R. gracilis (b), and subunit of glycine oxidase from B. subtilis (d), as well as model structure of subunit from T. variabilis (c) and 3D structures of dimeric DAAO from R. gracilis (e) anddimeric glycine oxidase from *B. subtilis* (f) by (Khoronenkova and Tishkov, 2008).

1.4.7 *DAAO Production and Molecular Biology*

DAAO can be produced through the fermentation of the original microorganism or by heterologous hosts, e.g. in *E. coli*. Directly comparing the data concerning the production of DAAO from different sources is an incredibly arduous task due in part to enzyme activity being measured using different assay, substrates, temperatures and pH values in various studies. In the past it was shown that *de novo* synthesis of DAAO in peroxisomes can be selectively induced by the presence of D- or D,L-amino acids. Synthesis is induced by D-alanine but the presence of the L-isomer prevents induction by inhibiting D-amino acid transport by a specific permease. *R. gracilis* is able to use D-alanine as both carbon and nitrogen; the maximal DAAO (0.5% of total soluble proteins in the crude extract) is obtained when D-alanine is the only source of nitrogen and glucose of carbon. Even in *T. variabilis* cells DAAO activity is inducible. The best results were obtained with *N*-carbonyl-D-alanine, *N*-acetyl-D-tryptophan, and *N*-chloroacetyl-D-α-aminobutyric acid. Comparing this with D-alanine, when 5mM *N*-carbamoyl-D-alanine (as D,L-form) is the single carbon source, a 1.5- to 4.2 fold higher specific activity was reached (650U/g dry cell vs. 175U/g dry cell). Zinc ions, which are involved in the synthesis of peroxisomal proteins, when present appear to play a significant role in *Tv*DAAO synthesis (Pilone, 2000, Pilone and Pollegioni, 2002, Pollegioni *et al*., 2007a).

Up to now, the best level of expression has been achieved for pkDAAO in *E. coli:* the enzyme reaches 40% of total soluble protein in the crude extract. However, due to the low activity of the mammalian DAAO, this value only corresponds to 200-300 DAAO units/L of fermentation broth. The low specific activity and weak binding of the coenzyme render pkDAAO unsuitable for industrial applications (Pilone, 2000).

1.5 *Oxidative Stress in Microorganisms*

Aerobic organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. Reactive by-products of oxygen, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (·OH), are generated continuously in cells grown aerobically. Most of such products derive from sequential univalent reductions of molecular oxygen catalysed by several membrane–associated respiratory chain enzymes. Experimental data indicates that, in *E. coli*, the respiratory chain can account for as much as 87% of the total H_2O_2 production (González-Flecha and Demple, 1997, Gonzalez-Flecha and

Demple, 1995). The leakage of single electrons from the bacterial respiratory chain was observed at the NADH dehydrogenase and ubiquinone sites, and was similar to that observed in eukaryotic mitochondria. Environmental agents such as ionizing, nearUV radiation, or numerous compounds that generate intracellular O_2^- (redox-cycling agents such as menadione and paraquat) can cause oxidative stress, which arises when the concentration of active oxygen increases to a level that exceeds the cell's defense capacity. Some immune cells which use the NADPH oxidase enzyme, upon invasion by pathogenic bacteria, also exploit oxidative stress as a weapon during phagocytosis. The biological targets for these highly reactive oxygen species are DNA, RNA, proteins and lipids. Much of the damage is caused by hydroxyl radicals generated from H_2O_2 via the Fenton reaction, which requires iron (or another divalent metal ion, such as copper) and a source of reducing equivalents (possibly NADH) to regenerate the metal. Lipids are major targets during oxidative stress. Free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation. A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins (Bulteau *et al*., 2001, Humphries and Szweda, 1998).

Microorganisms used in the biotechnology industry are exposed primarily to what is referred to as Oxidative stress: when the eqiulibrium between reactive oxygen species (ROS) and their subsequent elimination is shifted to the accumulation of elevated levels of ROS (Lushchak, 2011); in simple terms it is purely an imbalance between oxidants and antioxidants, with the favouring of the former (Sies, 1997).

Ultimately aerobic metabolism results in the production of oxidants, and though this is a normal outcome of such processes however, under pathophysiological conditions (those causing detrimental changes in physical and biochemical processes) as seen in fungi used in industrial processes previously described, oxidants are constantly fashioned and never eradicated (Chelikani *et al*., 2005). Examples of various ROS include hydrogen peroxide (H_2O_2); the superoxide anion (O_2^-) and the hydroxyl radical (OH^-) it should be noted that reactive nitrogen

species do occur (Sies, 1997), but for the purpose of this project they will not be discussed.

In fermenter vessels, water molecules are generated by the reduction of molecular oxygen in cells, resulting in the manufacturing of the three oxidants mentioned previously (Sies, 1997). Oxidants are also introduced by divergent forms of radiation including, but not exclusive to, X-rays and ultraviolet (UV) light (Schmidt *et al*., 1995, Sies, 1997). ROS can damage DNA, resulting ultimately in cell death due to mutagenesis of the DNA sugar-phosphate backbone (Rowe *et al*., 2008) which affects transcriptional and translational processes; cause lipid peroxidation which leads to membrane-leakage, thus allowing substances normally blocked from crossing the cell membrane to do so (Schuessel *et al*., 2006) and damage proteins which are involved in the synthesis of essential enzymes (Shenton and Grant, 2003).

Cells exposed to high levels of ROS have also been shown to have impaired growth (Moye-Rowley, 2003). Fortunately, in most instances of ROS attack upon cells, antioxidant enzymes are utilised to counteract the damaging effects: superoxide dismutase (SOD); catalase (CAT) and glutathione peroxidise (GPx) are just some of these enzymatic antioxidants (Frealle *et al*., 2006, Isobe *et al*., 2006, Pocsi *et al*., 2004). Conversely, there are non-enzymatic antioxidant mechanisms which cells can employ to overcome the negative consequences of high ROS levels such as the alternative respiratory pathway (Krause *et al*., 2004). One mechanism of this pathway is to attenuate the generation of ROS, as it is a non-proton pumping pathway and as such, does not contribute to ROS production (Krause *et al*., 2004); the alternative NADH:ubiquinone oxidoreductase (NADH dehydrogenase) enzymes are part of this alternative pathway and are possessed by many filamentous fungi.

A recent study by Li *et al*. (2008) has shown that the filamentous fungi *Aspergillus niger* can adapt to oxidative stress: by exposing the *A. niger* to a non-toxic level of the oxidant H_2O_2, *A. niger* could then adapt to this stressor at higher, more toxic levels, and depress its growth rates whilst simultaneously instigating the release of a number of antioxidant enzymes; a reduction of these antioxidants enzymes is correlated with an expansion in growth (Li *et al*., 2008). However, fungi are also capable of dealing with alternative stresses such as nutrient reduction, rapid temperature changes and variations in pH; certain genes are transcribed when such

reductions or fluctuations are detected and release a protein to a location known as the septal pore to reduce cytoplasmic leakage, so as to preserve internalised nutrients (Maruyama *et al*., 2010).

Basically, oxidative stress can detrimental to cells. In large scale industrial processes that require metabolites of cells, or other secretory intermediates and products, this could effectively reduce financial earnings of say, a pharmaceutical company running the processes. With cells unable to thrive in such highly hostile environments, like those presented by fermenter vessels, productivity and consumer satisfaction can suffer greatly and so it is essential to fully understand the physiological and biochemical effects of all stresses in cells, and, if possible, how to prevent or protect the cells from them.

1.6 *Process Development and Scale Up*

Current biotechnology applications require vast amounts of low-cost but high-quality products which can only be achieved if laboratory-scale developments of processes are carefully translated to full industrial scale (Lara *et al*., 2006). Environmental conditions are key to cell physiology, which in turn affects the yield and quality of the product and as such the culture environment should be identical during each step of the scale up process but unfortunately only a select number of parameters can be kept so after scaling up if geometric similarity is maintained between the vessels used at each stage. Consequently laboratory-scale, pilot-plant scale and production scale bioprocesses and cultures will all differ from one another (Lara *et al*., 2006). Whilst scaling-up can lead to an alteration in culture variability, it too can lead to a change in the level of homogeneity with accompanying environmental gradients, which can be predicted through comparing the times of relevant processes within the bioreactor. Cell physiology can be affected by temporary changes in environmental parameters and using computational fluid dynamics (CFD) and simulations, it is possible to predict these changes.

The culturing of fragile organism unable to withstand high aeration and agitation rates, and indeed cultures of high cell density, presents a challenge for the adequate transfer of oxygen to the bioreactor. Aerobic cultures are extremely demanding of oxygen, which must be continuously supplied to the reactor due the solubility of oxygen being extremely low in water. When the expected time for oxygen uptake is

less than the mixing time, dissolved oxygen gradients can develop (Silva *et al*., 2012), though reverse this and oxygen becomes the limiting mechanism and no gradients are expected.

In recent years, it has been demonstrated that correct protein folding and secretion are highly interrelated with environmental stress factors. While adaptive responses to such stresses have been studied in the model *yeast S. cerevisiae* little is known about their influence on the physiology of P. pastoris. Furthermore, such studies have generally not been performed under heterologous protein production conditions.

Aims

The aim of this study was to successfully develop bioprocesses to produce highly active DAAO in *E. coli* and *P. pastoris* for future industrial applications. The *E. coli* strain used will provide an alternative to expensive chemical inducers through the presence of a thermal induction system, and the *P. pastoris* strain in conjunction with a co-feed strategy will present a system capable of reaching high cell densities, with both producing highly active enzyme. Furthermore, an understanding of the effects of oxygen availability to the growing culture and subsequent enzyme production was gained.

Chapter Two

Materials and Methods

2 Materials and Methods

2.1 *Microorganisms and Culture Conditions*

2.1.1 *E. coli and P. Pastoris Strains*

All strains used in this experiment were provided by Ingenza (https://www.ingenza.com). Due to confidentiality agreements, how the strains were made cannot be discussed and very limited information on the constructs can be disclosed. All strain information below was provided when strains were received.

Escherichia coli pRES151DAAOWT BW2157 (Chapter Three and Chapter Five)

Pichia pastoris ING005-A9-1-MCB1 (Chapter Four and Chapter Five)

The source of the DAAO is *Trigonopsis variablis.*

E. coli BL21(DE3)/ pET28b His MAO-5N (Chapter 5)

P. pastoris MutS CBS 7435 His MAO-5N (Chapter 5)

2.1.1.1 *E. coli Master Cultures*

Master cultures were produced by carrying out flask fermentations with LB medium carrying 10 μg/ml of chloramphenicol (DAAO) or kanamycin (MAO-5N). At OD_{600nm} = 0.5 abs when cells are at the exponential phase of growth, aliquots of 0.8 ml of culture were transferred to 1 ml freezing vials (Sigma Aldrich, Dorset, UK.) containing 0.2 ml of glycerol and stored at – 80 °C. Cultures are revived by streaking them on LB agar plates (containing 10 μg/ml chloramphenicol) and put in to an incubator at 28 °C (DAAO) or 30 °C (MAO-5N) for 48 hours. A single colony was used to incubate a flask.

2.1.1.2 *P. pastoris Master Cultures*

Master cultures were produced by carrying out flask fermentations with MYGP medium carrying 100 μg/ml of zeocin. At OD_{600nm} = 6 - 10 abs when cells are at the exponential phase of growth, aliquots of 0.8 ml of culture were transferred to 1 ml freezing vials (Sigma Aldrich, Dorset, UK.) containing 0.2 ml of glycerol and stored at – 80 °C. Cultures are revived by streaking them on MYGP agar plates (containing 100 μg/ml zeocin) and put in to an incubator at 28 °C for 48 hours. A single colony was used to incubate a flask.

2.1.2 *Media Composition*

2.1.2.1 *Media Composition for E. coli Bioprocesses*

Inoculum (Table 2-1)

Table 2-1. E. coli Inoculum Composition

Salt Solution	60 ml
Glucose (50 % w/v)	6 ml
1 M Magnesium Sulphate	0.6 ml
Trace Elements Solution (Table 2-17)	0.6 ml
Chloramphenicol (10 mg/ml)	0.3 ml
Distilled H_2O	To a final volume of 300 ml

Batch Media A (Table 2-2)

Table 2-2. E coli Batch Media A Composition

Sodium Phosphate Monobasic Dihydrate	36 g
Ammonium Citrate Dibasic	5 g
Ammonium Sulphate	20 g
Potassium Phosphate Dibasic	146 g
Distilled H_2O	To a final volume of 9000 ml

Batch Media B (Table 2-3)

Table 2-3. E. coli Batch Media B Composition

Glucose	119 g
1 M Magnesium Sulphate	2 ml
Trace Elements (Table 2-6)	2 ml
Chloramphenicol (10 mg/ml)	10 ml
Distilled H_2O	To a final volume of 1000 ml

Glucose Feed (Table -2-4)

Table 2-4. E. coli Glucose Feed Composition

1 M Magnesium Sulphate	50 ml
Trace Elements (Table 2-6)	10 ml
Glucose	550.9 g
Yeast Extract	5 g
Distilled H_2O	To a final volume of 1000 ml

Salt Solution (Table 2-5)

Table 2-5. E. coli Salt Solution Composition

Ammonium Sulphate	10 g
Potassium Phosphate Dibasic	73 g
Sodium Phosphate	18 g
Ammonium Citrate Dibasic	2.5 g
Distilled H_2O	To a final Volume of 1000 ml

Trace Elements Solution (Table 2-6)

Table 2-6. E. coli Trace Elements Solution Composition

Calcium Chloride Dihydrate	0.5 g
Ferric Chloride	10 g
Zinc Sulphate Heptahydrate	0.18 g
Copper Sulphate Pentahydrate	0.16 g
Manganese Sulphate Monohydrate	0.15 g
Cobalt Chloride Hexahydrate	0.18 g
Disodium EDTA Dihydrate Salt	22.3 g
Distilled H_2O	To a final volume of 1000 ml

Lysogeny Broth (LB) Media

Table 2-7. Lysogeny Broth (LB) Media

Bacteriological Tryptone	10 g
Yeast Extract	5 g
Sodium Chloride	10 g
Distilled H2O	To a final volume of 1000 ml

2.1.2.2 *Media Composition for P. pastoris Bioprocesses*

Inoculum (Table 2-8)

Table 2-8. P. pastoris Inoculum Composition

Yeast Extract (Sigma)	2 g
Peptone	4 g
Potassium Phosphate Buffer 1M (pH 6)	20 ml
Yeast Nitrogen Base with Amino Acids (Sigma)	20 ml of a 134 g/L Stock
Biotin	0.4 ml of a 2 g/L Stock
Zeocin	0.2 ml of 100 mg/ml stock
Distilled H_2O	To a final volume of 200 ml

Batch Media A (Table 2-9)

Table 2-9. P. pastoris Batch Media A Composition

Phosphoric Acid (85%)	95 ml
Calcium Sulphate	1.36 g
Potassium Sulphate	28.59 g
Magnesium Sulphate Heptahydrate	23.17 g
Potassium Hydroxide	12.8 g
EDTA Disodium Salt Dihydrate	6 g
Sodium Chloride	2.2 g
Glycerol 400 g	400 g
Polypropylene Glycol 2025	1 ml
Distilled H_2O	To a final volume of 8500 ml

Batch Media B (Table 2-10)

Table 2-10. P. pastoris Batch Media B Composition

PTM[1] Trace Elements	43.5 ml
Yeast Nitrogen Base with Amino Acids	134 g
Distilled H_2O	To a final volume of 1500 ml

Batch Media C [Additional Supplementation] (Table 2-11)

Table 2-11. P. pastoris Batch Media C [Additional Supplementation] Composition

PTM[1] Trace Elements	43.5 ml
Yeast Nitrogen Base with Amino Acids	134 g
Adenine	400 mg
Arginine	200 mg
Aspartic Acid	1 g
Histidine	200 mg
Isoleucine	300 mg
Leucine	600 mg
Lysine	300 mg
Methionine	200 mg
Phenylalanine	400 mg
Threonine	3 g
Tryptophan	200 mg
Tyrosine	250 mg
Uracil	100 mg
Valine	1.5 g
Distilled H_2O	To a final volume of 1500 ml

Glycerol Feed (Table 2-12)

Table 2-12. P. pastoris Glycerol Feed Composition

Glycerol	500 ml
PTM[1] Trace Elements	12 ml
Distilled H_2O	To a final volume of 1000 ml

Methanol Feed (Table 2-13)

Table 2-13. P. pastoris Methanol Feed Composition

Methanol	1000 ml
PTM[1] Trace Elements	12 ml

Sorbitol Feed [2M] (Table 2-14)

Table 2-14. P. pastoris Sorbitol Feed Composition

Sorbitol	364.34 g
PTM[1] Trace Elements	12 ml
Distilled H_2O	To a final volume of 1000 ml

PTM1 Trace Elements (Table 2-15)

Table 2-15. P. pastoris PTM[1] Trace Elements Composition

Cupric Sulphate Pentahydrate	6 g
Sodium Iodide	0.08 g
Manganese Sulphate Monohydrate	3 g
Sodium Molybdate Dihydrate	0.2 g
Boric Acid	0.02 g
Cobalt Chloride Hexahydrate	0.92 g
Zinc Chloride	20 g
Ferrous Sulphate Heptahydrate	65 g
Biotin	0.2 g
Sulphuric Acid	5 ml
Calcium Sulphate	0.4 g
Distilled H_2O	To a final volume of 1000 ml

2.2 *Bioreactor Cultivations*

2.2.1 *E. coli Batch*

The reactor was inoculated with an LB flask of *E. coli* grown until the optical density of the culture at 600 nm reached $ODAbs_{600nm}$ 0.5 – 1. The shake flask culture was grown at 28 °C and 250 rpm. The temperature in the bioreactor was kept constant at 28 °C and the agitation rate was set at 300 rpm. Airflow was controlled at 1litre of air per litre of culture per minute (vvm) automatically, whilst pH was controlled by the automatic addition of 2 M H_2SO_4 and 25 % (v/v) NH_4OH.

2.2.2 *E. coli Fed Batch*

The bioreactor was filled with 10 L of medium and inoculated with 300 ml of culture grown at 28 °C to an $ODAbs_{600nm}$ = 0.5 – 1 abs. The temperature of the bioreactor was set at 28 °C throughout the batch phase. The pH was kept at 7 by automatic addition of 25 % (v/v) NH_4OH serving as both base and nitrogen source and 2 M H_2SO_4. The dissolved oxygen probe was calibrated with air and oxygen free nitrogen at 300 rpm. Unless otherwise stated, the DO2 set point was 30 % and for the aerated cultures it was maintained at the pre-set level by cascading the agitation rate from 300 rpm – 900 rpm and then from 1 vvm to 3 vvm of air while in the oxygen enriched cultures it was cascaded to stirring rate from 300 rpm to 900 rpm and then from 0 -100 % oxygen enrichment. In the oxygen limited cultures the DO2 was maintained by cascading the stirrer from 300 rpm to 900 rpm. When glucose was fully consumed the glucose feeding was turned on. To induce DAAO overexpression the temperature was increased from 28 °C to either 30 °C, 35 °C or 40 °C when the $OD_{Abs600nm}$ = 40, unless stated otherwise.

2.2.2.1 *Feed Profile*

0 minutes – 30 minutes → Start Feed at 0.15 ml/min

30 minutes to 1 hour → Change Feed to 0.3 ml/min

1 hour to 2 hours → Change Feed to 0.6 ml/min

2 hours to 3 hours → Change Feed to 0.8 ml/min

3 hours to Induction Time → Change Feed to 1.12 ml/min

At point of Induction Feed Rate changed to 0.8 ml/min

2.2.3 *P. pastoris Fed Batch*

The bioreactor was filled with 10 L of medium and inoculated with 200 ml of culture grown at 30 °C to an OD600nm = 6 – 10 abs. The temperature of the bioreactor was set at 30 °C throughout the runs. The pH was kept at 5 by automatic addition of 28 % NH4OH serving as both base and nitrogen source and 2 M H2SO4. The dissolved oxygen probe was calibrated with air and oxygen free nitrogen at 300 rpm. Unless otherwise stated, the DO2 setpoint was 30 % and for the aerated cultures it was maintained at the pre-set level by cascading the agitation rate from 300 rpm – 900 rpm and then from 1 vvm to 3 vvm of air while in the oxygen enriched cultures it was cascaded to stirring rate from 300 rpm to 900 rpm and then from 0 - 100 % oxygen enrichment. In the oxygen limited cultures the DO2 was maintained by cascading the stirrer from 300 rpm to 900 rpm. When glycerol was fully consumed the glycerol feeding was turned on. To induce DAAO overexpression the Methanol / Methanol: Sorbitol feed profiles were followed.

Table 2-16. P. pastoris Feeding Profile (Glycerol and Methanol)

Glycerol Feed	
Time from Start of Feed (h:m)	ml/min
0	1.05
0:30	1.20
0:60	1.40
1:45	1.60
2:30	1.80
3:00	2.00
3:45	2.10
4:15	2.30
4:45	2.50
5:15	2.70
5:45	2.90
6:00	3.00
6:30	2.10
7:30	1.05
8:30	0.00

Table 2-17. P. pastoris Feeding Profile (MeOH:Sorb Co Feed)

MeOH:Sorb 2:1		
Time from Start of Feed (h:m)	MeOH (ml/min)	Sorbitol (ml/min)
0	0.09	0.07
2:30	0.18	0.15
4:00	0.27	0.22
5:00	0.36	0.29
6:00	0.45	0.37
MeOH Sorb 1:1		
Time from Start of Feed (h:m)	MeOH (ml/min)	Sorbitol (ml/min)
0	0.09	0.15
2:30	0.18	0.29
4:00	0.27	0.44
5:00	0.36	0.59
6:00	0.45	0.73
MeOH Sorb 1:2		
Time from Start of Feed (h:m)	MeOH (ml/min)	Sorbitol (ml/min)
0	0.18	0.29
2:30	0.27	0.59
4:00	0.36	0.88
5:00	0.44	1.17
6:00	0.53	1.47

2.3 *Bioreactor*

2.3.1 *Braun Biostat C-DCU*

The Braun BIOSTAT C-DCU (B.Braun Biotech International, Switzerland) (Figure 2-1) is a sterilisable-in-place (SIP), stainless steel bioreactor with a maximum working volume of 15 L and a total volume of 22 L. The C15 -3 stainless steel culture vessel possesses a double wall heat exchanger with a side wall viewing port. The internal height to diameter ratio of the vessel is the standard ratio of 3:1. The vessel diameter measures 21 cm. Three adjustable six bladed Rushton turbines each with a diameter of 8.5 cm are located on the stirrer shaft. Four internal baffles with widths of 1.5 cm and lengths of 57 cm are present also. Air and water were supplied to the bioreactor from the main laboratory supplies. Sterile filtered air was sparged in to the vessel by means of a circular annular sparger at the bottom of the vessel in order to facilitate even distribution of air bubbles.

Accurate temperature control to within ± 0.1 °C of the temperature set point was accomplished through utilising the control loops of the DCU. The pH (Mettler Toledo Ltd., Leicester, UK) of the culture media was maintained to within ± 0.05 of the set point by DCU controlled operation of two peristaltic pumps supplying solutions of acid and base to the vessel via silicone rubber tubing. The dissolved oxygen tension (DOT) of the culture media was monitored by pO_2 electrode (Mettler Toledo Ltd., Leicester, UK). Calibration of the probe was performed by introducing oxygen free nitrogen (OFN) [0 % saturation value] and compressed air [100 % saturation value]. Calibration occurred at fermentation temperature and agitation speed. Polypropylene glycol 2025 was manually supplied to the vessel via a peristaltic pump as an anti-foam agent.

This reactor was used for both *E. coli* and *P. pastoris* batch and fed batch experiments for Chapter 3 (Page 64 – End of Chapter), Chapter 4 and Chapter 5 unless stated otherwise.

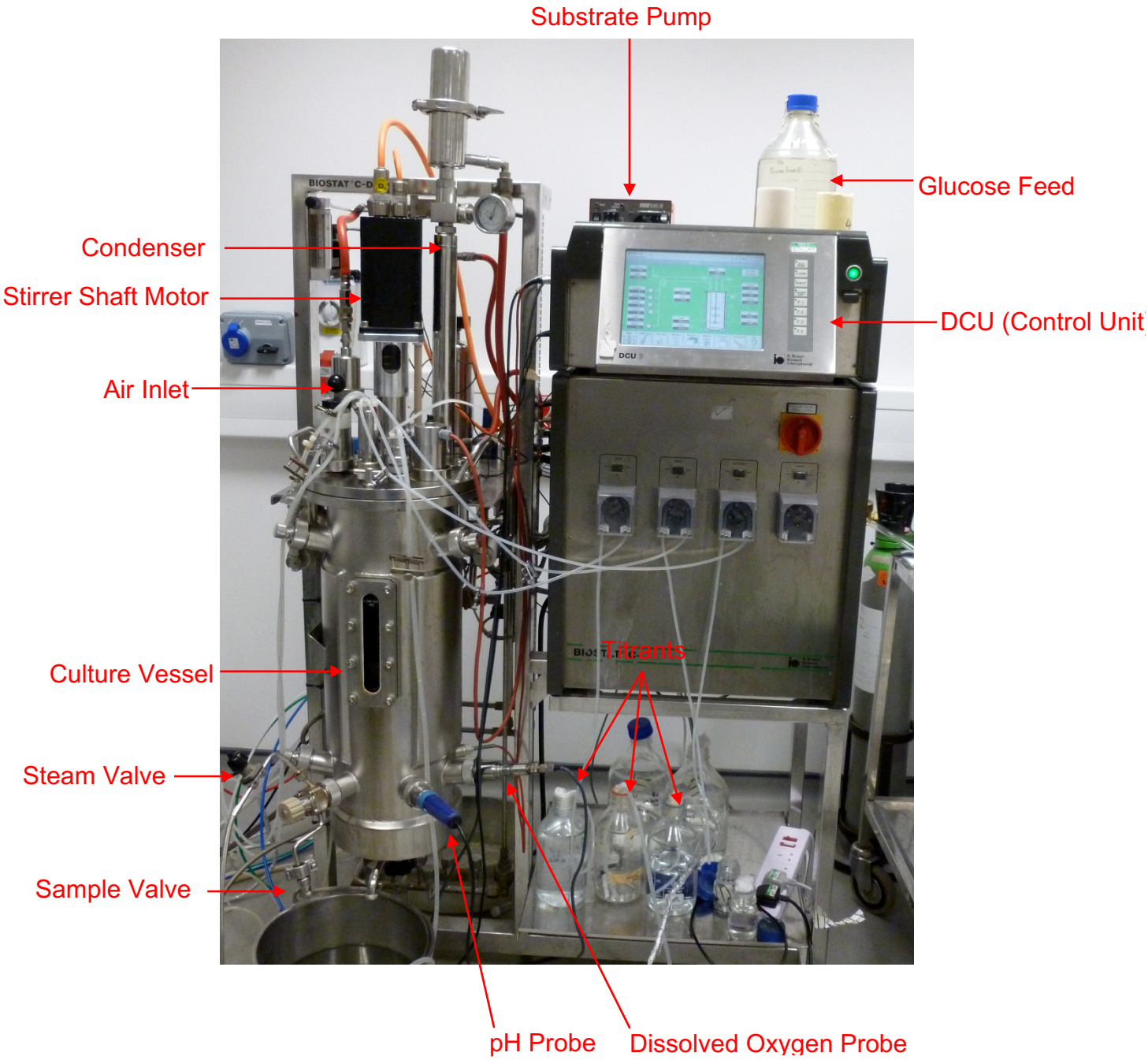

Figure 2-1. Operational Configuration of the Biostat C Bioreactor During an E. coli Fed Batch Process.

2.3.2 *Biostat Q DCU*

The Braun BIOSTAT Q-DCU (B.Braun Biotech International, Switzerland) (Figure 2-2) has four autoclavabe jacketed borosilicate glass bioreactor vessels each with a maximum working volume of 0.75 L and a total volume of 1 L. Rushton turbine impeller shafts are present in each vessel with a Teflon coated magnetic bar at the base used as a stirrer. The supply unit contains a temperature control unit, gas supply and DCU controlled pumps for the addition of acid and base.

The drive platform unit with integrated magnetic drive was used to agitate the cultures. The vessel temperature is maintained via a water jacket integrated in to the glass vessel. This is controlled by a thermostat unit heater and a circulation pump that supplies heated water (60 °C maximum) allowing for individual temperature control of each vessel. Accurate temperature control to within ± 0.1 °C of the temperature set point was accomplished through utilising the control loops of the DCU. Aeration was controlled by four rotamaters with a central pressure reducer installed in the supply unit. Air was supplied between 0.1 to 1 vvm through a circular annular sparger at the base of the vessel. Exhaust humidity was reduced using an exhaust cooler present on each vessel. The exhaust cooler supplied cooling water through additional water connections at the supply unit.

The pH (Mettler Toledo Ltd., Leicester, UK) of the culture media was maintained to within ± 0.05 of the set point by DCU controlled operation of two peristaltic pumps supplying solutions of acid and base to the vessel via silicone rubber tubing for each vessel. The dissolved oxygen tension (DOT) of the culture media was monitored by pO_2 electrode (Mettler Toledo Ltd., Leicester, UK). Calibration of the probe was performed by introducing oxygen free nitrogen (OFN) [0 % saturation value] and compressed air [100 % saturation value]. Calibration occurred at fermentation temperature and agitation speed. Polypropylene glycol 2025 was manually supplied to the vessels via a peristaltic pump as an anti-foam agent.

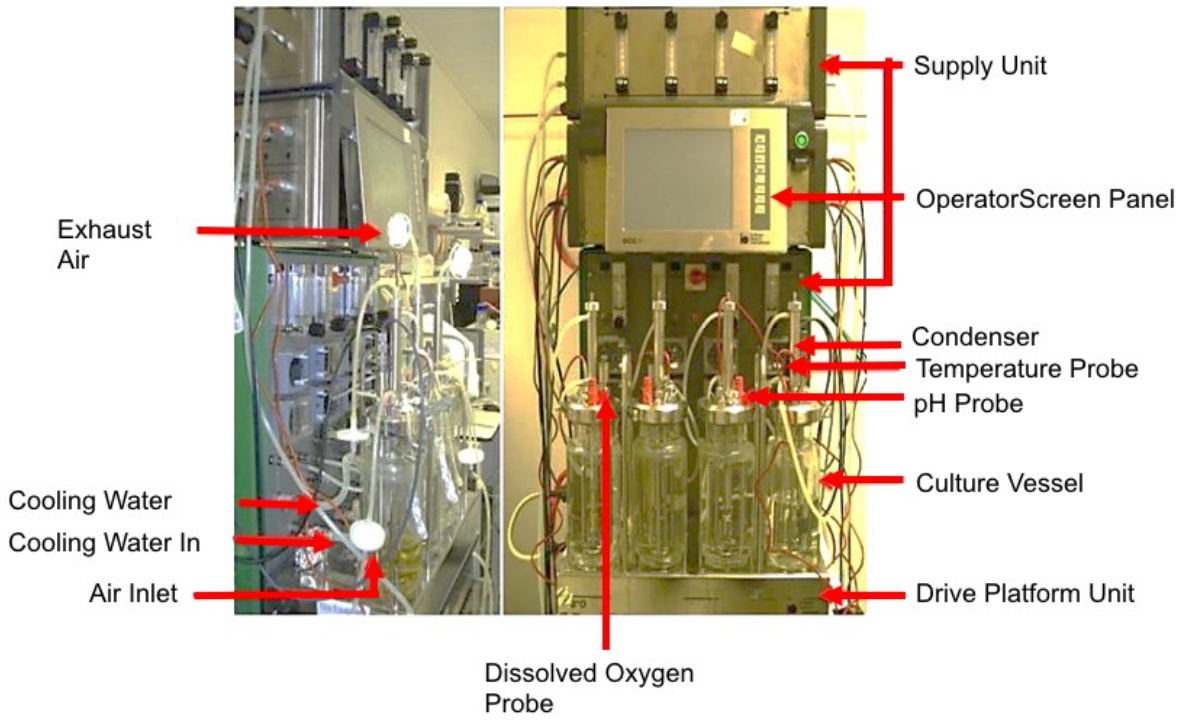

Figure 2-2. Operational Configuration of the Biostat Q Bioreactor during an E. coli Fed Batch Process.

This reactor was used for the initial *E. coli* work in Chapter 3 (Page 61-64).

2.4 *Analytical Equipment*

2.4.1 *YSI 2700 Analyser*

The YSI (Yellow Springs Instrument Company Inc) 2700 analyser (YSI Limited, Hampshire, UK) is a biochemistry analyser used to analyse the concentration of D-Glucose using YSI 2776 as a glucose-lactate standard in this project. YSI's enzyme sensor technologies are based on enzyme catalysed reactions leading to the production of hydrogen peroxide as a by-product which is electrochemically oxidised at the platinum anode of the YSI probe which ultimately produces a signal as a current. System was calibrated before each run using a Glucose standard supplied by YSI.

2.4.2 *Spectrophotometer*

For the measurement of optical density and the enzymatic activities a dual-light path UV-Vis spectrophotometer with a thermostatted rotary 7-cell changer was used (BioMate 5, Thermo Scientific, Hemel Hempstead, Hertfordshire, UK).

2.4.3 *Incubator*

Shake flask cultivations were performed in a rotary shaker (New Brunswick Scientific, Edison, USA).

2.4.4 *Gas Analyser*

The exit gas composition was measured using a digital gas analyser TANDEM PRO (Applikon Bioitechnology Ltd, Tewkesbury, Gloucestershire, UK). Two gases were used for calibration: 19 % Oxygen (v/v) and 1.75 % Carbon Dioxide (v/v).

2.4.5 *High Pressure Cell Homogeniser*

For the analysis of intracellular components including protein and D amino acid oxidase activity, cells had to be homogenised. This was carried out in a high pressure cell homogeniser (Model 400, Constant Systems Ltd., Warwick, UK). 5 ml of sample was transferred in to the piston chamber of the homogeniser and disrupted twice at 30 kpsi. The homogenate was collected and centrifuged in order to obtain a clear supernatant (Jouan BR4i, Thermo Electron Corporation, East Grindstead, West Sussex, UK) which was used in subsequent assays.

2.5 *Analytical Procedures*

2.5.1 *Dry Cell Weight for E. coli and P. pastoris*

Aliquots of 1 ml samples were pipetted in to pre-weighed Eppendorf tubes and centrifuged for 10 minutes at 12,000 rpm in a benchtop microcentrifuge (Eppendorf Microcentrifuge 5415D, Eppendorf AG, Hamburg, Germany). The supernatant was discarded and cells were re-suspended in water and centrifuged again after which the cells were re-suspended in water and centrifuged again, after which the Eppendorf with the cell pellet was dried in an oven at 100 °C for 24 hours. The Eppendorf was cooled in a desiccator for 2 hours and then weighed. The dry cell weigth was then calculated by subtracting the weight of the pre dried and weighed tubes. Performed in triplicate.

2.5.2 *Optical Density*

Appropriately diluted samples of each organism taken directly from the bioreactor at a particular time period was used to measure the optical density of the culture at 600nm.

2.5.3 *D-Amino Acid Oxidase Activity*

Samples of microorganisms were taken directly from the bioreactor at a particular time period. For extracellular DAAO samples, samples were centrifuged and supernatant used for analysis. For intracellular DAAO samples, samples were passed through the cell disrupter, centrifuged and supernatant used for analysis. DAAO activity was measured from intracellular and extracellular samples when necessary. The assay measures the release of H_2O_2 from the conversion of a substrate to a product. H_2O_2 is measured via a colorimetric reaction which occurs with the addition of horseradish peroxidase [HRP] (acting as a catalyst) and a substrate (4-aminoantipyrine [4-AAP]/2, 4, 6-tribromo-3-hydrozybenzoic acid [TBHBA]) oxidised to a coloured product. The absorbance of this coloured product is measured at 510 nm.

The assay stock solution can be seen in Table 2-18.

Table 2-18. Composition of DAAO Enzyme Assay Stock Solution

Potassium Phosphate Buffer (pH 7.6)	10 ml
Distilled H_2O	39 ml
TBHBA (In 1 ml of Dimethyl Sulphoxide)	20 mg
4 AAP	15.24 mg

The assay was performed in 1 ml cuvettes where 50 µl of appropriately diluted enzyme solution is added to the following in Table 2-19.

Table 2-19. Volumes of Final Assay Mix

Assay Stock Solution	500 µl
100 mM Substrate	100 µl
HRP Solution (5 mg/ml)	10 µl
Distilled H_2O	340 µl

The reaction is illustrated in Figure 2-3. The outcome of the reaction is illustrated in Figure 2-4.

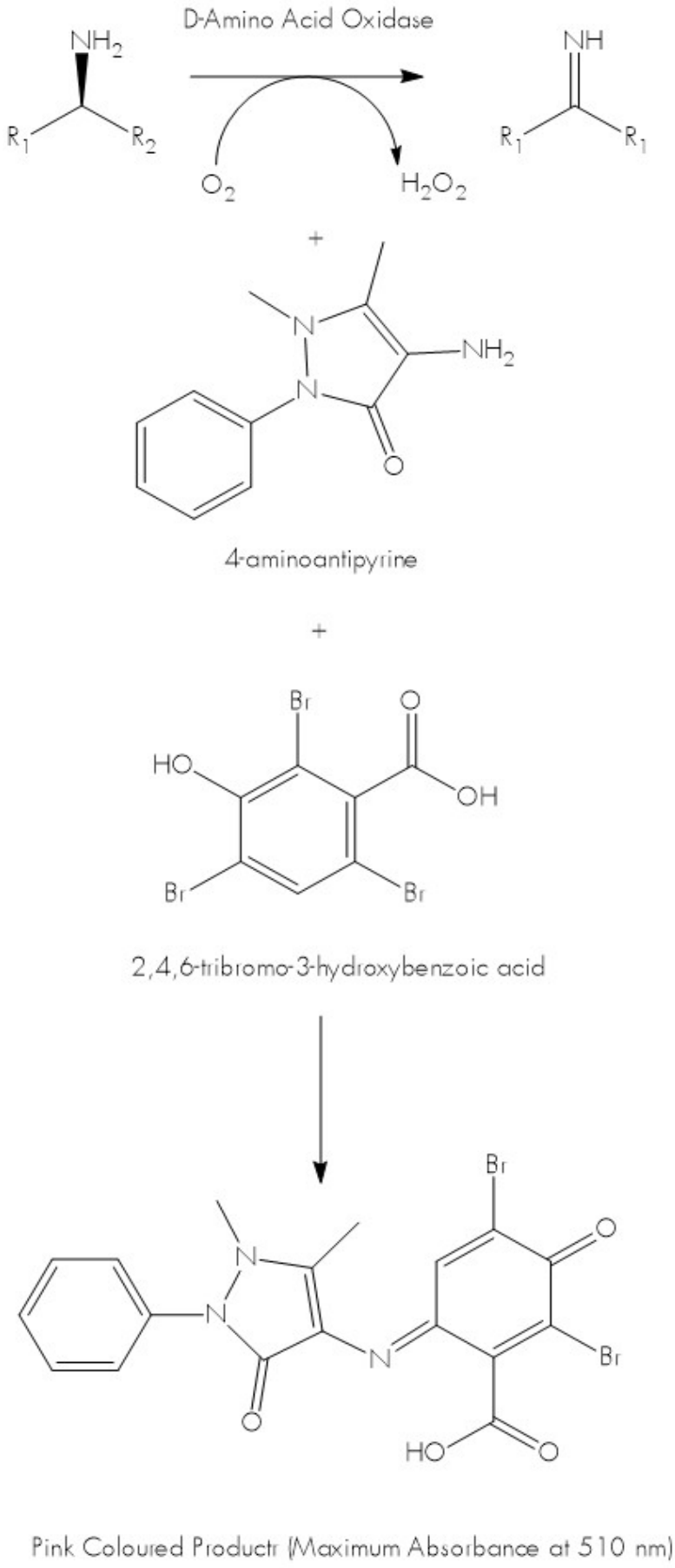

Figure 2-3. Principal of the DAAO Enzymatic Activity Assay

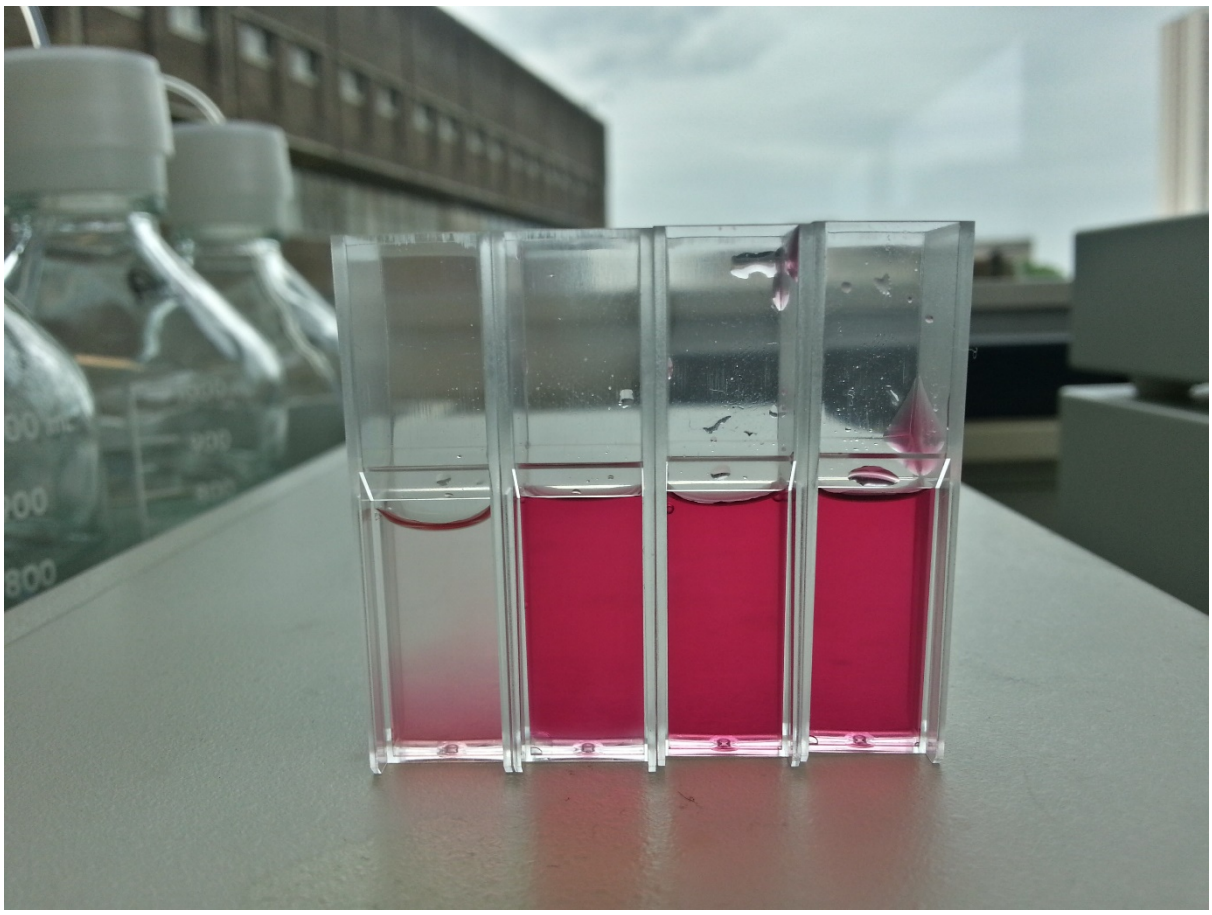

Figure 2-4. Principal of the DAAO Enzymatic Activity Assay Illustrating the Pink Colour Change.

The cuvettes are then immediately transferred to the spectrophotometer which has been set at 30 °C and the change in absorbance is monitored. Additionally, negative controls were also be run with a) no substrate and b) no oxidase enzyme to give the background rate of change in absorbance. This value obtained was subtracted from all observed rates of enzymatic reactions. Samples with commercially available DAAO were used to ensure the assay was working correctly.

The rate of change in absorbance at 510 nm was then used to calculate specific activities and other kinetic parameters such as k_{cat} and K_M. The extinction coefficient of the colorimetric product is 29,400 M^{-1} cm^{-1} and the path length of the 1 ml cuvette is 1 cm. Using Beer – Lambert law the change is the concentration of the colorimetric product and therefore the change in concentration of the substrate is :

$$Concentration\ Change\ (MS^{-1}) = Abs\ Units\ (s^{-1}) \div [29400\ [M^{-1}cm^{-1}] \times 1\ (cm)$$

2.5.4 *Glucose Determination*

2.5.4.1 *Enzymatic Kit Determination*

A commercial glucose concentration kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 716 251 035) was used to determine the glucose concentration in sample supernatants. The test principle is as follows: D-Glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP).

2.5.4.2 *YSI 2700 Determination*

A substrate for the analyte being measured enters the sample chamber and mixed and diluted. This substrate then diffuses through a thin polycarbonate membrane after which the substrate encounters an extremely thin layer of the appropriate oxidase enzyme where the following reaction occurs:

$$\text{D-Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} H_2O_2 + \text{D-GLucono-Delta-Lactc}$$

H_2O_2 diffuses towards the platinum anode in the probe assembly and this gives rise to the probe signal current by its oxidation:

$$H_2O_2 \xrightarrow[+\ 0.7\ V]{\text{Oxidised at Probe}} O_2 + 2H^+ + 2e^-$$

The substrate (analyte) concentration is proportional to the electron flow

$$\text{Substrate} \longrightarrow 2e^-$$

The machine compares the produced signal current to that from a known concentration standard and gives the final concentration results of the unknown.

In the presence of the enzyme G6P dehydrogenase (G6P-DH), G6P is oxidised by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH).

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The amount of NADPH formed in this reaction is stoichiometric to the amount of D-Glucose. The increase in NADPH is measured by means of its light absorbance at 340 nm.

2.5.5 *Acetate Determination*

A commercial acetate determination kit was used to measure acetate in culture supernatants (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 148 261 035). The test principal is as follows: Acetate is converted to acetyl-CoA in the presence of the enzyme acetyl-CoA Synthetase (ACS), adenosine-5'-triphosphate (ATP) and coenzyme A (CoA):

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{acetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

Acetyl-CoA reacts with oxaloacetate to citrate in the presence of citrate synthase (CS):

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + \text{H}_2\text{O} \xrightarrow{\text{CS}} \text{Citrate} + \text{CoA}$$

The oxaloacetate required for this reaction is formed from L – malate and nicotinamide-adenine dinucleotide (NAD) in the presence of L-malate dehydrogenase (L-MDH):

$$\text{L-Malate} + \text{NAD}^+ \underset{}{\overset{\text{L-MDH}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+$$

In this reaction NAD+ is reduced to NADH. The determination is based on the formation of NADH measured by the increase in light absorbance at 340 nm.

Because of the equilibrium of the preceding indicator reaction, the amount of NADH formed is not linearly (directly) proportional to the acetic acid concentration.

2.5.6 *Methanol Determination*

2.5.6.1 *YSI Determination*

This is a direct reading of methanol in solution at the enzyme sensor. The enzyme alcohol oxidase is immobilised in the enzyme membrane:

$$\text{Methanol} + O_2 \xrightarrow{\text{Alcohol Oxidase}} H_2O_2 + \text{Formaldehyde}$$

2.5.7 *Sorbitol Determination*

Sorbitol in culture supernatants was measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 670 057 035). The test principal is as follows: D-Sorbitol is oxidises by nicotinamide-adenine dinucleotide (NAD) to D-Fructose in the presence of the enzyme sorbitol dehydrogenase (SDH, also called polyol dehydrogenase), with the formation of reduced nicotinamide-adenine dinucleotide (NADH):

$$\text{D - Sorbitol} + NAD^+ \underset{}{\overset{\text{SDH}}{\rightleftharpoons}} \text{D-Fructose} + NADH + H^+$$

Under the assay conditions, the equilibrium of the reaction lies on the side of NAD+ and D-Sorbitol. However, they are favourably displaced as the formed NADH is removed in a subsequent reaction in which NADH reduces iodonitrotetrazolium chloride (INT) to a formazan in the presence of a diaphorase:

$$NADH + INT + H^+ \xrightarrow{\text{Diaphorase}} NAD^+ + \text{Formazar}$$

The absorbance of the formazan is measured at its maximum at 492 nm.

2.5.8 *Hydrogen Peroxide Determination*

H_2O_2 was measured by the FOX method (Wolff and Lester, 1994). In principle, hyperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange (XO; *o*-cresolsulfonepthalein 3', 3''-bis (methlyimino) diacetic acid, sodium salt) binds ferric ion with high selectivity to produce a coloured (blue-purple) complex with maximal absorbance at 560 nm:

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO\cdot + OH^-$$

$$Fe3+ + XO \longrightarrow Blue\text{-}PurpleComplex(560\ nm)$$

The recipe of the sorbitol-containing version of the water-soluble hydroperoxide assay, which in this study is referred to as FOX1, is as follows in Table 2-20.

Table 2-20. FOX1 Composition

Xylenol Orange	100 μM
Ammonium Ferrous Sulphate	250 μM
Sorbitol	100 μM
H_2SO_4	25 mM

50 μL of appropriately diluted sample was added to 950 μL of FOX1 reagent in a cuvette, vortexed, and incubated at room temperature for 5 minutes to allow colour development. The absorbance was read at 560 nm. The signal was read against an H_2O_2 standard curve measured at the same time along with samples and was linear in the 0 -100 mM concentration range. Distilled water was used as a blank. Performed in Triplicate

2.5.9 *Intracellular Enzyme Activities*

2.5.9.1 *Sampling*

For the intracellular enzyme activity from cell-free extracts, 10 ml of cells were collected immediately and transferred to 10 ml Falcon tubes. The cells were then centrifuged at 12,000 rpm for 10 minutes, the supernatant discarded and the pellet washed twice with water before being frozen at – 20 °C. After thawing, the cells were re-suspended in the same volume of protease inhibitor solution, and disrupted by a high-pressure cell homogeniser. Cell free extract was separated from cell debris by centrifugation at 4 °C, 12,000 rpm for 10 minutes. The clear supernatant was used to assay enzyme activities immediately.

2.5.9.2 *Superoxide Dismutase*

Reagents

- Solution A: 50mM Na_2CO_3 solution containing 0.1 mM EDTA
- mM Xanthine solution: 0.0152 g Xanthine (Sigma X4002) was dissolved in 100 mL solution A, stored at 4 °C for one week.
- mM Cytochrome *c*: 24.8 mg cytochrome *c* (Sigma C7752) was dissolved in 20 ml of solution A. aliquots were stored at – 20 °C.
- Xanthine Oxidase: Xanthine Oxidase (Sigma X1875) was diluted 200 times with solution A immediately before assay.

Procedure

SOD activity was measured by its ability to inhibit the reduction of cytochrome *c* with superoxide radical produced by the xanthine/xanthine oxidase system, which was described by Crapo *et al*., (1978). Operating concentrations were: 0.1 mM xanthine; 0.1 mM EDTA; 50 mM Na2CO3 (pH 10.2); 10 μm ferricytochrome *c*; and enough xanthine oxidase to cause ΔA_{550nm} = 0.025/min. 1 unit was defined as the amount of enzyme causing 50 % inhibition in a total reaction volume of 1.0 ml at room temperature. Superoxide dismutase standards showed that the inhibition correlated with enzyme units logarithmically in the range of 20 % to 80 %. The standard curve was used to calculate the units in the enzyme solution:

$$Activity\ (U/ml) = \frac{exp(\frac{Inhibition - 0.5439}{0.2367})}{Sample\ Volume\ (ml)} \times DF$$

2.5.9.3 *Catalase*

Catalase activity was determined by the decomposition of H_2O_2 at 240 nm within 30 seconds. One unit was defined as the amount of enzyme which catalyses the decomposition of 1 µmole of H_2O_2 per minute in a 3 ml solution of sodium phosphate buffer (50 mM, pH 7.0) at room temperature.

Reagents

50 mM phosphate buffer, pH 7.0. Stock solutions of 500 mM of monobasic and dibasic sodium phosphate were diluted in appropriate proportions. If necessary, 1 M NaOH solution or 1 M H_2SO_4 solution was used to adjust pH to 7.0 at room temperature.

H_2O_2 30 mM: diluted 0.34 ml of 30 % (w/w) H_2O_2 (Sigma H1009) with phosphate buffer to 100 ml.

Procedure

Enzyme solutions were diluted with phosphate buffer and equilibrated to room temperature before the assay. Then 2 ml of diluted enzyme solution was pipetted in to a silica cuvette, followed by the addition of 1 ml H_2O_2 solution (30 mM). The decrease in absorbance should be approximately A240 = 0.5 ± 0.005. 1 ml of phosphate buffer instead of H_2O_2 solution was used as a blank.

$$Activity\ (U/ml) = \frac{\frac{-\Delta_{240}/min}{0.0398}}{2} \times DF$$

2.5.10 *Intracellular Protein Content*

The intracellular protein content was determined using the Bradford Method as described by Sigma Aldrich.

The standard 3.1 ml Bradford assay consists of mixing 1 part of the protein sample with 30 parts of the Bradford Reagent. The sample may be a blank, a protein standard, or an unknown sample. The blank consists of buffer with no protein. The protein standard consists of a known concentration of protein, and the unknown sample is the solution to be assayed. Bradford assays are routinely performed at room temperature. Colour development begins immediately. The absorbance at 595 nm was recorded and the protein concentration was determined by comparison to a standard curve.

2.5.11 *Intracellular Protein Carbonyl Content*

Intracellular carbonyl has been extensively used as a bio marker for protein oxidation. In the present study, a spectroscopic method developed by Li *et al* (2008) has been adopted to quantify the carbonyl content in cell free extract with slight modification. Principally, protein carbonyl reacts with 2, 4-dinitrophenylhydrazine (DNPH), giving rise to 2, 4-dinitrophenyl (DNP) which was quantified by its absorbance at 370 nm (extinction coefficient = 22,000 M^{-1} cm^{-1}). Illustrated in Figure 2-5.

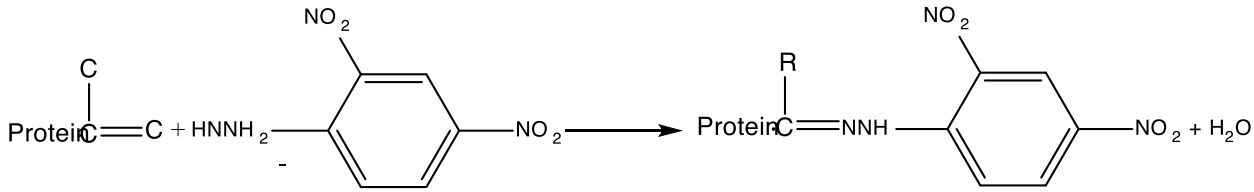

Figure 0-5. Principal of Protein Carbonylation

Reagents

2.5 M HCL Solution

10 % (v/v) TCA Solution

DNPH Solution in 2.5 M HCL

Ethanol:Ethyl Acetate (1:1)

6 M guanidine chloride in 20 mM potassium phosphate with pH adjusted to 2.3 by concentrated HCL.

Procedure

A protease inhibitor cocktail (Sigma P8215) was added to samples 1:100 (v/v) before the assay to prevent degradation of proteins. 0.1 ml of protein solution was incubated with 0.4 ml DNPH solution for 1 hour at room temperature. For the blank, 0nly 0.4 ml HCL was added to the sample. Samples were vortexed every 15 minutes to increase mixing. Afterward, 0.5 ml of 10 % ice-cold TCA was added to the Eppendorf and allowed to incubate on ice for 10 minutes. The samples were then centrifuged at 12,000 rpm for ten minutes to precipitate proteins. Supernatant was discarded to a waste reservoir. The protein pellets were washed three times by 0.5 ml ethanol:ethyl acetate solution to remove any excess DNPH. Again, the washing waste was discarded to another waste reservoir. 1 ml of 6 M guanidine chloride solution was added to the washed pellets and incubate in a water bath at 60 °C for 30 minutes followed by vigorous vortex mixing. The absorbance of the samples was monitored in a spectrophotometer at 370 nm against 1 ml 6 M guanidine chloride solution.

$$Protein\ Carbonyl\ (nmole/ml) = \frac{\frac{A_{370}}{22000} \times 10^6}{0.4}$$

2.5.12 *Intracellular Proteolytic (Protease) Activity*

Intracellular Protease Activity was measured using the Thermo Scientific™ Pierce™ Protease Activity Kit. The kit contains the following:

- Succinylated casein, 5 × 10mg, supplied as lyophilized salt-free powder
- TNBSA (2,4,6-trinitrobenzene sulphonic acid), 5% (w/v) in methanol, 2mL
- TPCK Trypsin, 50mg; specific activity: > 10,350 BAEE units/mg of protein
- BupH™ Borate Buffer Pack, yields 50mM borate, pH 8.5 after reconstitution with 500mL water.

The assay method uses succinylated casein and trinitrobenzenesulfonic acid (TNBSA). Succinylated casein is native casein that has been treated with succinic anhydride to block primary amines on the surface of the protein. In the presence of protease, the succinylated casein is cleaved at peptide bonds, thereby exposing primary amines (predominantly α-amines). TNBSA reacts with these exposed primary amines to produce an orange-yellow product whose intensity may be measured at 450nm. The increase in colour relative to sample without succinylated casein is a measure of protease activity in the sample.

Procedure

100µL of succinylated casein solution is added to one set of microplate wells. 100µL of assay buffer was also added to a duplicate set of wells to serve as blanks. 50µL of each unknown or standard sample was added to both succinylated casein wells and corresponding blank wells and these plates were then incubated for 20 minutes at room temperature. Afterwards 50µL of TNBSA working solution was added to each well and again, were incubated at room temperature for 20 mins. The absorbances of each wells was determined by a plate reader set to 450nm. For each well the change in absorbance at 450nm (ΔA450) was calculated by subtracting the A450 of the blank from that of the corresponding Casein well. This ΔA450 is the absorbance generated by the proteolytic activity of the protease.

Chapter Three

Recombinant Production of DAAO in *E. coli*

3 Recombinant Production of DAAO using *E. coli*

3.1 *Introduction*

Escherichia coli is an industrial workhorse in the field of recombinant protein expression. The genome of *E. coli* has been fully characterised and is believed to be well understood and the expression system is capable of being adapted for a multitude of inducible promoters. Despite the advances in other protein expression systems such as the baculovirus system, eukaryotic expression systems such as the methylotrophic yeast *Pichia pastoris* remains favoured over *E. coli* because of its innate abilities to perform post-translational modifications. *E. coli* is currently the only bacterial strain utilised industrially to produce biopharmaceuticals for human use. There has been a surge in interest of *E. coli* as an expression system over the last decade or so with applications in numerous areas of the pharmaceutical, agricultural, biotechnology and chemical industries. As an initial platform for laboratory investigations and as a comparative tool for other expression systems, *E. coli* is still the prokaryotic expression system of choice. In the current study we report upon the expression of an amine oxidase type enzyme using a thermally induced *E. coli* expression system.

Traditionally, expression of recombinant proteins in *E. coli* is carried out under control of the *lac* promoter, induced by the addition of IPTG to the reactor media. IPTG is both expensive and toxic, and often undesirable in the fermentation process. In order to overcome this, IPTG can be replaced by lactose, a product derived from the dairy industry. Unfortunately, the addition of lactose to the media can pose further challenges in the fermentation process because of the metabolisable nature of lactose; it is simultaneously a carbon source and inducer. Ultimately this leads to low volumetric yields and difficulties in process control. Thermal induction presents an alternative to the latter form of induction and is inexpensive and can be precisely controlled externally via the bioreactor control unit

The aim of Chapter Three was to study the effect of increasing the temperature during the thermal induction of E*. coli* to produce DAAO, with emphasis placed on dry cell weight accumulation and enzymatic activities and how these are affected by growth, and the physiology of the host organism. These parameters were

investigated using complex and defined media in order to ascertain suitability for industrial applications. DAAO production should only occur at induction temperatures or higher, and media complexity should not be detrimental to growth or activity of host cells.

3.2 *Results – Undefined Media for Growth of E. coli*

3.2.1 *Overview*

Lysogeny broth (LB) is traditionally used for the cultivation of various bacterial strains, and can be bought as a readily available powdered mix which can be made up very quickly. It contains a variety of growth complexes including B vitamins, minerals, salts, a paltry amount of carbohydrates and various utilisable carbon sources; all concentrations and compositions of each are hard to define and highly variable though LB allows for quick growth and a relatively straightforward process. In these forthcoming experiments, LB was used as a batch medium, this was followed by a glucose feed substituted with LB. Experiments were performed at temperatures ranging between 28 °C and 40 °C in order to induce thermal induction of the DAAO enzyme. Enzyme activities were measured, and the physiology of the host cells was monitored in order to ascertain the effects of media and temperature on recombinant protein production.

3.2.1.1 *E. coli Batch Fermentations – Temperature Range of 28 °C to 40 °C*

The Biostat Q multi vessel reactor was used to investigate whether there was premature expression of the DAAO enzyme at Temperatures below the theoretical induction temperature of 37 °C during the batch phase. Premature expression of the DAAO enzyme has the potential to act up on the D-alanine present in the cell wall of *E. coli* and negatively affect the growth of the host cells, and the overall process. It is essential to know this information before the implementation of a Fed-Batch process. Key measurements were Dry Cell Weight Accumulation and Specific and Total Enzymatic Activities. Figure 3-1 illustrates the Time Profile of DCW over four temperatures. Figure 3-2 illustrates the Enzymatic Activities over four temperatures.

Expression systems based on the strong P_L and/or P_R promoters in combination with the temperature-sensitive lambda repressor cI857 protein present important and convenient features for recombinant protein production. For example, they can be easily induced by simple and inexpensive schemes based on a temperature up-shift, without drawbacks associated to the addition of expensive and toxic chemical inducers, or to metabolically controlled promoters. Furthermore, heterologous gene expression is efficiently repressed at 30 °C, allowing the attainment of high cell densities before the induction phase, whereas increasing the temperature in the range of 39–42 °C results in increasing levels of recombinant protein production (Caspeta *et al*., 2013).

The results indicate that temperatures between 28 °C and 30 °C are optimal for growth. At 35 °C growth is initially higher than the other processes but declines after 12 hours. At 40 °C growth is lowest, and ceases after approximately 12 hours. Expression of the DAAO enzyme is low at temperatures between 28 °C and 30 °C, but increases at temperatures of 35 °C and above. This is the likely cause for the decrease in accumulation of DCW. As a result, 28 °C was chosen as the temperature for the batch phase of the fed batch process: it gave good DCW and the lowest enzymatic activities which is essential for the batch phase of the process.

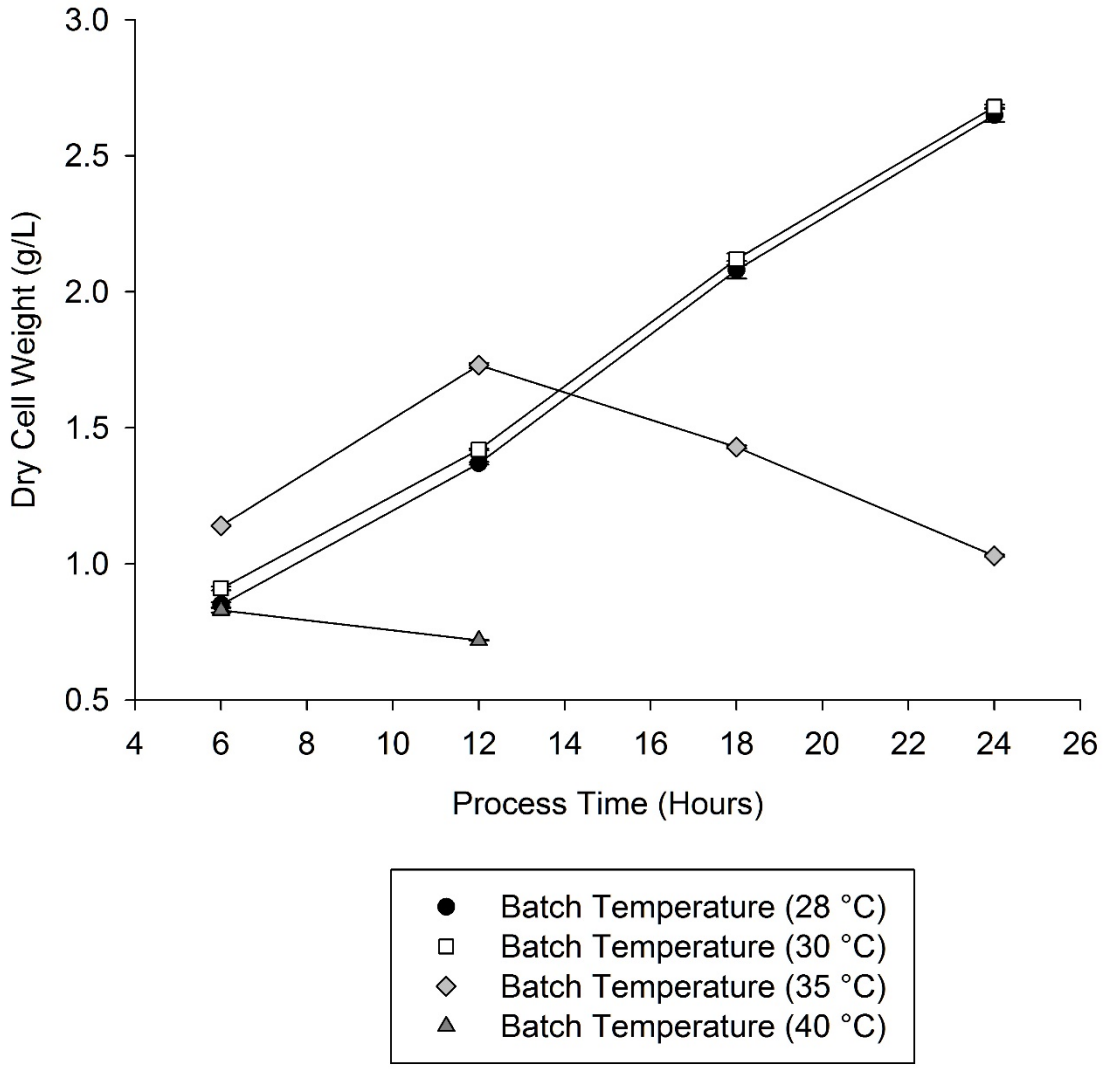

Figure 3-1. Time profiles of the Dry Cell Weight accumulation of E. coli pRES151DAAOWT BW2157 grown in LB media at 4 different temperatures. Culture Conditions: 28 °C, 30 °C, 35 °C, 40 °C and 300 – 900 rpm, 1 vvm – 3 vvm of compressed air for each experiment. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

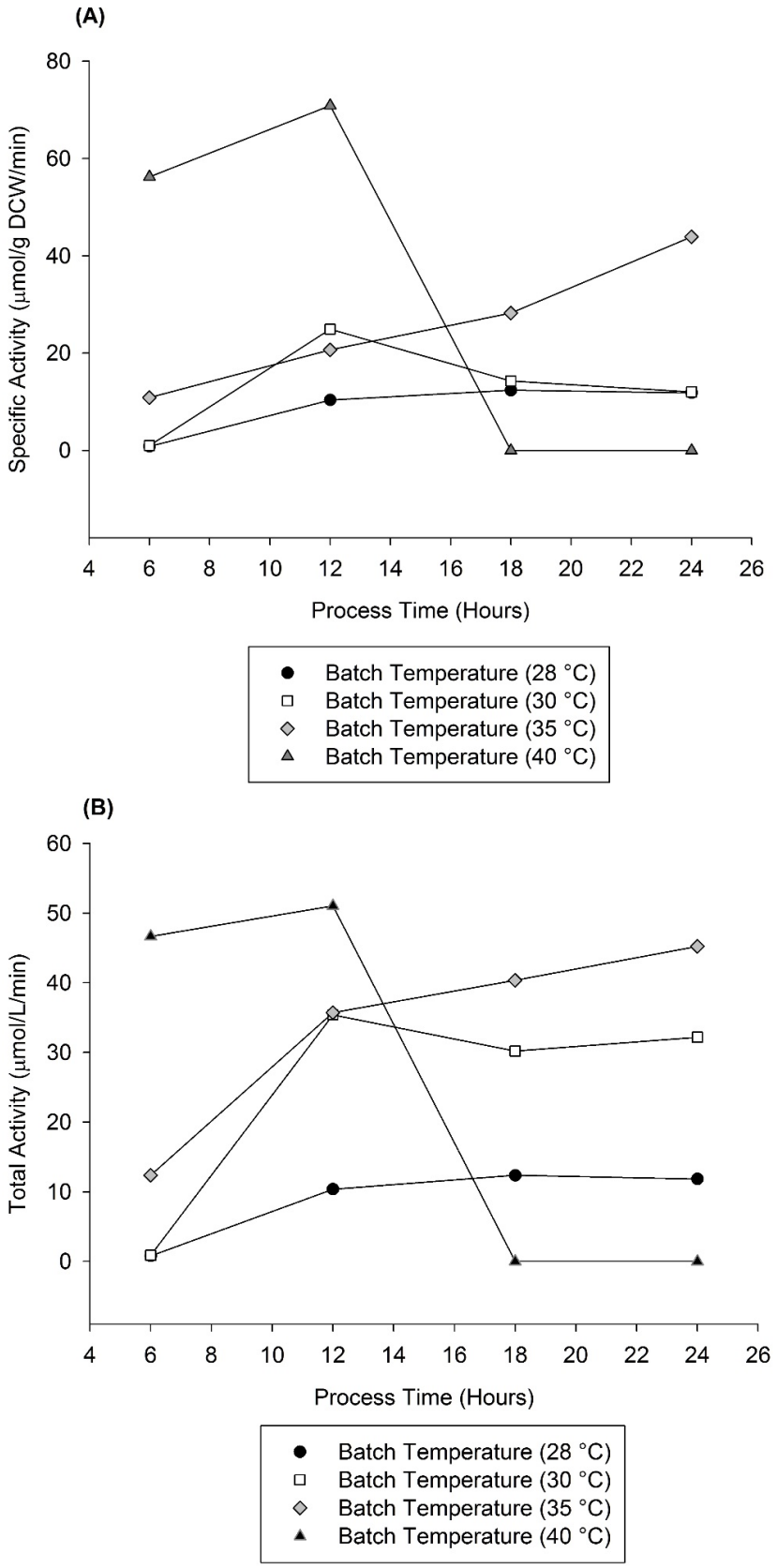

Figure 3-2. Time Profiles of the Specific and Total Activities of the DAAO produced using E. coli pRES151DAAOWT BW2157 grown in LB media at 28 °C, 30 °C, 35 °C, 40 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.2.1.2 *E.coli Fed Batch Fermentation – Constant Temperature of 28 °C*

Based on the Biostat Q work carried out previously, 28 °C was chosen as the temperature at which the processes would run before the onset of thermal induction as there was minimal promoter activation and subsequent enzyme production, which is essential for this process as DAAO has the potential to be toxic to growing cells.

In this experiment there was no induction and both the batch and fed-batch stages of the process were performed at a constant temperature of 28 °C as a baseline for future processes.

In Figure 3-3 it can be seen that 12 hours after inoculation, the cells reached a biomass of 2.88 g/L, with a corresponding acetate measurement of 0.12 g/L. The dissolved oxygen reached its pre-set level of 30 % at approximately 18 hours after inoculation. At this time point the biomass levels measured 2.95 g/L with a corresponding acetate measurement of 0.12 g/L. At approximately 20.5 hours the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 24 hours after inoculation, the dissolved oxygen measured 47 % and the glucose feeding program was initiated. At this time point the biomass measured 2.25 g/L with a corresponding acetate level of 0.14 g/L. 9 hours after the start of the glucose feed (a total time of 33 hours) the biomass of the culture measured 20.06 g/L with a corresponding acetate concentration of 0.24 g/L. Biomass levels continued to increase until the end of the process at 74 hours, with a final measurement of 49.38 g/L. The corresponding acetate measurement was 0.39 g/L.

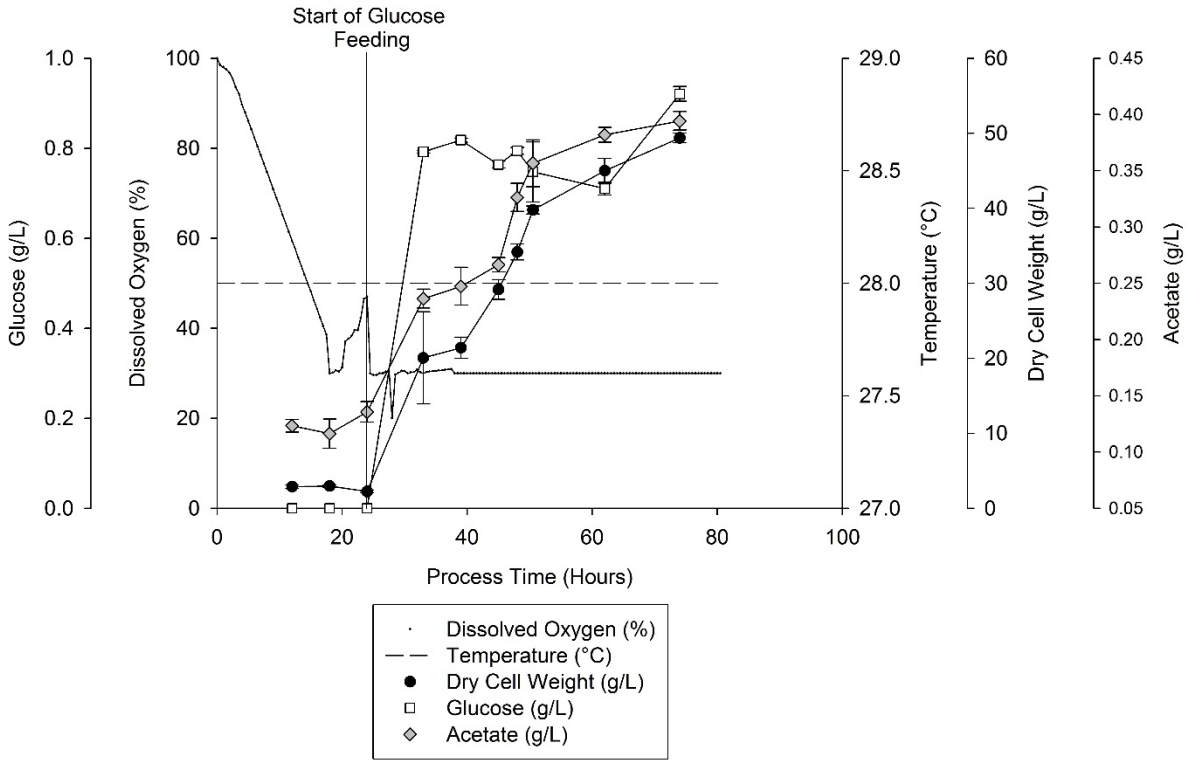

Figure 3-3. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Although the temperature of this experiment remained constant at 28 °C, the DAAO enzyme assay was performed in order to establish its expression during the feeding but before thermal induction. As seen in Figure 3-4 the maximum total DAAO activity was reached at 33 hours total process time. The total activities throughout the process remained between approximately 27 μmol/L/min and 41 μmol/L/min with the highest activities occurring during the glucose feeding program. Similarly, specific activities of the DAAO enzyme were found to be highest during the glucose feeding program with a maximum of 11 μmol/g DCW/min at 74 hours total process time.

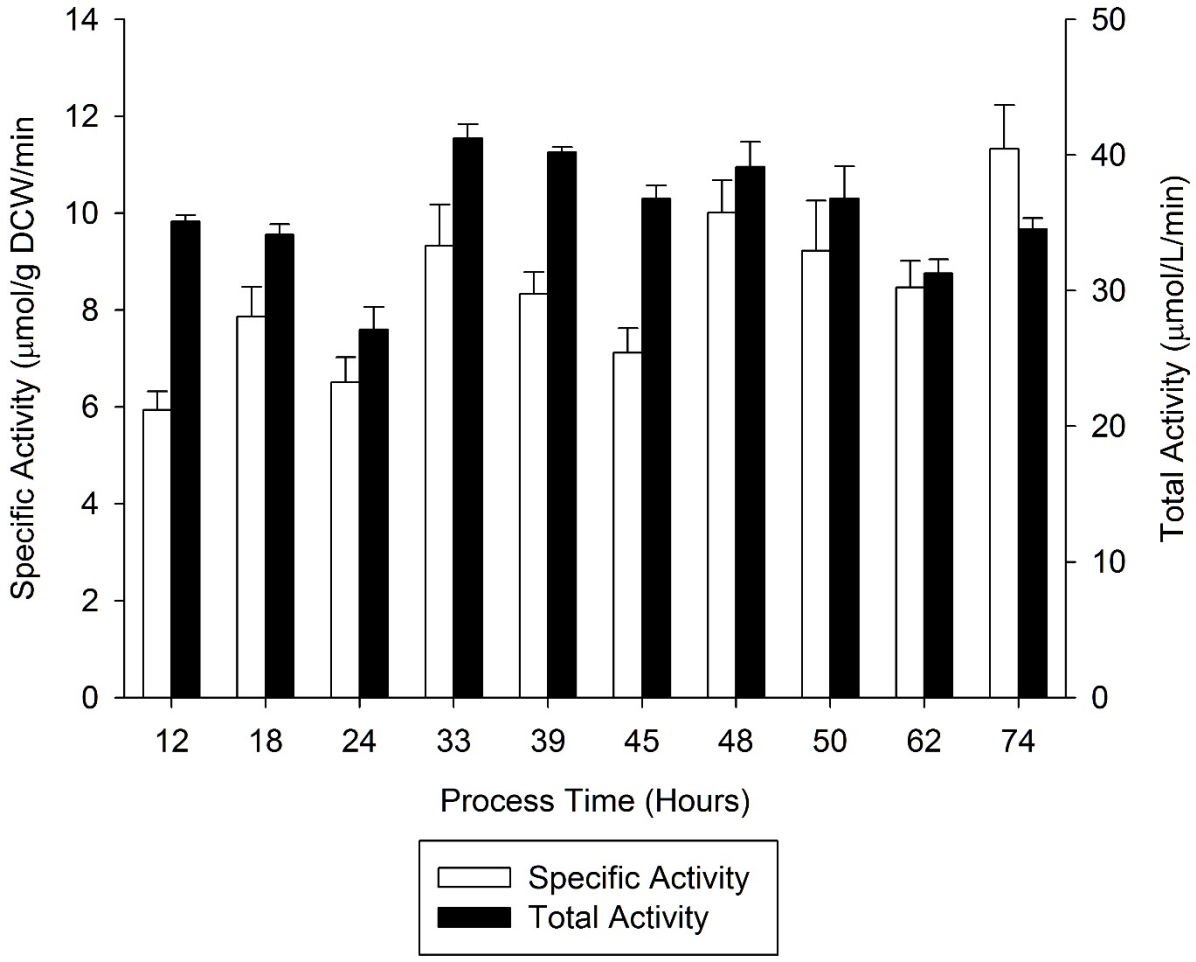

Figure 3-4. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media at a Constant Temperature of 28 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

As can be seen in Figure 3-5 the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, with a measured retention of 92 %. At the end of the process at 74 hours, plasmid retention measured 90 % indicating the culture had efficiently maintained the plasmid for DAAO expression.

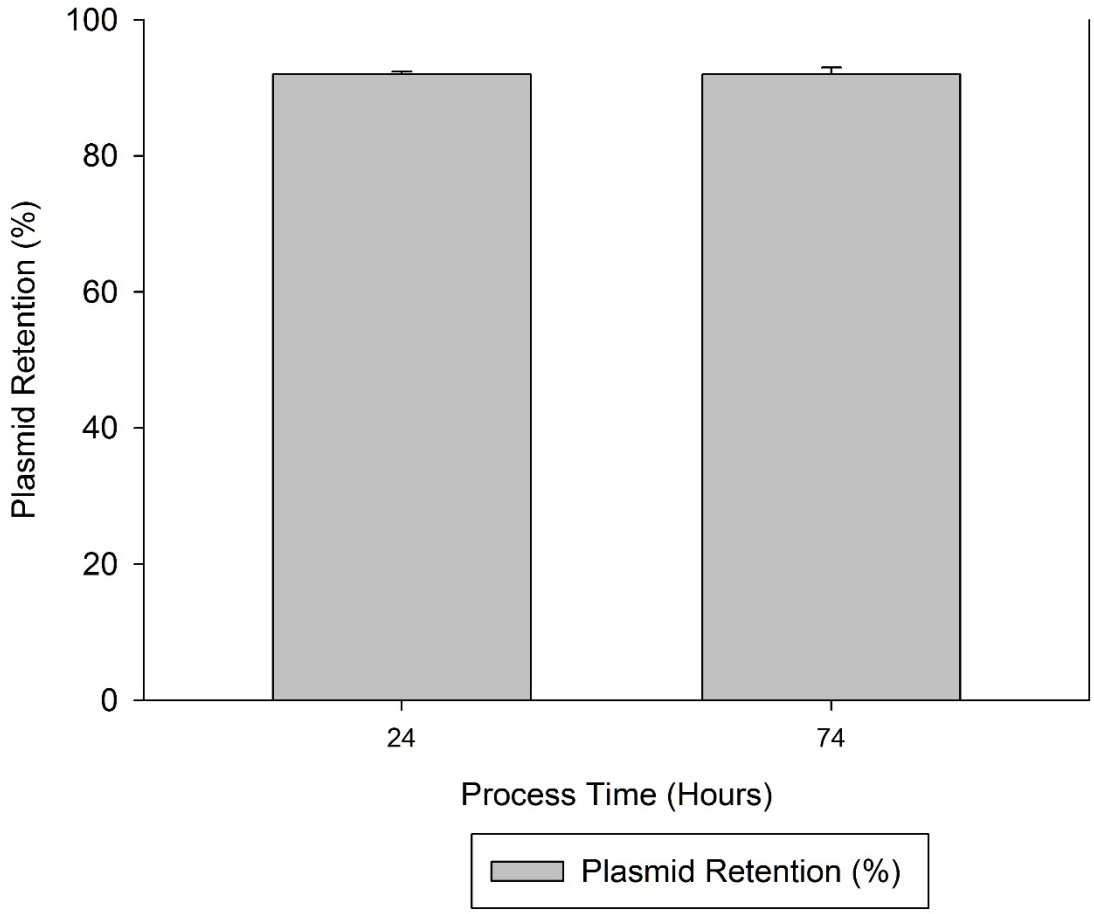

Figure 3-5. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air.All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.2.1.3 *E. coli Fed Batch Fermentation – Induction at 30 °C*

In this experiment thermal induction occurred at 30 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 30 °C was chosen as an induction temperature because evidence from the Biostat Q showed a higher incidence on enzymatic activity compared with 28 °C. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

In Figure 3-6 it can be seen that 12 hours after inoculation, the cells reached a biomass of 2.23 g/L, with a corresponding acetate measurement of 0.12 g/L. The dissolved oxygen reached its present level of 30 % at approximately 18 hours after inoculation. At this time point, the biomass measured 2.58 g/L with a corresponding acetate measurement of 0.11 g/L. At approximately 20 hours the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 24 hours after inoculation the dissolved oxygen measured 48.7 % and the glucose feeding program was initiated. At this time point the biomass measured 2.99 g/L with a corresponding acetate level of 0.14 g/L. 9 hours after the start of the glucose feed (a total time of 32 hours) the biomass of the culture measured 21.02 g/L with a corresponding acetate concentration of 0.28 g/L, Biomass levels continued to increase until 84 hours total process time, with a measurement of 51.05 g/L and a corresponding acetate concentration of 0.55 g/L. After this point, biomass decreased slightly to 49.3 g/L with a corresponding acetate level of 0.58 g/L.

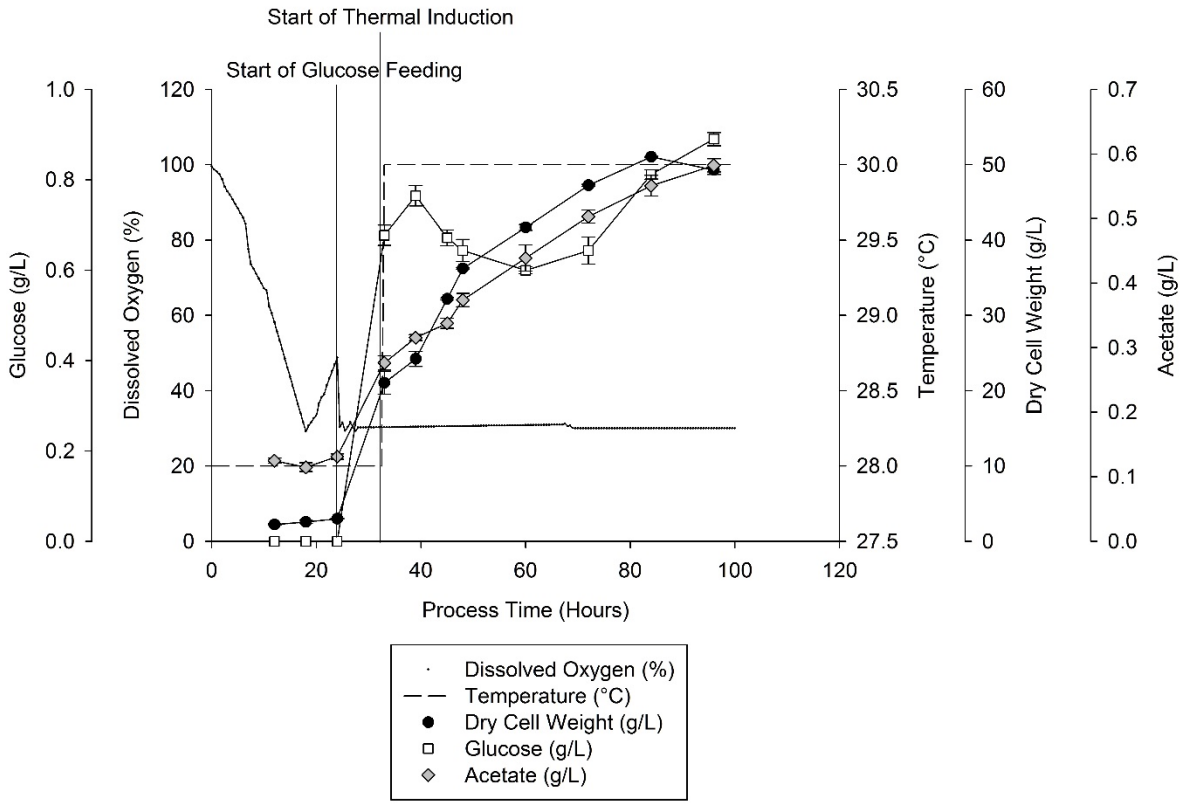

Figure 3-6. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C until Induction when changed to 30 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population

During this process, induction occurred when the temperature was raised from 28 °C to 30 °C. As seen in Figure 3-7 the maximum total DAAO activity was reached at 63 hours post induction (a Total Process Time of 96 hours). 6 hours post induction the specific DAAO activity and total DAAO activity measured 18.14 μmol/g DCW/ min and 439.31 μmol/L/min respectively. Pre induction (denoted as 0 hours) the specific DAAO activity and total DAAO activity measured 9.52 μmol/ g DCW/min and 32.88 μmol/L/min respectively. The specific DAAO activity peaked at 12 hours post induction and dropped continuously until 39 hours post induction, when it began to increase slightly. The total activities post induction continued to increase.

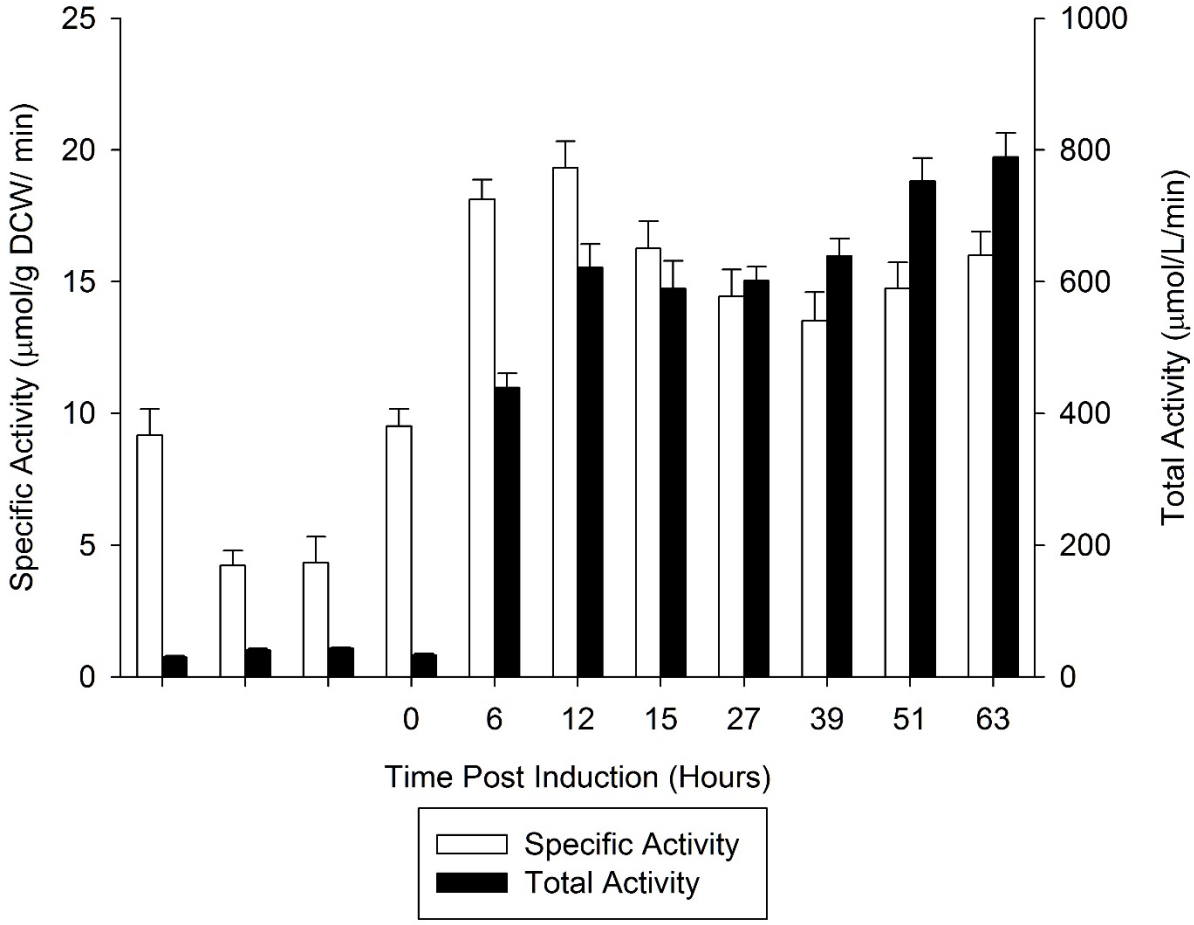

Figure 3-7. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media with induction at 30 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

As can be seen in Figure 3-8 the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, measuring 95 %. At the end of the process at 96 hours, the plasmid retention measured 84 %, indicating that there was some loss in plasmid retention.

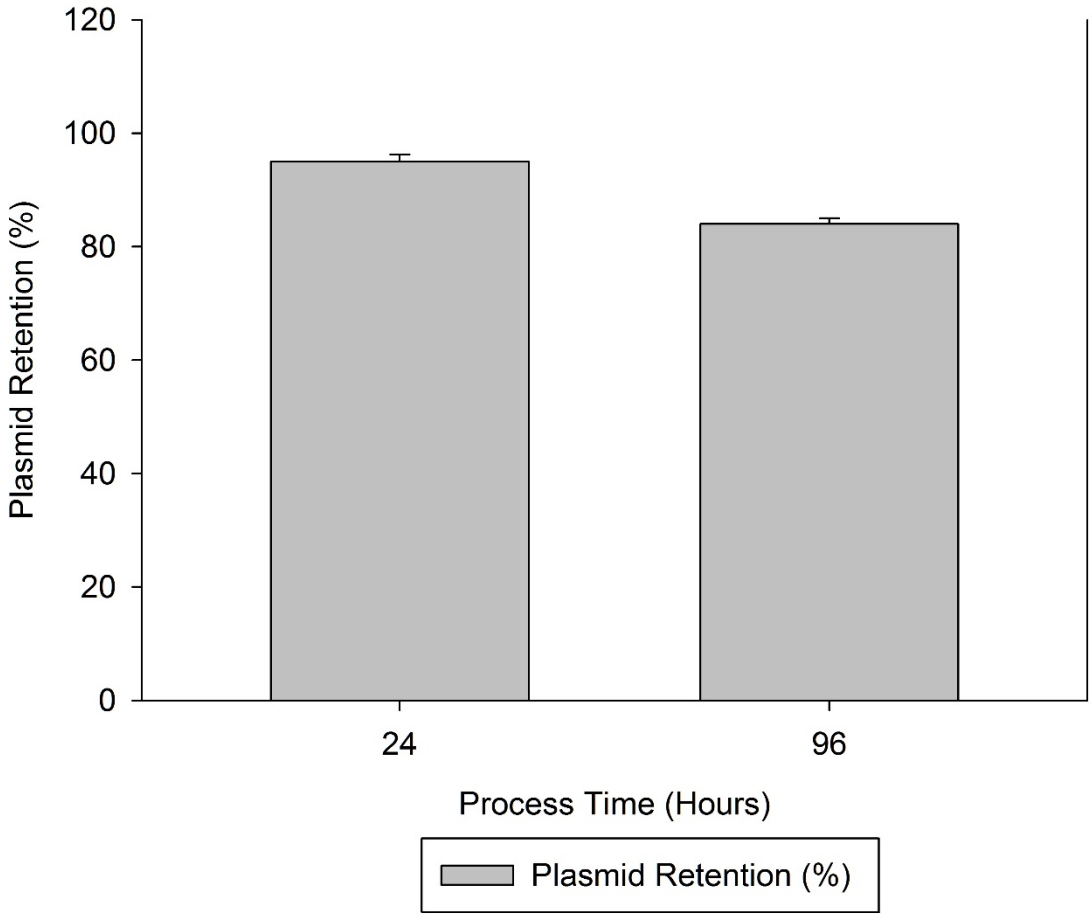

Figure 3-8. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 30 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.2.1.4 *E. coli Fed Batch Fermentation – Induction at 35 °C*

In this experiment thermal induction occurred at 35 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 35 °C was chosen as an induction temperature because evidence from the Biostat Q showed a higher incidence on enzymatic activity compared with 28 °C and 30 °C. 35 °C is also approaching 37 °C, the temperature where the promoter should theoretically have been unrepressed. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

In Figure 3-9 it can be seen that 12 hours after inoculation, the cells reached a biomass of 2.36 g/L with a corresponding acetate measurement of 0.17 g/L. The dissolved oxygen reached its pre-set level of 30 % at approximately 18 hours after inoculation. At this time point, the biomass measured 2.96 g/L with a corresponding acetate measurement of 0.17 g/L. At approximately 21 hours the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 24 hours after inoculation the dissolved oxygen measured 44.37 % and the glucose feeding program was initiated. At this time point the biomass measured 2.89 g/L with a corresponding acetate level of 0.15 g/L. 6 hours after the start of the glucose feeding (a total time of 31 hours) the biomass of the culture measured 22.13 g/L with a corresponding acetate level of 0.273 g/L. Biomass levels continued to increase for the remainder of the process, with a final measurement of 44.30 g/L at 96 hours total process time. The corresponding acetate measurement is 0.857 g/L.

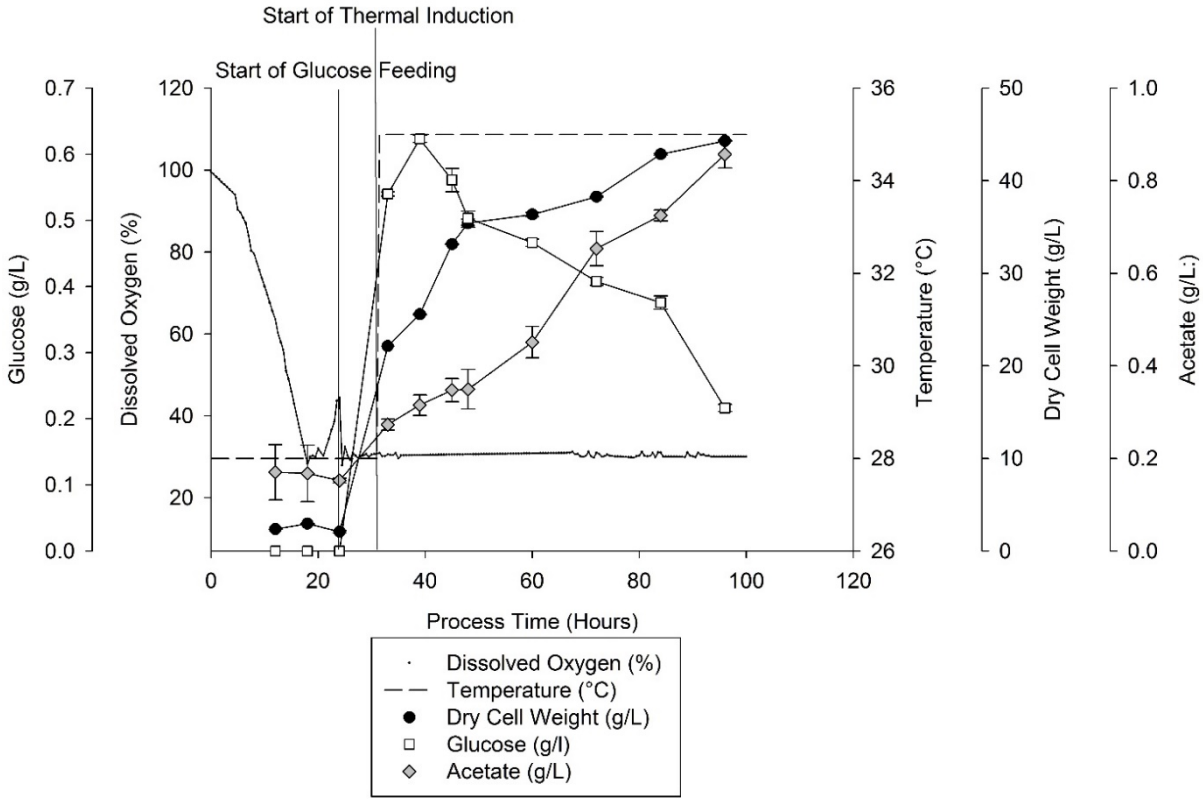

Figure 3-9. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C until Induction when changed to 35 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

During this process, induction occurred when the temperature was raised from 28 °C to 35 °C. As seen in Figure 3-10 the maximum total DAAO activity was reached at 51 hours post induction (a total time of 84 hours). 6 hours post induction the specific DAAO activity and the total DAAO activity measured 53.54 μmol/g DCW/min and 1342.81 μmol/L/min respectively. Pre induction (denoted as 0 hours) the specific DAAO activity and total DAAO activity measured 1.74 μmol/g DCW/min and 38.32 μmol/L/min respectively. The specific DAAO activity peaked at 67.54 μmol/g DCW/min at 39 hours post induction. From this point onwards, observed specific DAAO activity decreased.

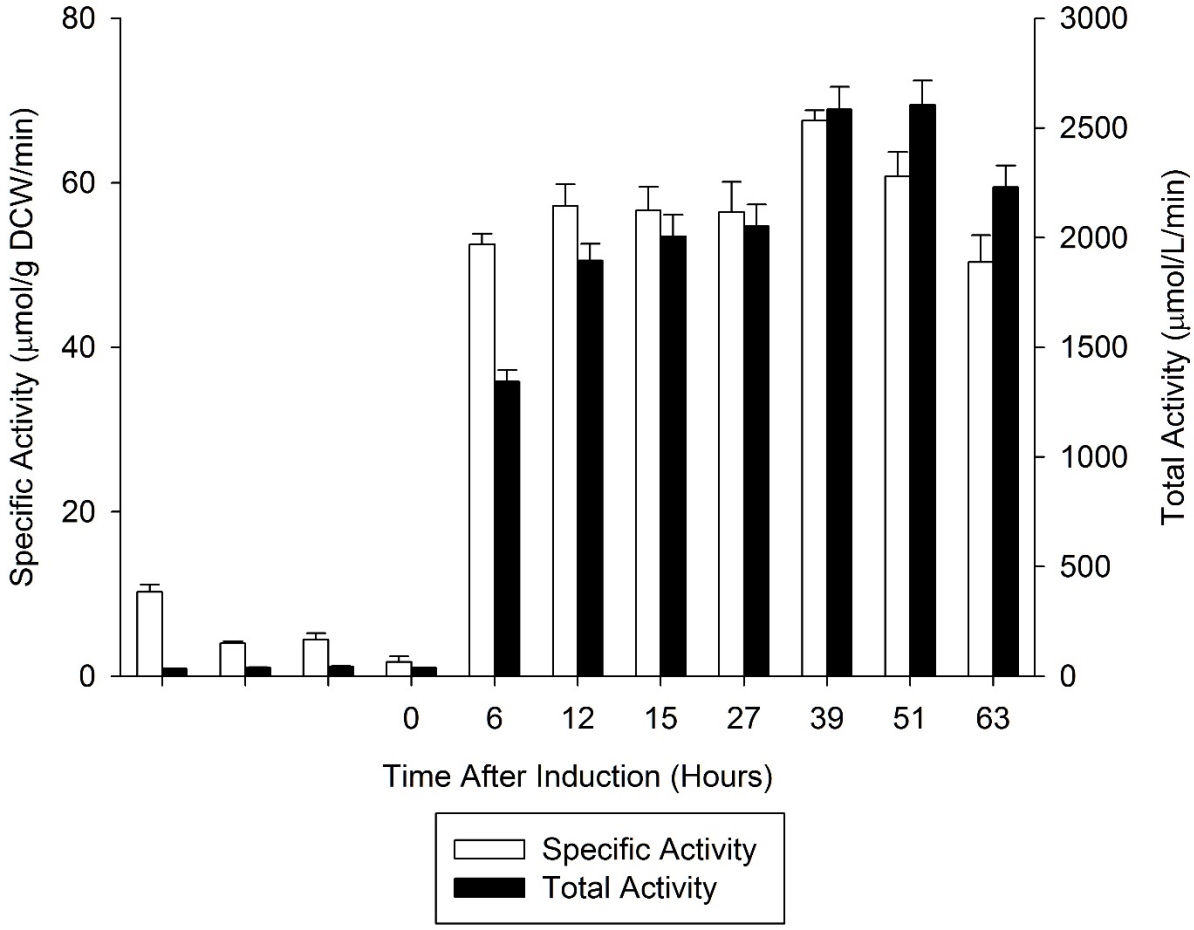

Figure 3-10. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 35 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

As can be seen in Figure 3-11 the plasmid retention of the culture was also measured during the experiment. At 24 hours the plasmid retention measured 93 %. At the end of the process, at 96 hours, the plasmid retention measured 78 % indicating that there was some loss in plasmid retention.

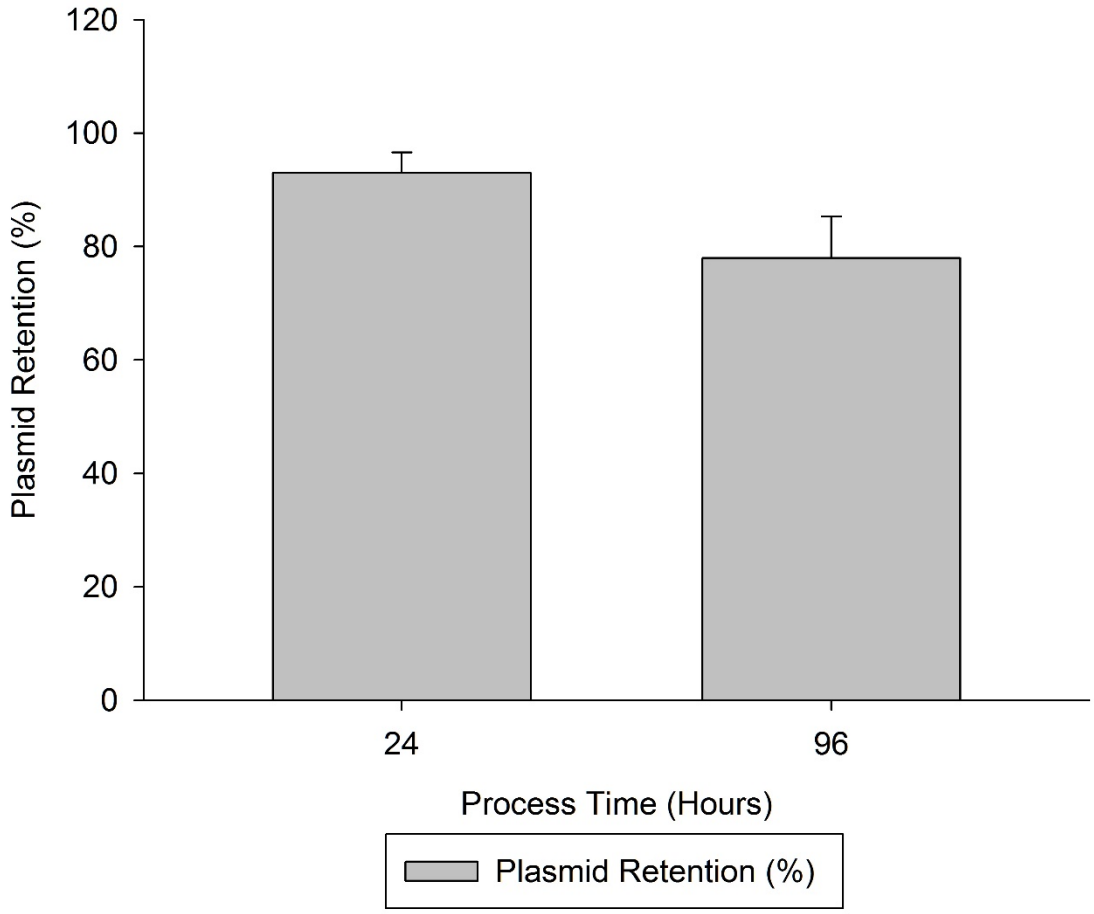

Figure 3-11. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 35 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.2.1.5 *E. coli Fed Batch Fermentation – Induction at 40 °C*

In this experiment thermal induction occurred at 40 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 40 °C was chosen as an induction temperature because it is above 37 °C, the promoter should theoretically be fully unrepressed. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

In Figure 3-12 it can be seen that 12 hours after inoculation, the cells reached a biomass of 2.13 g/L with a corresponding acetate measurement of 0.12 g/L. The dissolved oxygen reached its present level of 30 % at approximately 18.5 hours after inoculation. At this time point, the biomass measured 2.85 g/L with a corresponding acetate measurement of 0.14 g/L. At approximately 21 hours, the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 24 hours after inoculation, the dissolved oxygen measured 41.3 % and the glucose feeding program was initiated. At this time point the biomass measured 2.93 g/L with a corresponding acetate measurement of 0.16 g/L. 9 hours after the start of the glucose feed (a total of 33 hours) the biomass of the culture measured 22.10 g/L with a corresponding acetate concentration of 0.30 g/L. Biomass levels continued to accumulate until 72 hours total process time, at an overall peak measurement of 36.17 g/L with a corresponding acetate measurement of 0.81 g/L. After this point biomass levels began to decrease steadily with a final measurement of 27.63 g/L at 96 hours total process time.

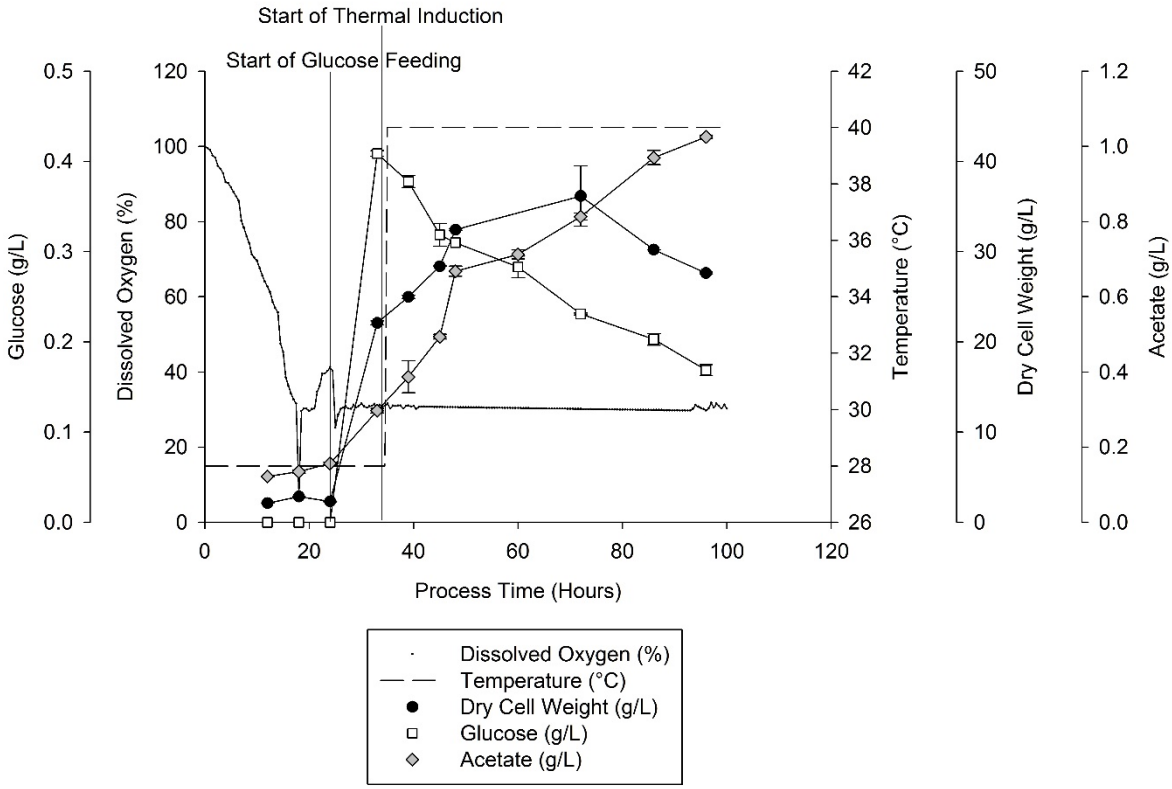

Figure 3-12. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C until Induction when changed to 40 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

During this process, induction occurred when the temperature was increased from 28 °C to 40 °C. As seen in Figure 3-13, the maximum total DAAO activity was reached at 51 hours post induction (a total process time of 84 hours). 6 hours post induction the specific DAAO activity and total DAAO activity measured 446.76 μmol/g DCW/min and 9875.31 μmol/L/min respectively. Pre induction (denoted as 0 hours) the specific DAAO activity and total DAAO activity measured 20.06 μmol/L/min and 43.42 μmol/L/min respectively. The specific DAAO activity peaked at 63 hours post induction with an observed activity of 539.2 μmol/g DCW/min. The total activity continued to increase throughout the induction phase.

Unlike the previous experiments, there was very low levels of enzyme activity in the extracellular media after approximately 12 hours of induction at 40 °C. At approximately 51 hours post induction, the extracellular activity was proportional to the activity of the enzyme derived intracellularly. This can be seen in Figures 3-14 [A] and [B].

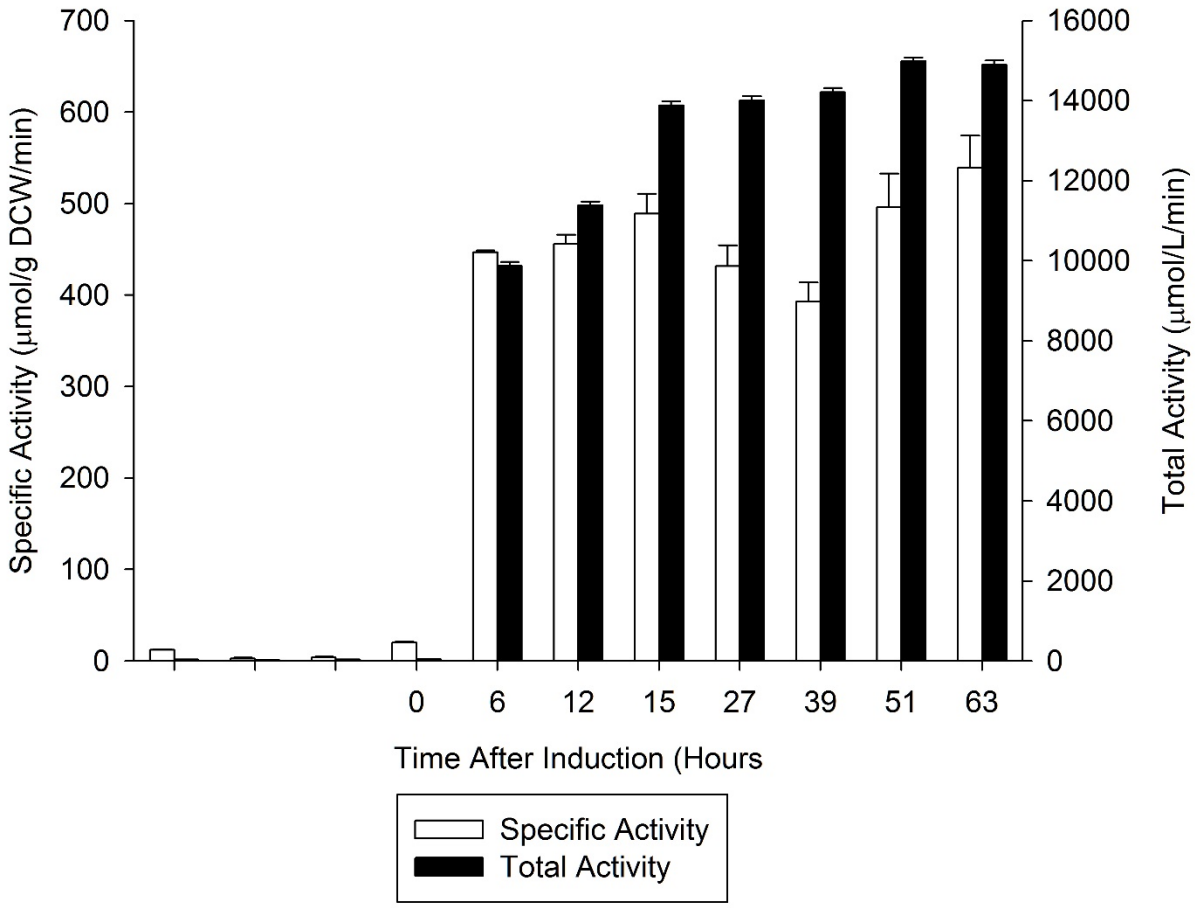

Figure 3-13. Time Profiles of the Specific and Total Activities of the(Intracellular) DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 35 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

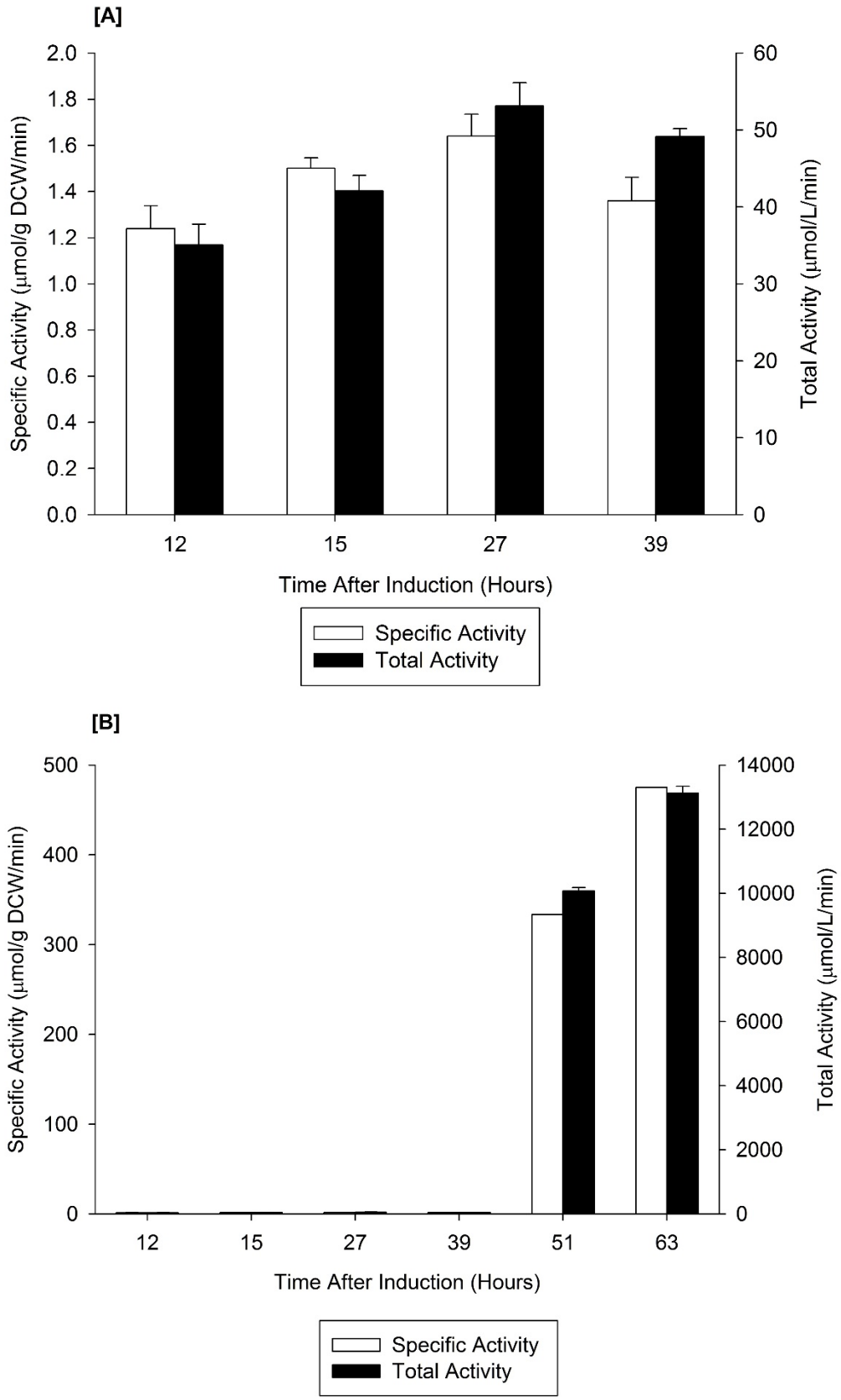

Figure 3-14. Time Profiles of the Specific and Total Activities of the DAAO Enzyme (Extracellular) produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 40 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population. Top graph is a zoomed in version of the bottom graph to provide more clarity as the values are to small for the scale.

As can be seen in Figure 3-15, the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, with a measured retention of 96 %. At the end of the process, at 92 hours, the plasmid retention measured 54 % indicating the culture had extreme difficulty in maintaining the plasmid.

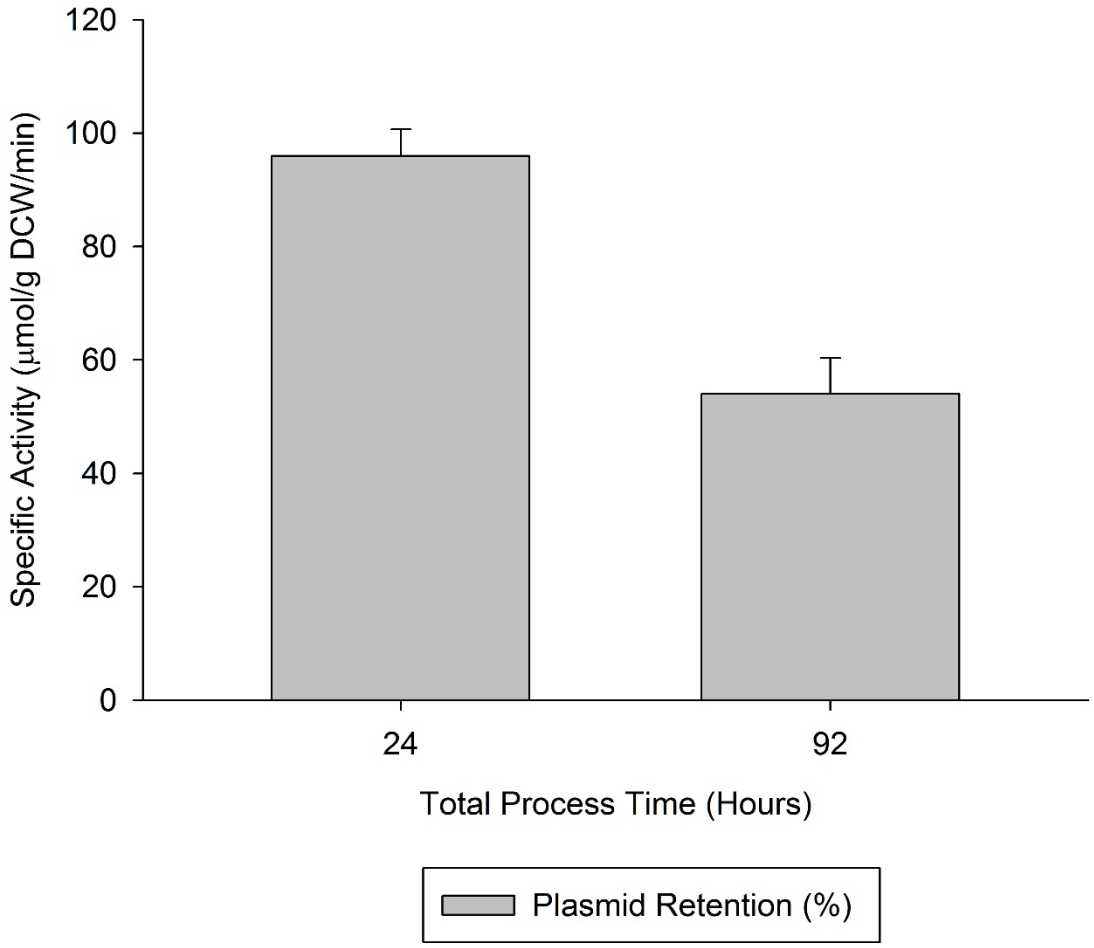

Figure 3-15. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 35 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.2.1.6 *E. coli Fed Batch Fermentation – Induction at 40 °C (Pre Induction 30 °C).*

In this experiment thermal induction occurred at 40 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 40 °C was chosen as an induction temperature because it is above 37 °C, the promoter should theoretically be fully unrepressed. However, the pre induction temperature was set to 30 °C in order to ascertain how premature expression of the DAAO enzyme would affect the process overall. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

During this process, the pre induction temperature was set at 30 °C, and induction commenced when the temperature was raised from 30 °C to 40 °C. In Figure 3-16 it can be seen that 6 hours after inoculation, the cells reached a biomass of 6.63 g/L with a corresponding acetate measurement of 0.32 g/L. At this same time point the dissolved oxygen levels reached the pre-set level of 30 %. 13 hours after inoculation, the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 15 hours after inoculation, the dissolved oxygen levels measured 41.25 % and the glucose feeding program was initiated. 3.5 hours after the start of the feeding (a total time of 18.5 hours) the biomass measured 23.27 g/L with a corresponding acetate measurement of 0.88 g/L. Biomass levels peaked at 33.95 g/L at 30.5 hours total process time with a corresponding acetate measurement 0f 1.40 g/L. From this point onwards, biomass accumulation decreased greatly, with a final measurement of 20.04 g/L at 54.5 hours total process time. The corresponding acetate concentration was 2.84 g/L.

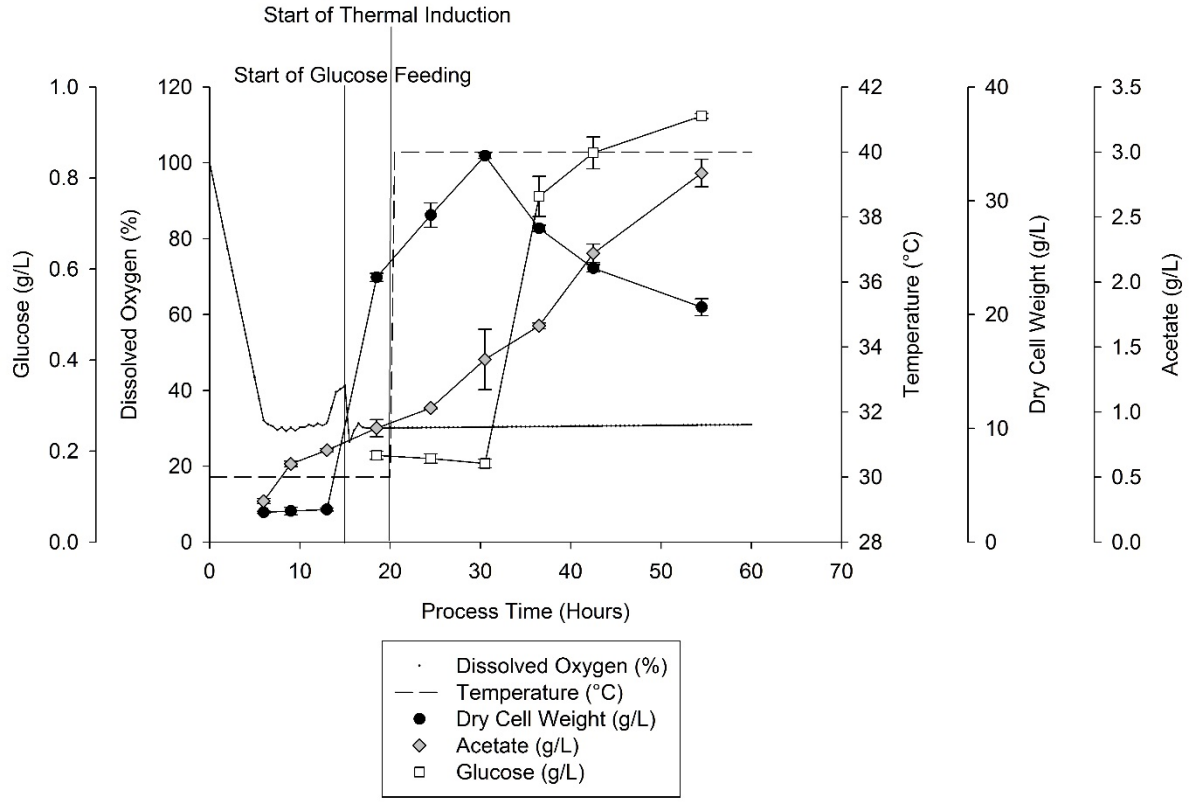

Figure 3-16. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 30 °C until Induction when changed to 40 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

During this process, induction occurred when the temperature was increased from 30 °C to 40 °C. As seen in Figure 3-17, the maximum total DAAO activity was reached at 12 hours post induction (a total process time of 30 hours). From this time point onwards, the total DAAO activities decreased dramatically, with a final specific DAAO activity and final total DAAO activity of 52.40 μmol/g DCW/min and 1081.53 μmol/L/min respectively at 36 hours post induction (a total process time of 54.5 hours).

Like the previous experiment where induction occurs at 40 °C there was detectable enzyme activity in the extracellular media after approximately 6 hours of induction at 40 °C. This can be seen in Figure 3-18.

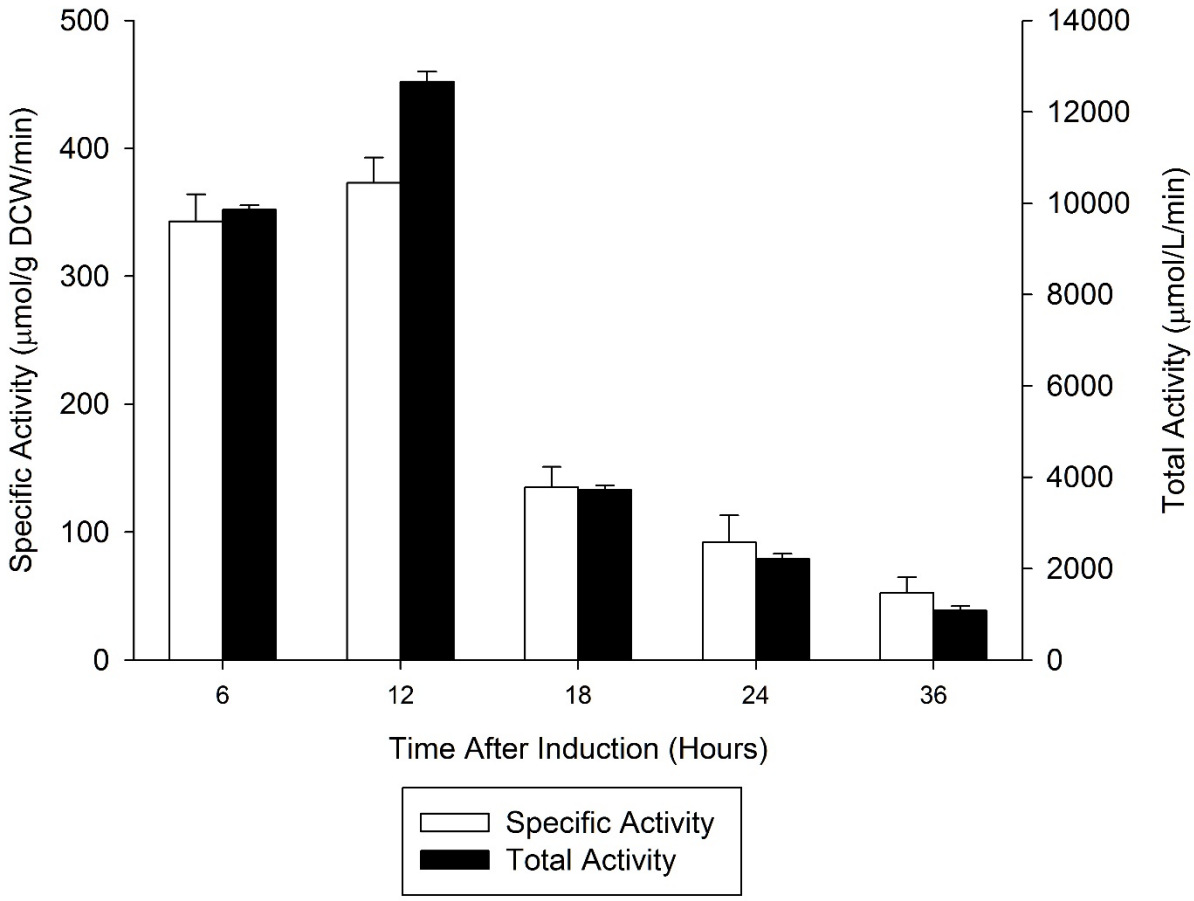

Figure 3-17. Time Profiles of the Specific and Total Activities of the (Intracellular) DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 40 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

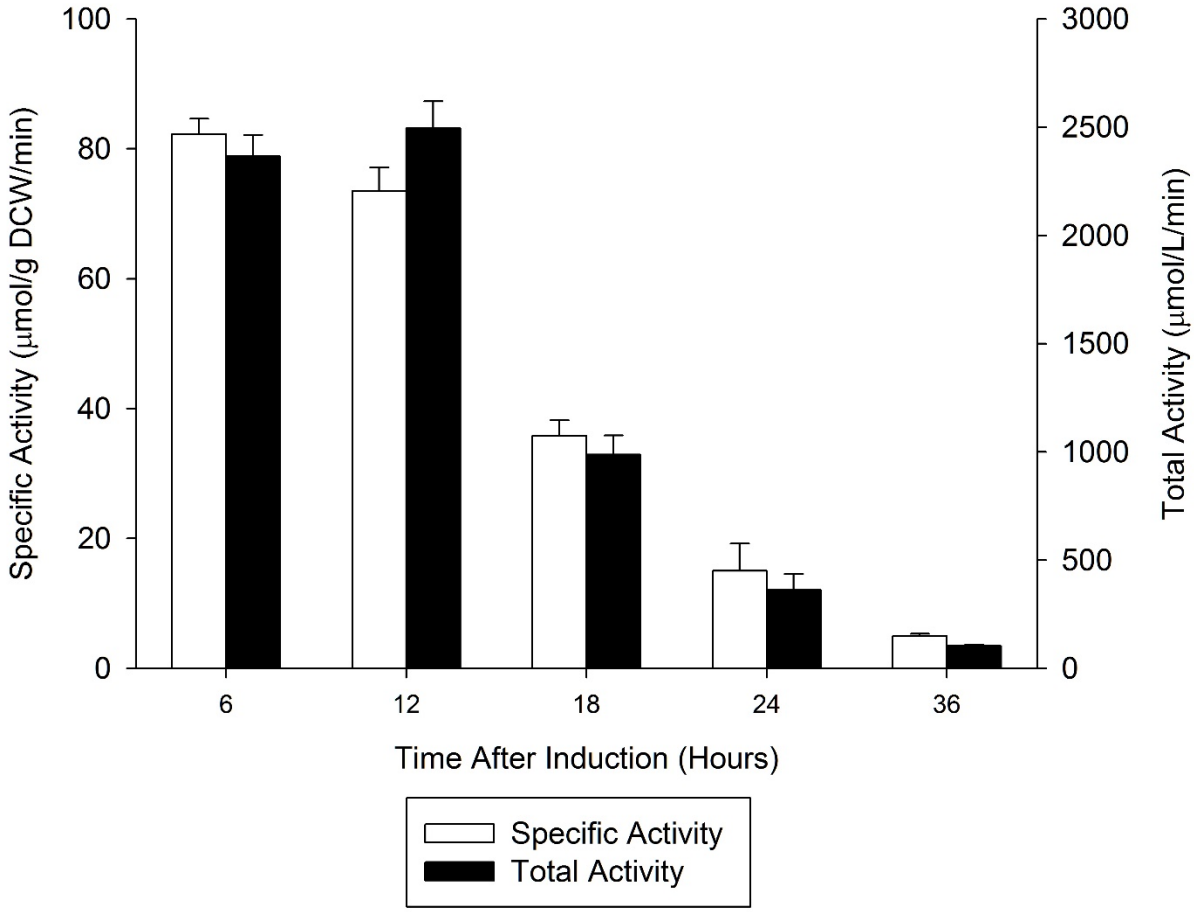

Figure 3-18. Time Profiles of the Specific and Total Activities of the (Extracellular) DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 40 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

As can be seen in Figure 3-19, the plasmid retention of the culture was also measured during the experiment. At 24 hours plasmid retention measured 90 °C. At the end of the process at 54.5 hours, the plasmid retention measured 39 % indicating that the culture had extreme difficulty in maintaining the plasmid.

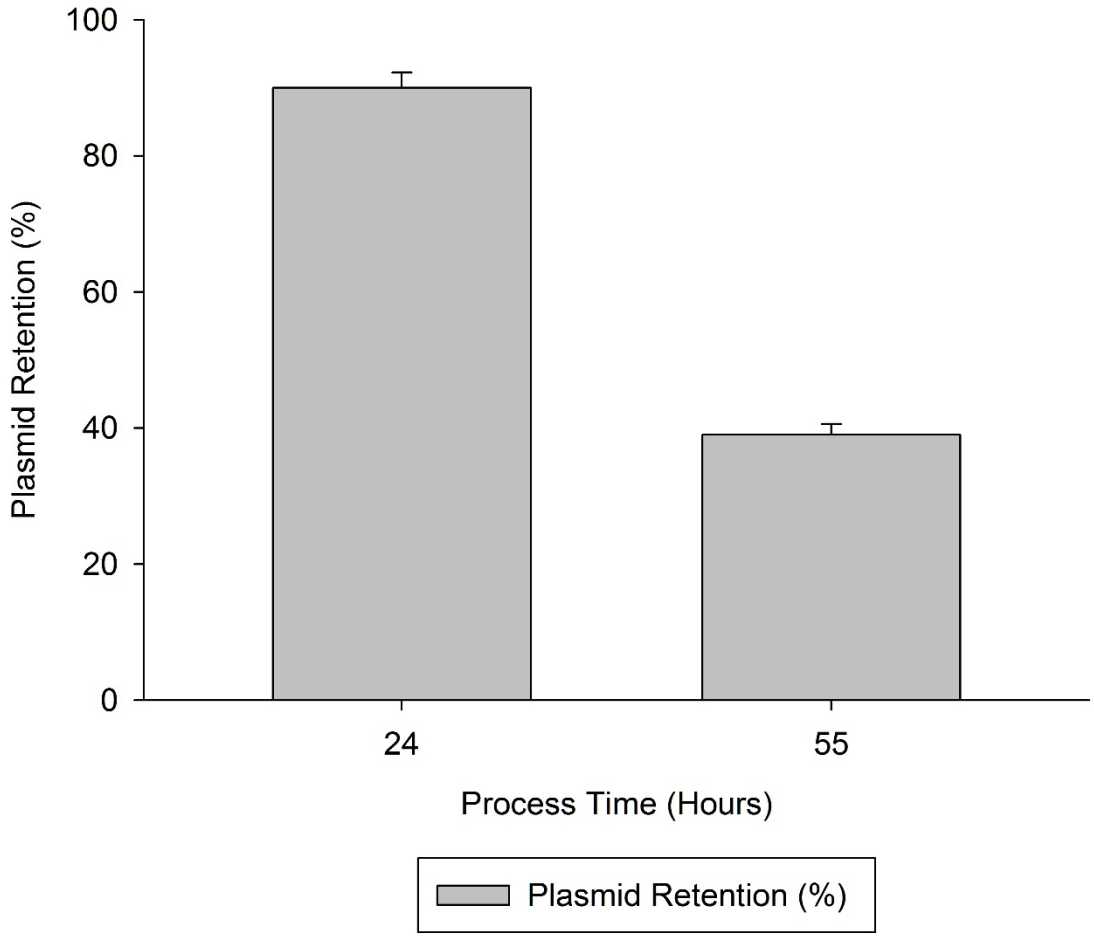

Figure 3-19*. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 30 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 40 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.*

3.3 Results – Defined Media for Growth of *E. coli*

3.3.1 *Overview*

With a defined medium, each constituent is known, as is the concentration of each. Traditionally, a defined medium is often simple, and contains a carbon/energy source as well as a source of nitrogen, and an inorganic salts solution. The most crucial quality of a defined medium over a complex medium, is that it is advantageous for research and laboratory scale experimentation where precision and reproducibility are fundamental to the work. However, defined media can be quite expensive when it is necessary to provide vitamins and other growth factors to the process. In these forthcoming experiments, a defined medium was used as a batch medium, this was followed by a glucose feed substituted with various defined media. The defined media chosen was deemed best based on shake flask work carried out previously with several defined media. Experiments were performed at temperatures ranging between 28 °C and 40 °C in order to induce thermal induction of the DAAO enzyme. Enzyme activities were measured, and the physiology of the host cells was monitored in order to ascertain the effects of media and temperature on recombinant protein production.

3.3.1.1 *E. coli Fed Batch Fermentation – Constant Temperature of 28 °C*

Based on the Biostat Q work carried out previously, 28 °C was chosen as the temperature at which the processes would run before the onset of thermal induction as there was minimal promoter activation and subsequent enzyme production, which is essential for this process as DAAO has the potential to be toxic to growing cells.

In this experiment there was no induction and both the batch and fed-batch stages of the process were performed at a constant temperature of 28 °C as a baseline for future processes.

In Figure 3-20 it can be seen that 12 hours after inoculation, the cells reached a biomass of 3.85 g/L. The dissolved oxygen reached its pre-set level of 30 % at approximately 18 hours after inoculation. At this time point the biomass measured 6.72 g/L with a corresponding acetate concentration of 0.03 g/L. 24 hours after inoculation, the biomass measured 9.98 g/L with a corresponding acetate concentration of 0.04 g/L. At approximately 29 hours, the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells and the glucose feeding program was initiated. 4 hours after the start of glucose feeding, the biomass measured 11.47 g/L with a corresponding acetate measurement of 0.1 g/L. The biomass levels continued to increase throughout the remainder of the experiment reaching a final biomass measurement of 49.32 g/L at 85 hours total process time.

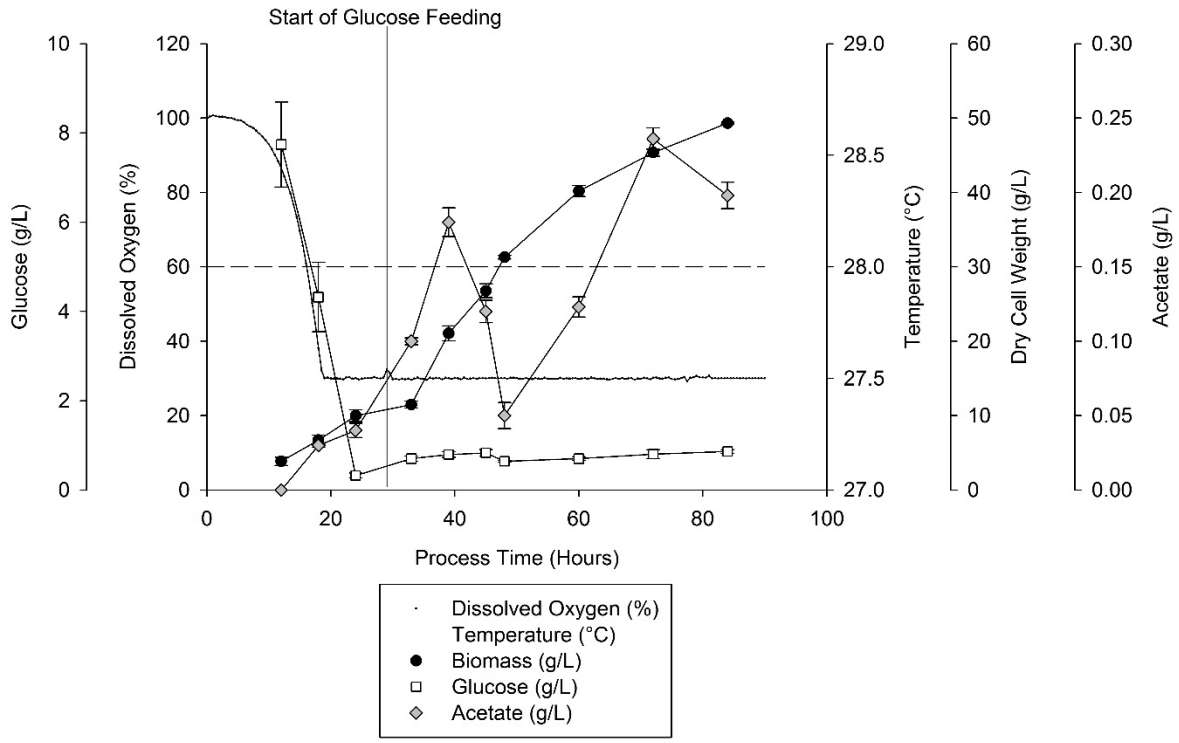

Figure 3-20. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in Defined media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

Although the temperature of this experiment remained constant at 28 °C, the DAAO enzyme assay was performed in order to establish its expression during the feeding but before thermal induction. As seen in Figure 3-21 the maximum total DAAO activity was reached at 48 hours total process time. The total activities throughout the process fluctuated between 18.63 μmol/L/min and 48.95 μmol/L/min. The highest specific DAAO activity was found at 12 hours total process time with a measurement of 7.68 μmol/g DCW/min. The specific DAAO activity continued to decline for the remainder of the experiment with a final measurement of 0.38 μmol/g DCW/min at 84 hours total process time.

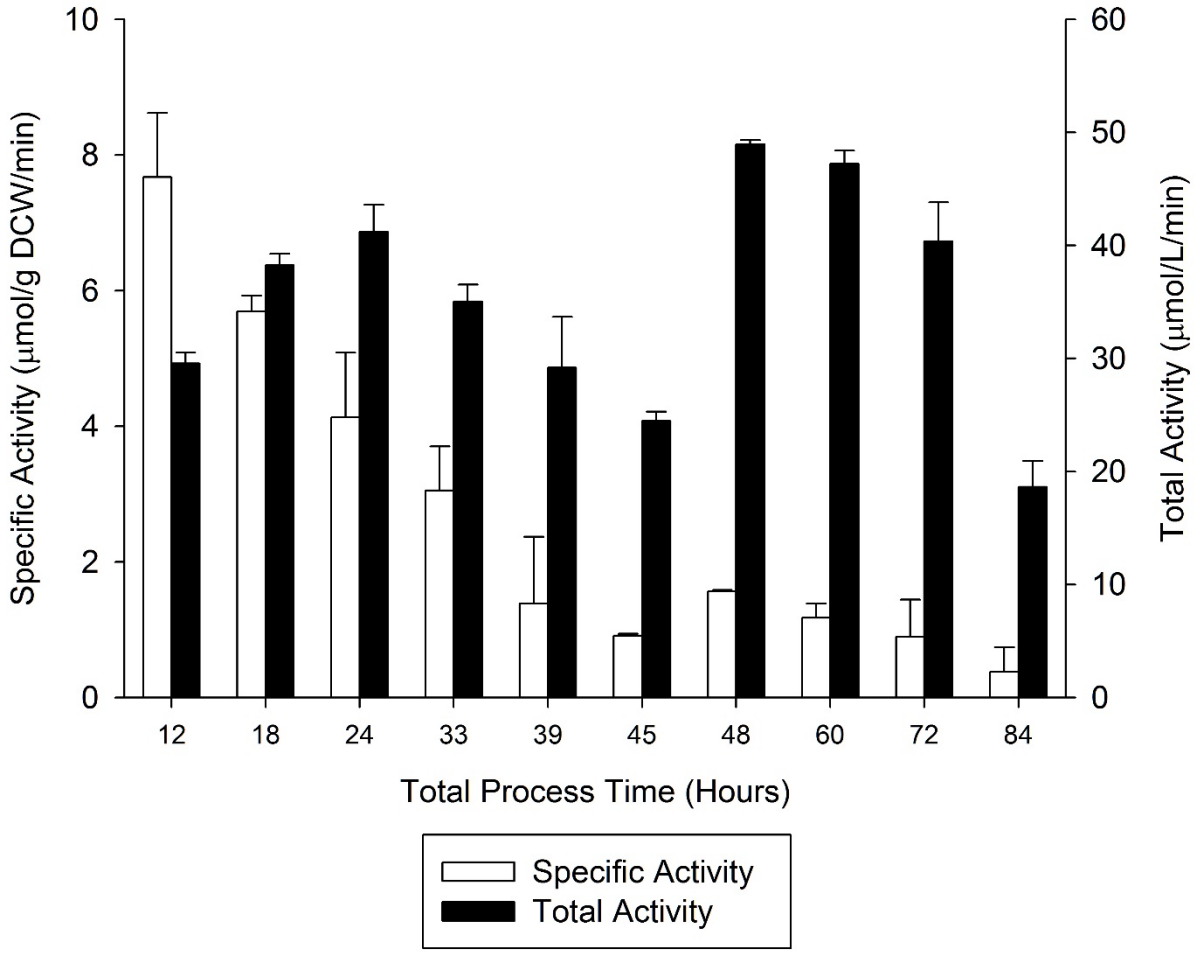

Figure 3-21. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in Defined Media media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

As can be seen in Figure 3-22, the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, with a measured retention of 95 %. At the end of the process at 96 hours, plasmid retention measured 91 % indicating the culture had efficiently maintained the plasmid for DAAO expression.

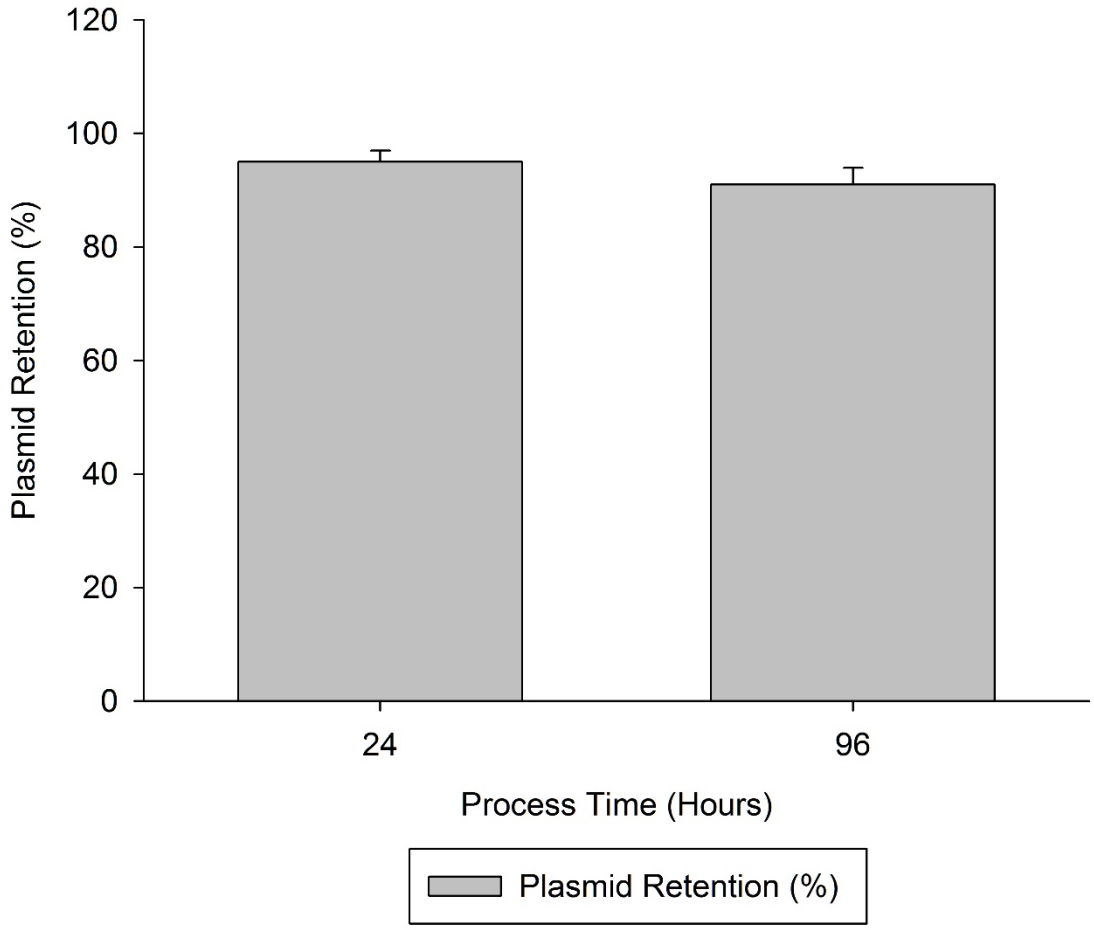

Figure 3-22. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.3.1.2 *E. coli Fed Batch Fermentation – Induction at 30 °C*

In this experiment thermal induction occurred at 30 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 30 °C was chosen as an induction temperature because evidence from the Biostat Q showed a higher incidence on enzymatic activity compared with 28 °C. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

In Figure 3-23 it can be seen that 12 hours after inoculation, the cells reached a biomass of 3.75 g/L. The dissolved oxygen reached its pre-set level of 30 % at approximately 18 hours after inoculation. At this time point, the biomass measured 9.84 g/L with a corresponding acetate measurement of 0.04 g/L. At approximately 32 hours after inoculation, the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 6 hours after the start of the glucose feeding the biomass measured 16.23 g/L with a corresponding acetate measurement of 0.02 g/L. At 40 hours thermal induction began when the temperature was increased from 28 °C to 30 °C. 5 hours after induction the biomass measured 21.10 g/L with a corresponding acetate measurement of 0.004 g/L. The biomass levels continued to increase throughout the remainder of the experiment, with a final biomass measurement of 50.00 g/L at 84 hours total process time.

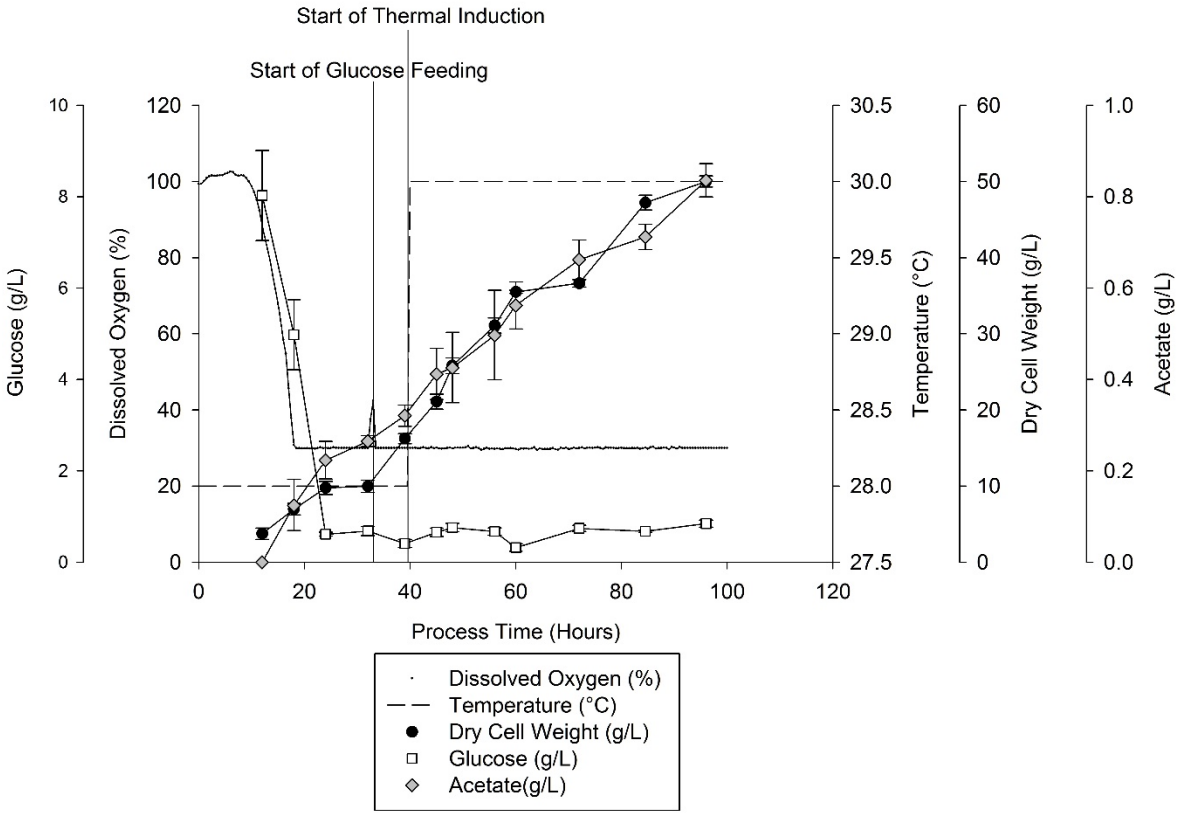

Figure 3-23. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C until Induction when changed to 30 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population

During this process, induction occurred when the temperature was increased from 28 °C to 30 °C. As seen in Figure 2-24 the maximum total DAAO activity was reached at 59 hours post induction (a total process time of 96 hours).The specific DAAO activity peaked at 8 hours post induction. The pre induction (denoted by 0 hours) specific DAAO activity and total DAAO activity measured 2.11 μmol/g DCW/min and 34.35 μmol/L/min respectively.

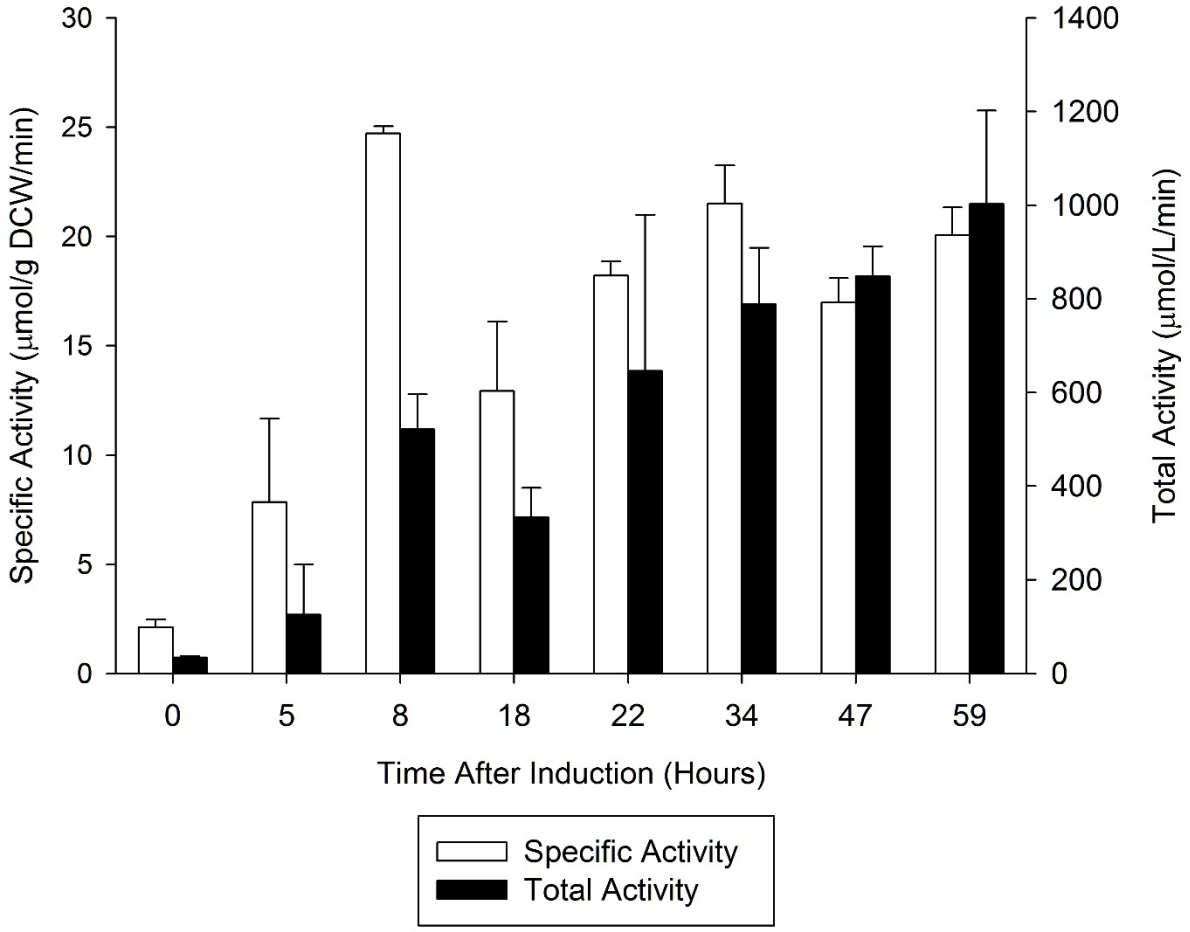

Figure 3-24. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 30 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

As can be seen in Figure 3-25 the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, with a measured retention of 94 %. At the end of the process at 96 hours, plasmid retention measured 87 % indicating the culture had efficiently maintained the plasmid for DAAO expression.

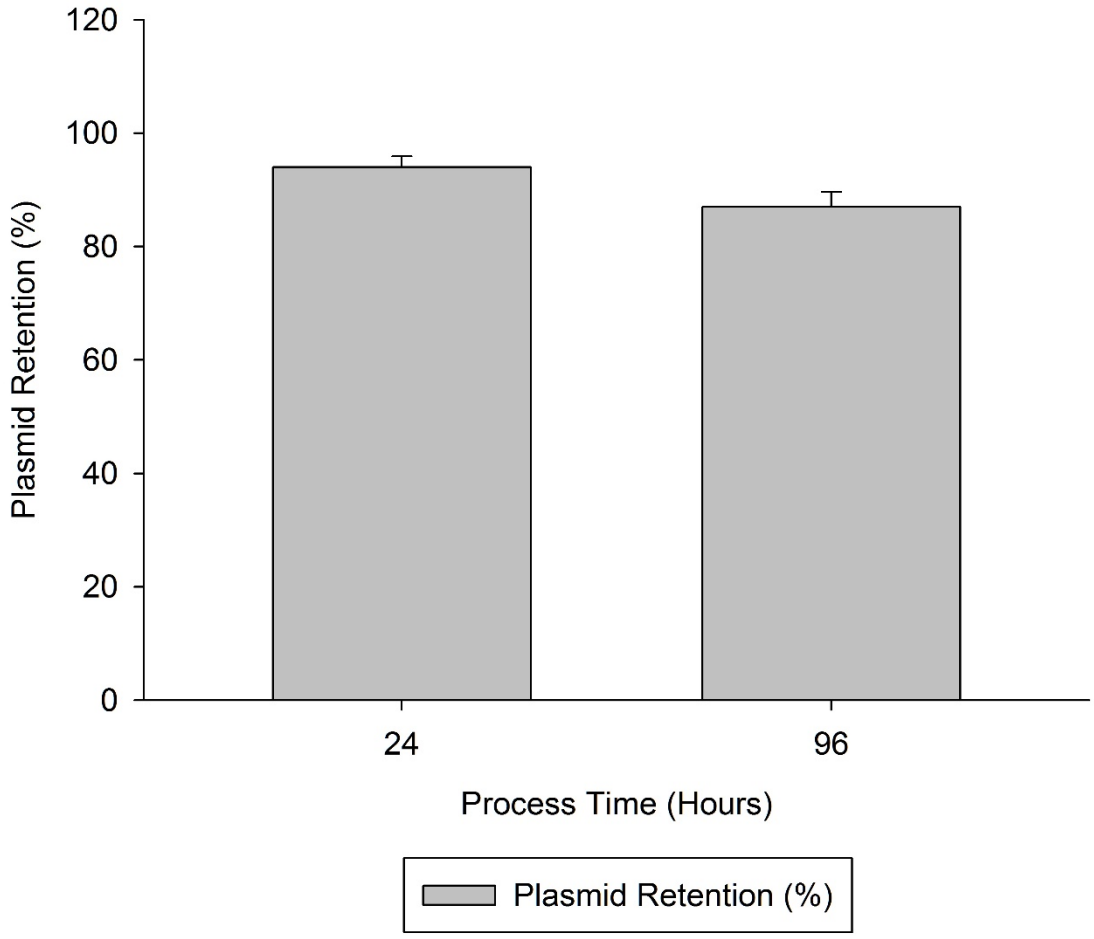

Figure 3-25. Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 30 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.3.1.3 *E. coli Fed Batch Fermentation – Induction at 35 °C.*

In this experiment thermal induction occurred at 35 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 35 °C was chosen as an induction temperature because evidence from shake flasks showed a higher incidence on enzymatic activity compared with 28 °C and 30 °C. 35 °C is also approaching 37 °C, the temperature where the promoter should theoretically have been unrepressed. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

It can be seen in Figure 26 that 12 hours after inoculation, the cells reached a biomass of 3.85 g/L. The dissolved oxygen reached its pre-set level of 30 % at approximately 23 hours after inoculation. At this time point, the biomass measured 9.84 g/L with a corresponding acetate measurement of 0.04 g/L. At approximately 31 hours after inoculation the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. 8 hours after the start of glucose feeding, the biomass measured 12.27 g/L with a corresponding acetate measurement of 0.02 g/L. At 40 hours, thermal induction began when the temperature was increased from 28 °C to 30 °C. 3 hours after induction the biomass measured 18.35 g/L with a corresponding acetate measurement of 0.004 g/L. The biomass levels continued to increase throughout the remainder of the experiment, with a final biomass measurement of 44.38 g/L at 84 hours total process time.

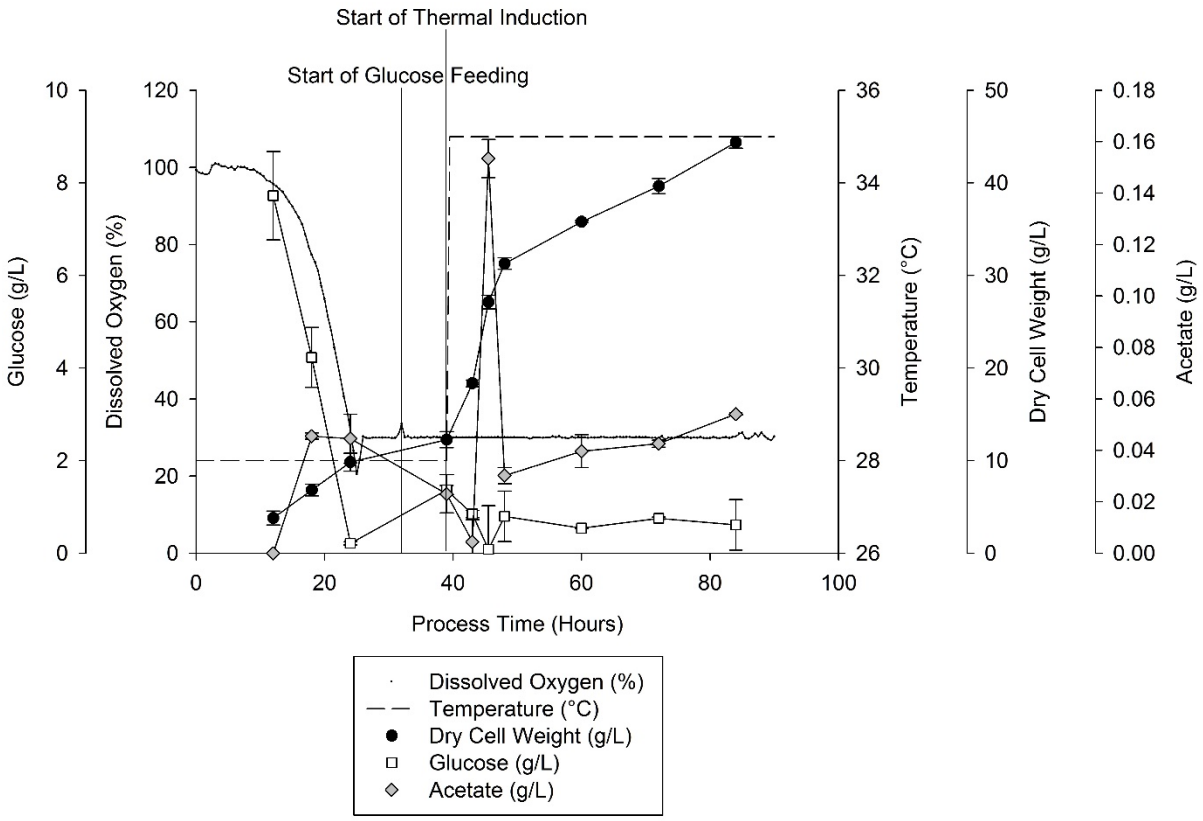

Figure 3-26. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C until Induction when changed to 35 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

During this process, induction occurred when the temperature was raised from 28 °C to 35 °C. As seen in Figure 3-27 the maximum total DAAO activity was reached at 21 hours post induction (a total time of 60 hours). 3 hours post induction the specific activity was 100 μmol/g DCW/min. 6 hours post induction the specific DAAO activity and the total DAAO activity measured 68.20 μmol/g DCW/min and 1143.53 μmol/L/min respectively. Pre induction (denoted as 0 hours) the specific DAAO activity and total DAAO activity measured 17.43 μmol/g DCW/min and 18.38 μmol/L/min respectively. The specific DAAO activity icreased to 56.05 μmol/g DCW/min at 45 hours post induction. The total activity measured 1604.84 μmol/L/min.

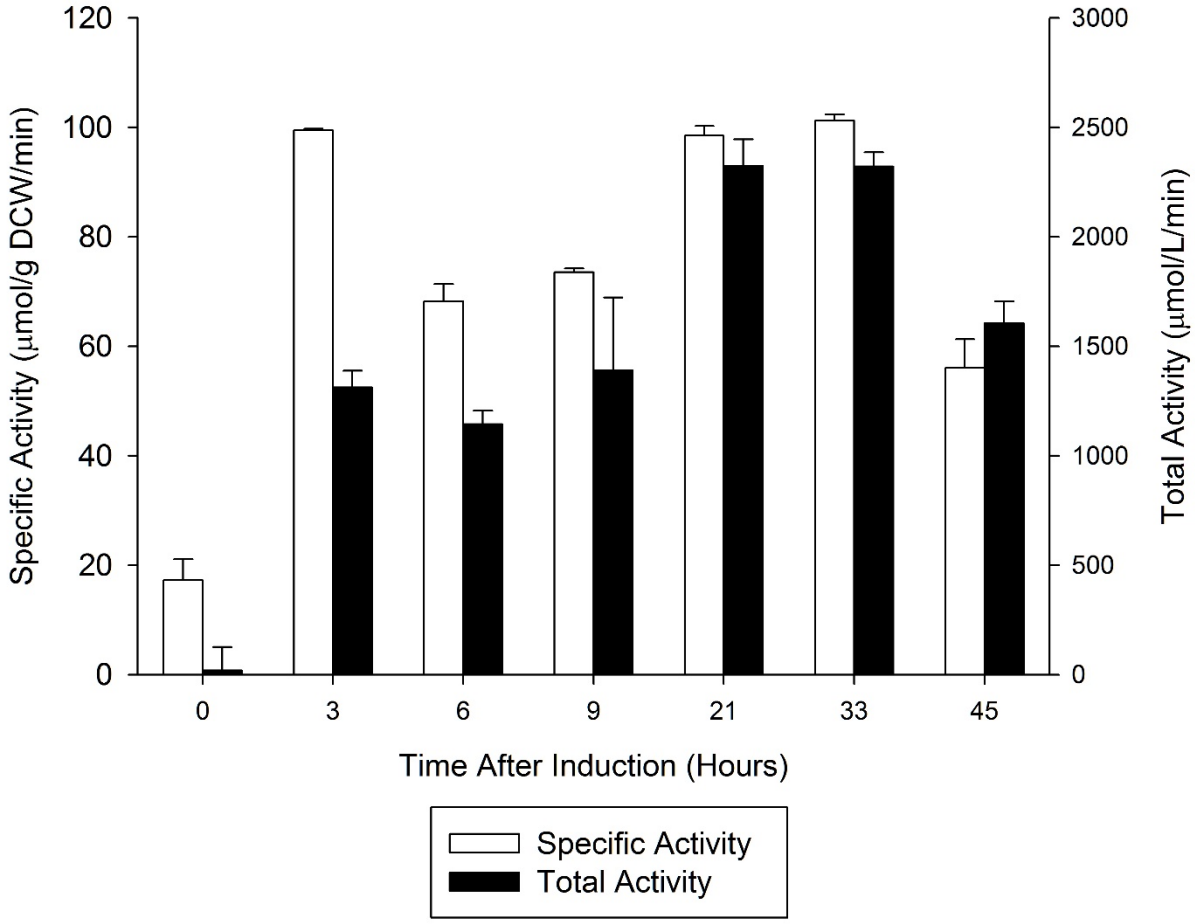

Figure 3-27. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 30 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

As can be seen in Figure 3-28 the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, with a measured retention of 96 %. At the end of the process at 96 hours, plasmid retention measured 72 % indicating the culture had efficiently maintained the plasmid for DAAO expression.

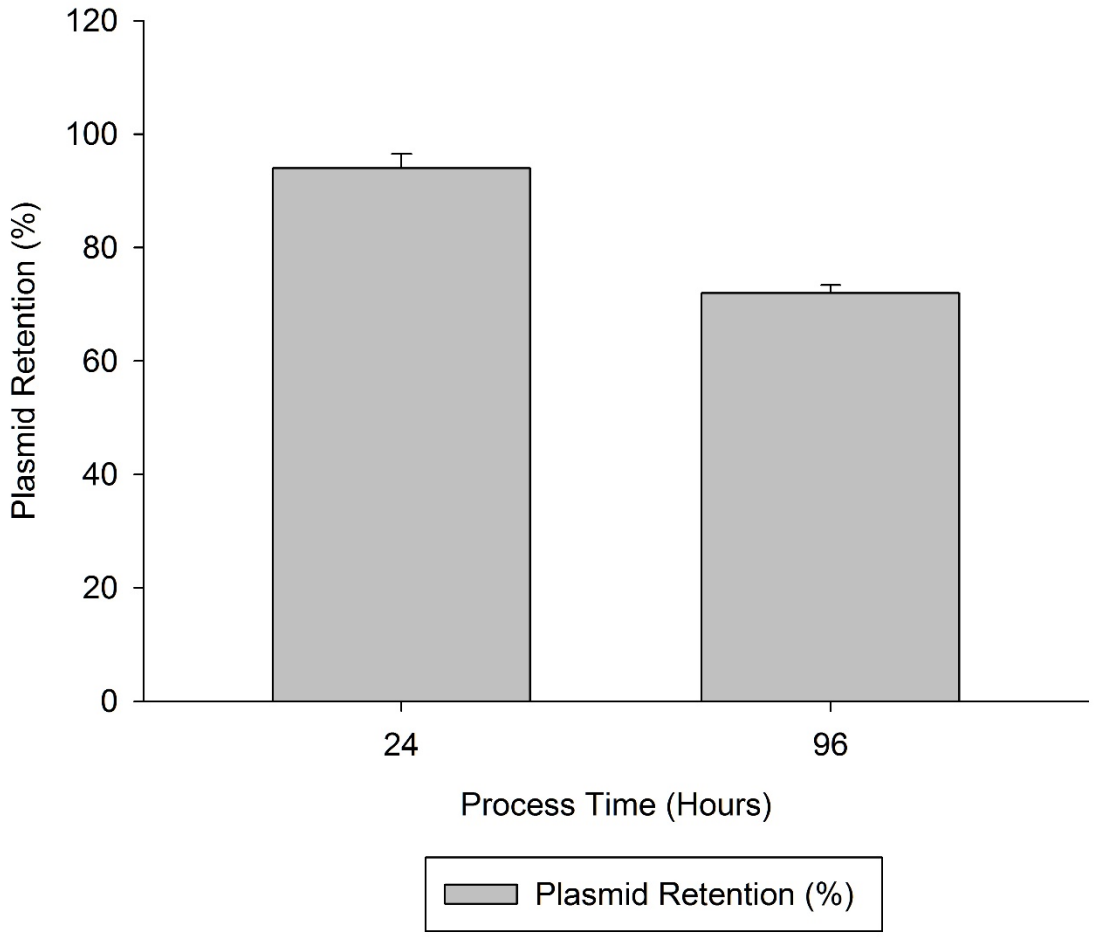

Figure 3-28. *Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 35 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.*

3.3.1.4 *E. coli Fed Batch Fermentation – Induction at 40 °C*

In this experiment thermal induction occurred at 40 °C when the OD (Abs_{600nm}) = ~ 40 and it remained at this temperature until the end of the process. 40 °C was chosen as an induction temperature because it is above 37 °C, the promoter should theoretically be fully unrepressed. However, the pre induction temperature was set to 30 °C in order to ascertain how premature expression of the DAAO enzyme would affect the process overall. It is essential to minimise expression of the DAAO enzyme because of its potential toxicity to growing host cells.

In Figure 3-29 it can be seen that 12 hours after inoculation, the cells reached a biomass of 3.95 g/L. The dissolved oxygen reached its pre-set level of 30 % at approximately 20 hours after inoculation. At 25.5 hours after inoculation, the biomass measured 9.76 g/L with a corresponding acetate measurement of 0.3 g/L. At approximately 28 hours after inoculation, the dissolved oxygen levels began to increase, indicating a decrease in the respiratory activity of the cells. The glucose feeding program was then initiated. 9 hours after the start of the glucose feeding the biomass measured 15.75 g/L with a corresponding acetate measurement of 0.45 g/L. At 39 hours thermal induction began when the temperature was increased from 28 °C to 40 °C. 6 hours after induction the biomass measured 20.09 g/L with a corresponding acetate measurement of 1.00 g/L. The biomass levels continued to increase for the first 21 hours of thermal induction, where the biomass measured 28.4 g/L with a corresponding acetate measurement of 1.90 g/L. Biomass continued to decrease after a total process time of 60 hours, with a final biomass measurement of 20.23 g/L at a process time of 96 hours.

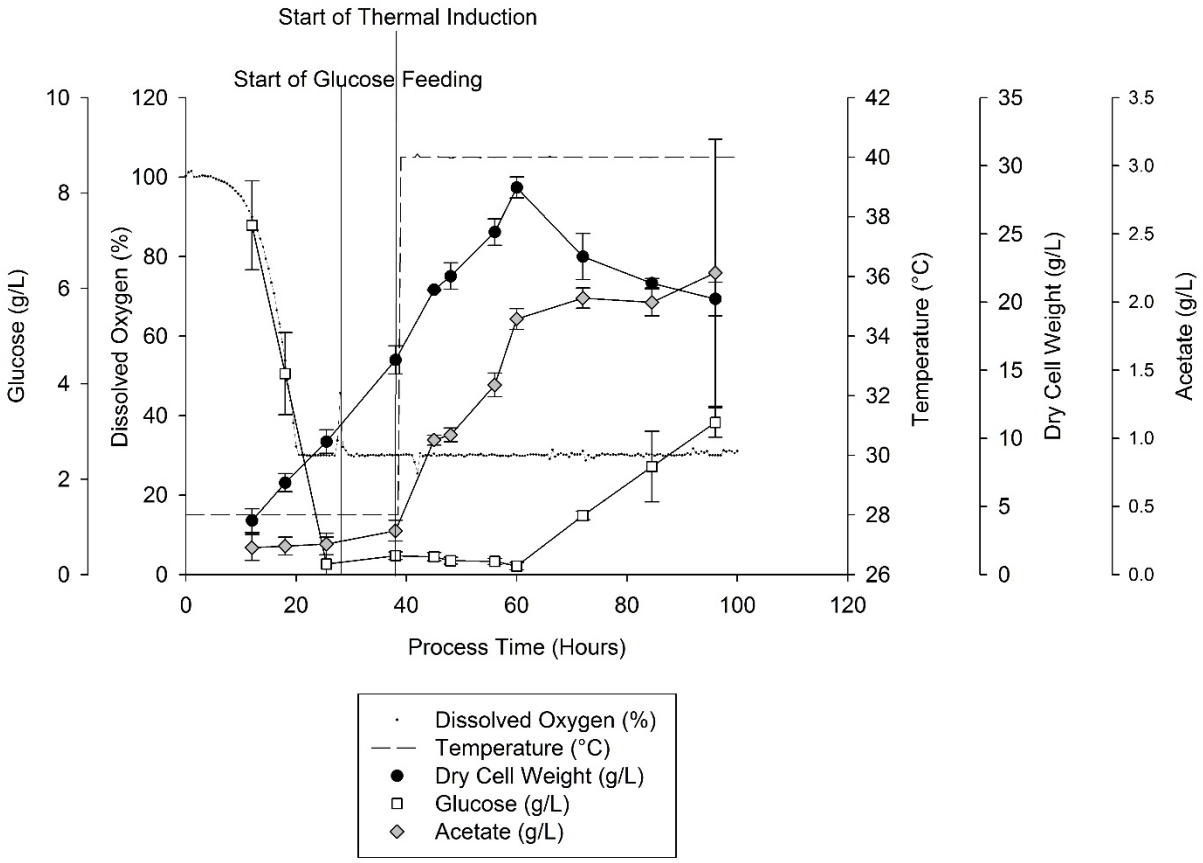

Figure 3-29. Time profiles of the Dissolved Oxygen, Temperature, Dry Cell Weight, Glucose and Acetate concentrations using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C until Induction when changed to 40 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

During this process, induction occurred when the temperature was increased from 28 °C to 40 °C. As seen in Figure 3-30, the maximum total DAAO activity was reached at 33 hours post induction (a total process time of 72 hours). 6 hours post induction the specific DAAO activity and total DAAO activity measured 58.35 μmol/g DCW/min and 1219.6 μmol/L/min respectively. Pre induction (denoted as 0 hours) the specific DAAO activity and total DAAO activity measured 9.85 μmol/L/min and 195.1 μmol/L/min respectively. The specific DAAO activity peaked at 57 hours post induction with an observed activity of 881.19 μmol/g DCW/min. The total activity decreased 33 hours after the start of induction. .

Unlike the previous experiments, there was very low levels of enzyme activity in the extracellular media after approximately 6 hours of induction at 40 °C. At approximately 33 hours post induction, the extracellular activity was being to be comparable to the activity of the enzyme derived intracellularly. This can be seen in Figures 3-31 [A] and [B].

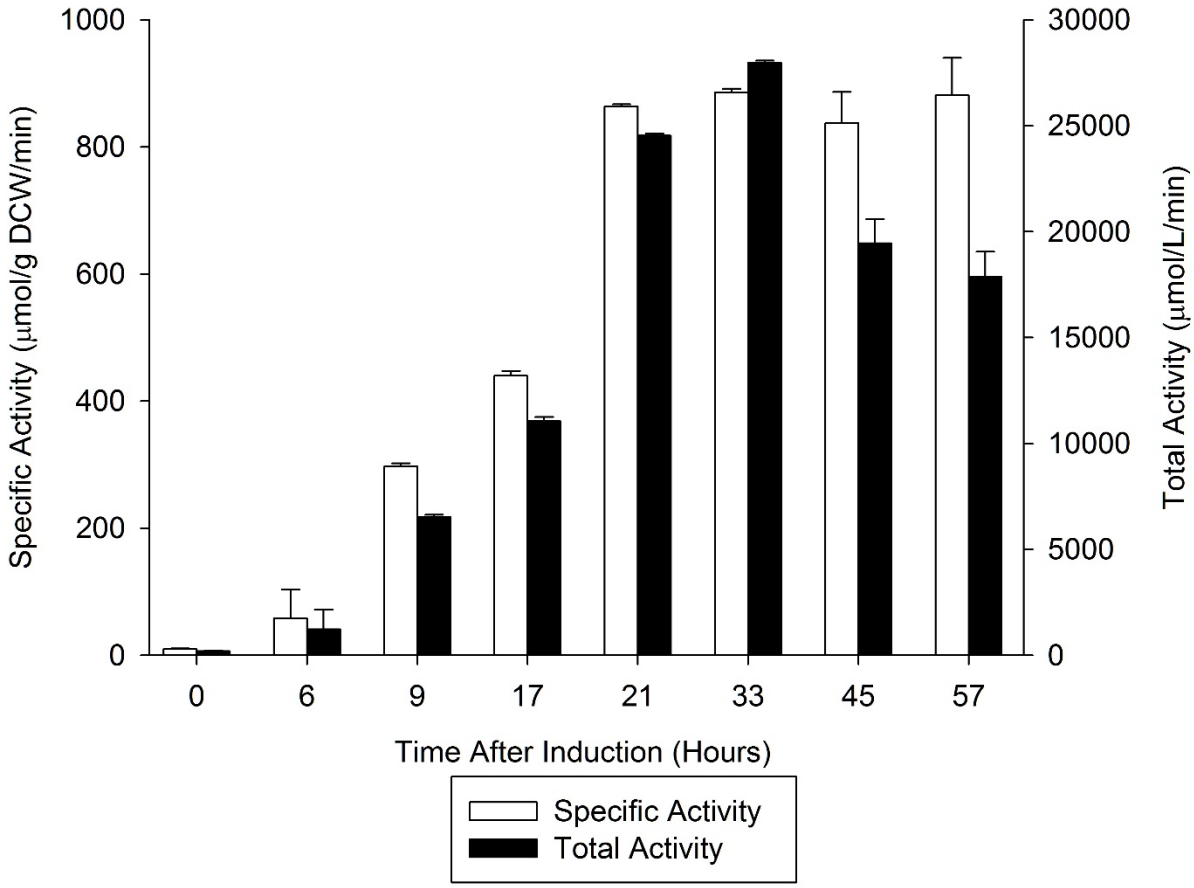

Figure 3-30. Time Profiles of the Specific and Total Activities of the (Intracellular) DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 40 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

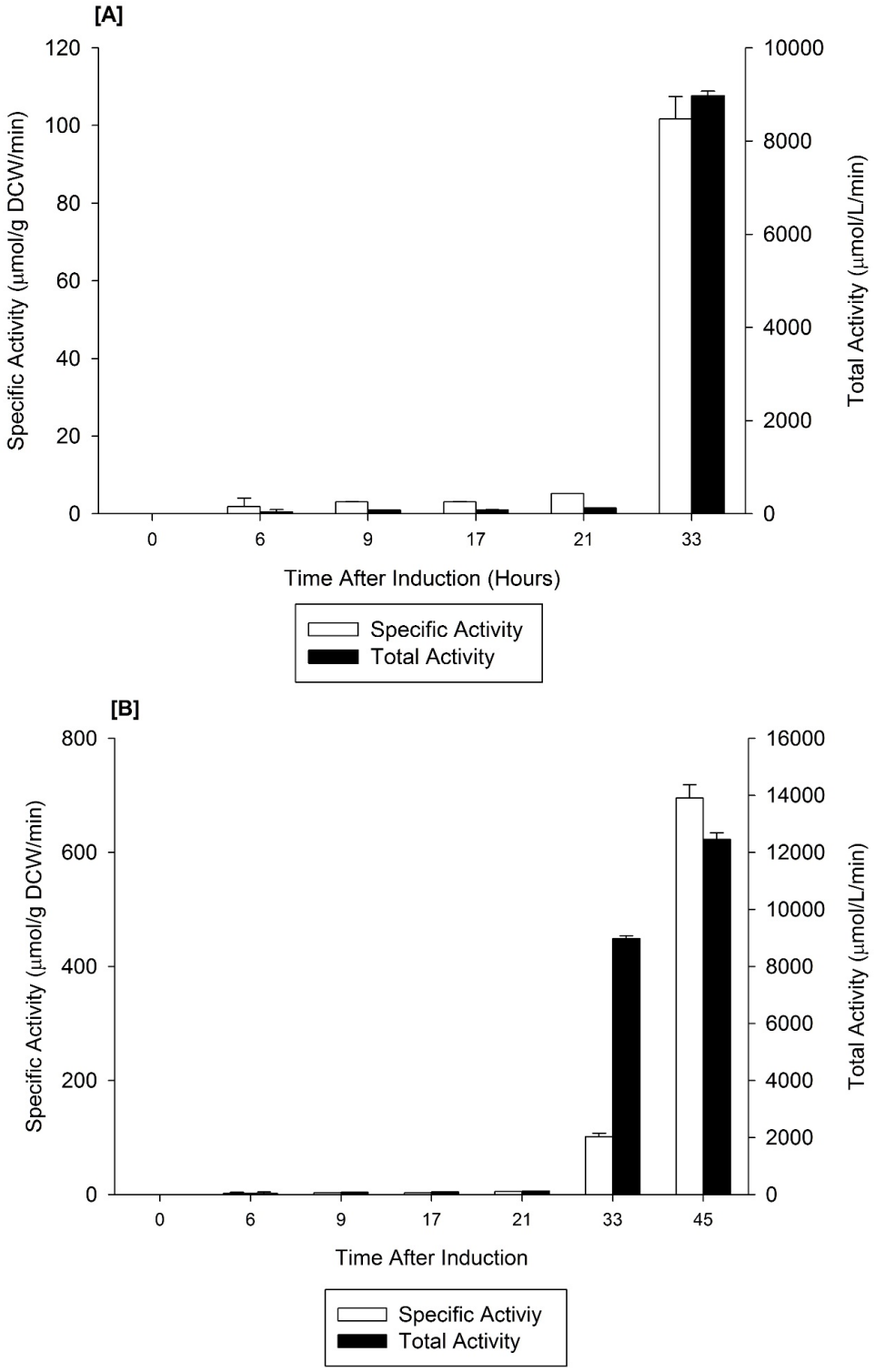

Figure 3-31. Time Profiles of the Specific and Total Activities of the (Extracellular) DAAO Enzyme produced using E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 30 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample populations.

As can be seen in Figure 3-32 the plasmid retention of the culture was also measured during the experiment. At 24 hours it was high, with a measured retention of 92 %. At the end of the process at 96 hours, plasmid retention measured 62 % indicating the culture had efficiently maintained the plasmid for DAAO expression.

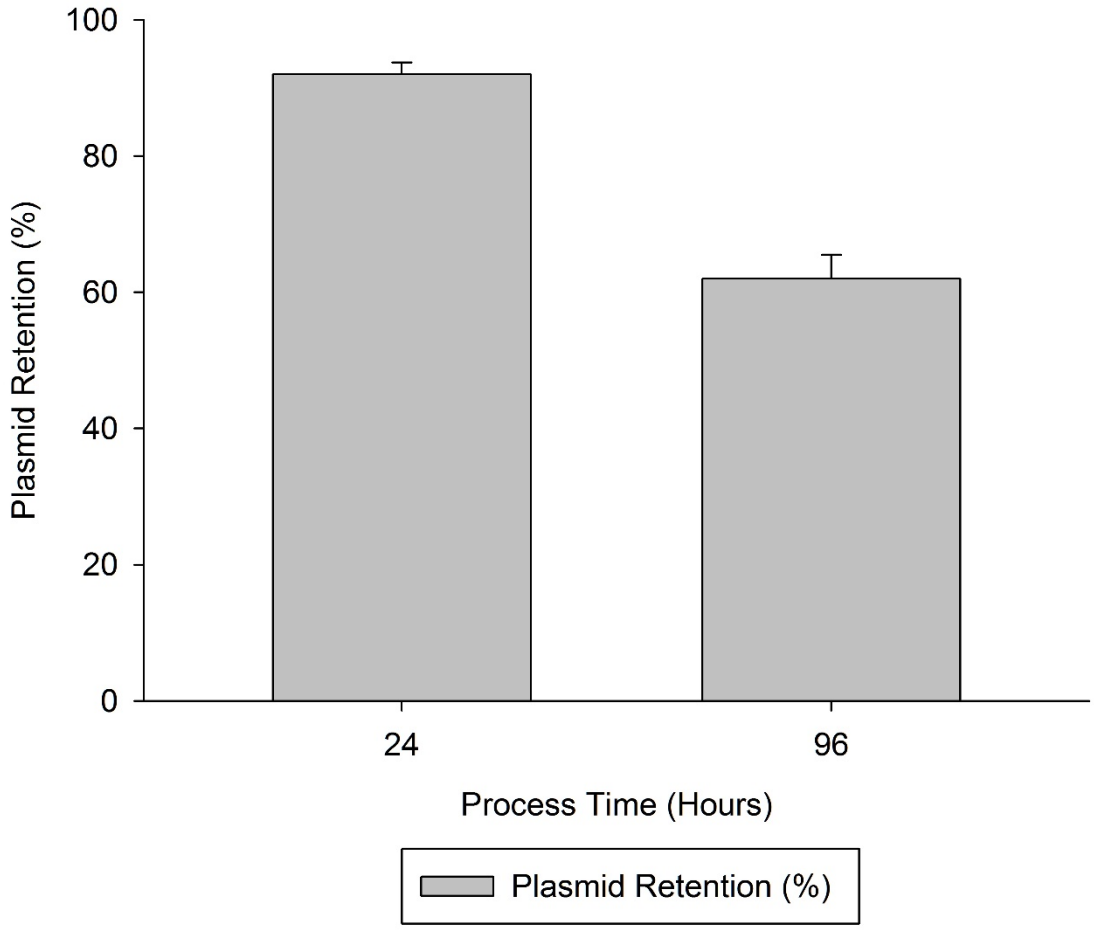

Figure 3-32. . Plasmid Retention of E. coli pRES151DAAOWT BW2157 grown in LB media. Culture Conditions: 28 °C, 300 – 900 rpm, 1 vvm – 3 vvm of compressed air. Induction at 35 °C. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

3.4 *Discussion*

Lysogeny broth (LB) is perhaps one of the most utilised culture mediums for bacterial cultivation in biology. As a general rule, LB is composed of 10 g of tryptone, 10 g of NaCl and 5 grams of yeast extract per litre of H_2O. Although its use is widespread, the chemical composition of LB remains vague, as a consequence of the constituents of some components being virtually impossible to govern. As an example, peptides and amino acids are provided by tryptone, a protein hydrolysate, and Yeast Extracts which contain undefined organic compounds. All things considered, this has the potential to cause batch variations.

However, the microbiology community as a whole still relies heavily upon LB because it is advantageous to the user; mainly the quick cultivation of bacteria and also that is can be bought directly from several different companies as a ready-made powder, easily dissolved in water. Using a defined media, means the scientists can requires additional time and effort meaning additional costs for scale up.

With regards to the LB fermentations, generally it can be seen that at 28 °C, before the start of glucose feeding, all of the cultures produced relatively similar biomass levels, and although there are slight variations, these are not significant enough to suggest batch variability is of concern. The same can be said after the commencement of the glucose feeding, with all cultures producing relatively similar biomass levels (Figure 3-1). Similarly, the biomass levels between the fermentation run at a constant temperature of 28 °C (Figure 3-3) and the fermentation where induction occurred at 30 °C (Figure 3-6) remain virtually identical. Biomass levels using defined media also remained similar at 28 °C (Figure 3-20) and at 30 °C (Figure 3-23).

During the fermentation where induction occurred at a temperature of 35 °C there was a lag in growth between 48 hours and 72 hours (Figure 3-9) which was absent from the earlier fermentations using LB. This similar trend can also be seen in the fermentation where induction occurred at 40 °C (Figure 3-12). Furthermore, there is a distinct decrease in biomass accumulation levels between both the 30 °C fermentation and 35 °C and 40 °C fermentations. Interestingly, the biomass levels drop quite drastically after the 72 hour point in the 40 °C fermentation (Figure 3-12). Using a defined media, biomass levels at 35 °C (Figure 3-26) are comparable to LB

(Figure 3-9). However, using a defined media, at 40 °C (Figure 3-29) biomass levels are much less in comparison to LB at 40 °C (Figure 3-12).

Isopropyl-β-D-thiogactopyranoside (IPTG) is a widely used chemical inducer for recombinant protein production in E. coli, but it is very expensive to use at industrial scale and highly toxic to humans (Aucoin *et al*., 2006). An IPTG alternative is the dissacharide sugar lactose which is capable of activating the same promoters as IPTG whislt also acting as an additional carbon source for the bacterium. The addition of these chemicals to the reactor media requires careful regulation of the feeding rates. Thermal induction presents an alternative to the latter form of induction and is inexpensive and can be precisely controlled externally via the bioreactor control unit. The most commonly used thermal induction promoters are based upon the $P_L$and/or P_R promoters from the lamda phage repressor cI857 protein (Caspeta *et al*., 2013, Valdez-Cruz *et al*., 2011, Vélez *et al*., 2013).

Theoretically, with this system recombinant gene expression is efficiently repressed at 30 °C, which facilitates the acquistion of high cell densities before the induction phase. A subsequent increase in temperature in the range of 39 °C to 42 °C causes a surge in recombinant protein production. Unfortunately cells which are exposed to an upshift in temperature above 37 °C are often prone to reduced growth rates as a consequence of the overexpression of foreign proteins (Caspeta *et al*., 2013, Valdez-Cruz *et al*., 2011).

During the glucose batch phase of the processes the temperature of the reactor was maintained at 28 °C initially during the growth phase to restrict premature expression of the DAAO enzyme which can be toxic to the growing *E. coli* culture: it is generally agreed that the recombinant expression of DAAO in the growing culture has the potential to convert the intracellular store of D-alanine which is a prerequisite of the cell wall, to its constituent components and thus leave the cell unable to perform cross-linkage of the peptidoglycan (Mejia *et al*., 1995) which will result in cell lysis.

Using LB as media, the growth rate of the 40 °C induction process measured 0.004 h^{-1} during induction versus the 0.02 h^{-1} growth rate of the 30 °C induction

process and the 0.01 h-1 growth rate of the 35 °C induction process. Previous studies into analysing temperature effects on growth and promoter induction have proved that increasing the temperature of a culture from 30 °C to 42 °C caused a decrease in growth rate to 0.025 h^{-1} from 0.77 h^{-1} during a fed batch process for recombinant protein production (Aucoin *et al*., 2006).

Using a the defined media, the growth rate of the culture during induction at 40 °C measured 0.005 h^{-1} versus the 0.02 h^{-1} during the 30 °C and 35 °C induction processes.

Comparing the results of the enzymatic activities of the 28 °C process with the 30 °C may indicate that the culture was operating under leaky promoter conditions, using both defined and undefined media. There was a 3.25 fold increase in specific enzymatic activity and a 17.7 fold increase in total enzymatic activity using undefined media at 30 °C (Figure 3-7) over 28 °C (Figure 3-4) after 12 hours of temperature elevation even though at 30 °C gene expression should be highly repressed. Using the defined media there was a 4.34 fold increase in specific enzymatic activity and an 8.7 fold increase in total enzymatic activity after 18 hours of temperature elevation at 30 °C. (Figure 3-24)

It should also be noted that the biomass levels of the 28 °C and 30 °C induction processes were similar and that enzyme activity noted was a result of promoter leakage. Using the temperature sensitive cI857 repressor allows control of gene expression by changing the growth temperature: at 30 °C the cI857 repressor is functional and it turns off expression of the gene, but at 42 °C the repressor is inactivated so expression of the gene ensues. Because the CI857 is known generally to function when temperatures are raised above 30 °C, ie transcription is repressed at 30 °C or below, it can be assumed that the CI857 promoter in the E. coli system used in the current study is not being regulated fully and thus causing transcription of the DAAO enzyme (Jechlinger *et al*., 1999).

Furthermore at 35 °C there was a 9.62 fold increase in specific activity and a 54 fold increase in total activity using undefined media (Figure 3-10). Using defined media there was a 9.62 fold increase in specific activity and a 54 fold increase in total activity (Figure 3-27). (Caspeta et al., 2013) indicate that in a λ P_L/CI857 system expression of a recombinant protein was highly repressed at 30 °C whereas

temperature increases to 36 °C, 38 °C and 40 °C resulted in increased induction levels. Moreover, at 35 °C the CI857 repressor started to denature and detaches from the promoter allowing commencement of gene transcription, explaining why the 35 °C induction process has higher activity than the 30 °C process (Vélez *et al*., 2013).

Statistically the specific activities of the 28 °C process and the 30 °C induction process were significant for undefined media (P value = 0.033) and for defined media (P value = 0.008). The 35 °C and 40 °C induction process specific activities were statistically significant compared to the 28 °C process (P values = 0.009 and 0.005 respectively) using undefined media. Using defined media, the specific activities at the aforementioned temperatures were significant (P values 0.001 and 0.016 respectively).

Statistically the total activities of the 28 °C process and the 30 °C induction process were significantly different (P value = 0.001) using undefined media. The 35 °C and 40 °C induction process total activities were statistically significant also (P values = 0.001 and 0.001respectively. Using defined media, the total activities of the 30 °C, 35 °C and 40 °C processes are also statistically significant (P value = 0.005; P value = 0.003 and P value = 0.008) respectively.

The enzymatic activity of DAAO per gram of DCW using LB at 30 °C was higher than at 28 °C. At 35 ° and 40 °C these activities were higher still (Figures 3-4, 3-7, 3-10 and 3-13, 3-14, 3-17 and 3-18). The same trend can be seen with the enzymatic activities during the processes using the defined medium (Figures 3-21, 3-24, 3-27, 3-30 and 3-31). The lowering of Yields of DCW per gram of Glucose, with each subsequent temperature increase, coupled with the increase of enzymatic activities with each subsequent temperature increase, is indicative that carbon flow is being directed toward plasmid maintenance and promoter activation and the cellular stress responses associated with the production of recombinant proteins, particularly when the temperature is raised to above 30 °C during induction.

An unwelcome consequence of fed batch *E. coli* fermentations is the formation of acetate as a by-product. Studies have shown that acetate concentrations measuring as low as 0.5 g/L can be detrimental to the growth rates of the culture and indeed impede protein generation (Voulgaris *et al*., 2011). Acetate is

a major concern during growth of *E. coli* in media containing glucose as a carbon source, particularly when growth is performed for recombinant protein production. Acetate accumulation never exceeded 0.5 g/L (Figure 3-3, 3-6, 3-9, 3-12, 3-20, 3-23, 3-26 and 3-29). Acetate appeared to have no effect upon the specific and total activities of the enzyme during the induction phase using either defined or undefined media (Figures 3-4, 3-7, 3-10, 3-13, 3-17, 3-18, 3-21, 3-24, 3-27, 3-30 and 3-31).

The thermal induction system employed in this study presents a more appealing option for the production of an amine oxidase. Voulgaris *et al*. (2011) attempted to produce a monoamine oxidase (MAO), a similar enzyme to DAAO, in an IPGT/Lactose inducible *E. coli* system but found acetate accumulation to be a problem. In an aerated fed batch fermentation (DO = 30%) acetate accumulation was in excess of 0.5 g/L, peaking at a maximum of 1.2 g/L post induction. In order to overcome this, subsequently oxygen enriched fermentations were employed resulting in acetate being maintained at below 0.5 g/L. Unfortunately it was found that extremely low enzymatic activities were being produced which was attributed by the authors to the negative effect of increased oxygen availability to overcome acetate production. Additionally, since recombinant protein production is itself frequently considered a stress (Hoffmann and Rinas, 2001, Hoffmann and Rinas, 2004) the authors also proposed this might be synergistic with the high oxidative stress imposed upon the culture and further contribute to the low enzymatic activities. Recombinant proteins are produced in much higher amounts than native proteins but naturally do not have acceptably suitable chaperones when compared to those of native proteins and therefore they are more exposed to the consequences of oxidation such as inappropriate folding, reduced activity and inclusion body formation.

In a thermally induced system, where the culture is essentially exposed to heat shock, heat shock proteins are produced and synthesis of chaperone molecules is reported to be upregulated (Vostiar *et al*., 2003). The principal function of these heat shock proteins is the maintenance of protein quality, chaperoning the folding of proteins. They also act to prevent the aggregation and re-solubilisation of aggregated proteins and to assist in the degradation of proteins incapable of folding, or simply mis-folded (Roy *et al*., 2012). The occurrence of a heat shock like response in the host cell following induction of recombinant protein synthesis has been well

documented for many years in a number of alternative recombinant protein systems (Hsu *et al*., 2014, Harcum and Haddadin, 2006, Hou *et al*., 2013). Legitimate induction of a heat shock like response is seen when a temperature sensitive promoter like the λP_L and P_r promoters in conjunction with the thermos-sensitive CI857 repressor is employed for recombinant protein production (Merten, 2001, Enfors, 2004).

Enzymes such as DAAO are prone to oxidative damage (Imlay, 2013) amino acid side chains of proteins undergo modification by reactive oxygen species at the cellular level resulting in alterations of the basic structure which can cause changes in their basic functions; in the case of an enzyme it can be a reduction in activity (Cabiscol *et al*., 2000). *T. variabilis* DAAO has methionine and cysteine residues which are prone to oxidisation to –SOH or SO_2H under oxidative conditions which results in substantial stereo chemical changes which prevent the binding of the FAD cofactor to the apoenzyme and therefore it is imperative to minimise the oxygen availability in the cultures (Dib *et al*., 2007). Alonso *et al*. (1999) discovered that decreasing the aeration rate of a fed batch *E. coli* fermentation increased DAAO activity and decreased the percentage of the apoenzyme form. The effects of oxygen availability are discussed in much further detail in Chapter 5.

3.5 *Conclusions*

Highly active DAAO was expressed successfully in thermally induced fed-batch cultures of *E. coli* using both defined and complex media. A constant temperature of 28 °C produced almost consistent results at 28 °C, particularly the DAAO activities. At 30 °C DAAO activities were higher earlier in the process when using LB as opposed to defined media, but the defined media reached higher activities overall, in the latter stages of the process, much quicker. At 35 °C using defined media, DAAO activities were higher and achieved earlier in the process than in comparison to LB. Using 40 °C the highest activities were achieved using defined media, much higher than using LB, and they were achieved much quicker in the process than with LB.

However, at 40 °C regardless of media used, low activity enzymatic activity was found in the extracellular reactor media, indicating that cells were becoming unstable due to the elevated temperatures and overexpression of the DAAO. It can be concluded that the optimum medium for DAAO production was the defined

medium, and although potentially more time consuming to make the individual components, maximum DAAO activities were higher than with LB medium, and were also achieved considerably earlier in the process, theoretically allowing a quick turn over of each process and allowing for more high quality product to be produced faster which at an industrial scale would be more enviable. Unfortunately, with the induction system used in these experiments, there is potential for promoter leakage which encourages enzyme production prematurely which has the potential to damage the growing cultures and thus creating the potential for processes failures.

With this being said, fed-batch as opposed to a continuous fermentation would be recommended. Although continuous fermentations present low labour costs and allow for good utilization of the bioreactor system, the nature of this expression system in this study means that continuous would not be viable, because the DAAO expressed is toxic to the cells and would render a process ineffective after just 24 hours of induction, as evidenced in the results mentioned previously.

Chapter Four

Recombinant Production of DAAO in *P. pastoris*

4 Recombinant Production of DAAO in *P. pastoris*

4.1 *Introduction*

Pichia pastoris is an exceptionally popular expression system exploited frequently for the production of recombinant proteins. Possessing what is often considered the most powerful of inducible promoters, the tightly regulated alcohol oxidase 1 gene (AOX1), *P. pastoris* is at the forefront of controlled expression of non-native genes. Unlike other yeast expression systems, particularly *S. cerevisiae* which tends to embrace respirative more than fermentative growth, *P. pastoris* favours the latter which allows the accumulation of biomass levels in excess of 160 g/L of dry cell weight. *P. pastoris,* like expression systems such as *E. coli and S. cerevisiae,* can secrete low level toxic by-products like acetate and ethanol which can be both growth and protein limiting ultimately resulting in much lower product yields (Inan and Meagher, 2001). The major advantage that *P. pastoris* has over prokaryotic expression systems is its innate ability to perform post translational modifications such as disulphide bond formation, glycosylation and proteolytic processing.

A high cell density *P. pastoris* fermentation is a multi-step process, consisting of approximately 4 phases. Phase 1 is a glycerol batch phase, the purpose of which is to maximise biomass accumulation whilst simultaneously inhibiting the expression of genes necessary for recombinant protein production. Phase 2 is a glycerol fed-batch phase employed to further enhance biomass accumulation whilst carefully allowing for repression of genes of the enzymes necessary for methanol catabolism. Phase 3, for some processes often *the* final stage, involves the induction of recombinant protein expression via a carefully controlled methanol feed, which simultaneously acts as a carbon source. Phase 3 can also be a transition phase whereby the feed rate of glycerol is decreased slowly as the methanol feed rate is simultaneously increased. In the latter scenario Phase 4 would be a methanol only induction phase discussed previously.

Traditionally the expression media found in the literature contains Yeast Nitrogen Base (YNB), a highly nutrient rich and ultimately costly media. Two forms of YNB: one with amino acid and one without. Utilising this expensive media compromised the economic profitability of scaling up to an industrial scale for the production of the enzyme used in this study. An attempt was therefore made to replace YNB with the

less costly alternative Yeast Extract (YE) so as to ascertain the effects this may have on both biomass and enzymatic activities.

The *Pichia* strain exploited in these experiments is a slow methanol utilisation strain which can lead to slower growth rates and so to overcome this a mixed feeding strategy was employed; methanol and sorbitol were fed simultaneously, ensuring various ratios of carbon from each source were maintained at a set rate. Sorbitol is a non-repressing carbon source and thus high cell densities can be achieved without compromising recombinant protein production.

4.1.1 *Standard Fermentations Based on Invitrogen™ Life Technologies Protocol*

To begin with, a typical *P. pastoris* fed batch protocol developed by Invitrogen™ Life Technologies was used and treated as a benchmark to ascertain how this affects growth and recombinant protein production. Methanol feed rates for both a Mut^+ and Mut^S strain were followed to gain a greater understanding of how this impacts the particular strain used in this study.

4.1.1.1 *High Methanol Feed Rate (Mut^+ Strain)*

In Figure 4-1 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 34.7 g/L at 31 hours. The methanol feed was begun at 32 hours. After that point there was, as expected, a decrease in growth rate as well as biomass accumulation. After 2 hours of feeding with methanol a biomass concentration of 24.9 g/L was measured. 19 hours after feeding with methanol, biomass concentrations measured 30.5 g/L at 51 hours and peaked at 39.3 g/L at 57 hours. From this point onwards the biomass concentrations began to slowly decrease before a final recording of 25.3 g/L was recorded at 100 hours. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.03 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.0002 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 5.5 hours process time the dissolved oxygen reached its pre-set level of 30 %. After 2 hours of methanol feeding the measured concentration of methanol was 16.3 g/L and this increased to 51 g/L at 51 hours process time before dropping to 40 g/L at 57 hours process time and increasing for the remainder of the process until a final concentration of 95.5 g/L was measured.

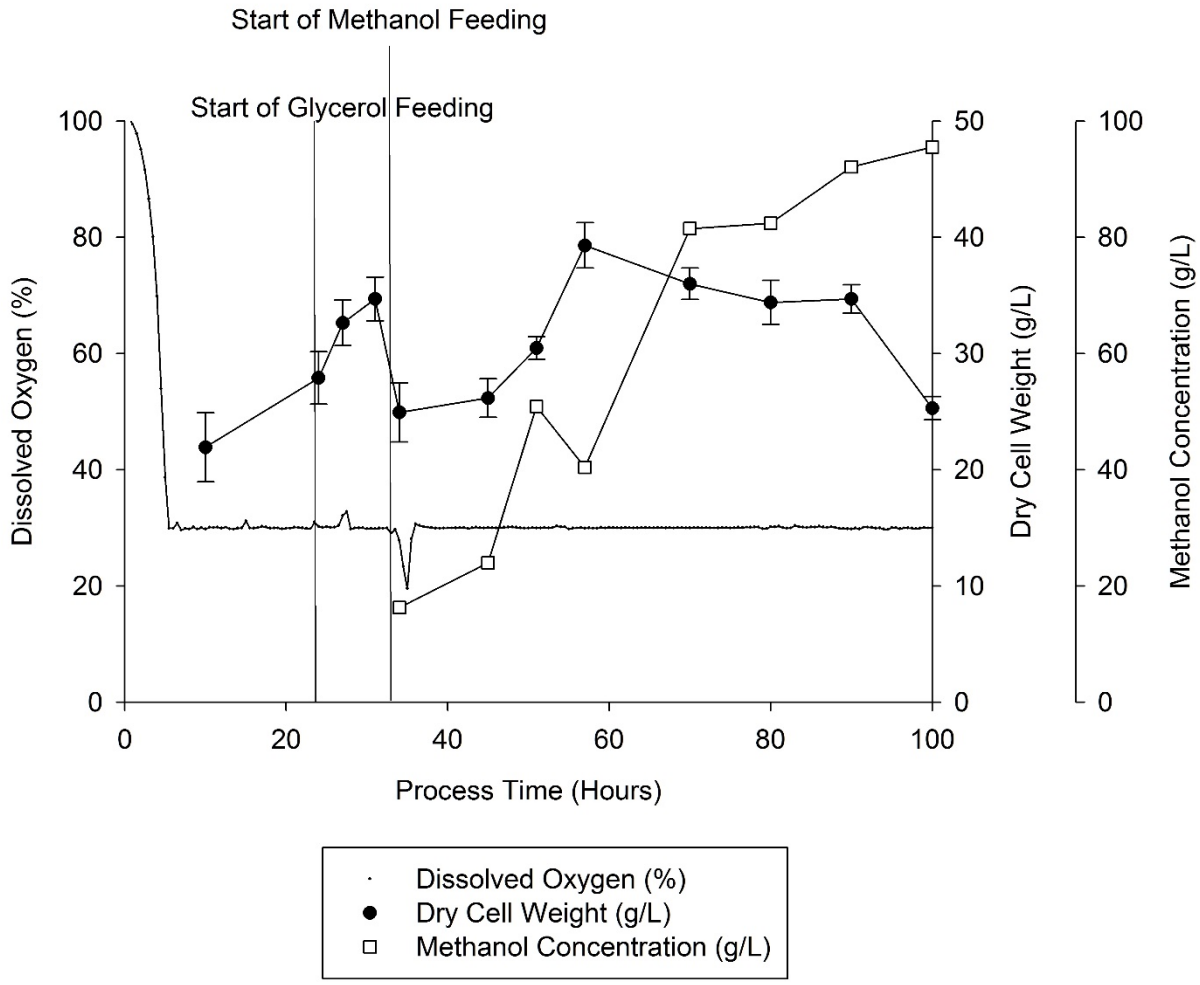

Figure 4-1. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a high MeOH feed rate. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-2 it can be seen that both specific and total D-amino acid oxidase activities exhibited a sharp decrease after the commencement of methanol induction. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 0.12 μmol/g DCW/min and 4.04 μmol/L/min respectively. At the end of the process (68 hours post induction) the specific activity measured 0.03 μmol/g DCW/min and the total 0.3 μmol/L/min.

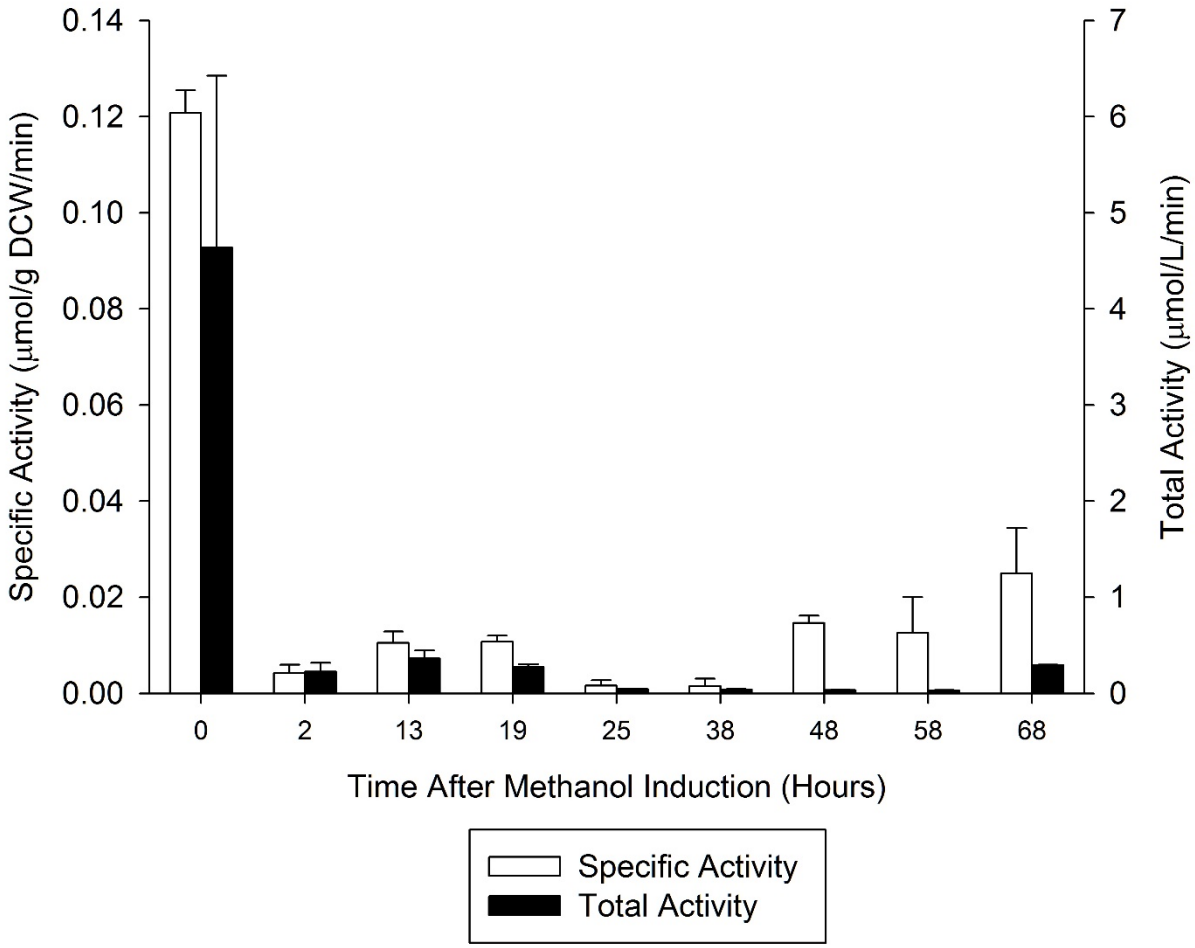

Figure 4-2. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a high MeOH feed rate. 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.1.2 *Low Methanol Feed Rate (Mut^S Strain)*

In Figure 4-3 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 35.6 g/L at 31 hours. The methanol feed was begun at 33 hours. After that point there was, as expected, a decrease in growth rate but unlike the previous experiment, there was biomass accumulation. For the first 17 hours after feeding with methanol, biomass concentrations essentially plateaued, measuring between 40.7 g/L and 41.9 g/L during this period. At 57 hours process time the biomass concentration peaked at a concentration of 46.0 g/L. After this point, biomass concentrations measured between 41.0 g/L and 39.5 g/L for the remainder of the process. Again biomass accumulation had plateaued. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.04 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.0005 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 5.5 hours process time the dissolved oxygen reached its pre-set level of 30 %. After 1 hour of methanol feeding the measured concentration of methanol was 6.4 g/L and this increased slowly to 25.6 g/L at 70 hours process time before increasing rapidly to 86.6 g/L at 80 hours process time until a final concentration of 90.5 g/L was measured at 100 hours process time.

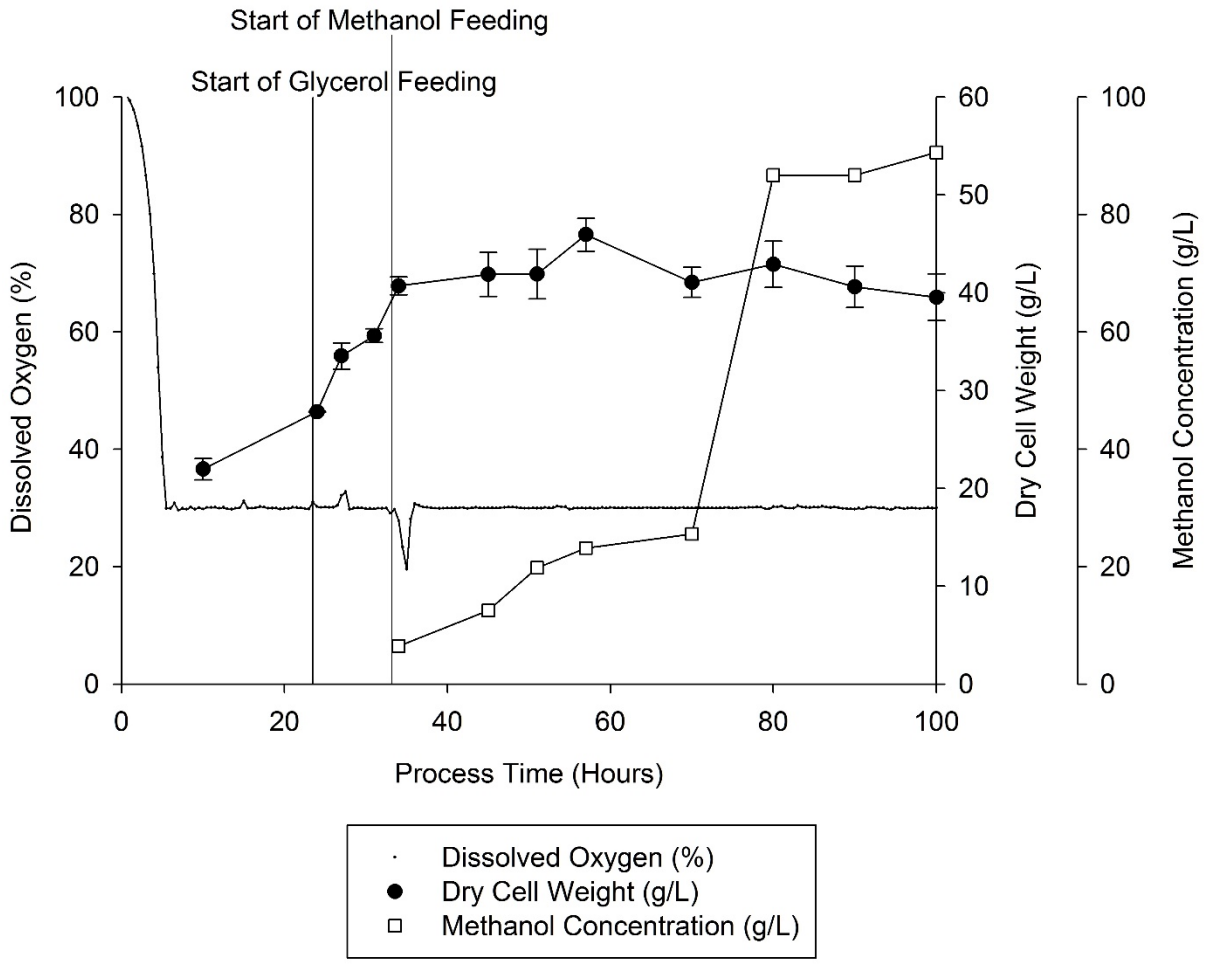

Figure 4-3. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a low MeOH feed rate. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-4 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 3.14 μmol/g DCW/min and 159.2 μmol/L/min respectively. 2 hours after methanol feeding specific and total activities of the culture exhibited were 4.33 μmol/g DCW/min and 209.81 μmol/L/min respectively. At the end of the process (68 hours post induction) the specific activity measured 0.0112 μmol/g DCW/min and the total 0.1838 μmol/L/min.

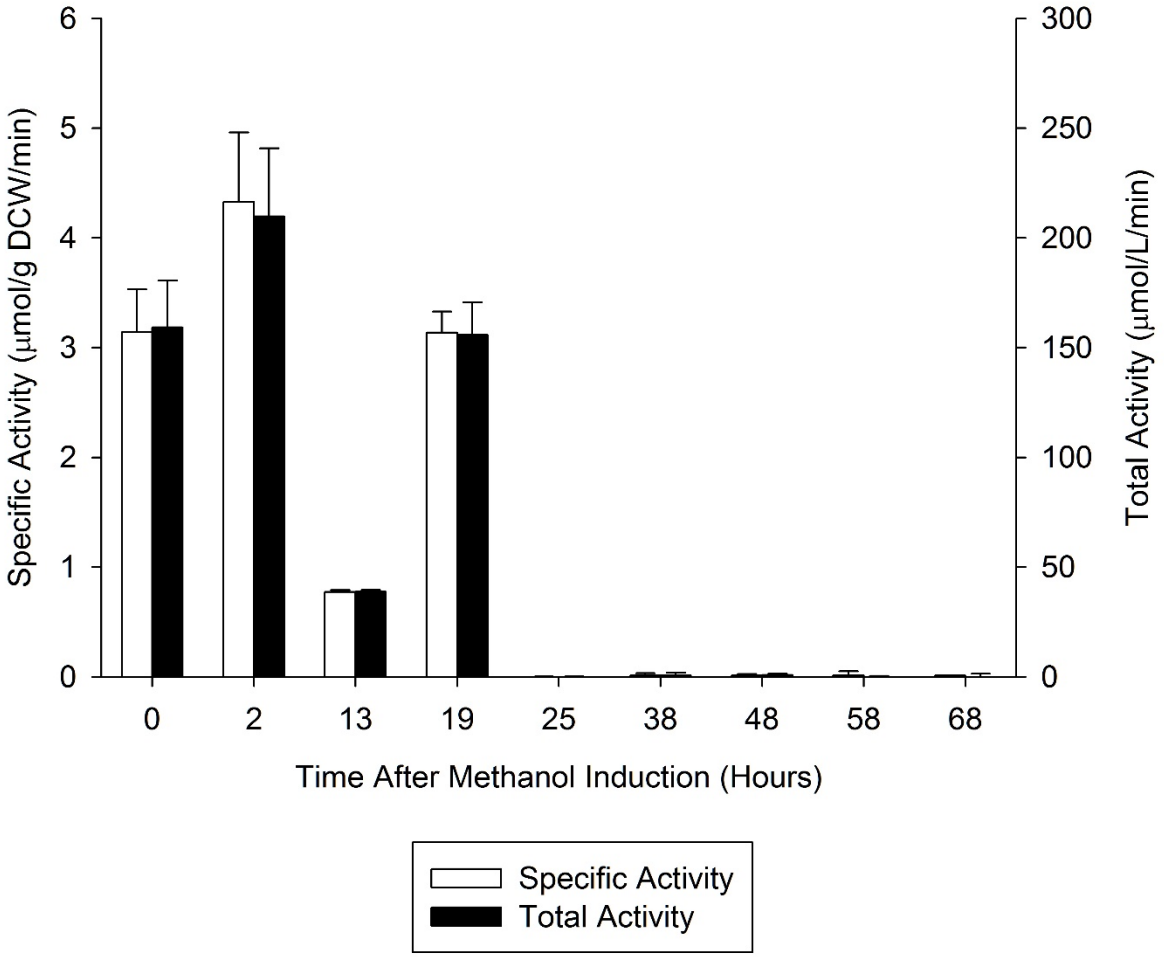

Figure 4-4. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a low MeOH feed rate. 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.2 *Implementation of an adapted Invitrogen™ Life Technologies MutS Protocol*

Voulgaris *et al*., (2011) investigated the recombinant expression of monoamine oxidase (MAO) in a methanol inducible strain of *P. pastoris* using an adapted Invitrogen™ Life Technologies protocol (See Appendix). A further reduced feeding rate of methanol in comparison to the Mut^S protocol used previously, and the introduction of a glycerol/methanol transition phase, discussed previously with a much slower increment glycerol feed were the main focus of this protocol and it was used for the expression of DAAO in this study.

4.1.2.1 *Implementation of an adapted Invitrogen™ Life Technologies Mut^S Protocol with no Media Supplementation*

In Figure 4-5 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 46.63 g/L at 31 hours. The methanol feed was begun at 32 hours. After that point there was an increase in biomass concentration, with a recording of 49.53 g/L after 2 hours of feeding with methanol. Between 34 and 45 hours process time there was a decrease in biomass concentration with a recording of 45.8 g/L. From this point onwards biomass began to increase for the remainder of the process, reaching a peak of 54 g/L at approximately 80 hours total process time at which it plateaued for the remainder of the process. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.07 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.001 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 13.5 hours process time the dissolved oxygen reached its pre-set level of 30 %. After 2 hours of methanol feeding the measured concentration of methanol was 6.03 g/L; this decreased to 5.8 g/L 13 hours after the start of methanol feeding. From this point onwards, the methanol concentration in the media the decreased to 4.5 g/L and never exceed 5.00 g/L for the remainder of the process.

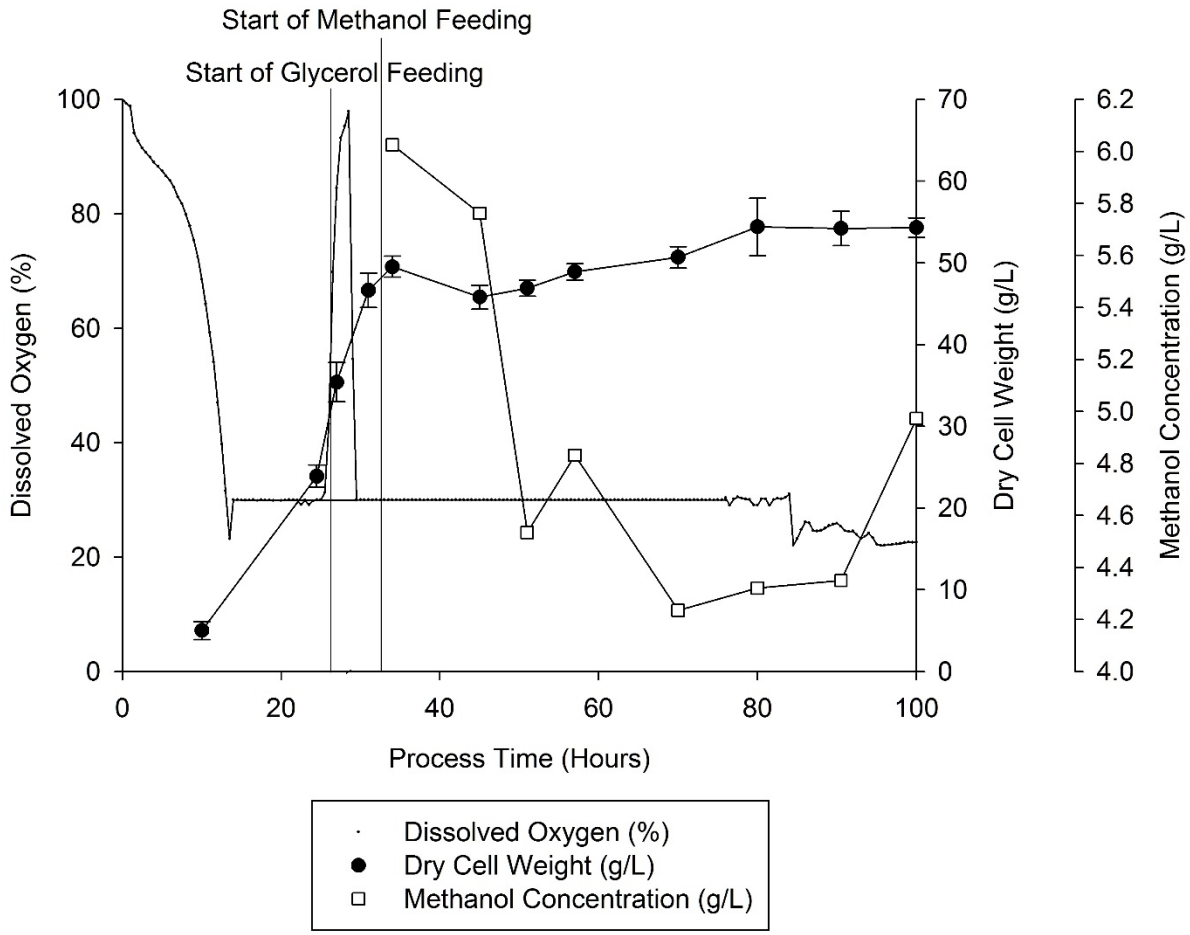

Figure 4-5. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with an adapted Invitrogen MeOH feed rate with no media supplementation. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-6 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction and throughout the remainder of the process. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 2.72 μmol/g DCW/min and 146.38 μmol/L/min respectively. 2 hours after methanol feeding specific and total activities of the culture exhibited were 25.72 μmol/g DCW/min and 2389.06 μmol/L/min respectively. At the end of the process (68 hours post induction) the specific activity measured 90.93 μmol/g DCW/min and the total 5529.35 μmol/L/min.

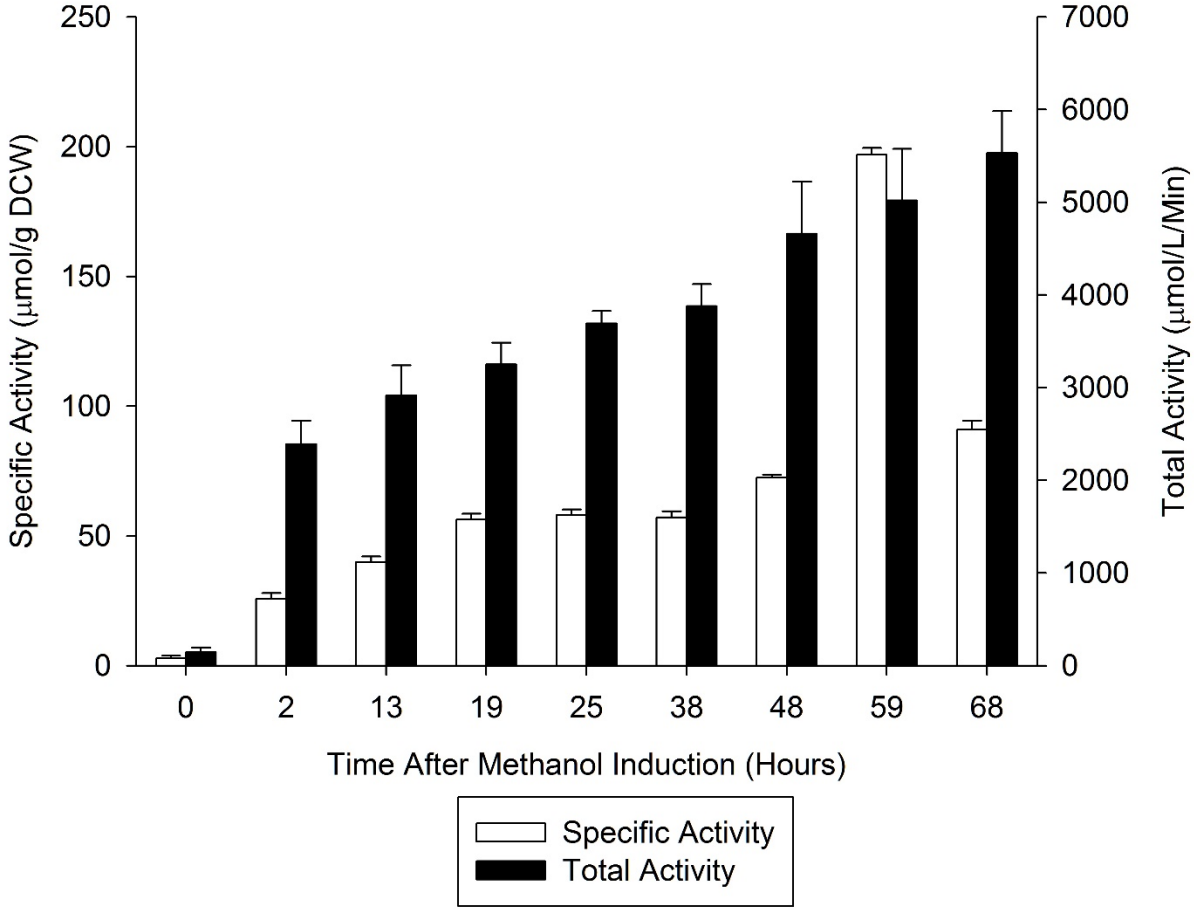

Figure 4-6. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with an adapted Invitrogen MeOH feed rate with no media supplementation. 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.2.2 *Implementation of an adapted Invitrogen™ Life Technologies Mut^S Protocol with Media Supplementation: Yeast Extract*

The 1st attempt at trying to improve the results achieved previously was to supplement the glycerol batch media with YE, as an alternative to the more expensive YNB. As seen in Figure 4-7 reached a concentration of 49.75 shortly before the onset of the methanol induction (31 hours). 2 hours after methanol feeding biomass concentrations measured 4.10 g/L (34 hours). Unlike the previous experiment biomass continued to increase throughout the methanol feeding phase, reaching a peak of 55.8 g/L at 100 hours. The specific growth rate after the beginning of glycerol feeding but before methanol induction was 0.09 h^{-1} while after methanol induction it fell to 0.003 h^{-1}.

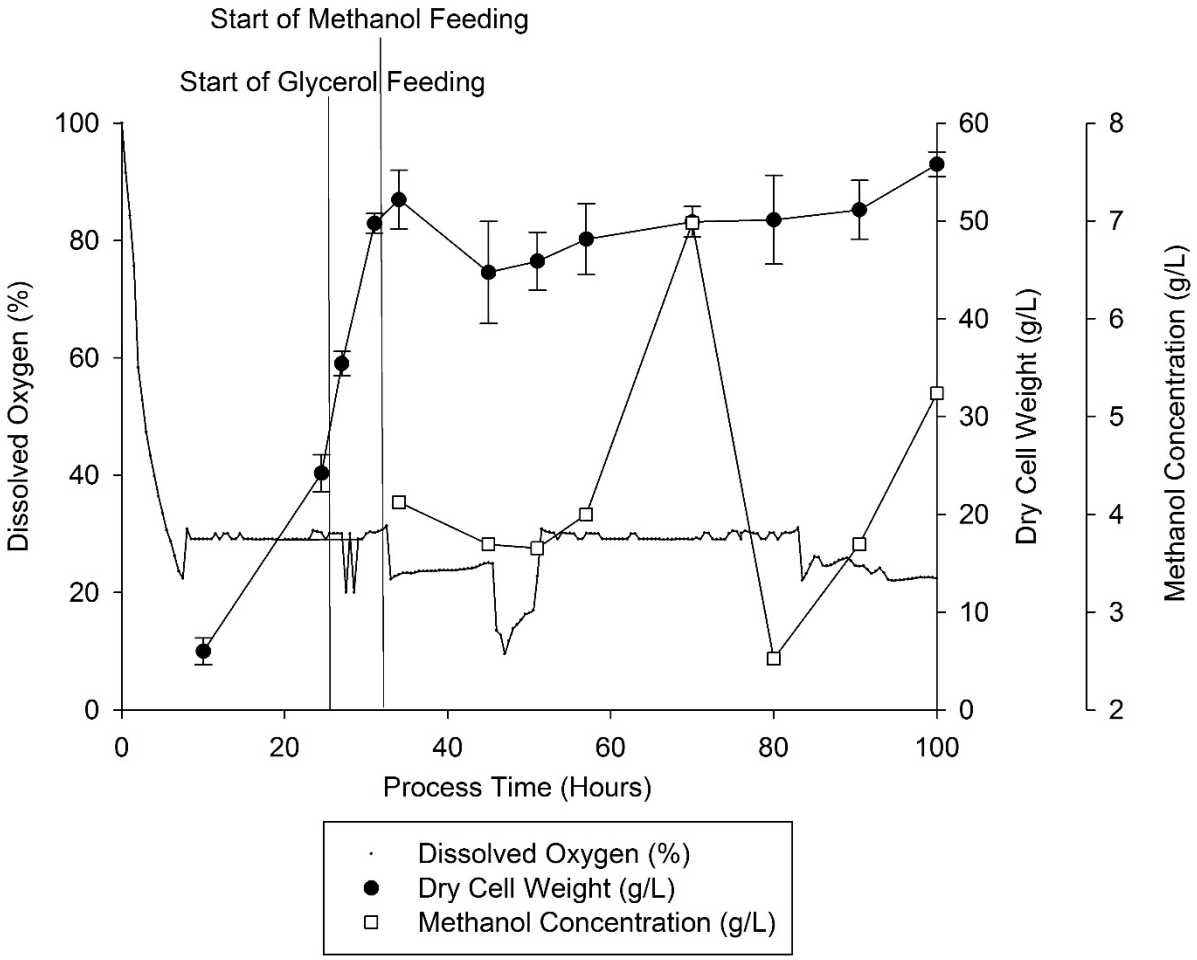

Figure 4-7. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with an adapted Invitrogen MeOH feed rate with media supplementation: Yeast Extract. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-8 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction and throughout the remainder of the process. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 2.61 μmol/g DCW/min and 139.28 μmol/L/min respectively. 2 hours after methanol feeding specific and total activities of the culture exhibited were 127.72 μmol/g DCW/min and 3595.06 μmol/L/min respectively. At the end of the process (68 hours post induction) the specific activity measured 199.23 μmol/g DCW/min and the total 9909.35 μmol/L/min.

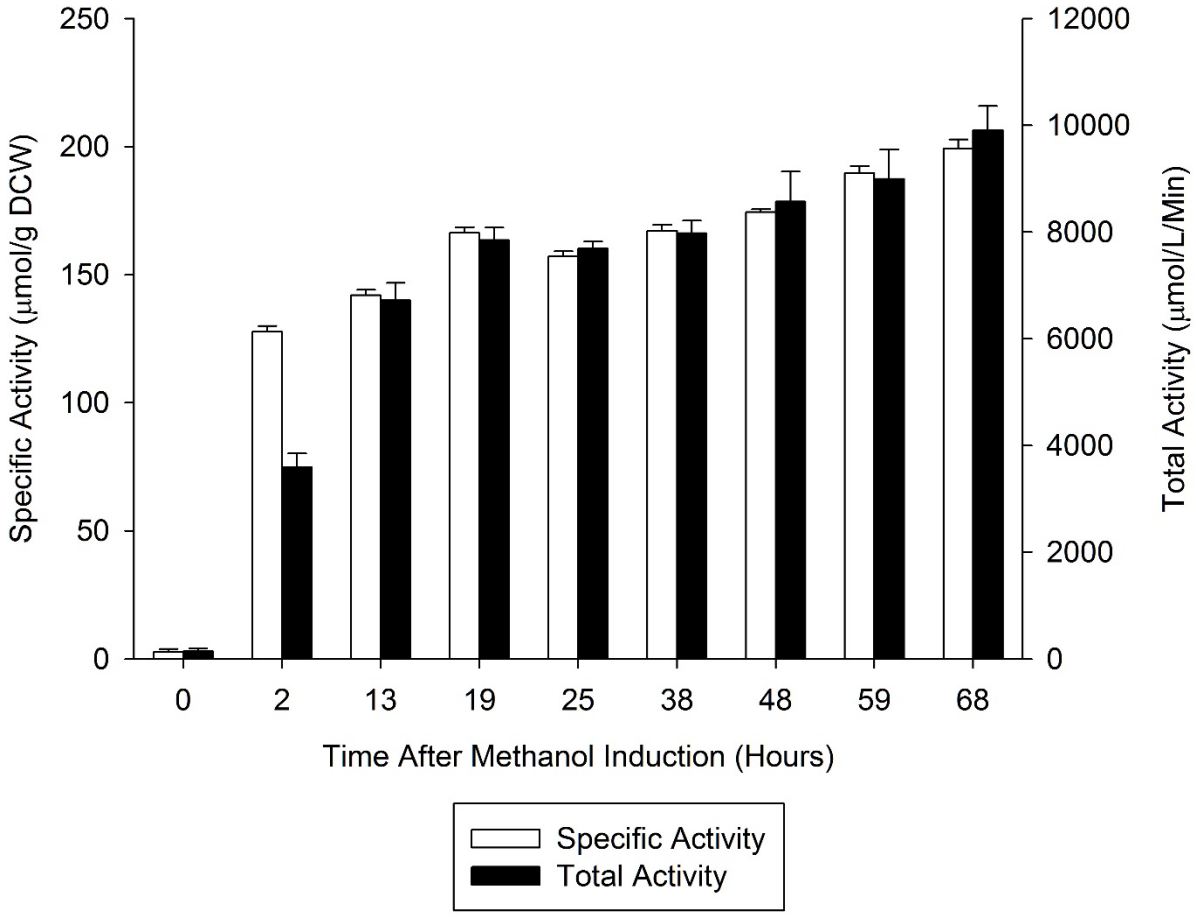

Figure 4-8. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with an adapted Invitrogen MeOH feed rate with media supplementation: Yeast Extract. 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.2.3 *Implementation of an adapted Invitrogen™ Life Technologies Mut^S Protocol with Media Supplementation: Yeast Nitrogen Base*

For comparison and again to try and improve the results achieved previously the batch media was supplemented with the more expensive and costly, but highly nutrient rich YNB. In Figure 4-9 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 47.93 g/L at 31 hours. The methanol feed was begun at 32 hours. Like the previous experiment, biomass continued to increase throughout the methanol feeding phase, reaching a peak of 59.71 g/L at 100 hours. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.1 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.005 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 11 hours process time the dissolved oxygen reached its pre-set level of 30 %. After 2 hours of methanol feeding the measured concentration of methanol was 4.50 g/L. Apart from peaking at 5.23 g/L (45 hours total process time) and 5.32 g/L (90.5 hours total process time) methanol levels were maintained below 5 g/L for the majority of the process.

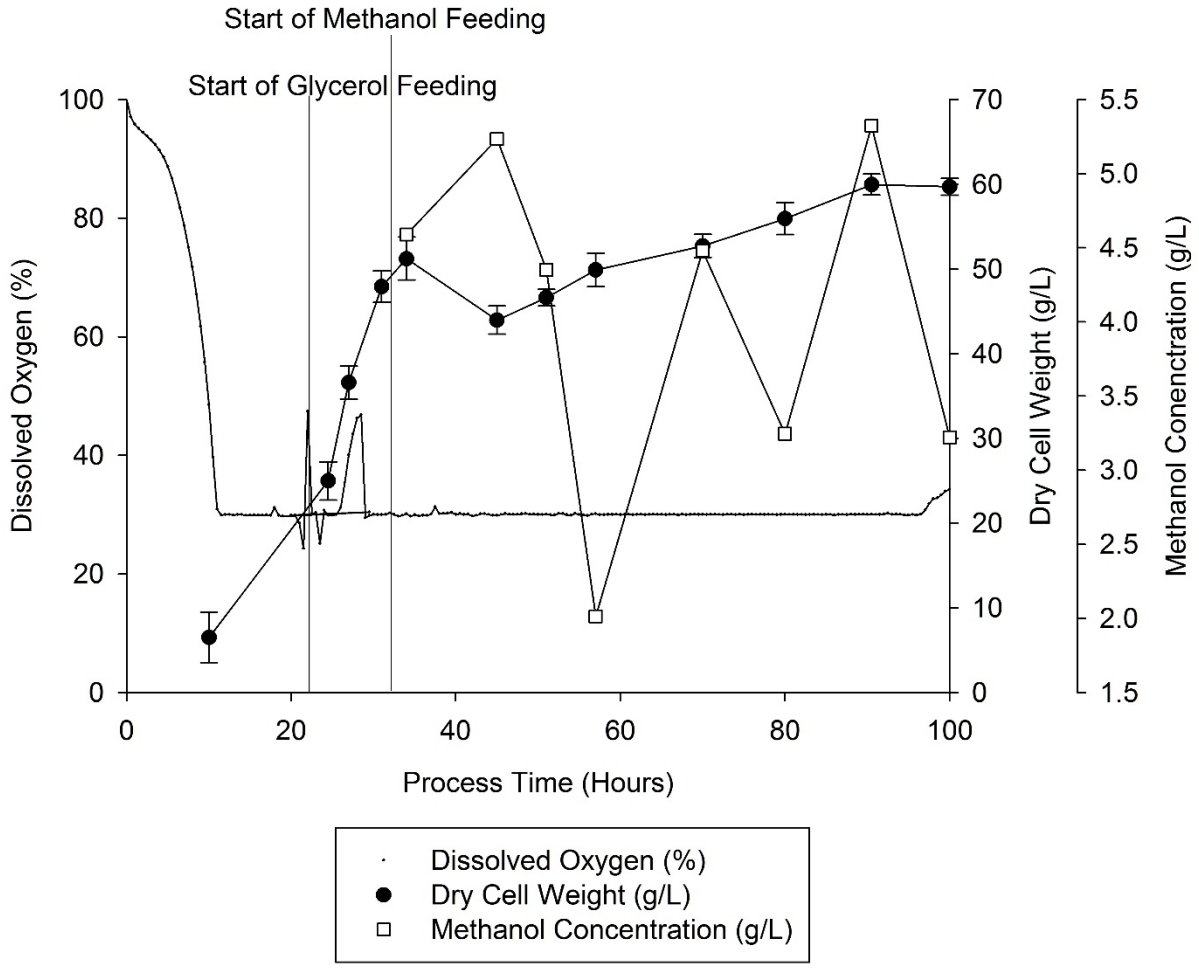

Figure 4-9. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with an adapted Invitrogen MeOH feed rate with media supplementation: Yeast Nitrogen Base. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-10 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction and throughout the remainder of the process. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 5.71 μmol/g DCW/min and 225.4 μmol/L/min respectively. 2 hours after methanol feeding specific and total activities of the culture exhibited were 177.72 μmol/g DCW/min and 5432.06 μmol/L/min respectively. At the end of the process (68 hours post induction) the specific activity measured 207.23 μmol/g DCW/min and the total 12009.4 μmol/L/min.

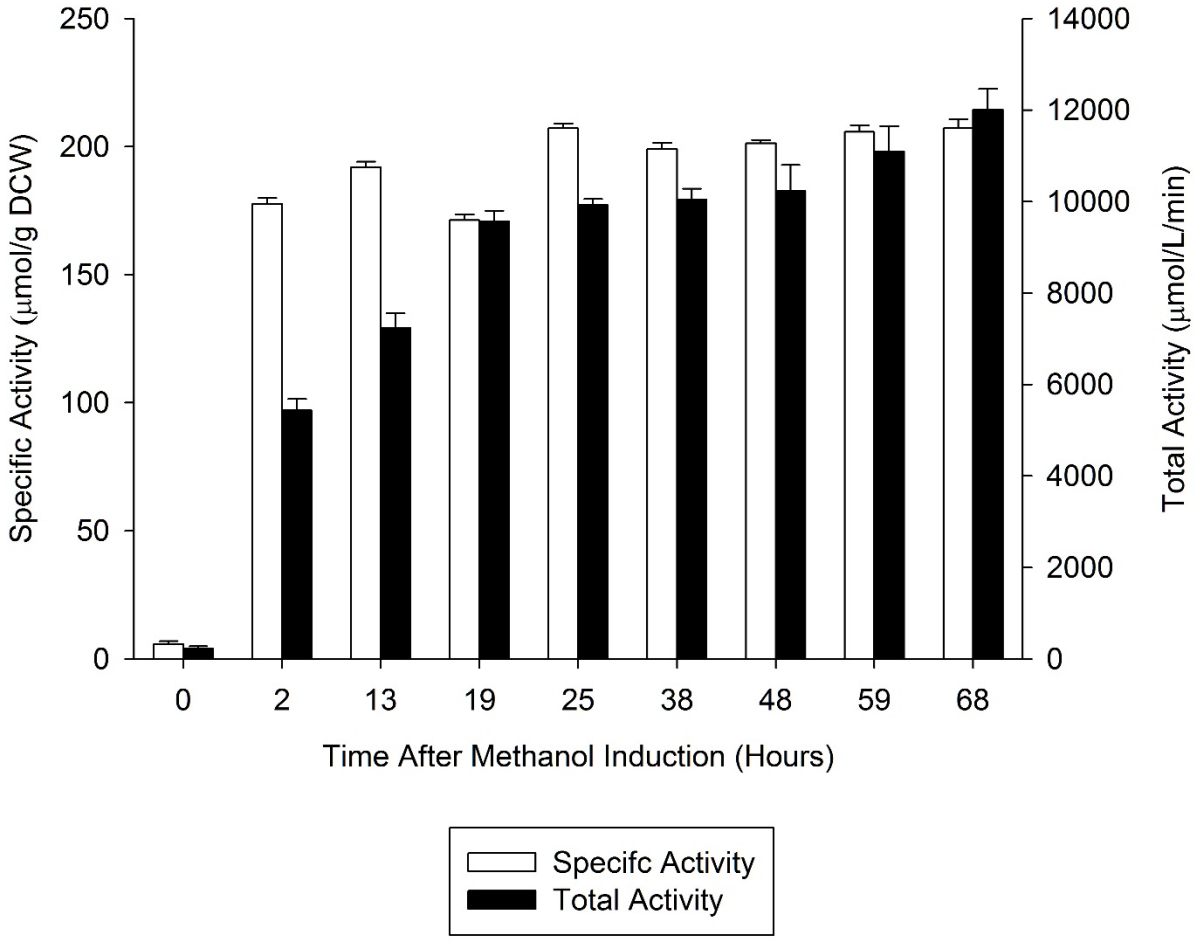

Figure 4-10. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with an adapted Invitrogen MeOH feed rate with media supplementation: Yeast Nitrogen Base. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.2.4 *Implementation of an adapted Invitrogen™ Life Technologies Mut^S Protocol with Media Supplementation: Yeast Nitrogen Base + Additional Amino Acid Supplementation (Sigma® Protocol)*

The YNB used in this study contains only 3 amino acids: Histidine, Methionine and Tryptophan. In order to understand how increasing the availability of other amino acids would affect the overall process media supplementation with more essential amino acids was carried out in this study.

As seen in Figure 4-11 biomass reached a concentration of 46.50 shortly before the onset of the methanol induction (34.5 hours). 2 hours after methanol feeding biomass concentrations measured 5.04 g/L (38 hours). 7 hours after the start of the methanol feed the biomass levels dropped to 4.01 g/L. Biomass concentrations reached a peak of 69.4 g/L at 90 hours. The specific growth rate after the beginning of glycerol feeding but before methanol induction was 0.03 h^{-1} while after methanol induction it fell to 0.01 h^{-1}.

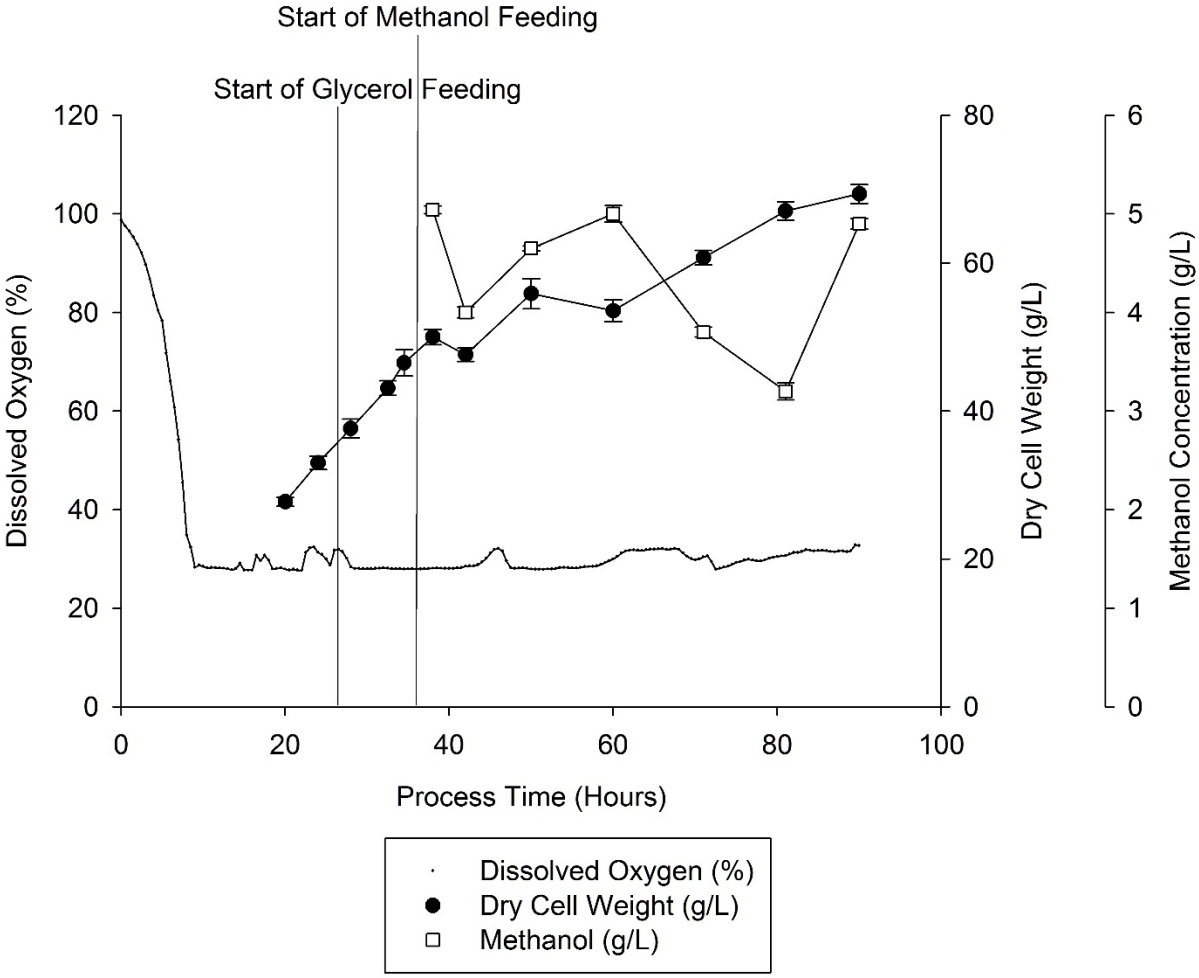

Figure 4-11. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with an adapted Invitrogen MeOH feed rate with media supplementation: Yeast Nitrogen Base + Additional Amino Acid Supplementation (Sigma Protocol). Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-12 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction 24 hours after the start of methanol feeding. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 6.14 μmol/g DCW/min and 76.24 μmol/L/min respectively. 2 hours after methanol feeding specific and total activities of the culture exhibited were 179.7μmol/g DCW/min and 8982.71 μmol/L/min respectively. At the end of the process (55 hours post induction) the specific activity measured 189.30 μmol/g DCW/min and the total 12693.3 μmol/L/min.

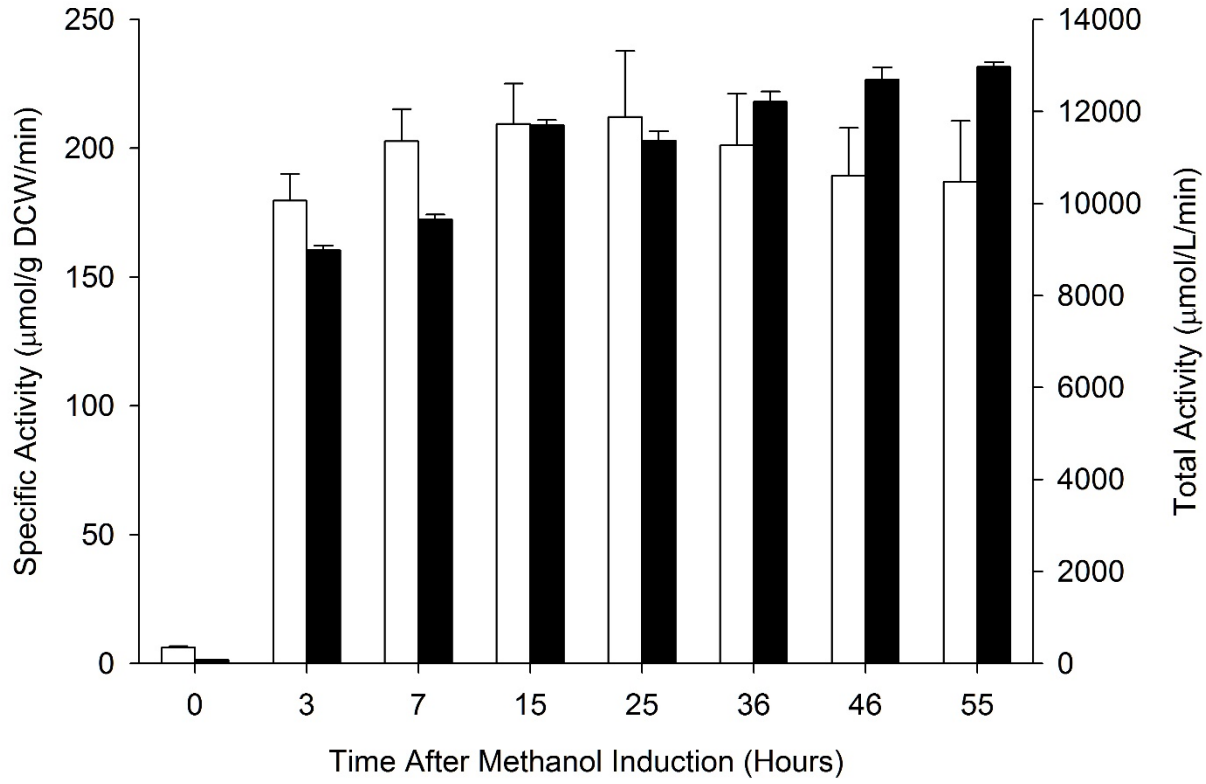

Figure 4-12. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with an adapted Invitrogen MeOH feed rate with media supplementation: Yeast Nitrogen Base + Additional Amino Acid Supplementation (Sigma Protocol). Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.3 *Implementation of a Co-Feeding Strategy*

Voulgaris *et al*., (2011) developed a co-feeding strategy for the production of monoamine oxidase in a methanol induced strain of *Pichia*. These protocol were implemented in this study. Batch media was also supplemented with YNB as this yielded the best enzyme activities and growth in the previous experiments.

4.1.3.1 *Methanol: Sorbitol Co-Feed – Ratio 2:1*

In Figure 4-13 it can be seen that the biomass concentration 3 hours after the start of the MeOH/Sorb co-feed was 34.7 g/L. Like the previous experiment, biomass continued to increase throughout the MeOH/Sorb co-feeding phase, reaching a peak of 57.4 g/L at 67 hours. The specific growth rate during the MeOH/Sorb co-feeding was 0.008 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 8.5 hours process time the dissolved oxygen reached its pre-set level of 30 %. After 4 hours of MeOH/Sorb co-feeding the measured concentration of methanol was 1.4 g/L and this increased to 19.0 g/L at 41.5 hours. Methanol remained below 5 g/L until 83 hours total process time when it measure 15.6 g/L.

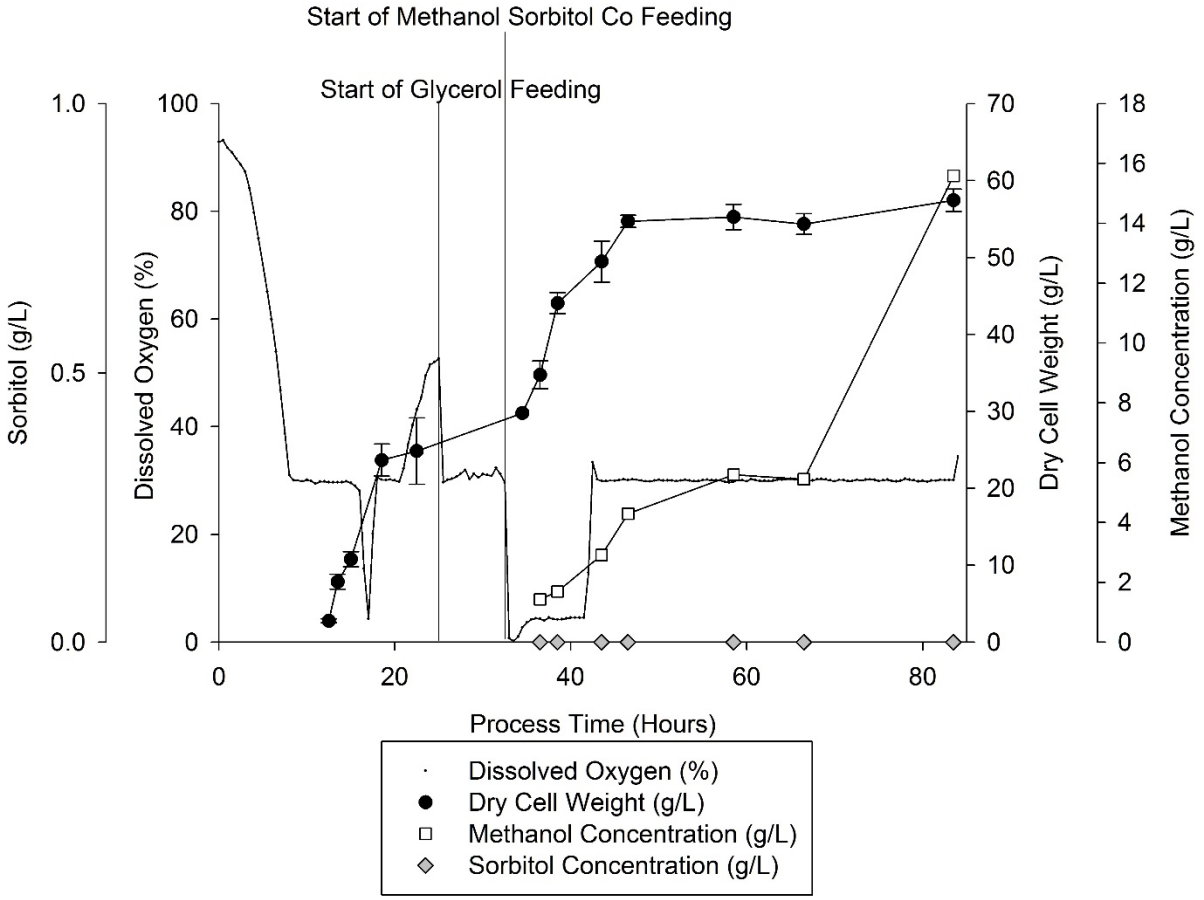

Figure 4-13. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [2:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-14 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction for the first three hours after the start of methanol feeding. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 0.125 μmol/g DCW/min and 26.0 μmol/L/min respectively. 12 hours after methanol feeding specific and total activities of the culture exhibited were 138.9 μmol/g DCW/min and 7675.35 μmol/L/min respectively. At the end of the process (52 hours post induction) the specific activity measured 202.59 μmol/g DCW/min and the total 13256.10 μmol/L/min.

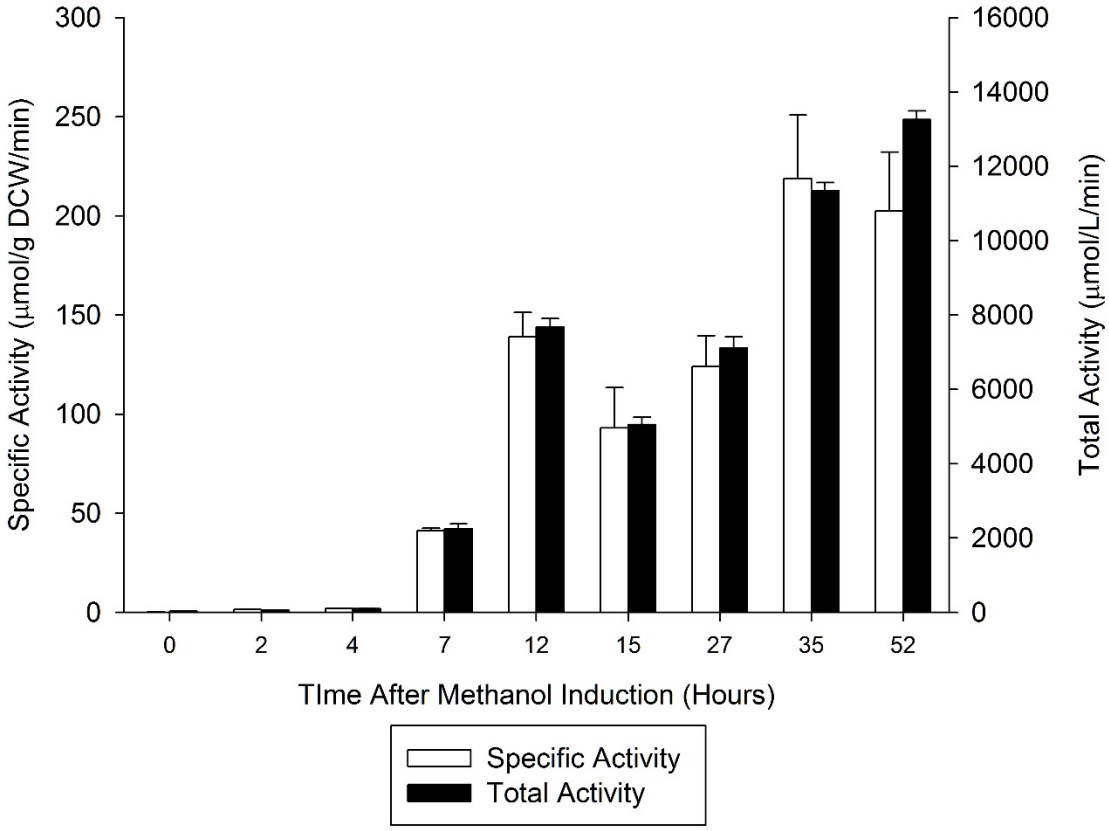

Figure 4-14. *Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a MeOH Sorbitol co feed [2:1]. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.*

4.1.3.2 *Methanol: Sorbitol Co-Feed – Ratio 1:1*

In Figure 4-15 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 69.4 g/L at 34 hours. The MeOH/Sorb co-feed was begun at 36 hours. After the start of the MeOH/Sorb co-feed biomass increased to a maximum of approximately 80 g/L. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.09 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.0008 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 9 hours process time the dissolved oxygen reached its pre-set level of 30 %. Methanol concentration never exceeded 5 g/L throughout the process.

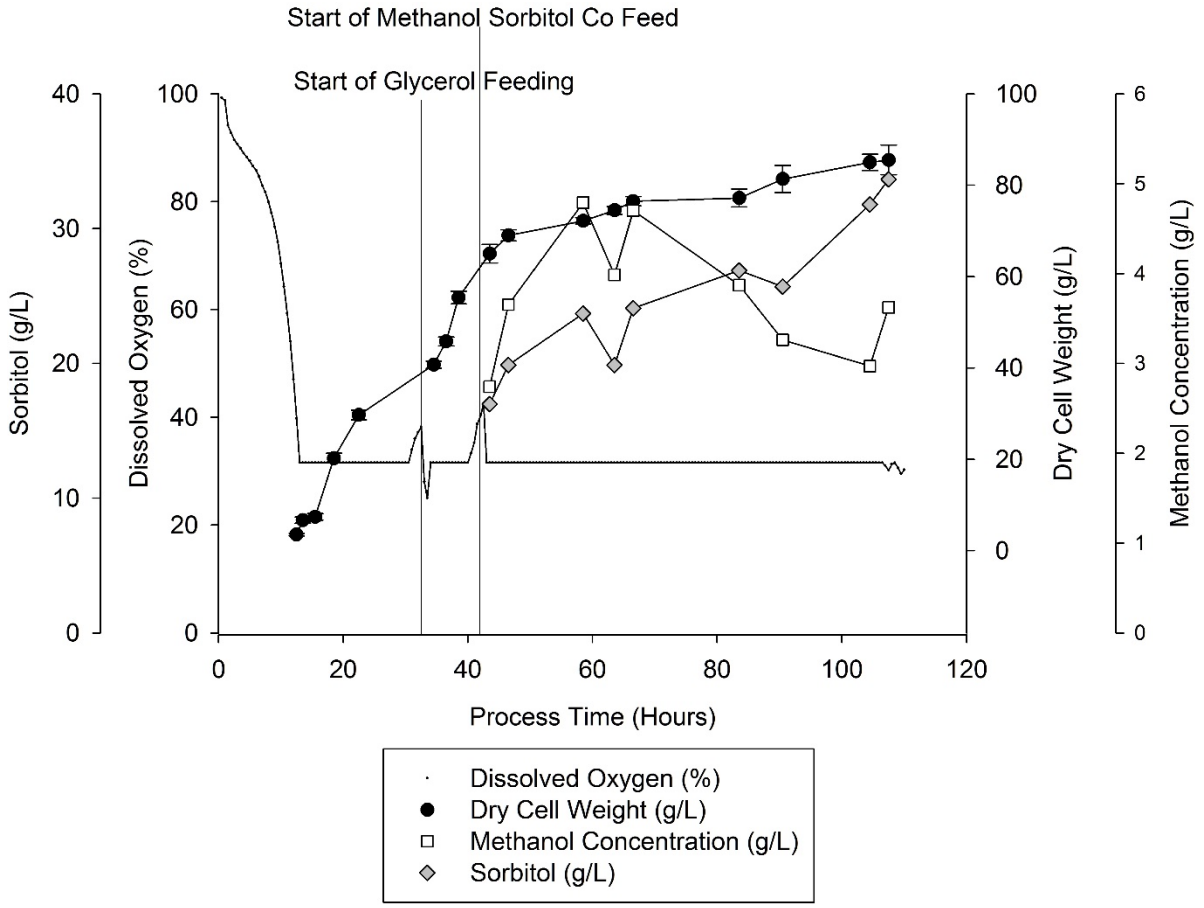

Figure 4-15. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-16 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction for the first three hours after the start of methanol feeding. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 9.30 μmol/g DCW/min and 76.6 μmol/L/min respectively. 6 hours after methanol feeding specific and total activities of the culture exhibited were 112.0 μmol/g DCW/min and 5800.4 μmol/L/min respectively. At the end of the process (67 hours post induction) the specific activity measured 221.36 μmol/g DCW/min and the total 18952.1 μmol/L/min.

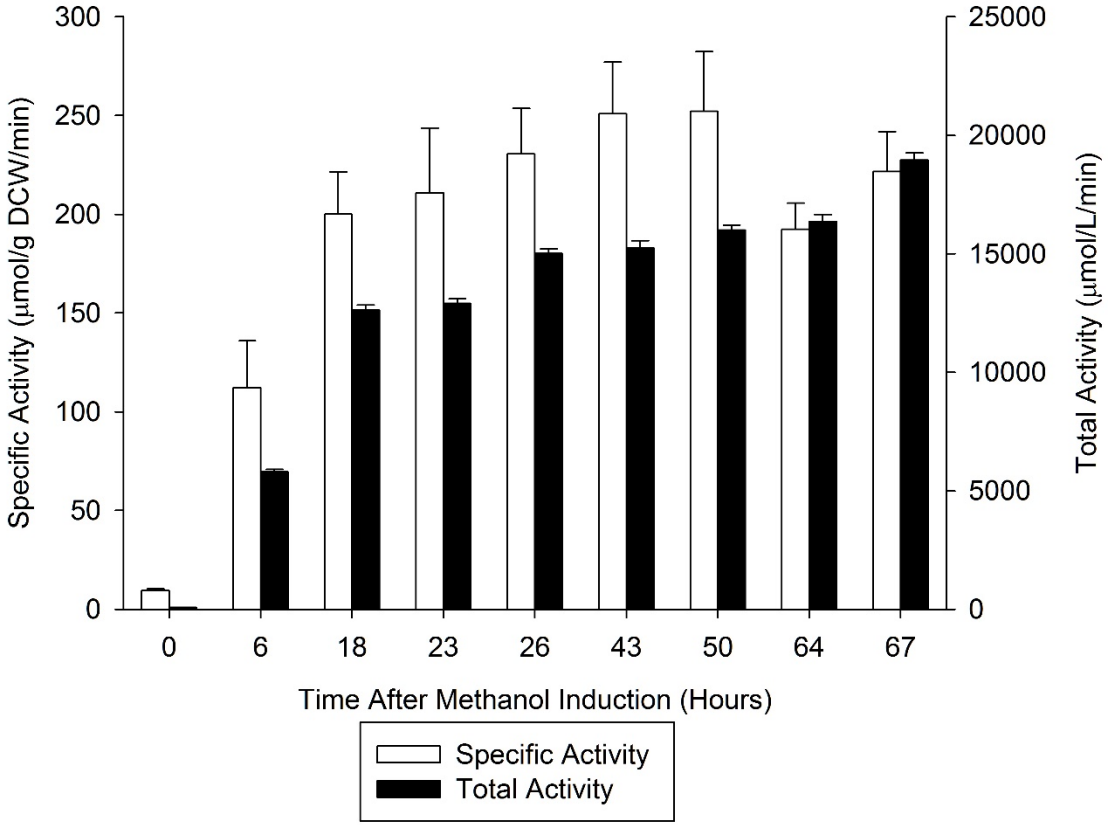

Figure 4-16. *Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a MeOH Sorbitol co feed [1:1]. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.*

4.1.3.3 *Methanol: Sorbitol Co-Feed – Ratio 1:2*

In Figure 4-17 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 58.8 g/L at 43.5 hours. The MeOH/Sorb co-feed was begun at 44 hours. Like the previous experiment, biomass continued to increase throughout the MeOH/Sorb co-feeding phase, reaching a peak of 83.8 g/L at 63.5 hours. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.02 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.003 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 10.5 hours process time the dissolved oxygen reached its pre-set level of 30 %. Methanol measured above 5 g/L at 15 and 20 hours post induction, but remained below 5 g/L for the remainder of the process.

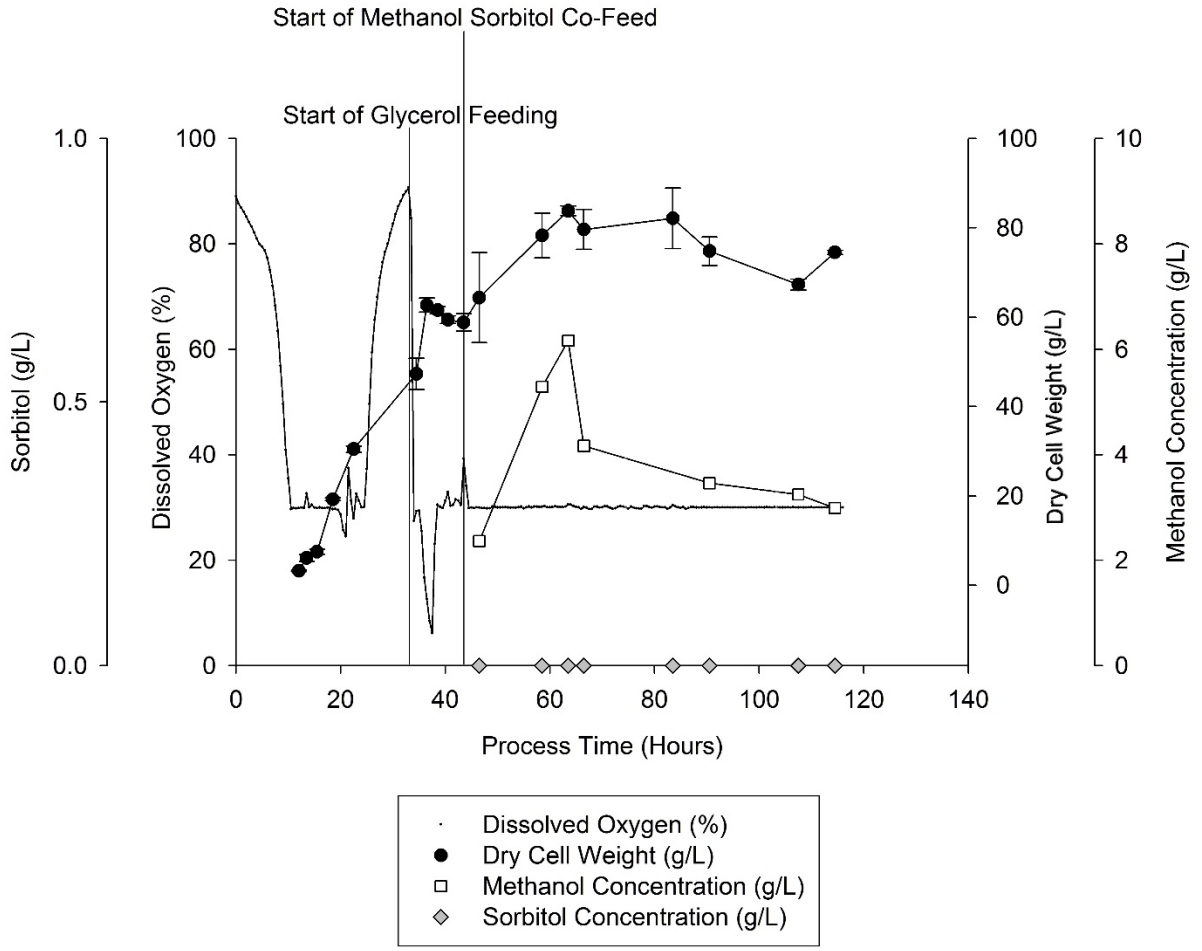

Figure 4-17. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:2]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 4-18 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction for the first twenty hours after the start of methanol feeding. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 2.735 μmol/g DCW/min and 31.7 μmol/L/min respectively. 20 hours after methanol feeding specific and total activities of the culture exhibited were 120.4 μmol/g DCW/min and 9421.04 μmol/L/min respectively. From here on in both specific and total activities began to decrease with specific and total activities measuring 96.0 μmol/g DCW/ and 7879.21 μmol/L/min. Both specific and total activities continued to increase 40 hours after induction and peaked at 41 hours post and at 71 hours post induction the specific activity measured 175.8 μmol/g DCW/min and the total 13412.9 μmol/L/min.

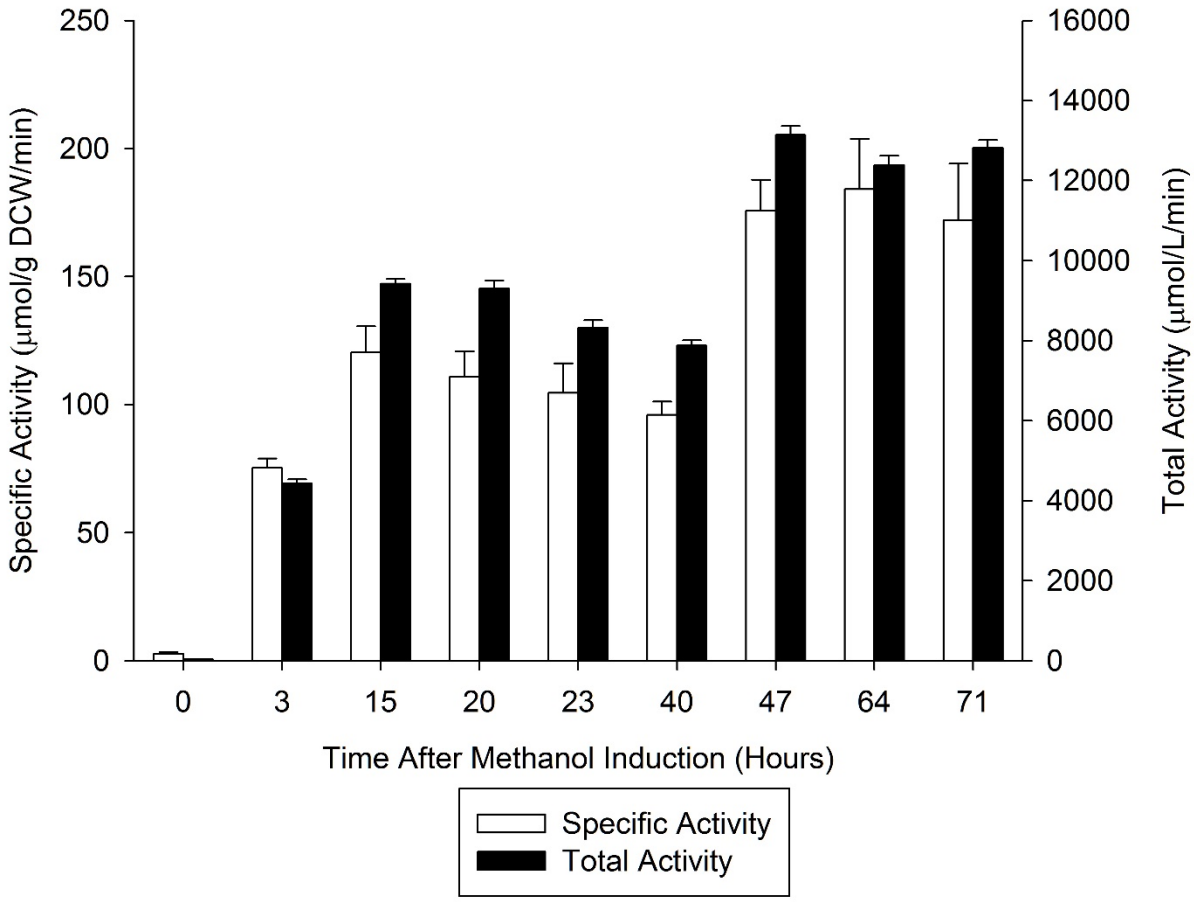

Figure 4-18. *Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a MeOH Sorbitol co feed [1:2]. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.*

It is clear from Figure 4-19 that with the introduction of a MeOH/Sorb co-feed during the induction phase there was not a statistically significant decrease in the respiratory quotient of the cultures. The average respiratory quotient of the cultures after the start of the MeOH/Sorb co-feed were 0.751, 0.884 and 0.925 for the 2:1, 1:1 and 1:2 ratios respectively.

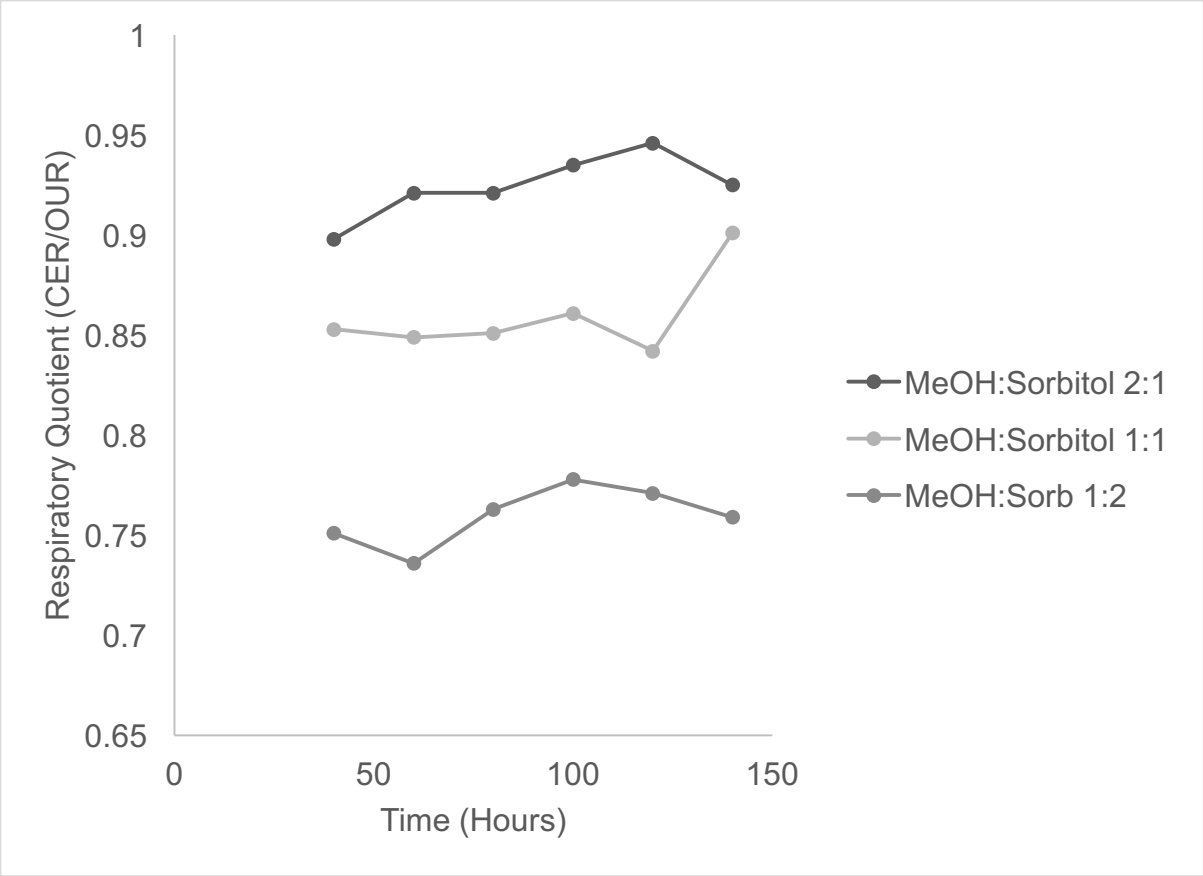

Figure 4-19. Time profiles of the Respiratory Quotients of P. pastoris ING005-A9-1-MCB1 with a MeOH Sorbitol co feed [1:2] + [1:1} + [2:1]. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 30 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

4.1.4 *Discussion*

Biomass concentrations between the glycerol only and glycerol YE/YNB fermentations were not significant during the batch phase of the fermentation process. After the start of the methanol feed during the glycerol only fermentation, growth of the culture slowed and biomass accumulation remained very low. Conversely in the YE and YNB fermentations, the biomass accumulation continued right through the process, never decreasing at any time point. However, biomass levels in the YNB fermentation were much higher than in the YE fermentation, and indeed the glycerol only fermentation. Such discrepancies can be attributed to nutrient limitation in both the glycerol only and glycerol and YE fermentations. Nevertheless, such finding occurred long after the preliminary incorporation of the supplements had been done in the batch media. The YNB contains a sufficient nitrogen source in the form of ammonium sulphate (5 g/L, which is the equivalent of 1.06 g of nitrogen/L) and the amino acids histidine (10 mg/L), methionine (20 mg/L) and tryptophan (20 mg/L). Furthermore, it also contains approximately 2 mg/ml of each of the vitamins. Conversely the composition of YE is not well characterised which ultimately presents issues with variability amongst batches. YE is a particularly excellent source of vitamins and amino nitrogen. The total nitrogen contained within yeast extract is approximately 10 % - 12 % (w/w) whilst amino nitrogen corresponds to 5.1 % (w/w) and therefore this will yield approximately 1.30 g/L to 1.70 g/L of total nitrogen in solution and approximately 0.70 g/L is derived from amino nitrogen in solution. In particular YE is an excellent source of B-complex vitamins. Considering that a 25 % (v/v) solution of ammonia is used as a source of nitrogen during pH control and that YE is rich in B-complex vitamins, the discrepancy between the biomass in the YE fermentation compared with the YNB fermentation is unlikely due to nitrogen source limitation.

Another possible suggestion for the discrepancy in biomass levels could potentially be down to a limitation of a particular amino acid(s), namely one seldom used by the organisms but one repeatedly found in the amino acid sequence of the recombinant protein being produced; examples of which include tryptophan, histidine and methionine. Carnicer *et al*. (2009) reported that a *P. pastoris* culture growing in a chemostat culture will usually contain approximately 1.40 %. 1.89 % and 0.79 % of these amino acids respectively, in its overall amino acid constituents and if we take

in to consideration that the DAAO enzyme used in this study has an amino acid sequence consisting of approximately 2.7 %, 5.3 % and 4.2 % of these amino acids respectively it can be assumed that a lack of or reduction in these amino acids can negatively affect cellular metabolism. For this to be confirmed, there should theoretically be a reduction in the activity of the DAAO enzyme. It can be seen that supplementing the YNB media with additional amino acids, both essential and non-essential led to a slight decrease in biomass accumulation compared with the YNB only but a slight increase in both specific and total enzymatic activities indicating that an overall limitation in amino acids can negatively affect enzyme activities but not necessarily biomass accumulation. There is a clearly illustrated trend that as the complexity of the media used increases, so to do the specific and total activities of the DAAO enzyme.

A lack of vitamin solution may also be a potential point of contention for the discrepancy in biomass levels and/or reduced enzymatic activities obtained with just YE and not YNB. Vitamins like biotin, folic acid and thiamine can be utilised for the metabolism of carbon sources like methanol and sorbitol which YE should theoretically contain in some quantity or another. However, the need for a vitamin solution seems unnecessary as the introduction of a sorbitol feed as seen in figure 4.12 led to both increased biomass levels and enzymatic activities, compared with a methanol only feed and thus it can be concluded that additional vitamins were unnecessary for growth.

As can be seen in Figures 4-1 and 4-2 methanol accumulation appeared to have detrimental effects on both biomass accumulation and DAAO enzymatic activities. If we take Figures 4-5, 4-7 and 4-9 into consideration, in which methanol accumulation did not occur, we see that biomass levels continued to increase throughout the process and that enzymatic activities were higher than those seen in Figures 4-1 and 4-2. Moreover, we see also see that enzymatic activities were higher in Figures 4-6, 4-8 and 4-10 compared with Figures 4-2 and 4-4. This suggests that methanol accumulation does have as serious a consequence on the activities of the enzyme during synthesis.

Methanol is toxic to cells because alcohol oxidase oxidises methanol to formaldehyde and hydrogen peroxide, which can ultimately accumulate; when

methanol levels remain low, such toxicity is abrogated as this process occurs in the peroxisome which essentially isolates the remainder of the cell from the presence of hydrogen peroxide which is reduced to H_2O and O_2 via dismutation by peroxisomal catalases. Cytosolic NAD^+-dependent formate dehydrogenases and NAD^+-dependent formaldehyde dehydrogenases can convert formaldehyde to CO_2. Additionally, formaldehyde can enter the xylose – 5'- phosphate pathway.

The biochemical reactions associated innately with recombinant protein production are obliquely and unequivocally affiliated with the overall growth of the cell and thus a feeding strategy which ultimately governs the specific growth rate is perhaps one of the most imperative determinants for aggrandizing recombinant protein production. When methanol is utilised as both the solitary carbon source for energy, and indeed as the inducer for recombinant protein production, the growth rate of a MutS strain of *P. pastoris* is kept very low to ensure that the methanol is maintained below a hazardous concentration. In this study, a non-repressive carbon source called sorbitol was employed in the hopes of increasing carbon flux through the cell in order to ascertain the effects on biomass accumulation and enzymatic activities.

As can be seen from figure 4-7 a carbon to carbon (MeOH/Sorb) ratio of 1:2 yielded the best biomass concentrations overall, due in part to their being approximately 200 % more carbon available to the cells in comparison to a feed of methanol as the solitary carbon source. Moreover, the available carbon was 150 % more than that of a carbon to carbon (MeOH/Sorb) ration of 2:1 and 100 % more than that of a carbon to carbon (MeOH/Sorb) ration of 1:1. From results section 4.1.3 it is clearly demonstrated that increased carbon flux has an explicit effect on biomass accumulation as a result of alterations in growth rates.

The 1:1 process clearly gave the best enzymatic activities, both specific and total. In the 1:1 process, at 2 hours post MeOH/Sorb feeding, the specific activity of the cultures is 10% greater than the methanol only feed in Figure 4-10 and the corresponding total activity is 40 % greater. After 34 hours of feeding, the specific activity is 17 % greater than the methanol only feed in figure 4-10 and the corresponding total activity is 24 % greater.

In *E. coli* when the growth rate exceeds a certain threshold, recombinant protein production is greatly reduced because carbon and energy is routed toward the formation of biomass and product. If we look at the results in Figure 4-17 for the 1:2 process it can be seen that the DAAO activity is greatly reduced but biomass accumulation is much higher in comparison to the 1:1 ratio and it is very possible that what is seen in *E. coli* is happening in this *Pichia* expression system.

In this study, the maximum specific growth rate = 0.003 h-1 (MeOH/Sorb ratio 1:2) gave the highest biomass concentrations; notwithstanding the maximum specific recombinant enzyme activity was achieved at a specific growth rate = 0.0008 h-1, this is in concurrence with the results published by de Lamotte *et al*. (2001) and Issaly *et al*. (2001) who discovered that maximum biomass accumulation was achieved at a specific growth rate of 0.1 h-1 and recombinant protein production occurred at a specific growth rate of 0.09 h-1. It should be noted that in each of these studies that additional supplementation occurred in the feeding media (vitamins and trace elements, and YE respectively). Furthermore Ye *et al*. (2011) tested two different specific growth rates (0.03 h-1 and 0.07 h-1) and obtained the highest production of their recombinant protein at the lowest specific growth rate. Interestingly, Arnau *et al*. (2011) found that irrespective of any economic reasons to use sorbitol or glycerol as co-substrates, one of the key advantages pf using glycerol instead of sorbitol is a higher specific growth rate (μ = 0.18 h-1 vs 0.02 h-1) and the subsequent productivity of the bioprocess for a Mut+ strain. However, they noted that for a MutS phenotype the potential advantage is ineffective. At the highest μ tested, proteolytic activity was found to be at its highest. Garcia-Ortega *et al*. (2013) demonstrated that transcription appears to the preeminent cause of limited recombinant protein production in co-fed cultures of *P. pastoris* under growth limiting conditions. The lowest specific growth rate produced the lowest FAB production, and said production was virtually abated at 40 hours of operation.

Rebnegger *et al*. (2014) investigated how growth rates regulate protein synthesis and secretion, mating and stress responses in *Pichia*. They show that the genes encoding for the cellular response UPR (unfolded protein response) are up regulated as the specific growth rate increases and conclude it is the foremost regulatory reaction for increasing cellular proliferation. These cellular responses are induced upon overexpression of the UPR regulator *HAC1.* Furthermore they

demonstrate that there is a greater requirement for proteosomal activity at very high growth rates due to the increased turnover of cell cycle regulators and other regulatory proteins at higher growth rates. However, a study in 2005 by Pakula *et al*. (2005) suggests that upregulation of the UPR in the fungal expression system *Trichoderma reesei* at high growth rates reduces the formation and secretion of recombinant proteins whilst Rebnegger *et al* (2014) conclude that higher growth rates are beneficial for the secretion of recombinant proteins. The work in this study conforms to the formers findings in regards to protein synthesis, but the latters in regards to cellular growth and proliferation.

4.2 *Conclusion*

It has been frequently reported in the literature that the implementation of a co-substrate feed can reduce the time necessary for the maximum expression of the recombinant protein of interest. The highest enzymatic activities in this study were achieved at or after 23 hours post induction in the 1:1 feed ratios and 47 hours post induction for the 1:2 ratio. Essentially the 1:1 feed ratio helped to reduce the process time overall whilst providing the best productivities and thus this reduction can help to significantly reduce the costs associated with a scaled up industrial process. In the 1-1 ration, enzyme activities remained high and constant until they peaked further at the end of the process.

Chapter Five

The Effect of Oxygen Availability on the Physiology of *P. pastoris* During Recombinant DAAO Production, and Effects on Enzymatic Activity

5 The Effect of Oxygen Availability on the Physiology of *P. pastoris* during recombinant DAAO Production, and effects on Enzymatic Activity

5.1 *Introduction*

The reduction of molecular O_2 can be toxic to all organisms. O_2 is continuously reduced to two H_2O molecules during cellular respiration. The constant cycle requires four electrons; partial reduction of O_2 nevertheless results in the generation of powerful oxidants, called Reactive Oxygen Species (ROS) including Superoxide ($O_2^{\cdot-}$), Hydrogen Peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). Previous research carried out by Voulgaris *et al, (2011)* suggested that oxidative stress (the imbalance between the formation of ROS and limited antioxidant defence) was the root cause of reduced enzymatic activity of an industrially important amine oxidase, Monoamine Oxidase (MAO) produced in fed batch *E. coli* fermentations. The fed batch process utilised by Voulgaris *et al* (2011) resulted in toxic yields of the by-product acetate, and to overcome this they used oxygen enrichment to abate the production of acetate which, as mentioned previously, resulted in the low MAO activities.

In Chapter Five, the effects of oxygen availability upon the physiology of *P. pastoris (*and subsequent enzymatic activities of DAAO) were investigated. The investigations were carried out with dissolved oxygen tensions of 5 % (critically anaerobic), 30 % and 40 % (highly aerobic) achieved with O_2 enrichment. Also investigated was the effect of oxygen availability upon the physiology of a *P. pastoris* strain engineered to overexpress an amine oxidase called Monoamine Oxidase, and the enzymatic activities of MAO.

Due to time constraints, only samples of DAAO from *P. pastoris* were then sent for mass spectrometry in order to understand the structural changes to the enzyme under highly oxygen rich conditions.

5.2 *Results (D-Amino Acid Oxidase Strain)*

5.2.1 *P. pastoris Fed Batch – Dissolved Oxygen of 40 % with Oxygen Enrichment*

In this experiment, the dissolved oxygen tension was maintained at 40 % and was achieved by sparging the reactor vessel with pure oxygen as required in order to ascertain how an excess of oxygen affects cell physiology and enzyme activity. This fermentation was performed using a MeOH/Sorbitol Co-Feed of 1:1.

In Figure 5-1 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 41.395 g/L at 36 hours. The MeOH/Sorb co-feed was begun subsequently. 3 hours after the start of the MeOH/Sorb co-feed biomass increased to 55.73 g/L and increased to 72.70 g/L 11 hours after the start of the co-feed. For the remaining 35 hours of the co-feed, the biomass slowly accumulated to a final concentration of 82.23 g/L. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.2 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.08 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 8 hours process time the dissolved oxygen reached its pre-set level of 40 %. Methanol concentration never exceeded 5 g/L throughout the process.

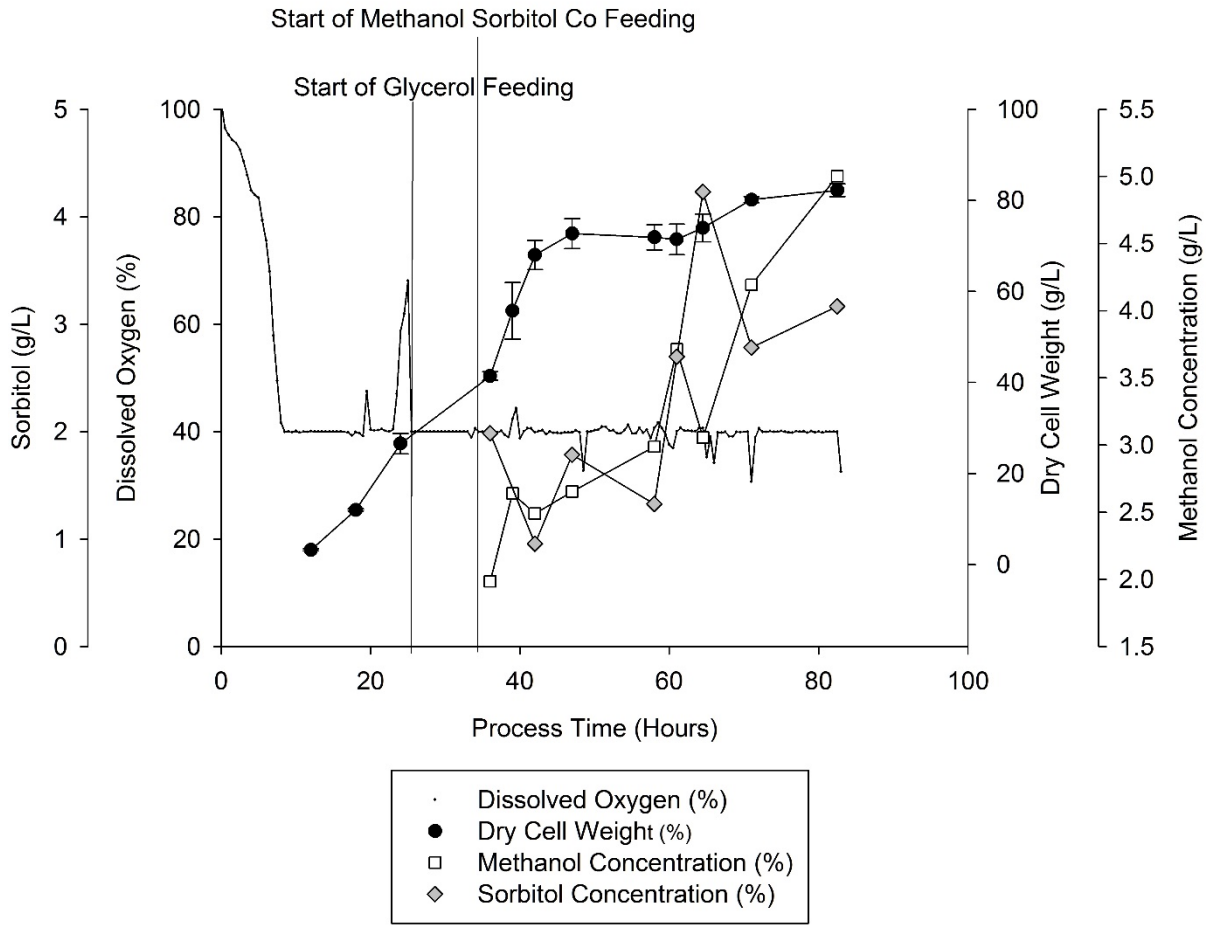

Figure 5-1. Time profiles of the dissolved oxygen, biomass, sorbitol and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1].. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 40 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 5-1 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction with the specific and total activities peaking at 395.62 μmol/g DCW min and 26901.80 μmol/L/min. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 7.35 μmol/g DCW/min and 50.14 μmol/L/min respectively. From this time point until 22 hours post induction, both specific and total activities began to drop, measuring 144.30 μmol/g DCW/min and 10307.70 μmol/L/min respectively at this time point. 25 hours after the start of co-feeding specific and total activities of the culture exhibited increased slightly to 204.00 μmol/g DCW/min and 15089.3 μmol/L/min respectively. Both specific and total activities again decreased after this time point and at the end of the process (47 hours post induction) the specific activity measured 161.50 μmol/g DCW/min and the total 13280.50 μmol/L/min.

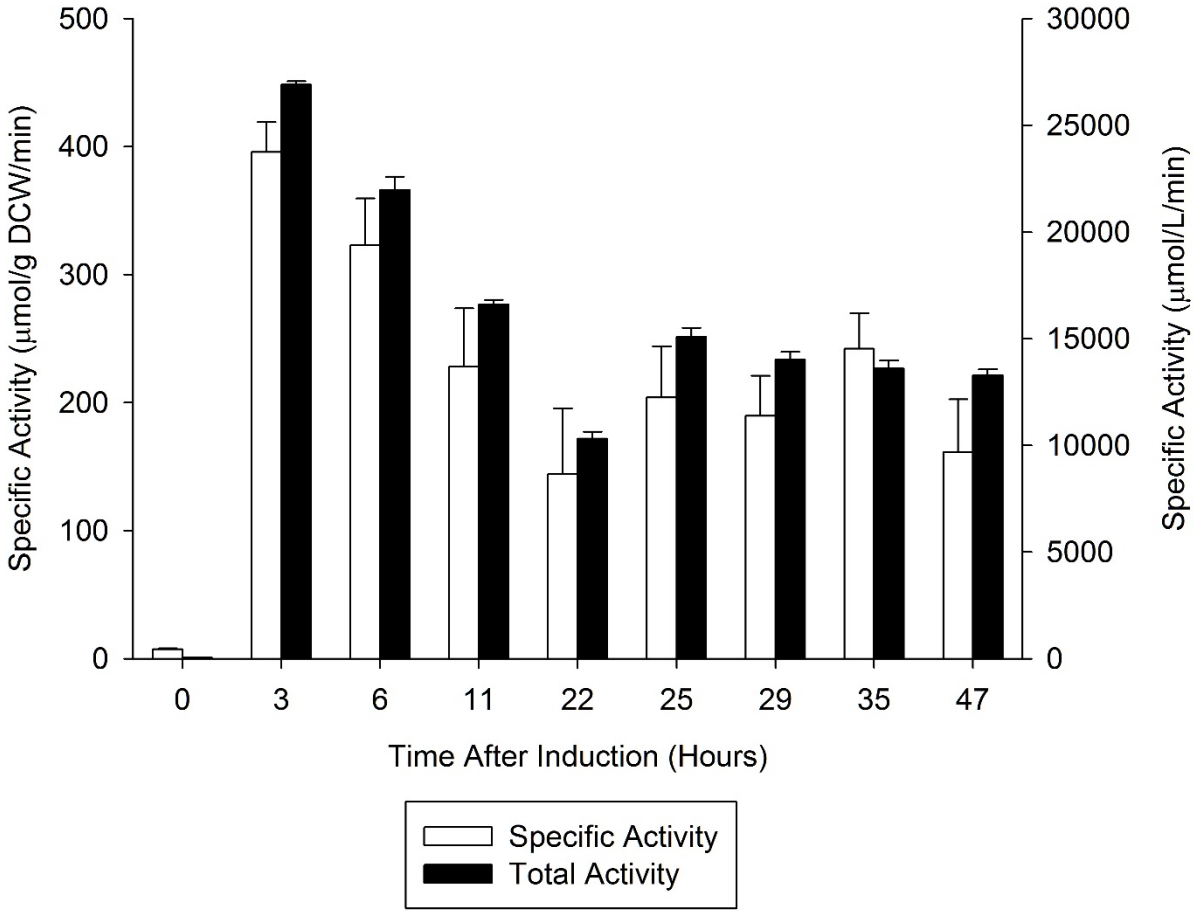

Figure 5-2. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a MeOH Sorbitol co feed [1:1]. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 40 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.2.2 *P. pastoris Fed Batch – Dissolved Oxygen of 5 %*

In this experiment, the dissolved oxygen tension was maintained at 5 % and was achieved by sparging the reactor vessel with pure oxygen as required in order to ascertain how an excess of oxygen affects cell physiology and enzyme activity. This fermentation was performed using a MeOH/Sorbitol Co-Feed of 1:1.

In Figure 5-3 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 57.60 g/L at 38 hours. The MeOH/Sorb co-feed was begun at 40 hours. 2 hours after the start of the MeOH/Sorb co-feed biomass increased to 65.10 g/L. For the remaining 50 hours of the co-feed, the biomass slowly accumulated to a final concentration of 81.60 g/L. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.5 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.06 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 11.5 hours process time the dissolved oxygen reached its pre-set level of 5 %. Methanol concentration never exceeded 5 g/L throughout the process.

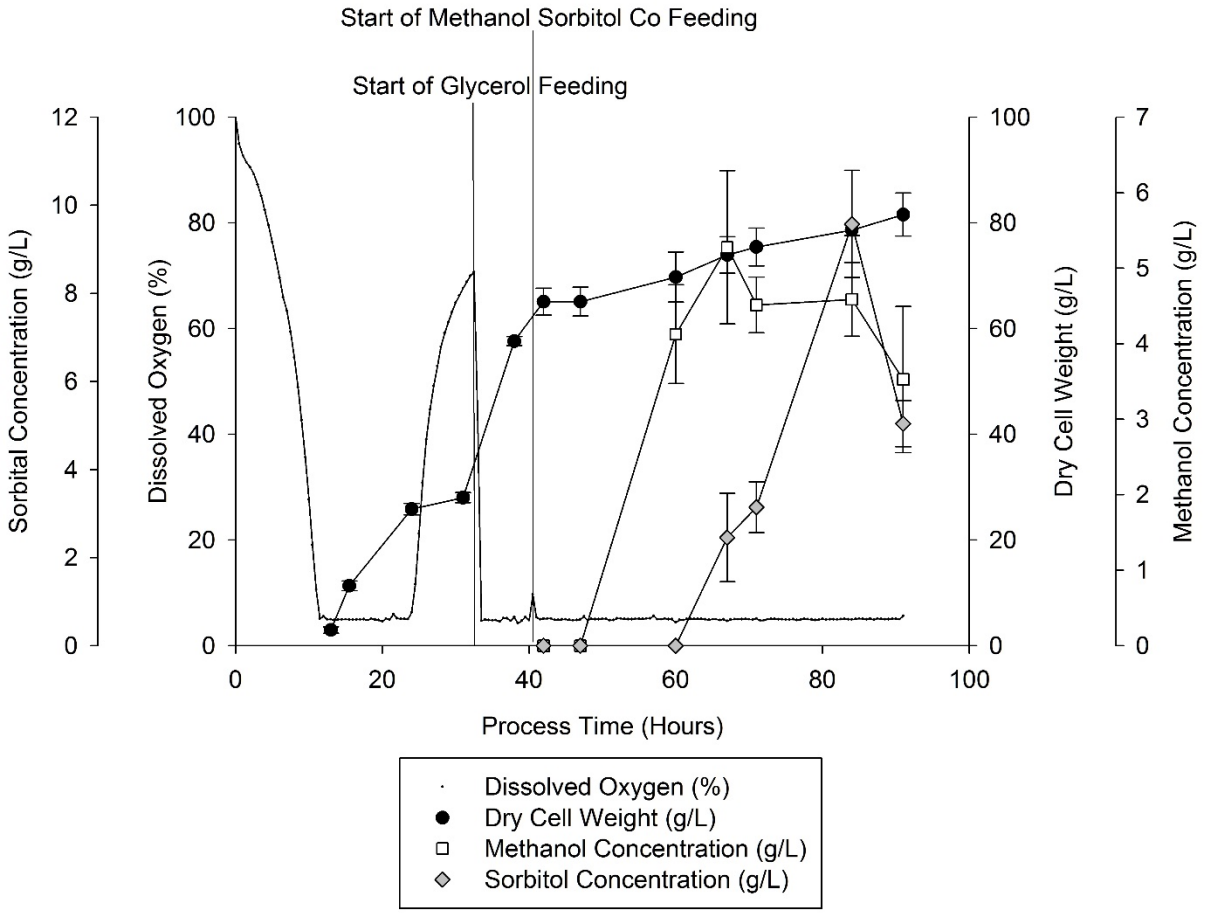

Figure 5-3. Time profiles of the dissolved oxygen, biomass, sorbitol and methanol concentrations from growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 5 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 5-4 it can be seen that both specific and total D-amino acid oxidase activities increased after the commencement of methanol induction with the specific and total activities peaking at 80.42 μmol/g DCW min and 4643.61 μmol/L/min. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 4.80 μmol/g DCW/min and 33.66 μmol/L/min respectively. From this time point until 22 hours post induction, both specific and total activities began to increase further, measuring 236.84 μmol/g DCW/min and 15148.30 μmol/L/min respectively at this time point. 25 hours after the start of co-feeding specific and total activities of the culture exhibited decreased to 138.56 μmol/g DCW/min and 9666.55 μmol/L/min respectively. Both specific and total activities again decreased after this time point and at the end of the process (50 hours post induction) the specific activity measured 138.19 μmol/g DCW/min and the total 10866.10 μmol/L/min.

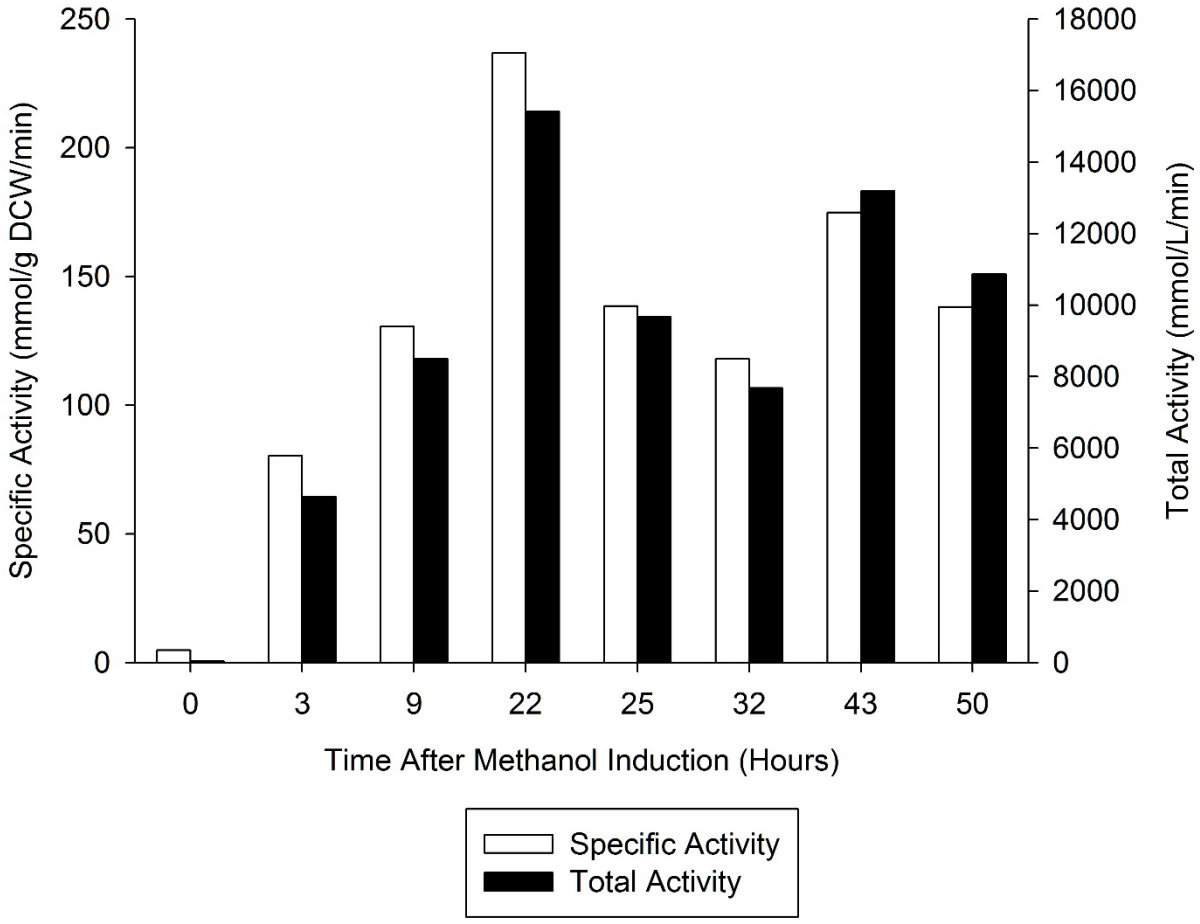

Figure 5-4. Time Profiles of the Specific and Total Activities of the DAAO Enzyme produced using P. pastoris ING005-A9-1-MCB1 with a MeOH Sorbitol co feed [1:1]. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 5 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.2.3 *Presence of Anti-Oxidant Enzymes and Protein Oxidation Markers*

5.2.3.1 *Protein Carbonylation*

Protein Carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. It usually refers to a process that forms reactive ketones or aldehydes that can be reacted by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones (Section 2.3.11). Direct oxidation of side chains of lysine, arginine, proline, and threonine residues, among other amino acids, in the primary protein Carbonylation reaction produces DNPH detectable protein products. This was used as a simple method to detect the levels of intracellular protein carbonylation to give an indication of how oxygen availability might be affecting the production of DAAO and other proteins. Samples from 5 % and 40 % fermentations were analysed. Samples from the 1:1 MeOH/Sorbitol co-feed in Chapter 4 were also analysed for intracellular protein Carbonylation as a comparison for an oxygen availability of 30 %.

Figure 5-5 shows the protein carbonyl content of the *P. pastoris* cultures grown with dissolved oxygen settings of 5 %, 30 % and 40 %. With each subsequent increase of oxygen availability, there is an increase in the presence of protein carbonyls, highest at 40 % pO_2 and lowest at 5 % pO_2. Protein Carbonylation is somewhat hard to induce, though usually associated with a reduction in protein function; proteins which have been carbonylated are often damaged beyond repair and can only be removed by the action of proteolytic enzymes which were subsequently measured in this series of experiments (Section 2.3.12). The results can be seen in Figure 5-6 The intracellular proteolytic activity increases with each increase in oxygen availability, with the 40 % pO_2 experiment having the highest activities overall.

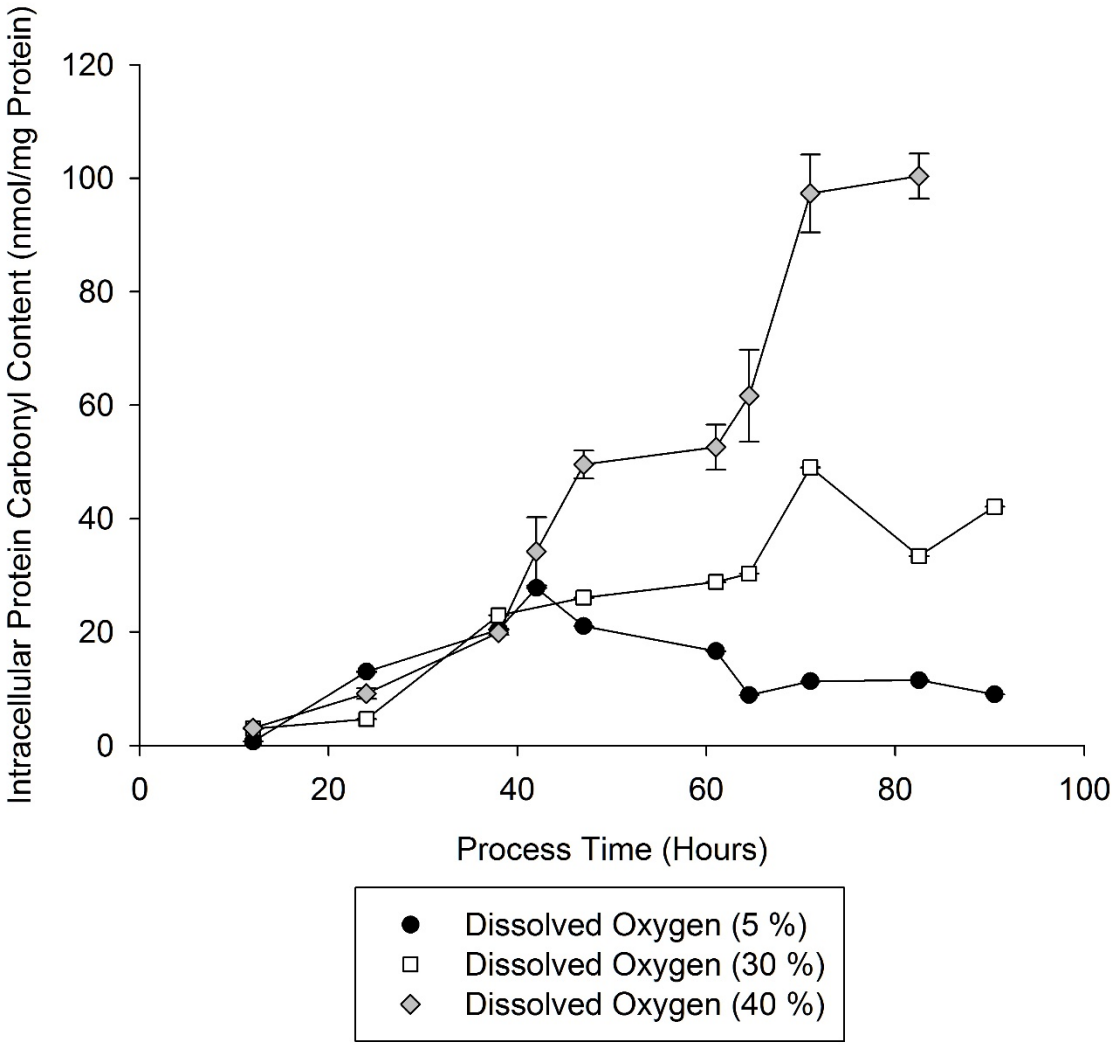

Figure 5-5. Intracellular Protein Carbonyl Content of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population. Intracellular protein carbonyl content for 30 % dissolved oxygen analysed from Chapter 4.

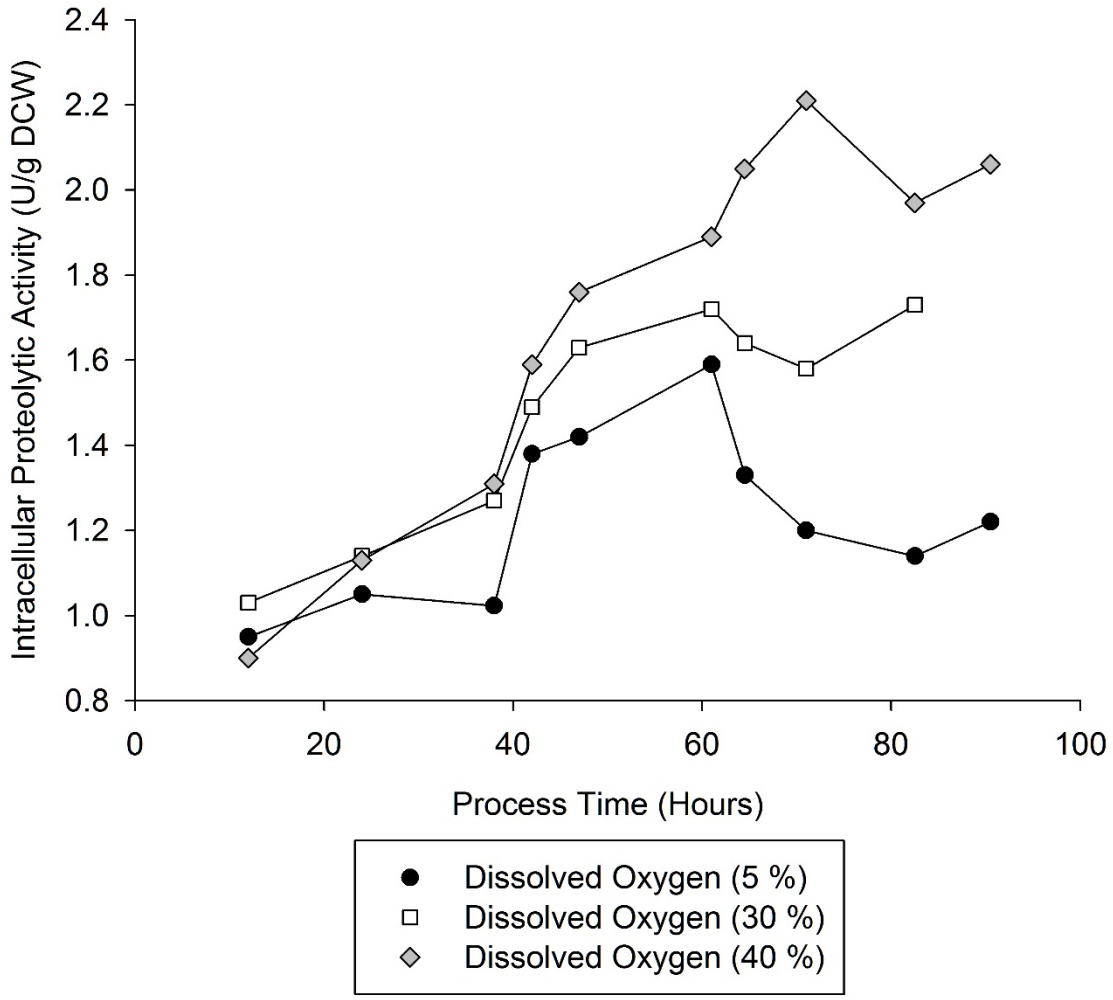

Figure 5-6. Intracellular Proteolytic Activity of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population. Intracellular protein carbonyl content for 30 % dissolved oxygen analysed from Chapter 4.

5.2.3.2 *Catalase (Cat)*

Catalase is an intracellular enzyme which degrades H_2O_2 in to H_2O in order to protect cells from damage through oxidative stress and it is one of the main antioxidant enzymes. H_2O_2 is an inevitable by-product of all living organisms that gain energy through the process of respiration when glucose and oxygen are degraded. Another potential source of H_2O_2 is enzyme derived: both MAO and DAAO can produce H_2O_2. The mitochondria is the key source of H_2O_2 production. Inside the mitochondria, there is interminable generation of the superoxide anion caused by electron leakage, and inadequate reduction of the superoxide anion gives rise to H_2O_2. The production of catalase was monitored at during the 5 %, 30 % and 40 % fermentations to ascertain how this correlated with any reduction in enzymatic activities. Results can be seen in Figure 5-7.

As can be seen, the highest catalase activities were found when the oxygen availability was high (40 %), and lowest when under oxygen limited conditions which indicates there is a high turnover of ROS at 40 % pO_2.

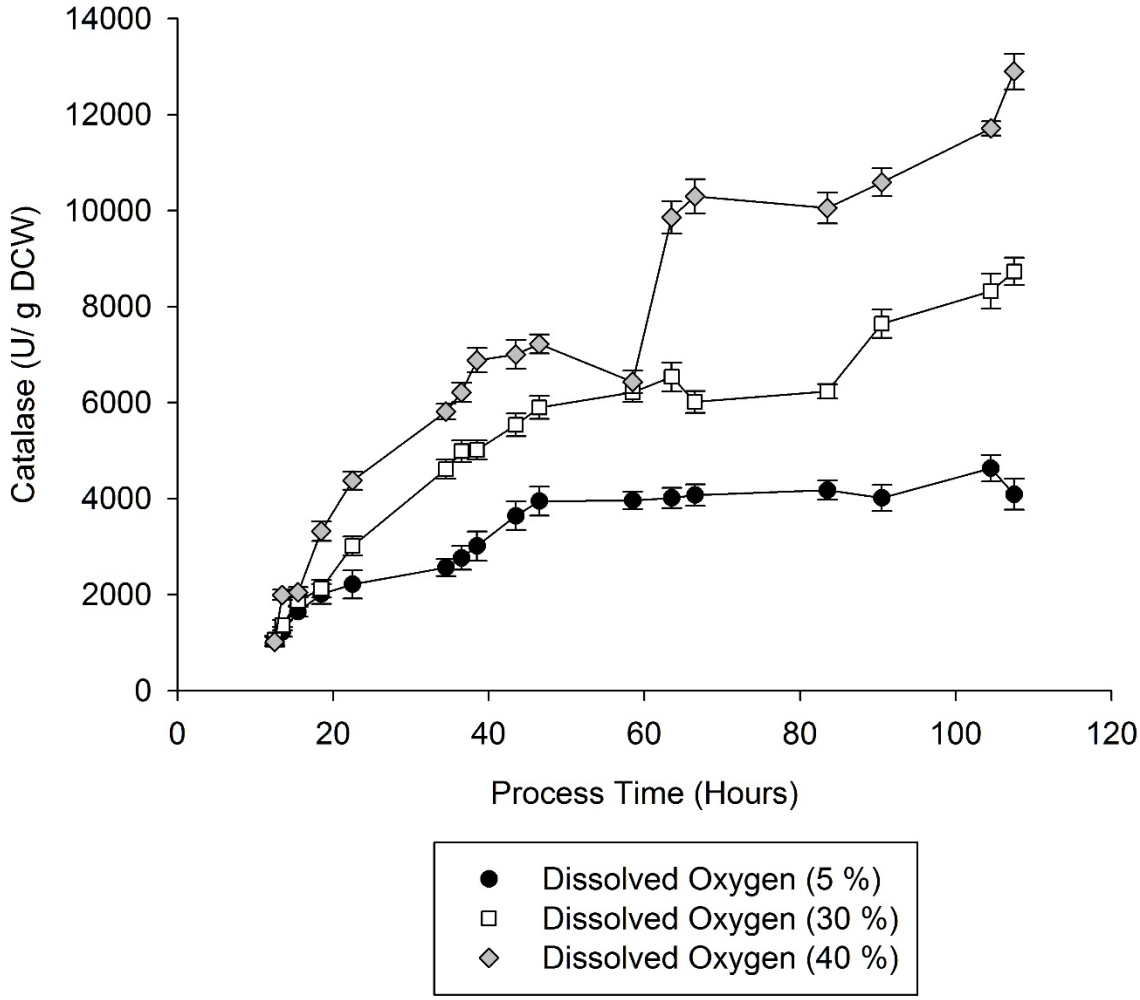

Figure 5-7. Catalase activity of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population. Intracellular protein carbonyl content for 30 % dissolved oxygen analysed from Chapter 4.

5.2.3.3 *Superoxide Dismutase (SOD)*

Superoxide Dismutase (SOD) is the enzyme responsible for catalysing the conversion of $O_2^{\cdot-}$ in to H_2O_2. To ascertain the levels of $O_2^{\cdot-}$ in the fermentations, SOD activity was determined. This gives some insight in to the role it may play in the damage to the DAAO enzyme. The results can be seen in Figure 5-8.

SOD levels for both the 30 % pO_2 and 40 % pO_2 processes are relatively similar, though slightly higher for the latter. At 5 % pO_2 the SOD are much lower, suggesting $O_2^{\cdot-}$ generation is far reduced than at 30 % pO_2 and 40 % pO_2.

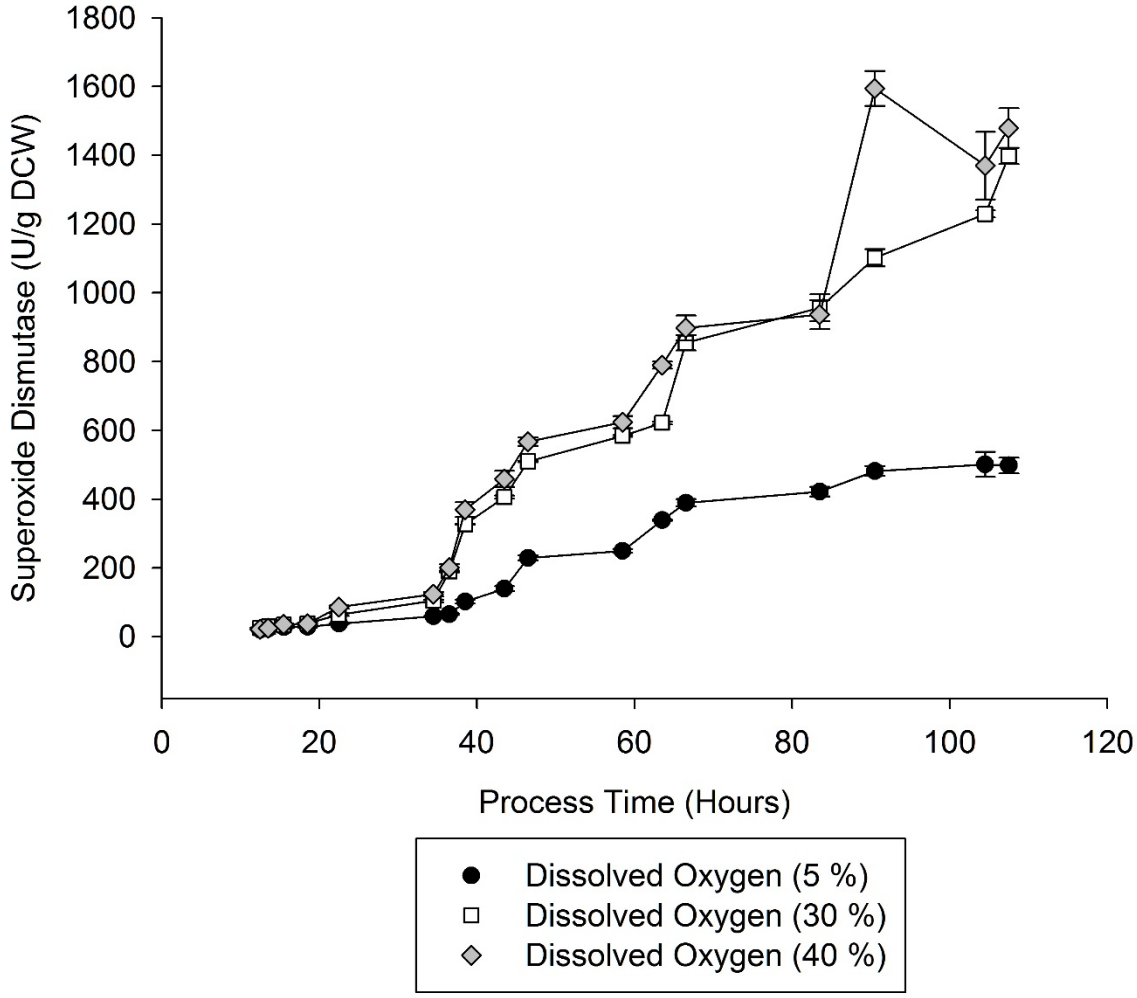

Figure 5-8. Superoxide Dismutase Activity of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population. Intracellular protein carbonyl content for 30 % dissolved oxygen analysed from Chapter 4.

5.2.3.4 *Ethanol and Acetate Production*

P. pastoris can produce ethanol as a by-product during the glycerol fed batch, and mixed feed induction stage of a typical, high cell density fermentation, regardless of the phenotype of the strain. Ethanol can be converted to acetate, and acetate can subsequently block the AOX1 promoter. Ethanol production can be quite prevalent when *P. pastoris* is grown under oxygen limiting conditions. Acetate concentrations during the fermentations performed under varying pO2 concentrations were measured and can be seen in Figure 5-9. Ethanol concentrations could not be quantified.

Acetate accumulation is very high at 5 % pO2 suggesting that ethanol is being produced at a higher rate under highly anaerobic conditions, and is subsequently being converted to acetate. A well understood method of reducing acetate formation is via oxygen enrichment, and this correlates with the very low acetate concentrations at 30 % pO_2 and 40 % pO_2.

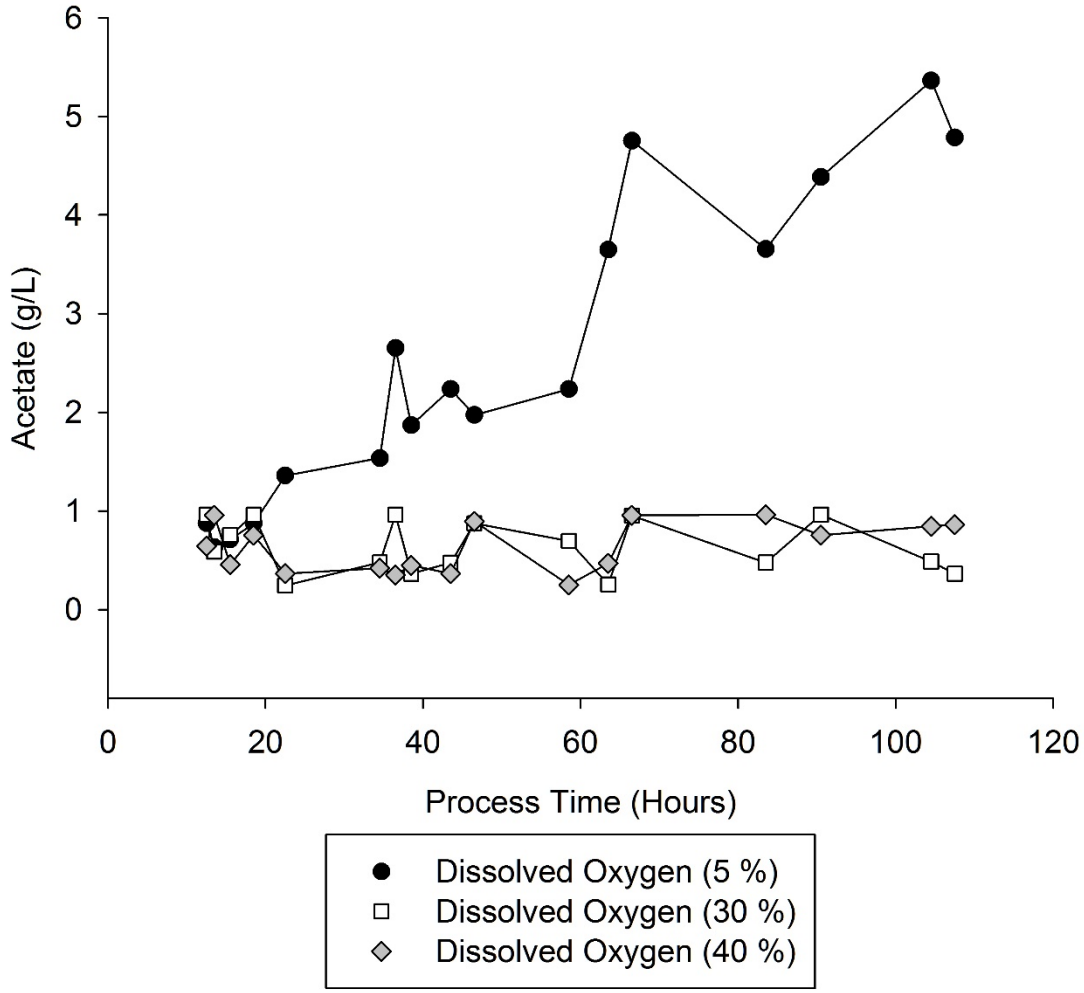

Figure 5-9. Acetate Concentrations of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population. Intracellular protein carbonyl content for 30 % dissolved oxygen analysed from Chapter 4.

5.2.4 *Tandem Sequencing (MS/MS) Mass Spectrometry of Protein Samples*

In order to perform Tandem Sequencing (MS/MS) Mass Spectrometry protein samples had to be run on a protein gel. *TvDAAO* has a molecular weight (mw) of approximately 37kD to 40 kD and thus molecular weight markers (MWM) in the range of 10 kD to 150 kD were run alongside the protein samples. The results of this can be seen in Figure 5-10. The protein sample from the 5 % pO2 fermentation has bands which appear in the 37 kD range indicating the presence of *TvDAAO.* The protein sample from the 40 % pO2 fermentation has bands which appear out with the 37 kD but this does not mean the protein isn't present; the MW of proteins can be altered if the structure has been altered/damaged for e.g. by oxidation. Since these bands were the only ones close to the 37 kD range, these were used for MS analysis.

Analysis was performed by Dr. Gemma Warren at Aston University, Birmingham as a paid service.

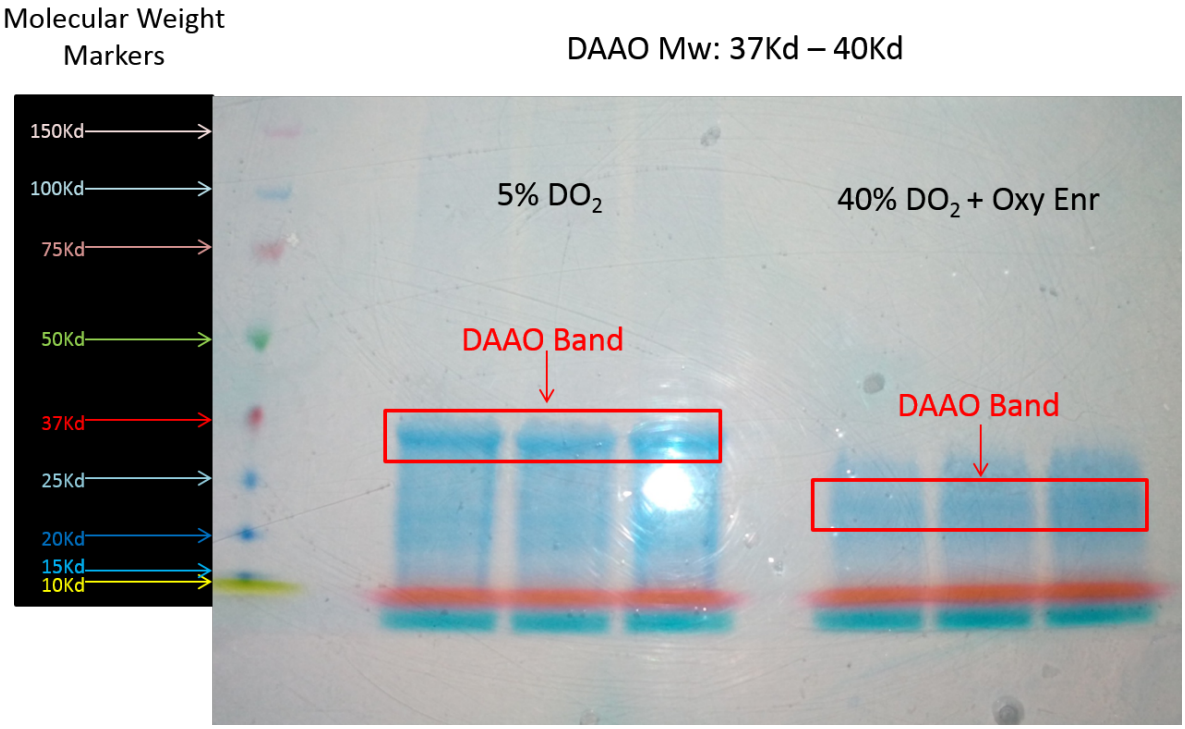

Figure 5-10. SDS-PAGE with DAAO Samples and Molecular Weight Markers obtained from growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate.

5.2.4.1 *Results from Mascot (Mass Spec Analysis)*

(A) Summary

D-amino-acid oxidase from Trigonopsis variabilis (OXDA_TRIVR) was the most abundant protein identified in all samples, with an average of 48% sequence coverage.

Two methionine oxidations were identified by Mascot analysis and confirmed by sequencing. The amount of modified vs. unmodified peptide was calculated to give an estimate of the percentage of modification.

(B) Comments

The estimated concentration of DAAO in the 5% and 40% samples was different which might affect the ability to detect the same peptides in both samples. The samples were analysed for the presence of oxidation of MWHP, dioxidation of CW, trioxidation of C, Lys->Allysine. Search parameters were widened to try and find additional modifications. Some were found in one or two samples but there were no other modifications that were present in all samples.

(C) Sequence of DAAO

Matched peptides shown in bold, peptides containing modifications shown in red, modified amino acids underlined.

```
1   MAKIVVIGAG VAGLTTALQL LRKGHEVTIV SEFTPGDLSI GYTSPWAGAN
51  WLTFYDGGKL ADYDAVSYPI LRELARSSPE AGIRLISQRS HVLKRDLPKL
101 EVAMSAICQR NPWFKNTVDS FEIIEDRSRI VHDDVAYLVE FRSVCIHTGV
151 YLNWLMSQCL SLGATVVKRR VNHIKDANLL HSSGSRPDVI VNCSGLFARF
201 LGGVEDKKMY PIRGQVVLVR NSLPFMASFS STPEKENEDE ALYIMTRFDG
251 TSIIGGCFQP NNWSSEPDPS LTHRILSRAL DRFPELTKDG PLDIVRECVG
301 HRPGREGGPR VELEKIPGVG FVVHNYGAAG AGYQSSYGMA DEAVSYVERA
351 LTRPNL
```

(D) Peptide 1

Peptide sequence: ENEDEALYI**M**TR

Modification: Met245 Oxidation (highlighted in sequence above)

In most samples both a 2+ and a 3+ charge state was detected and analysed.

Average % modification in 5% DO samples: 15%

In the samples treated with 40% DO XICs could not be calculated for the unmodified peptide. In some samples the unmodified peptide could not be detected either by Mascot or in the raw data. In the samples where it could be detected XICs could not be calculated due to overlapping peaks.

(E) Peptide 2

Peptide sequence: NSLPFMASFSSTPEK

Modification: Met226 Oxidation (highlighted in sequence above)

Average% modification in 5% DO samples: 60%

Average modification in 40% DO samples: 55%

The unmodified peptide could only be identified in two of the three 40% samples

A potential tryptophan oxidation and dioxidation was identified but it was only present in two of the 5% DO samples so couldn't be analysed further.

5.3 *Results (Monoamine Oxidase Strain)*

The aim of this section was to determine the effects of varying oxygen concentrations on the physiology and enzyme activity in a different strain of *P. pastoris* and a structurally similar enzyme to DAAO. MAO had previously been found to be oxidised under high oxygen environments.

5.3.1 *P. pastoris Fed-Batch – Dissolved Oxygen of 40 % with Oxygen Enrichment*

In this experiment, the dissolved oxygen tension was maintained at 40 % and was achieved by sparging the reactor vessel with pure oxygen as required in order to ascertain how an excess of oxygen affects cell physiology and enzyme activity. This fermentation was performed using a MeOH/Sorbitol Co-Feed of 1:1.

In Figure 5-11 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 63 g/L at 24 hours. The MeOH/Sorb co-feed was begun subsequently. 2 hours after the start of the MeOH/Sorb co-feed biomass increased to 65 g/L and increased to 74.70 g/L 12 hours after the start of the co-feed. For the remainder of the co-feed, the biomass slowly accumulated to a peak concentration of 94.60 g/L. After the start of methanol induction the specific growth rate decreased to 0.006 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 7 hours process time the dissolved oxygen reached its pre-set level of 40 %. Methanol concentration never exceeded 5 g/L throughout the process.

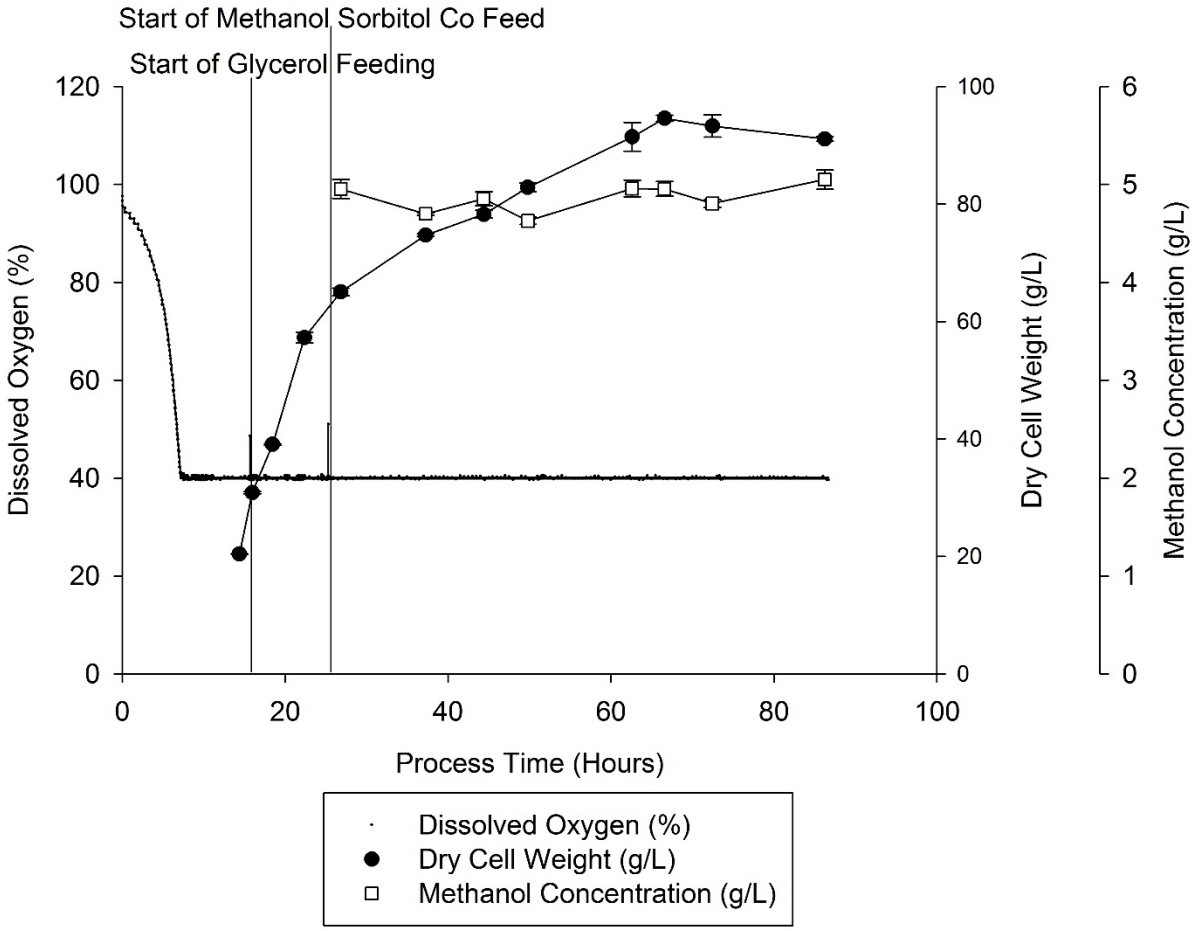

Figure 5-11. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris MutS CBS 7435 His MAO-5N. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = 40 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

In Figure 5-12 it can be seen that both specific and total Monoamine oxidase activities increased after the commencement of methanol induction with the specific and total activities peaking at 173.92 μmol/g DCW min and 16000.00 μmol/L/min 12 hours after induction. Pre-induction specific and total activities of the culture, used as a baseline, exhibited were 1.39 μmol/g DCW/min and 79.37 μmol/L/min respectively. Both specific and total activities again decreased after 12 hours of induction and at the end of the process (61 hours post induction) the specific activity measured 108.43 μmol/g DCW/min and the total 8100.00 μmol/L/min.

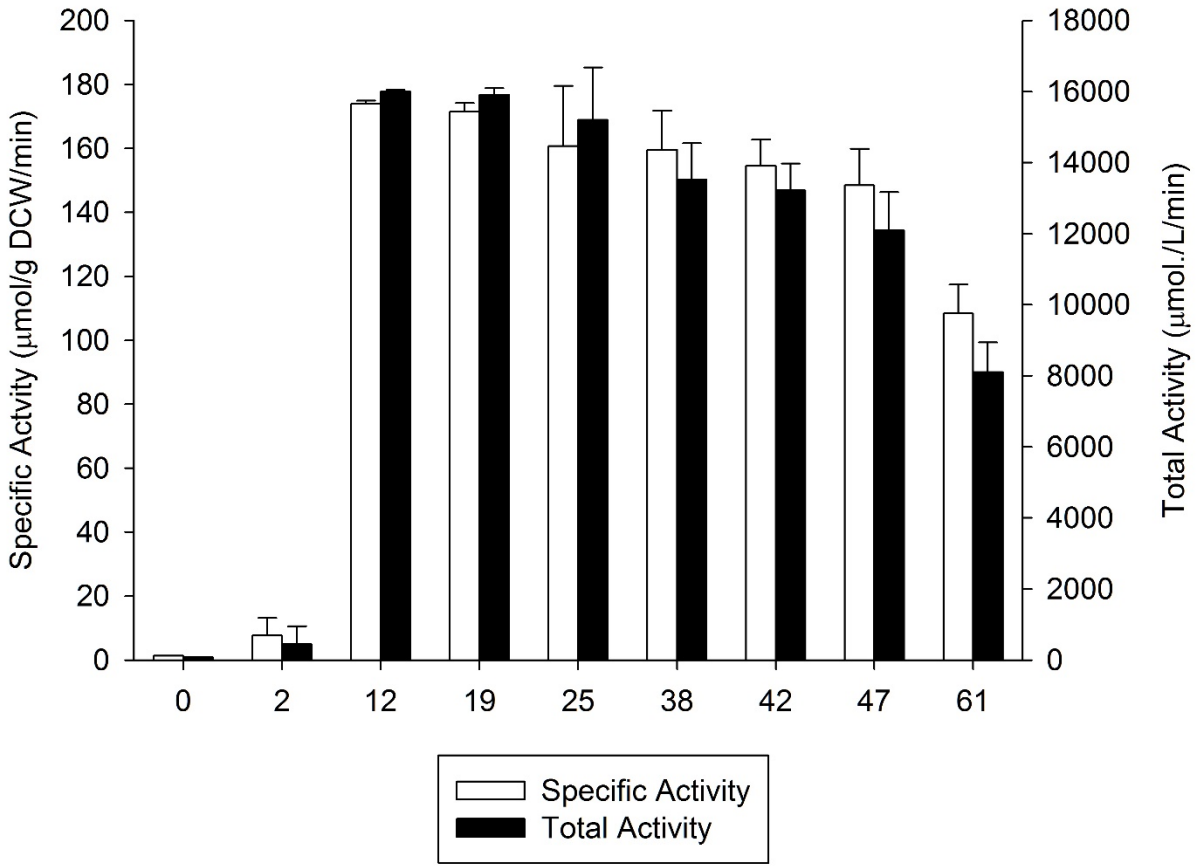

Figure 5-12. Time Profiles of the Specific and Total Activities of the MAO Enzyme produced using P. pastoris MutS CBS 7435 His MAO-5N. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 40 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.3.2 *P. pastoris Fed-Batch – Dissolved Oxygen of 5%*

In this experiment, the dissolved oxygen tension was maintained at 5 % and was achieved by sparging the reactor vessel with pure oxygen as required in order to ascertain how an excess of oxygen affects cell physiology and enzyme activity. This fermentation was performed using a MeOH/Sorbitol Co-Feed of 1:1.

In Figure 5-13 it can be seen that the biomass concentration very rapidly increased during the glycerol feeding, leading to a biomass concentration of 57.3 g/L at 23 hours. The MeOH/Sorb co-feed was immediately. 15 hours after the start of the MeOH/Sorb co-feed biomass increased to 63.20 g/L. Biomass peaked at 66 g/L 21 hours after induction. The specific growth rate after the start of the glycerol fed-batch but before methanol feeding was 0.09 h^{-1}. After the start of methanol induction the specific growth rate decreased to 0.001 h^{-1}. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 8.5 hours process time the dissolved oxygen reached its pre-set level of 5 %. Methanol concentration never exceeded 5 g/L throughout the process.

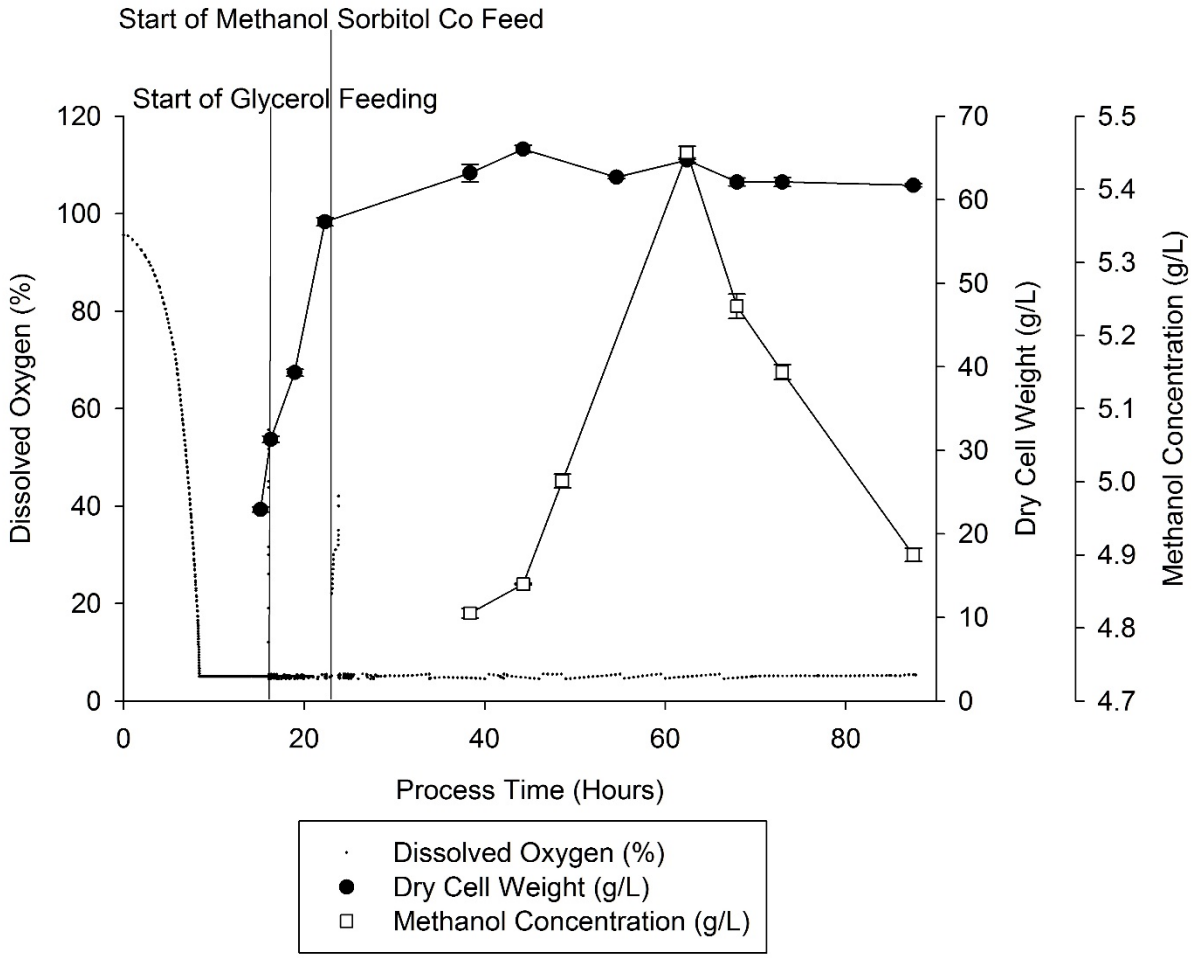

Figure 5-13. Time profiles of the dissolved oxygen, biomass and methanol concentrations from growing P. pastoris MutS CBS 7435 His MAO-5N. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point 5 %, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

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In Figure 5-14 it can be seen that both specific and total monoamine oxidase activities increased for 21 hours after the commencement of methanol induction with the specific and total activities peaking at 169.5 μmol/g DCW min and 11198.00 μmol/L/min. From this time point until 22 hours post induction, both specific and total activities began to decrease and at the end of the process (64 hours post induction) the specific activity measured 119.64 μmol/g DCW/min and the total 7385.71 μmol/L/min.

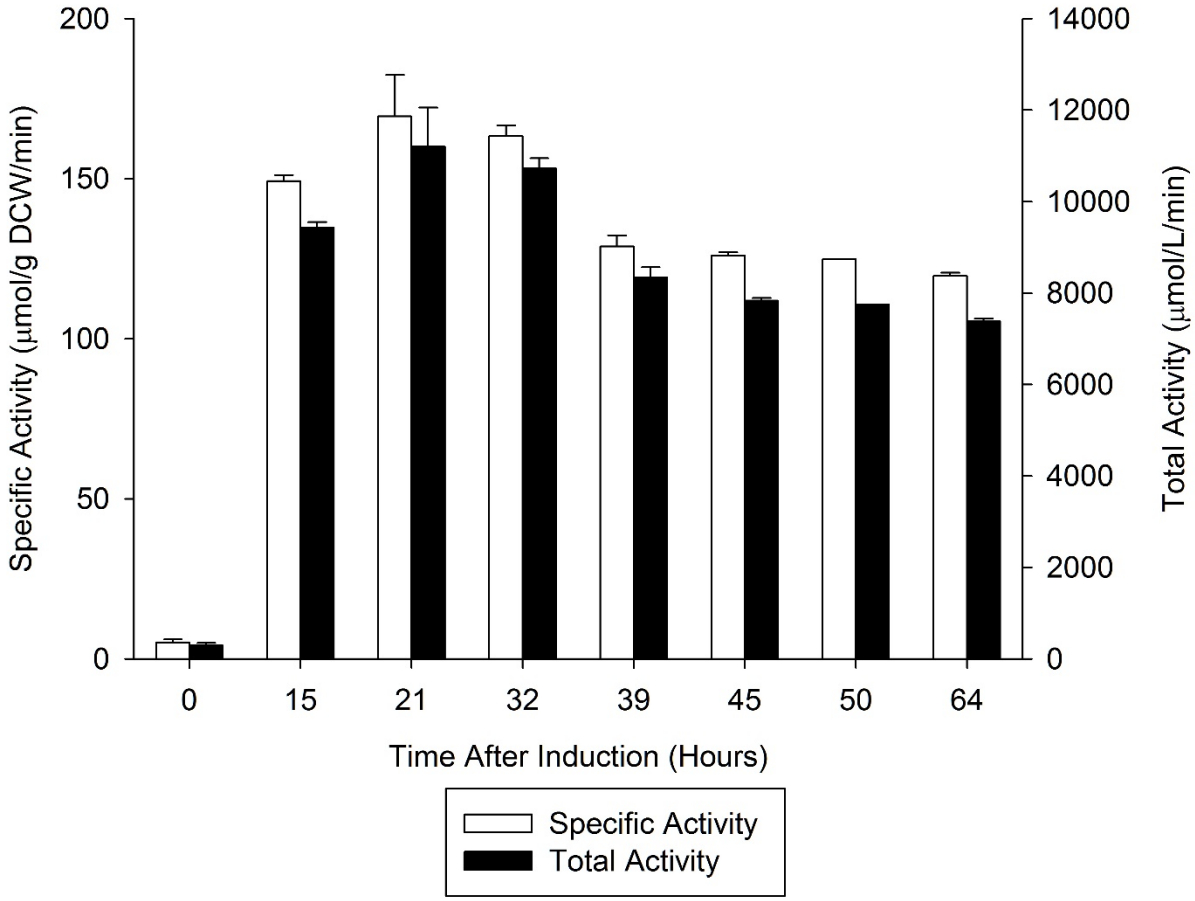

Figure 5-14. Time Profiles of the Specific and Total Activities of the MAO Enzyme produced using P. pastoris MutS CBS 7435 His MAO-5N. Bioreactor cultivations,30 °C, pH 5.8, DO2 Set point = 5 %, 300 – 900 rpm and 1 - 3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.3.3 *Presence of Anti-Oxidant Enzymes and Protein Oxidation Markers*

5.3.3.1 *Protein Carbonylation*

Protein Carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. It usually refers to a process that forms reactive ketones or aldehydes that can be reacted by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Direct oxidation of side chains of lysine, arginine, proline, and threonine residues, among other amino acids, in the primary protein Carbonylation reaction produces DNPH detectable protein products. This was used as a simple method to detect the levels of intracellular protein Carbonylation to give an indication of how oxygen availability might be affecting the production of amine oxidases and other proteins. Samples from 5 % and 40 % fermentations were analysed.

Figure 5-15 shows the protein carbonyl content of the *P. pastoris* ING005-A9-MCB1cultures grown with dissolved oxygen settings of 5 %, 30 % and 40 %. With each subsequent increase of oxygen availability, there is an increase in the presence of protein carbonyls, highest at 40 % pO_2 and lowest at 5 % pO_2. Protein Carbonylation is somewhat hard to induce, though usually associated with a reduction in protein function; proteins which have been carbonylated are often damaged beyond repair and can only be removed by the action of proteolytic enzymes. The intracellular proteolytic activity increases with each increase in oxygen availability, with the 40 % pO_2 experiment having the highest activities overall.

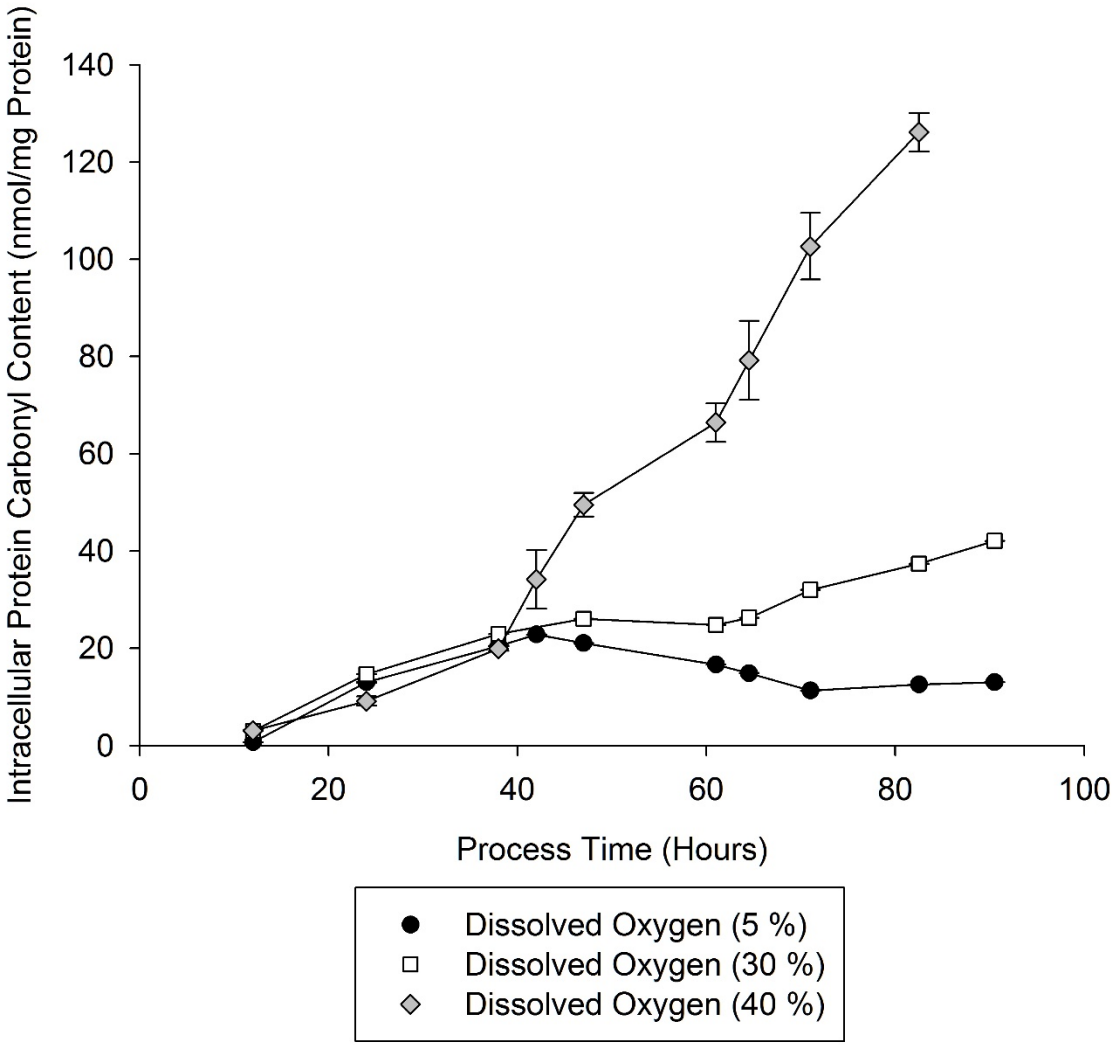

Figure 5-15. Intracellular Protein Carbonyl Content of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.3.3.2 *Catalase (CAT)*

Catalase is an intracellular enzyme which degrades H_2O_2 in to H_2O in order to protect cells from damage through oxidative stress and it is one of the main antioxidant enzymes. H_2O_2 is an inevitable by-product of all living organisms that gain energy through the process of respiration when glucose and oxygen are degraded. Another potential source of H_2O_2 is enzyme derived: both MAO and DAAO can produce H_2O_2. The mitochondria is the key source of H_2O_2 production. Inside the mitochondria, there is interminable generation of the superoxide anion caused by electron leakage, and inadequate reduction of the superoxide anion gives rise to H_2O_2. The production of catalase was monitored at during the 5 %, 30 % and 40 % fermentations to ascertain how this correlated with any reduction in enzymatic activities. Results can be seen in Figure 5-16.

As can be seen, the highest catalase activities were found when the oxygen availability was high (40 %), and lowest when under oxygen limited conditions which indicates there is a high turnover of ROS at 40 % pO_2.

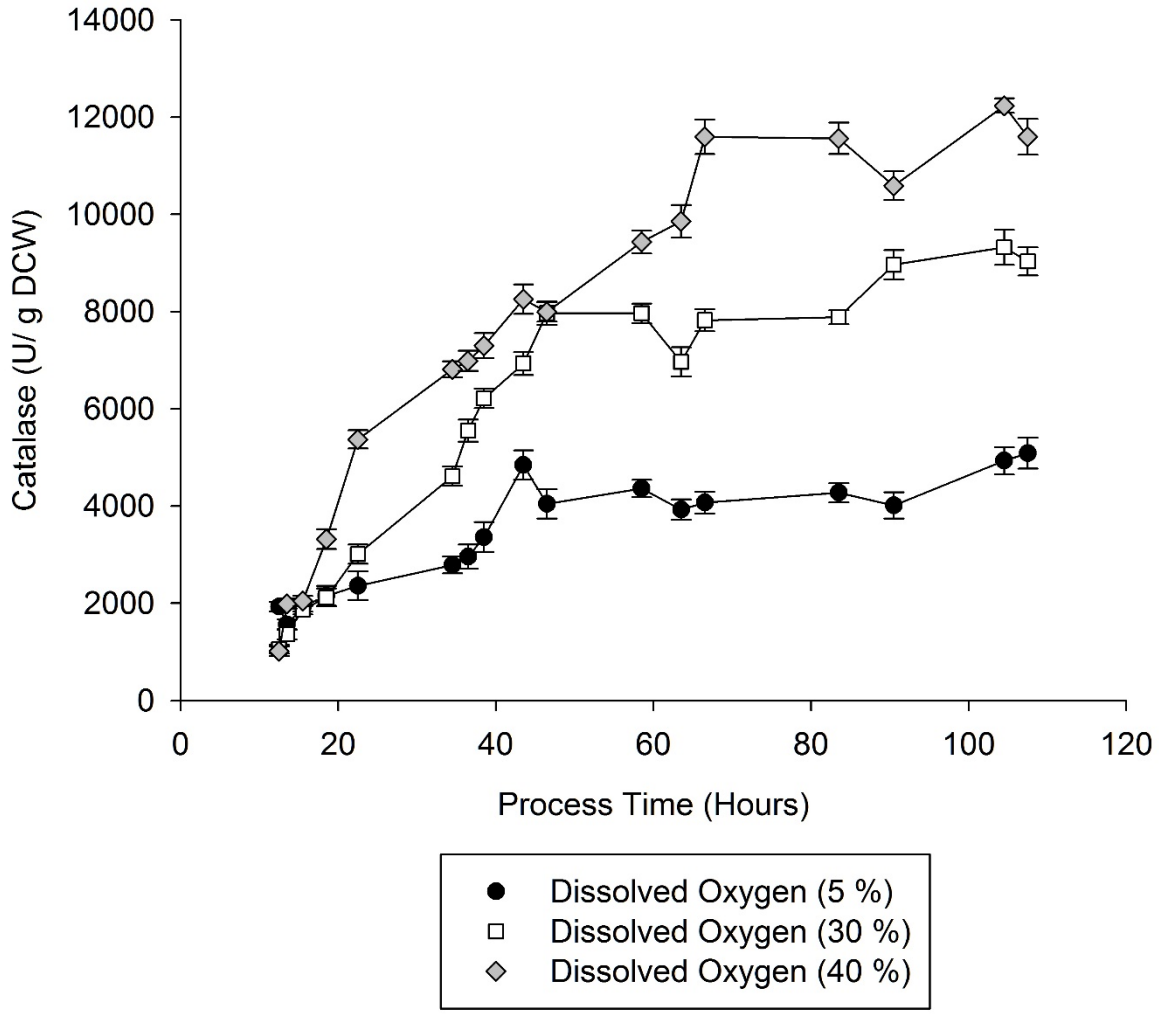

Figure 5-16. of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.3.3.3 *Superoxide Dismutase (SOD)*

Superoxide Dismutase (SOD) is the enzyme responsible for catalysing the conversion of $O_2^{\cdot -}$ in to H_2O_2. To ascertain the levels of $O_2^{\cdot -}$ in the fermentations, SOD activity was determined. This gives some insight in to the role it may play in the damage to the MAO enzyme. The results can be seen in Figure 5-17.

SOD levels for 40 % pO_2 processes are considerably higher than the 30 % pO2 process for the first 80 hours of the process, though towards the end of the process measurements are of a similar level. At 5 % pO_2 the SOD are much lower, suggesting $O_2^{\cdot -}$ generation is far reduced than at 30 % pO_2 and 40 % pO_2.

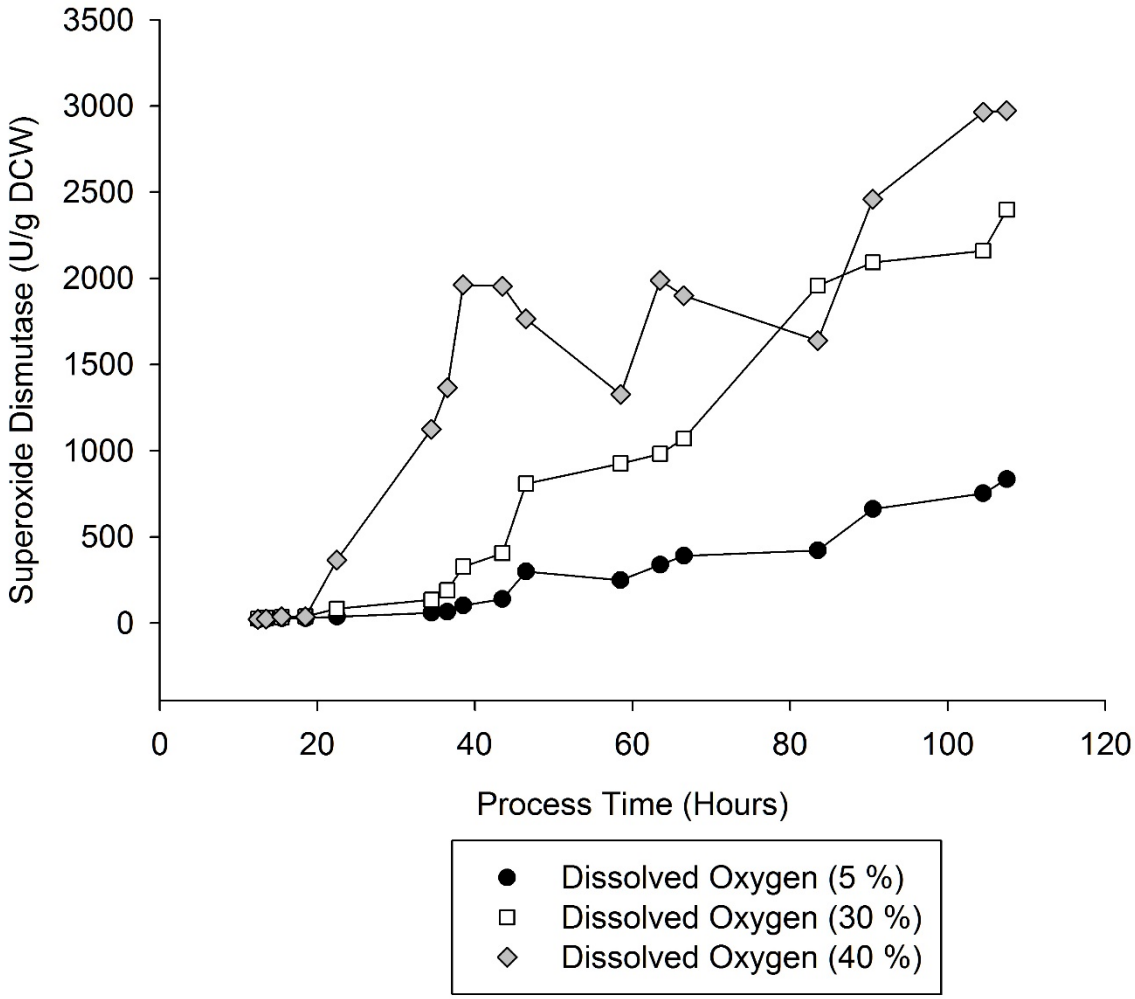

Figure 5-17. Superoxide Dismutase Activity of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.3.3.4 *Ethanol and Acetate Production*

P. pastoris can produce ethanol as a by-product during the glycerol fed batch, and mixed feed induction stage of a typical, high cell density fermentation, regardless of the phenotype of the strain. Ethanol can be converted to acetate, and acetate can subsequently block the AOX1 promoter. Ethanol production can be quite prevalent when *P. pastoris* is grown under oxygen limiting conditions. Acetate concentrations during the fermentations performed under varying pO2 concentrations were measured and can be seen in Figure 5-18. Ethanol concentrations could not be quantified.

Acetate accumulation is very high at 5 % pO2 suggesting that ethanol is being produced at a higher rate under highly anaerobic conditions, and is subsequently being converted to acetate. A well understood method of reducing acetate formation is via oxygen enrichment, and this correlates with the very low acetate concentrations at 40 % pO_2. At both 30 % pO2 and 40 % pO2, acetate measurements remain consistent throughout, and appear to plateau at around 40 hours total process time.

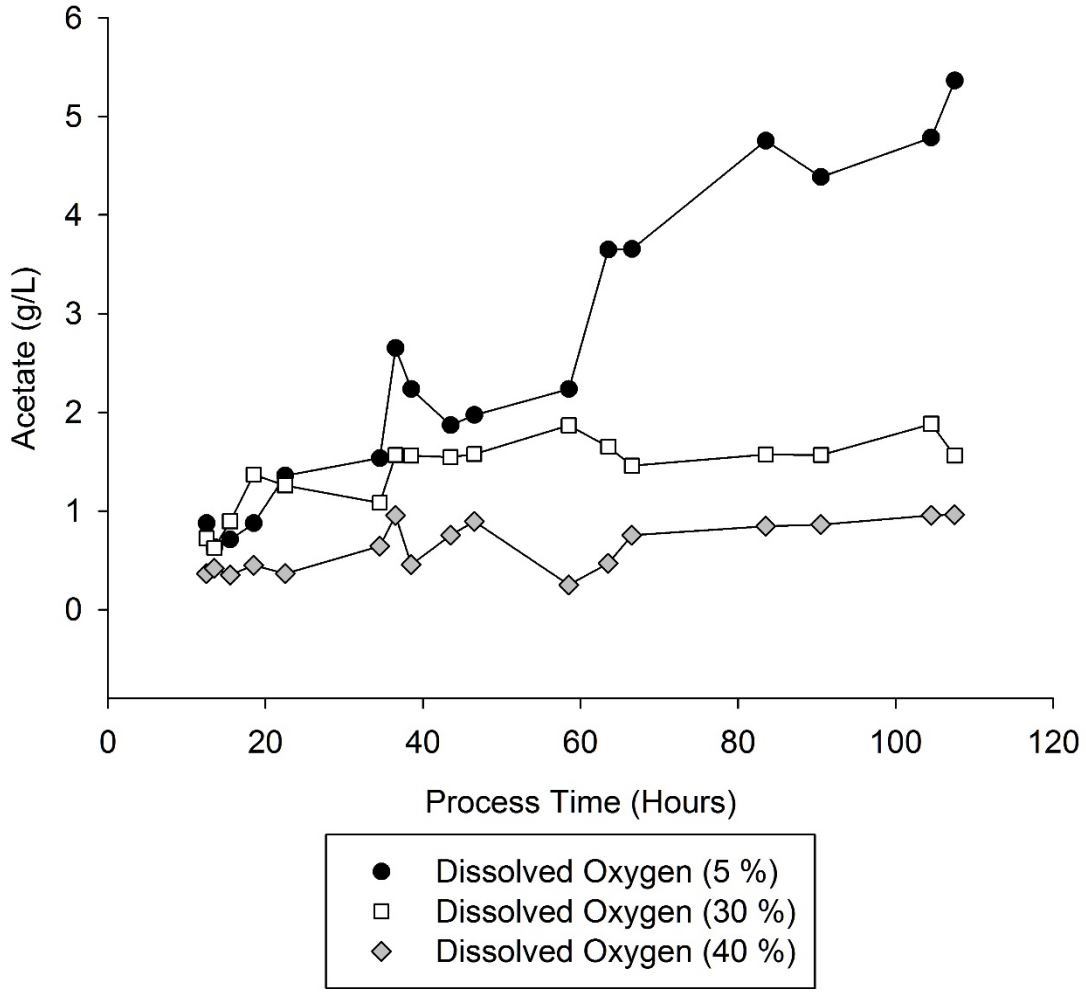

Figure 5-18. Acetate Concentrations of growing P. pastoris ING005-A9-MCB1with a MeOH Sorbitol co feed [1:1]. Culture conditions: Bioreactor cultivations, 30 °C, pH 5.8, DO2 Set point = as indicated, 300 – 900 rpm and 1 -3 vvm cascaded DO_2. All analysis methods were carried out in triplicate. Error bars were calculated from the standard deviation of the sample population.

5.4 *Discussion*

Oxidative stress is a phenomenon caused when there is an imbalance between ROS generation and their subsequent elimination from cells (de Arruda Grossklaus *et al*., 2013). Protein oxidative modifications are an extreme form of post translational modifications resulting from interactions between ROS and amino acids residues of proteins. Said modifications fall under two distinct categories: reversible and irreversible (Cai and Yan, 2013). MAO and DAAO are enzymes which are prone to oxidative damage by ROS as they possesses methionine and cysteine residues which are prone to oxidisation (Imlay, 2013). The role of Met in proteins is not well understood (Kim *et al*., 2014).

Methionine is an essential amino acid which plays a crucial role in numerous biochemical processes, particularly during the initiation of protein synthesis. It is also an important anti oxidising agent in itself (Xipsiti and Nicolaides, 2013). The most critical characteristic of methionine residues in proteins is that it can be reversibly oxidised or reduced (Kim *et al*., 2014). The oxidation of methionine is a major factor in the development and progression of several diseases in higher organisms. For example, the oxidation of the nine methionine residues of the eukaryotic Ca_2^+-sensor calmodulin (CaM), which is responsible for the activation of the membranous adenylyl cyclase 1 (AC1), associated with memory and learning, can ultimately result in neuropathogenicity (Lubker *et al*., 2015). Furthermore, it has been shown that the pathogenesis of cardiovascular disease (CVD) is hastened by the oxidation of methionine residue 148 (M148) of the apolipoprotein A-1 (ApoA-1), a protein that can bind lipids (oil-soluble substances such as fat and cholesterol) to form lipoproteins. ApoA-1 activates lecithin-cholesterol acyltransferase (LCAT) which is a protein coding gene that esterifies cholesterol on high density lipoproteins. LCAT is also essential for reverse cholesterol transport (Yassine *et al*., 2014). The latter study utilised mass spectrometry to measure the post translational modifications (PTM) of proteins and defined the transitions for monitoring the ratio of oxidized M148 to its unmodified peptide in ApoA-I using multiple reaction monitoring (MRM) tandem mass spectrometry (Yassine *et al*., 2014).

Under aerobic conditions, approximately 6 % of protein-bound Met residues can be extemporaneously oxidized though not all Met residues in a protein are equally responsive to oxidation, suggesting that the location of the Met residue in the

structure of the protein can determine its responsiveness to oxidants (Drazic and Winter, 2014). Human Growth Hormone (HGH) is a single chain protein consisting of 191 amino acid residues containing three methionine residues at positions Met^{14}, Met^{125} and Met^{170}; Met^{14} and Met^{125} are partially exposed on the surface of the protein and easily oxidized by chemical treatment with H_2O_2. Conversely, Met^{170} is partially buried and not easily oxidized. It was shown that oxidation of Met^{14} and Met^{125} did not induce conformational changes to the structure of recombinant HGH but did cause enhanced aggregation of the protein at 37 °C (Mulinacci *et al*., 2013).

Based on the MS results performed on the DAAO enzyme in this Chapter, two Met oxidations have been discovered: Met^{245} and Met^{226}. For peptide 1 (Met^{245} oxidation), the amount of unmodified peptide in the 40% pO_2 samples could not be quantified. This was due to it not being identified in the samples. This could mean that the treatment with 40% pO_2 causes the oxidation of the Methionine in the majority of the protein so there is very little unmodified peptide in the samples. However, the absence of the unmodified protein doesn't mean it wasn't present in the sample, just that it wasn't detected by the mass spectrometer.

For peptide 2 (Met^{226} oxidation), there doesn't appear to be a significant difference in the amount of modified peptide in the 5% and 40% pO_2 samples. However the unmodified peptide was not detected in one of the three 40% pO_2 samples, so the average was taken from the two samples where it was detected.

This is a good indication that oxidative modifications to these peptides are responsible for the reduction in enzymatic activities that can be seen between the 30 % pO_2 and 40 % pO_2 oxygen enriched fermentations. The difference between the 5 % and 30 % fermentations is most likely due to the accumulation of acetate in the cultures, as a result of the ethanol production as a by-product; since there were no oxidative modifications found to the peptides when the fermentation was performed with a pO_2 of 5 %. Tryptophan (Try) is one of nine essential amino acids and plays a key role in general protein synthesis, serotonin synthesis and kynurenine production, oxidation of which can cause damage to proteins associated with the pathogenesis of age-related disease (El Refaey *et al*., 2015). Interestingly, a potential tryptophan oxidation and dioxidation was found in the MS analysis of the DAAO enzyme, though further analysis could not be performed. ROS usually attacks Try at the indole ring,

producing several well-known tryptophan oxidant products. These oxidants cannot be removed from the cell via the cellular machinery and more often than not, change the structure of the protein irreversibly, thus affection the functionality of the protein (El Refaey *et al*., 2015, Todorovski *et al*., 2011). There are very few articles on the oxidation of Try in pharmaceutical proteins (Ji *et al*., 2009). Unfortunately, there is no definitive evidence that the potential Try oxidation plays any role whatsoever in the reduction of enzymatic activity of DAAO.

As we know, DAAO is a flavoenzyme which catalyses the oxidative deamination of D-Amino Acids to α-keto acids and the by-product H_2O_2, effectively meaning it is responsible for the generation of ROS which can in turn, theoretically oxidise the amino acid resides of the DAAO enzyme itself. In higher organisms, the ROS generation of DAAO has been implicated in the pathogenesis of spinal ROS and chronic pain (Lu *et al*., 2012). DAAO is also a potent neurotransmitter (Yamanaka *et al*., 2012).

Like Met and Try, Cysteine (Cys) is an amino acid responsible for protein structure and function. Like Met, the amino acid side chains of Cys present in proteins are prone to a multitude of oxidative post translational modifications (Ox-PTM) and such Ox-PTMs have been implicated in the pathogenesis of cardiovascular diseases (Chung *et al*., 2013). In particular, the thiols of cysteine residues are the most common targets of intracellular oxidation. In the presence of extensive ROS, SOH can undergo irreversible oxidations leading to permanent alterations of protein structure and function (Garcia-Santamarina *et al*., 2014). Interestingly, MS analysis of DAAO failed to find any Cys modifications, even though it is as easily oxidised as Met, which suggests Met residues are the key targets for protein oxidation in DAAO in this study. To our knowledge this is the first time these Met residues have been highlighted as the targets for protein oxidation in DAAO.

No microorganism is indifferent to the presence of oxygen. For many it is essential for life, for some it is toxic even in small quantities, while in others it brings about fundamental changes in metabolism. Because of the differences in metabolism that leads to great increases in available energy when cells are grown aerobically the supply of oxygen is most important for efficient use of the carbon source. Under anaerobic conditions a part of the carbohydrate is not oxidized

completely and accumulates as one of several by products like ethanol, acetic acid and lactic acid. There appears to be no significant difference between overall biomass accumulation between the 5 % pO_2, 30 % pO_2 and 40 % pO_2 fermentations, though at 30 % the final few measurements are higher than at 5 % pO_2 and 40 % pO_2. Furthermore, there is a clear accumulation of acetate at 5 % pO2 indicating that carbon is not being fully utilised for growth and recombinant protein production.

At 30 % pO2 the total activity of the enzyme continues increase throughout the fermentation, and the specific activity takes a slight dip at around 50 hours post induction. Specific activities of the 5 % pO2 process are much lower than those at 30 %, most likely due to the acetate production and subsequent blocking of the AOX1 promoter. Interestingly, there is a drastic increase after only a few hours of induction in activities of the 40 % pO2 process, perhaps because there is an increased flux of carbon to plasmid maintenance and cellular proliferation. However, there is a sharp drop in enzymatic activities at 40 % pO2 after this time point, due to the oxidation of the methionine residues of the DAAO enzyme as discussed previously.

Similarly, there is evidence of oxidative stress in *P. pastoris* cultures producing MAO. CAT and SOD activities are much higher at 40 % pO2 than at 5 % pO2. Furthermore, there is a clear decrease in enzymatic activities at 40 % pO2. The lower activities at 5 % pO2 are again attributable to the production of ethanol and its subsequent conversion to acetate. There is clear evidence of damage to MAO at 40 % pO2, but MS analysis is required to identify the direct cause of this. It is most likely due to oxidation of Met residues of the protein. Increased oxygen availability was beneficial to overall biomass accumulation as evidenced by the 40 % pO2 data but has a negative impact on enzyme activity.

5.5 *Conclusion*

The aim of this work was to study the effect of Oxygen Availability on physiology of fed batch *P. pastoris* cultures and the enzymatic activities of DAAO and MAO. Low oxygen availability (5 %) caused an accumulation of the toxic by product ethanol, which was in turn converted to acetate which blocked the AOX1 promoter and produced low activity enzyme. High Oxygen Availability (40 %) produced a state of Oxidative Stress as indicated by increased protein Carbonylation and proteolytic activity, as well as increased levels of catalase in response to the increased levels of

the ROS H_2O_2. There was also a higher level of SOD in response to increased superoxide production. The state of oxidative stress successfully caused a reduction in enzymatic activities which can be attributed to the confirmed oxidation of two Met residues of the DAAO enzyme and speculative oxidation to unknown Met residues in MAO.

Chapter 6

Conclusions and Future Work

6 Conclusions and Future Work

6.1 *Conclusions*

- Regarding the expression of DAAO in *E. coli pRES151DAAOWT BW2157*, this work shows that thermal induction is a cheaper, and less invasive method for the induction of recombinant protein expression in comparison to more common inducers like IPTG.
- It has been demonstrated that at high temperatures like 40 °C, there is a problem with cell lysis during the thermal induction of *E. coli pRES151DAAOWT BW2157* producing DAAO, which is not desirable because DAAO can act upon the D-alanine present in the cell walls of *E. coli*.
- The temperature before the onset of induction of *E. coli pRES151DAAOWT BW2157* cells producing DAAO is crucial. When grown at 30 °C and Induced at 40 °C there is a severe drop in biomass accumulation, as well as a significant reduction of DAAO activities because there is a premature expression of DAAO at 30 °C which negatively affects the overall process. This is not the case when grown at 28 °C and induced at temperatures above 30 °C.
- Interestingly, growth at 30 °C means the pO_2 reaches its pre-set level of 30 % earlier, and that the glucose feed can begin earlier, meaning theoretically the process can finish quicker. As is the case in this study though, growth at 28 °C produces overall better results, though the process takes slightly longer. Though in an industrial scale a quicker turn around between processes is desirable, with the strain of *E. coli pRES151DAAOWT BW2157* used in this study, this would not be feasible due to the sensitivity of the strain to temperature and premature thermal induction.
- Overall, there is no immediate difference between the activities of the enzyme when the strain *E. coli pRES151DAAOWT BW2157* is grown on LB or defined media. However, using a defined media means that the highest activities are achieved earlier meaning the overall time for the process completion is reduced. Furthermore, defined media reduces the potential for process variability, and means the operator can know exactly the composition of media during each process which provides benefits for quality control at industrial scale.

- The optimisation of the *P. pastoris ING005-A9-1-MCB1* protocol for the production of DAAO showed that the key factor to a successful process was the amount of carbon supplied to the cells in the feed during the induction phase and from which source. The Invitrogen protocols presented too high a level of methanol to the cells. A further reduction of methanol fed to the cells as seen in the Voulgaris adapted protocol was also too high. Furthering reduce the feed rate, as seen in this study, was shown to be most effective, and kept methanol at non-toxic levels during the processes.
- The use of sorbitol as an additional feed improved both growth and the recombinant enzyme activity when carbon flux was directed to both growth and recombinant protein expression, and the growth rate achieved here was the optimum value for the expression of DAAO.
- Higher carbon feeding caused higher growth rates which resulted in higher biomass levels, and due to the presence of highly nutrient rich supplements, there was no reduction in the activity of the enzyme using a co-feed of MeOH:Sorbitol 1:1.
- It is possible to reduce the cost of recombinant protein production in *E.coli* by substituting more expensive inducers like IPTG with thermal induction, and defined media which is generally cheaper than complex media, and has no detrimental effects to recombinant DAAO activity. However, the best activities for *P. pastoris ING005-A9-1-MCB1* were the result of supplementing the media with highly nutrient rich components and implementing a co-feed.
- The effects of oxygen availability upon the structure and function of DAAO were successfully investigated. Key methionine oxidations were identified in DAAO produced under highly aerobic conditions in *P. pastoris ING005-A9-1-MCB1* and there was evidence of damage to MAO in *P. pastoris MutS CBS 7435 His MAO-5N* also. Evidence was also found in DAAO produced in *E. coli.*

6.2 *Future Work*

The work presented in this thesis has provided several insights in to the production of DAAO using various host organisms understanding various factors that may improve the processes utilised. However, there are more detailed studies that can be carried out to further improve the results obtained in this study, which can potentially improve the production of other recombinant proteins. For Example:

Regarding the Work in Chapter Three:

- Utilising Design of Experiments (DoE) can provide useful insights in to feed rates; media composition and induction cell densities during the expression of DAAO in *E. coli*.
- Trying to reduce the cell lysis at high temperatures in *E. coli* during DAAO production, or trying to utilise this to improve downstream processing could be beneficial, though the toxicity of the DAAO enzyme to the host cell may present some additional challenges.

Regarding the Work in Chapter Four:

- Applying Metabolic Flux analysis to the *P. pastoris* cultures in this study can provide additional, and incredibly useful information, regarding the carbon flux during fed batch strategies of an industrially important enzyme. Any process bottlenecks can be identified, and it can be seen if carbon flux needs to be shunted to other cellular pathways to improve the activities of DAAO without compromising other necessary cellular activities.
- Engineering a strain of *P. pastoris* which can secrete the DAAO enzyme extracellularly has the potential to reduce downstream processing, and unlike *E. coli*, DAAO would not be toxic to the growing host cells.

Regarding the Work in Chapter Five:

- Applying MS analysis to the DAAO produced in *E. coli* would provide further information about the structurcal changes to the enzyme which may affect functionality. It would give a strong indication, if any, of the potential differences between the DAAO enzymes produced in prokaryotic and eukaryotic host organisms.

- The identification of the modification(s) can potentially provide us with solutions to overcome the negative effects of oxygen availability. Amino acid residues prone to Oxidative modifications could theoretically be substituted with residues which are less prone to oxidation, provided these are not detrimental to the overall catalytic activity of the DAAO enzyme. Moreover, is these oxidative modifications happen to be reversible, then there is every opportunity to restore the activity of the enzyme once it has been recovered after downstream processing has taken place.

Chapter 7

References

7 References

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Appendices

Appendix I – Voulgaris *P. pastoris MeOH/Sorbitol/Glycerol Feed Profile (Adapted From Invitrogen)*

glycerol feed	
Time from start of feed (h)	ml/min
0	1.07
0.5	1.24
1	1.42
1.75	1.60
2.51	1.78
3	1.96
3.75	2.13
4.25	2.31
4.75	2.49
5.25	2.67
5.75	2.84
6	3.02
6.33	2.13
7.33	1.07
8.33	0.00

MeOH Sorb 2:1		
Time from start of feed (h)	MeOH (ml/min)	Sorbitol (ml/min)
0	0.18	0.15
2.5	0.27	0.22
4	0.36	0.29
5	0.44	0.37
6	0.53	0.44

MeOH Sorb 1:1		
Time from start of feed (h)	MeOH (ml/min)	Sorbitol (ml/min)
0	0.18	0.29
2.5	0.27	0.44
4	0.36	0.59
5	0.44	0.73
6	0.53	0.88
MeOH Sorb 1:2		
Time from start of feed (h)	MeOH (ml/min)	Sorbitol (ml/min)
0	0.18	0.59
2.5	0.27	0.88
4	0.36	1.17
5	0.44	1.47
6	0.53	1.76

Appendix II – Invitrogen *P. pastoris Protocol*

Invitrogen life technologies

Pichia Fermentation Process Guidelines

Overview

Introduction

Pichia pastoris, like *Saccharomyces cerevisiae*, is particularly well-suited for fermentative growth. *Pichia* has the ability to reach very high cell densities during fermentation which may improve overall protein yields.

We recommend that only those with fermentation experience or those who have access to people with experience attempt fermentation. Since there are a wide variety of fermenters available, it is difficult to provide exact procedures for your particular case. The guidelines given below are based on fermentations of both Mut^+ and Mut^S *Pichia* strains in a 15 liter table-top glass fermenter. Please read the operator's manual for your particular fermenter before beginning. The table below provides an overview of the material covered in these guidelines.

Fermentation Parameters

It is important to monitor and control the following parameters throughout the fermentation process. The following table describes the parameters and the reasons for monitoring them.

Parameter	Reason
Temperature (30.0°C)	Growth above 32°C is detrimental to protein expression
Dissolved oxygen (>20%)	*Pichia* needs oxygen to metabolize glycerol and methanol
pH (5.0-6.0 and 3.0)	Important when secreting protein into the medium and for optimal growth
Agitation (500 to 1500 rpm)	Maximizes oxygen concentration in the medium
Aeration (0.1 to 1.0 vvm* for glass fermenters)	Maximizes oxygen concentration in the medium which depends on the vessel
Antifoam (the minimum needed to eliminate foam)	Excess foam may cause denaturation of your secreted protein and it also reduces headspace
Carbon source (variable rate)	Must be able to add different carbon sources at different rates during the course of fermentation

* volume of oxygen (liters) per volume of fermentation culture (liters) per minute

continued on next page

Version B
053002

Overview, continued

Recommended Equipment

Below is a checklist for equipment recommendations.

- A jacketed vessel is needed for cooling the yeast during fermentation, especially during methanol induction. You will need a constant source of cold water (5-10°C). This requirement may mean that you need a refrigeration unit to keep the water cold.
- A foam probe is highly recommended as antifoam is required.
- A source of O_2--either air (stainless steel fermenters at 1-2 vvm) or pure O_2 (0.1-0.3 vvm for glass fermenters).
- Calibrated peristaltic pumps to feed the glycerol and methanol.
- Automatic control of pH.

Medium Preparation

You will need to prepare the appropriate amount of following solutions:

- Fermentation Basal Salts (page 11)
- PTM_1 Trace Salts (page 11)
- ~75 ml per liter initial fermentation volume of 50% glycerol containing 12 ml PTM_1 Trace Salts per liter of glycerol.
- ~740 ml per liter initial fermentation volume of 100% methanol containing 12 ml PTM_1 Trace Salts per liter of methanol.

Monitoring the Growth of *Pichia pastoris*

Cell growth is monitored at various time points by using the absorbance at 600 nm (OD_{600}) and the wet cell weight. The metabolic rate of the culture is monitored by observing changes in the concentration of dissolved oxygen in response to carbon availability (see next page).

Dissolved Oxygen (DO) Measurement

Introduction

The dissolved oxygen concentration is the relative percent of oxygen in the medium where 100% is O_2-saturated medium. *Pichia* will consume oxygen as it grows, reducing the dissolved oxygen content. However, because oxygen is required for the first step of methanol catabolism, it is important to maintain the dissolved oxygen (DO) concentration at a certain level (>20%) to ensure growth of *Pichia* on methanol. Accurate measurement and observation of the dissolved oxygen concentration of a culture will give you important information about the state and health of the culture. Therefore, it is important to accurately calibrate your equipment. Please refer to your operator's manual.

Maintaining the Dissolved Oxygen Concentration (DO)

1. Maintaining the dissolved oxygen above 20% may be difficult depending on the oxygen transfer rates (OTR) of the fermenter, especially in small-scale glass vessels. In a glass vessel, oxygen is needed to keep the DO above 20%, usually ~0.1-0.3 vvm (liters of O_2 per liter of fermentation culture per minute). Oxygen consumption varies and depends on the amount of methanol added and the protein being expressed.
2. Oxygen can be used at 0.1 to 0.3 vvm to achieve adequate levels. This can be accomplished by mixing with the air feed and can be done in any glass fermenter. For stainless steel vessels, pressure can be used to increase the OTR. Be sure to read the operator's manual for your particular fermenter.
3. If a fermenter cannot supply the necessary levels of oxygen, then the methanol feed should be scaled back accordingly. Note that decreasing the amount of methanol may reduce the level of protein expression.
4. To reach maximum expression levels, the fermentation time can be increased to deliver similar levels of methanol at the lower feed rate. For many recombinant proteins, a direct correlation between amount of methanol consumed and the amount of protein produced has been observed.

Use of DO Measurements

During growth, the culture consumes oxygen, keeping the DO concentration low. Note that oxygen is consumed whether the culture is grown on glycerol or methanol. The DO concentration can be manipulated to evaluate the metabolic rate of the culture and whether the carbon source is limiting. The metabolic rate indicates how healthy the culture is. Determining whether the carbon source is limiting is important if you wish to fully induce the *AOX1* promoter. For example, changes in the DO concentrations (DO spikes) allow you to determine whether all the glycerol is consumed from the culture before adding methanol. Secondly, it ensures that your methanol feed does not exceed the rate of consumption. Excess methanol (> 1-2% v/v) may be toxic.

Manipulation of DO

If carbon is limiting, shutting off the carbon source should cause the culture to decrease its metabolic rate, and the DO to rise (spike). Terminate the carbon feed and time how long it takes for the DO to rise 10%, after which the carbon feed is turned back on. If the lag time is short (< 1 minute), the carbon source is limiting.

Fermenter Preparation and Glycerol Batch Phase

Inoculum Seed Flask Preparation

Remember not to put too much medium in the baffled flasks. Volume should be 10-30% of the total flask volume.

1. Baffled flasks containing a total of 5-10% of the initial fermentation volume of MGY or BMGY are inoculated with a colony from a MD or MGY plate or from a frozen glycerol stock.
2. Flasks are grown at 30°C, 250-300 rpm, 16-24 hours until OD_{600} = 2-6. To accurately measure OD_{600} > 1.0, dilute a sample of your culture 10-fold before reading.

Glycerol Batch Phase

1. Sterilize the fermenter with the Fermentation Basal Salts medium containing 4% glycerol (see page 11).
2. After sterilization and cooling, set temperature to 30°C, agitation and aeration to operating conditions (usually maximum rpm and 0.1-1.0 vvm air), and adjust the pH of the Fermentation Basal Salts medium to 5.0 with 28% ammonium hydroxide (undiluted ammonium hydroxide). Add aseptically 4.35 ml PTM_1 trace salts/liter of Fermentation Basal Salts medium.
3. Inoculate fermenter with approximately 5-10% initial fermentation volume from the culture generated in the inoculum shake flasks. Note that the DO will be close to 100% before the culture starts to grow. As the culture grows, it will consume oxygen, causing the DO to decrease. Be sure to keep the DO above 20% by adding oxygen as needed.
4. Grow the batch culture until the glycerol is completely consumed (18 to 24 hours). This is indicated by an increase in the DO to 100%. Note that the length of time needed to consume all the glycerol will vary with the density of the initial inoculum.
5. Sampling is performed at the end of each fermentation stage and at least twice daily. We take 10 ml samples for each time point, then take 1 ml aliquots from this 10 ml sample. Samples are analyzed for cell growth (OD_{600} and wet cell weight), pH, microscopic purity, and protein concentrations or activity. Freeze the cell pellets and supernatants at -80°C for later analysis. Proceed to **Glycerol Fed-Batch Phase**, page 5.

Yield

A cellular yield of 90 to 150 g/liter wet cells is expected for this stage. Recombinant protein will not yet be produced due to the absence of methanol.

Glycerol Fed-Batch Phase

Introduction

Once all the glycerol is consumed from the batch growth phase, a glycerol feed is initiated to increase the cell biomass under limiting conditions. When you are ready to induce with methanol, you can use DO spikes to make sure the glycerol is limited.

Glycerol Fed-Batch Phase

1. Initiate a 50% w/v glycerol feed containing 12 ml PTM_1 trace salts per liter of glycerol feed. Set the feed rate to 18.15 ml/hr /liter initial fermentation volume.
2. Glycerol feeding is carried out for about four hours or longer (see below). A cellular yield of 180 to 220 g/liter wet cells should be achieved at the end of this stage while no appreciable recombinant protein is produced.

Note

The level of expressed protein depends on the cell mass generated during the glycerol fed-batch phase. The length of this feed can be varied to optimize protein yield. A range of 50 to 300 g/liter wet cells is recommended for study. A maximum level of 4% glycerol is recommended in the batch phase due to toxicity problems with higher levels of glycerol.

Important

If dissolved oxygen falls below 20%, the glycerol or methanol feed should be stopped and nothing should be done to increase oxygen rates until the dissolved oxygen spikes. At this point, adjustments can be made to agitation, aeration, pressure or oxygen feeding.

Proteases

In the literature, it has been reported that if the pH of the fermentation medium is lowered to 3.0, neutral proteases are inhibited. If you think neutral proteases are decreasing your protein yield, change the pH control set point to 3.0 during the glycerol fed-batch phase (above) or at the beginning of the methanol induction (next page) and allow the metabolic activity of the culture to slowly lower the pH to 3.0 over 4 to 5 hours (Brierley, *et al.*, 1994; Siegel, *et al.*, 1990).

Alternatively, if your protein is sensitive to low pH, it has been reported that inclusion of casamino acids also decreases protease activity (Clare, *et al.*, 1991).

Methanol Fed-Batch Phase

Introduction

All of the glycerol needs to be consumed before starting the methanol feed to fully induce the *AOX1* promoter on methanol. However, it has been reported that a "mixed feed" of glycerol and methanol has been successful to express recombinant proteins (Brierley, *et al.*, 1990; Sreekrishna, *et al.*, 1989). It is important to introduce methanol slowly to adapt the culture to growth on methanol. If methanol is added too fast, it will kill the cells. Once the culture is adapted to methanol, it is very important to use DO spikes to analyze the state of the culture and to take time points over the course of methanol induction to optimize protein expression. Growth on methanol also generates a lot of heat, so temperature control at this stage is very important.

Mut$^+$ Methanol Fed-Batch Phase

1. Terminate glycerol feed and initiate induction by starting a 100% methanol feed containing 12 ml PTM_1 trace salts per liter of methanol. Set the feed rate to 3.6 ml/hr per liter initial fermentation volume.
2. During the first 2-3 hours, methanol will accumulate in the fermenter and the dissolved oxygen values will be erratic while the culture adapts to methanol. Eventually the DO reading will stabilize and remain constant.
3. If the DO cannot be maintained above 20%, stop the methanol feed, wait for the DO to spike and continue on with the current methanol feed rate. Increase agitation, aeration, pressure or oxygen feeding to maintain the DO above 20%.
4. When the culture is fully adapted to methanol utilization (2-4 hours), and is limited on methanol, it will have a steady DO reading and a fast DO spike time (generally under 1 minute). Maintain the lower methanol feed rate under limited conditions for at least 1 hour after adaptation before doubling the feed. The feed rate is then doubled to ~7.3 ml/hr/liter initial fermentation volume.
5. After 2 hours at the 7.3 ml/hr/liter feed rate, increase the methanol feed rate to ~10.9 ml/hr per liter initial fermentation volume. This feed rate is maintained throughout the remainder of the fermentation.
6. The entire methanol fed-batch phase lasts approximately 70 hours with a total of approximately 740 ml methanol fed per liter of initial volume. However, this may vary for different proteins.

Note: The supernatant may appear greenish. This is normal.

Yield

The cell density can increase during the methanol fed-batch phase to a final level of 350 to 450 g/liter wet cells. Remember that because most of the fermentation is carried out in a fed-batch mode, the final fermentation volume will be approximately double the initial fermentation volume.

Fermentation of Mut^S *Pichia* Strains

Since Mut^S cultures metabolize methanol poorly, their oxygen consumption is very low. Therefore, you cannot use DO spikes to evaluate the culture. In standard fermentations of a Mut^S strain, the methanol feed rate is adjusted to maintain an excess of methanol in the medium which does not exceed 0.3% (may be determined by gas chromatography). While analysis by gas chromatography will insure that nontoxic levels of methanol are maintained, we have used the empirical guidelines below to express protein in Mut^S strains. A gas chromatograph is useful for analyzing and optimizing growth of Mut^S recombinants.

continued on next page

Methanol Fed-Batch Phase, continued

Mut^S Methanol Fed- Batch Phase

The first two phases of the glycerol batch and fed-batch fermentations of the Mut^S strains are conducted as described for the Mut$^+$ strain fermentations. The methanol induction phases of the Mut$^+$ and Mut^S differ in terms of the manner and amount in which the methanol feed is added to the cultures.

1. The methanol feed containing 12 ml PTM_1 trace salts per liter of methanol is initiated at 1 ml/hr/liter initial fermentation volume for the first two hours. It is then increased in 10% increments every 30 minutes to a rate of 3 ml/hr which is maintained for the duration of the fermentation.
2.. The vessel is then harvested after ~100 hours on methanol. This time may be varied to optimize protein expression.

Harvesting and Lysis of Cells

Introduction

The methods and equipment listed below are by no means complete. The amount of cells or the volume of supernatant will determine what sort of equipment you need.

Harvesting Cells and Supernatant

For small fermentations (1-10 liters), the culture can be collected into centrifuge bottles (500-1000 ml) and centrifuged to separate the cells from the supernatant.

For large fermentations, large membrane filtration units (Millipore) or a Sharples centrifuge can be used to separate cells from the supernatant. The optimal method will depend on whether you need the cells or the supernatant as the source of your protein and what you have available.

Supernatants can be loaded directly onto certain purification columns or concentrated using ultrafiltration.

Cell Lysis

We recommend cell disruption using glass beads as described in *Current Protocols in Molecular Biology*, page 13.13.4. (Ausubel, *et al.*, 1990) or *Guide to Protein Purification* (Deutscher, 1990). This method may be tedious for large amounts of cells. For larger amounts, we have found that a microfluidizer works very well. French pressing the cells does not seem to work as well as the glass beads or the microfluidizer.

References

Introduction

Most of the references below refer to papers where fermentation of *Pichia* was performed. Note that some of these are patent papers. You can obtain copies of patents using any of the following methods.

- **Patent Depository Libraries.** U. S. patents and international patents granted under the Patent Cooperation Treaty (PCT) are available on microfilm. These can be copied and mailed or faxed depending on length. There is a fee for this service. The reference librarian at your local library can tell you the location of the nearest Patent Depository Library.
- **Interlibrary Loan.** If you are not near a Patent Depository Library, you may request a copy of the patent through interlibrary loan. There will be a fee for this service.
- **U. S. Patent Office.** Requests may be made directly to the Patent Office, Arlington, VA. Please call 703-557-4636 for more information on cost and delivery.
- **Private Library Services.** There are private companies who will retrieve and send you patents for a fee. Two are listed below:

  Library Connection: 804-758-3311
  Rapid Patent Services: 800-336-5010

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Recipes

Fermentation Basal Salts Medium

For 1 liter, mix together the following ingredients:

Phosphoric acid, 85%	(26.7 ml)
Calcium sulfate	0.93 g
Potassium sulfate	18.2 g
Magnesium sulfate-$7H_2O$	14.9 g
Potassium hydroxide	4.13 g
Glycerol	40.0 g
Water	to 1 liter

Add to fermenter with water to the appropriate volume and sterilize.

PTM_1 Trace Salts

Mix together the following ingredients:

Cupric sulfate-$5H_2O$	6.0 g
Sodium iodide	0.08 g
Manganese sulfate-H_2O	3.0 g
Sodium molybdate-$2H_2O$	0.2 g
Boric Acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate-$7H_2O$	65.0 g
Biotin	0.2 g
Sulfuric Acid	5.0 ml
Water	to a final volume of 1 liter

Filter sterilize and store at room temperature.

Note: There may be a cloudy precipitate upon mixing of these ingredients. Filter-sterilize as above and use.