Production of novel amine oxidases from microorganisms

Ioannis Voulgaris

A thesis submitted to the University of Strathclyde in accordance with the regulations governing the award of the degree of

Doctor of Philosophy

Strathclyde Fermentation Centre
Strathclyde Institute of Pharmacy and Biomedical Sciences
University of Strathclyde
Glasgow, Scotland

January, 2011

Copyright declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

Acknowledgment

First of all, I would like to express my sincere gratitude to my supervisors, Prof. Brian McNeil and Dr. Linda Harvey, Dr. S. Alison Arnold and Dr. Robert Speight for their continuous support, encouragement and guidance throughout my studies.

I am also thankful to my colleagues and friends in the Strathclyde Fermentation Centre, Dr. Andrew O'Donnell, Dr. Li Qiang, Dr. Mariana Fazenda, Dr. Payal Roy Choudhury, Dr. Manal Eshelli, Ebtihaj Jambi (my best and craziest lab-mate in the world), Tantima Kumlung, Ivo Kretzers and the chemists Melissa Black and Pete Gardner. I am also thankful to the lab technician, Walter McEwan, for the excellent technical support.
I also want to thank Ingenza Ltd. for making this project reality and in particular Dr. Nicole Barnes, Dr. Fraser Brown, Dr. Reuben Carr, Dr. Ian Fotheringham, Dr. Ian Archer and Sarah Graham for all the help so kindly game me.

Special thanks to my wonderful friends who supported me and helped to have a wonderful time during my work in Glasgow, Konstantinos Liaskos, Konstantinos Kaliarntas, Solon Karagiannopoulos, Ilias Tsagas, and Yiorgos Perikleous. Last but not least many thanks to my parents for their continuous love and support.

I am grateful for the plasmid pET28b (MAO-5N) which was a kind gift from Dr. Gideon J. Grogan and Dr. Kate Atkin (Structural Biology Laboratory, Department of Chemistry, University of York) and for the *P.pastoris* construct which was kindly donated by Dr. Sandra Abad and Prof. Anton Glieder (Institute of Molecular Biotechnology, Graz University of Technology). The *A. niger* strain B-1D was a gift from Prof. David Archer (University of Nottingham)

Table of Contents

List of figures

List of tables

List of abbreviations

AO: Amine oxidase
AOX: Alcohol oxidase
CER: Carbon dioxide evolution rate
DAAO: D-amino acid oxidase
DCW: Dry cell weight
DMSO: Dimethyl sulfoxide
DO: Dissolved oxygen
ETC: Electron transport chain
ER: Endoplasmic reticulum

FAD: Flavin adenine dinucleotide

GSH: Glutathione

HPLC: High-performance liquid chromatography

HRP: Horseradish peroxidase

IPTG: Isopropyl-β-D-1-thiolgalactopyranoside

LPS: Lipopolysaccharide

MAO: Monoamine oxidase

MAO-5M: Mutant of the wild type MAO-N carrying three 5 amino acid mutations to increase substrate specificity against aromatic amines.

Mut^-, Mut^s, Mut^+ : Methanol utilisation phenotype (negative, slow, positive)

NIRS: Near infrared spectroscopy

OD_{600}: Optical Density at 600nm

OUR: Oxygen utilisation rate

PAO: Polyamine oxidase

PTM_1: *Pichia* trace metals

RQ: Respiratory quotient

SCP: Single cell protein

SSAO: Semicarbazide-sensitive amine oxidase

TBHBA: 2,4,6-tribromo-3-hydroxybenzoic acid

TPQ: Topa quinone

UPR: Unfolded protein response

YE: Yeast extract

YNB: Yeast nitrogen base

Abstract

With the advent of molecular biology and genetic transformation the production of high value proteins in recombinant host cells has become an everyday reality. However, the establishment of the optimum conditions for every target protein requires to take into account the nature of the product, and needs experimentation. In this study the over-expression of an industrially important monoamine oxidase was attempted using different expression systems, both homologous and heterologous, and the optimum conditions for the industrial production were established. Amine oxidases exhibit stereoselectivity, and, therefore, can potentially be used for the deracemisation of non-optically pure mixtures of amines leading to optically pure amines of high value in the fine chemical and pharmaceutical areas. Initially, different strains of *A.niger* were evaluated regarding their ability to produce the industrially important monoamine oxidase (MAO-N), while later optimisation of the culture conditions took place. These were achieved by the use of the appropriate nitrogen source, incorporation of an amine oxidase inhibitor to reduce inducer's breakdown and consequently boost amine oxidase expression, and the induction of the culture at the appropriate time. Over-expression of the genetically modified MAO-5N in *E.coli* was studied under different experimental setups with the aim of improving both the activity of the produced enzyme leading to relatively low-cost scalable processes. Towards this aim, substitution of the expensive inducer IPTG with the less expensive lactose took place, while changes in the feed profile were implemented. Moreover, the examination of the combined effects of induction biomass levels and oxygen availability, through the use of air and oxygen enriched air, in the culture led to significant increase of the enzyme activity and gave important insights about the sensitivity of this enzyme under highly oxidative conditions. Undoubtedly, *E.coli* remains the main workhorse for the production of recombinant proteins, nevertheless, the use of methylotrophic yeasts is gaining significant ground and for that reason a *P.pastoris* system intracellularly expressing the MAO-5N was investigated. Appropriate monitoring and control of the bioprocess

along with optimisation of the carbon flux led to significantly higher volumetric MAO activities and better biotransformation capacity of the whole cells than the previously used *E.coli* systems.

The aforementioned improvements result in more efficient processes, where both the overall productivity is increased with a simultaneous reduction of the cost which ultimately leads to production of cheaper valuable compounds. Moreover, the present study extends our knowledge on the nature and physiology of monoamine oxidase production by microbial organisms and suggest a solution which can be implemented for the industrial manufacture of other oxidase type enzymes.

Chapter 1

Literature review and aims

1.1. Literature review

1.1.1. Amine oxidases

1.1.1.1. Introduction to Amine Oxidases

The amine oxidases form a large group of enzymes which are widely distributed in nature. They are found in higher organisms like mammals as well as in plants and microorganisms (fungi and bacteria). All the enzymes of the amine oxidase group catalyze the oxidative deamination of amines to form the corresponding aldehydes, ammonia and hydrogen peroxide, as shown in the following reaction:

$$RCH_2NH_2 + H_2O + O_2 \longrightarrow RCHO + NH_3 + H_2O_2$$

Figure 1.1. Reaction catalysed by amine oxidases.

Amine oxidases are usually divided into two groups based on the chemical nature of the attached co- factor; the flavine adenine dinucleotide (FAD)-containing enzymes (EC. 1.4.3.4), and the copper containing amine oxidases (EC. 1.4.3.6). The latter group contains also topa – quinone (TPQ) as a co-factor (Klinman, 1996), which is formed post-translationally by modification of a tyrosine unit in the amino acid chain (Janes et al, 1990).

The nomenclature of the amine oxidase enzymes is somewhat confusing, as the various amine oxidase enzymes exhibit different specificities and preferences towards substrates. Moreover, the varying nature of the co-factor also adds to the complexity. As early as (1928), Hare described an enzyme that catalyzed the oxidative deamination of tyramine. This was the first mention of the enzyme now known as monoamine oxidase (or amine oxidase). It was later found by Blaschko et al. (1937) that this enzyme did not act on diamines, such as putrescine (1,4-diaminobutane) and cadaverine (1,5 – diaminopentane). Additionally, this enzyme was inactive against histamine, which was found to be deaminated by the then

known histaminase. Histaminase of pig kidney was also active against aliphatic diamines like putrescine and cadaverine (Zeller, 1951). Therefore, the term histaminase has now been replaced by the term diamine oxidase, however, it is a fact that many so called monoamine oxidases act on many diamines, and conversely diamine oxidases may act on many monoamines (Blaschko, 1963).

As discussed above, a way of classification of amine oxidases is by the co-factor they are bound to. FAD and TPQ containing enzymes can be both classified as monoamine oxidase and diamine oxidase depending on the substrates they prefer to act on. In the case of the mammals FAD containing monoamine oxidases, there are two subcategories, the MAO A and the MAO B. This further classification took place due to the different chemical properties, in particular the different affinity for different substrates, and the differing response of these enzymes to various inhibitors. The MAO A preferentially oxidizes the substrate β- phenethylamine, while MAO B preferentially accepts substrates, such as serotonin and norepinephrine (noradrenaline). However, these compounds are not entirely specific for the respective MAO form (Ochiai et al, 2006). Therefore, classification by means of responses to specific inhibitors has been more convenient for the identification of different types of amine oxidase. The differentiation of the two forms by the use of inhibitors is based on Johnston (1968) and Knol and Magyar (1972) which state that clorgyline can specifically inhibit the MAO A and deprenyl can specifically inhibit the MAO B. The TPQ amine oxidases are not inhibited by the above compounds, but they are almost completely inhibited by semicarbazide (Ochiai et al, 2006), this is the reason why their name is very often abbreviated as SSAO, which stands for semicarbazide sensitive amine oxidase. It is worth noting that MAO A and MAO B are different proteins encoded by distinct genes in the human genome. They are also clearly distinct in their primary amino acid sequences and the molecular weights of the subunits of purified human MAO A and MAO B (Weyler et al, 1990; Weyler & Salach, 1985).

1.1.1.2. Amine oxidases in microorganisms

Research into amine oxidases has mainly targeted mammalian enzymes, due to their importance in the metabolism of aromatic amines, including neurotransmitters, such as serotonin, adrenaline, histamine and dopamine. This area has received great attention, and has led to the development of new drugs (MAO inhibitors) for the treatment of Parkinsonism and Alzheimer's disease, as well as for the alleviation of depression (Shih & Thompson, 1999).

The existence of amine oxidase enzymes in microorganisms is very widespread, and in many cases, more than one kind of amine oxidase enzyme is simultaneously expressed in these organisms (Frébort et al, 1997). This fact makes the study of the function and physiological roles of microbial amine oxidases more complicated. The simplest organism in which an amine oxidase type enzyme is found is the archaebacterium *Methanosarcina barkery,* which is indicative of the appearance of these enzymes at a very early stage in microbial evolution (Yagodina et al, 2002). Among the microorganisms which possess amine oxidase enzymes are the fungal genera *Aspergillus, Penicillium, Fusarium, Coccidiodes*, *Armilaria*, *Trichoderma* (Frébort et al, 1997), *Brettanomyces, Debaryomyces, Hansenula, Hanseniaspora, Pichia, Schwanniomyces*, *Saccharomyces* (Walt, 1962), *Candida* (Haywood & Large 1981) *Trigonopsis, Pichia* (Green et al, 1983), and the bacterial genera *Micrococcus, Klebsiella, Escherichia, Pseudomonas*, *Brevibacterium, Enterobacter, Salmonella, Serratia, Proteus* (Murooka et al, 1979), *Arthrobacter* (Levering et al, 1981).

It is worth mentioning that both flavin and copper containing amine oxidases can be expressed and sometimes co – expressed in one microorganism (Frébort et al, 1997). Figure 1.2. shows the classification of the various amine oxidases found in microorganisms.

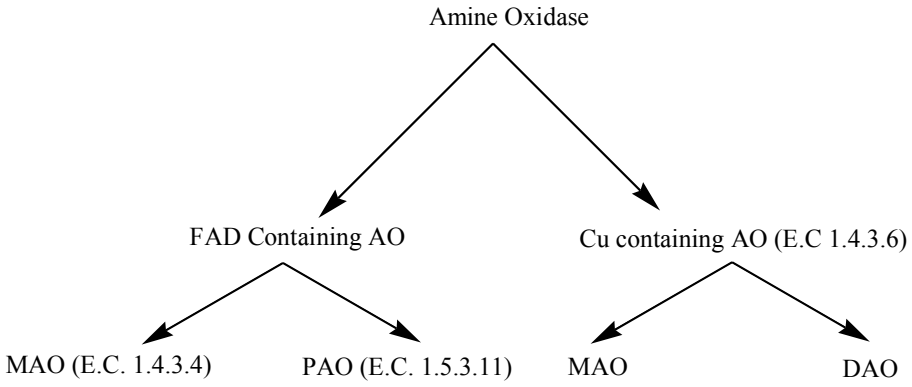

Figure 1.2. Classification scheme for the microbial amine oxidases.

1.1.1.3. Nitrogen metabolism and amine oxidase expression in fungi

The preferred nitrogen sources for most prokaryotes, and lower eukaryotes, are ammonium, glutamate and glutamine. However, in the absence of these compounds, cells can utilize a variety of secondary nitrogen compounds as sole sources of nitrogen. The utilization of these secondary nitrogen sources is highly regulated, and requires the co-ordinated synthesis of enzymes and permeases which are subject to nitrogen metabolite repression, which ensures that these activities are functional only when supplies of the preferred nitrogen sources are limited (Debusk & Ogilvie, 1984). Nitrogen control activates the expression of the genes which encode the aforementioned proteins, only in the absence of the primary nitrogen source. The *de novo* synthesis of the permeases and catabolic enzymes of a particular pathway is controlled at the level of transcription, and often requires two distinct positive signals: the first of which indicates the absence of primary nitrogen source (nitrogen de-repression), and the second of which is a pathway- specific signal and indicates the presence of a substrate or an intermediate of that pathway. This process enables the selective expression of just the enzymes of a specific catabolic pathway, from many potential candidates within the nitrogen regulatory circuit (Marzluf, 1997).

Among the enzymes that can be expressed by the nitrogen regulatory mechanism are those responsible for the assimilation of nitrate (nitrate and nitrite reductase), urea

(urea aminohydrolase), various amino acids (both L and D – amino acids). However, in the case of D – amino acid utilisation, the enzymes which are responsible for the conversion of the amino acids to the utilizable ammonium ion form, namely the D - amino acid oxidases, these do not strictly follow the rules of the nitrogen regulatory system, as the ammonium which is a primary nitrogen source does not repress the expression of the D amino acid oxidase mRNA, but acts at the subsequent translational level, which is in contrast to the enzymes of the nitrogen regulatory mechanism (Molla et al, 2003). On the other hand, the L – amino acid oxidase is under the control of the nitrogen regulatory system (Sikora & Marzluf, 1982).

Other compounds that can be utilized by the nitrogen regulatory system are amines. Though not well investigated, the utilization of amines by microorganisms seems to be controlled by the nitrogen regulatory system. It has been found that only the presence of an amine can induce the expression of the respective amine oxidase when ammonium is absent. It has been reported that ammonium strongly represses the expression of amine oxidase enzymes by fungi (Schilling & Lerch, 1995b; Zwart et al, 1980).

1.1.1.4. Fungal metabolism of amines and amine oxidase

As regards methylamine and other methylated nitrogen sources it has been reported that in the case of methylotrophic yeasts such as *Pichia pastoris*, *Hansenula polymorpha*, *Candida boidinii*, the utilization of such nitrogen sources can take place through the methanol dissimilation pathway of formaldehyde and formate dehydrogenases. Methylamine is oxidized to formaldehyde, hydrogen peroxide and ammonia by the amine oxidase, and then the peroxisomal enzymes oxidize formaldehyde to carbon dioxide with an overall gain of two NADH molecules from one processed formaldehyde molecule. The toxic hydrogen peroxide is degraded by peroxisomal catalase. As can be seen in figure 1.3., the formaldehyde produced by the methylotrophic cells inside the peroxisomes can also be used as a carbon and energy source through the xylulose monophosphate pathway (van Dijken et al, 1987). That is to say, the formaldehyde molecules are condensed with xylulose 5-

phosphate molecules, and are converted into the C3-compounds dihydroxyacetone and glyceraldehyde 3-phosphate which subsequently enter the glycolysis pathway (Hartner & Glieder, 2006). The previous conversion is catalysed by a transketolase called dihydroxyacetone synthase (Sakai et al, 1998).

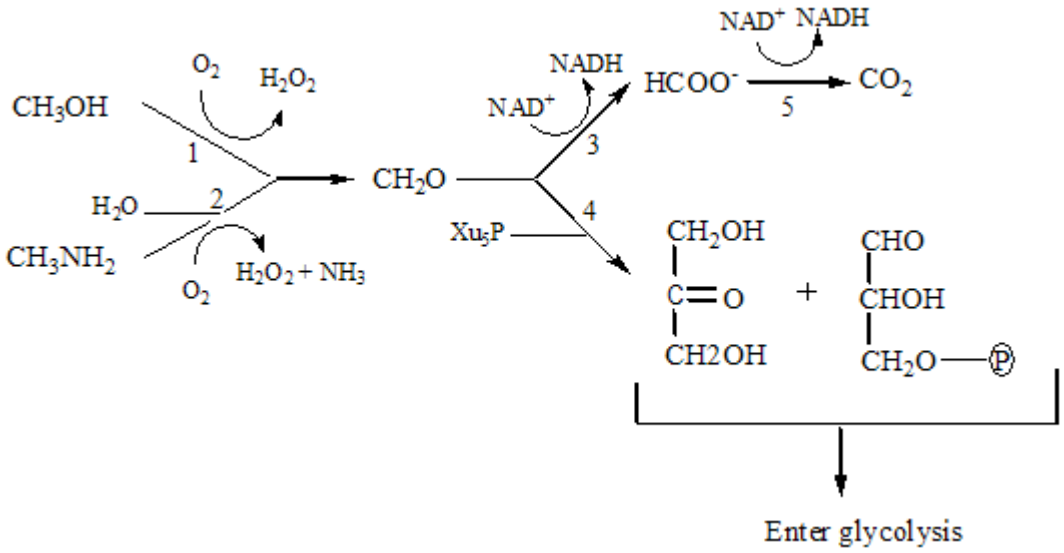

Figure 1.3. The pathway for the utilisation of methanol and methylamine by fungi. The enzymes involved are: 1.alcohol oxidase, 2. amine oxidase, 3. formaldehyde dehydrogenase, 4. dihydroxyacetone synthase, 5. formate dehydrogenase

The cultivation of the *C.utilis* and *H.polymorpha* cells in media containing methylamine, or other alkylated amines such as dimethylamine and trimethylamine as sole nitrogen source, leads to an increase of the number and volume of peroxisomes (Zwart et al., 1980, Zwart & Harder 1983; Faber 1995). However, in the case of the filamentous fungus *A.niger* the increase in the number of peroxisomes and the increase in their size induced by n-butylamine is much lower (Frébort et al, 2000). These peroxisomes contain high levels of amine oxidase and catalase (Zwart et al, 1980). Catalase synthesis was mainly regulated at the transcriptional level (Sakai et al, 1998) even though some regulation at the post-translational level, which was detected at the import into peroxisomes, has been reported (Horiguchi et al, 2001).

Methylamine induces the transcription of formaldehyde and formate dehydrogenases even in the presence of glucose, as long as ammonia is absent (Lee et al., 2002; Shen et al., 1998; Nakagawa et al., 2004).

It is important to mention that growth of methylotrophic yeast in the presence of methylamine and ammonia is not possible, due to the fact that the MAO is strongly repressed; therefore the amount of formaldehyde is not sufficient to induce the methylotrophic routes of carbon assimilation (Zwart et al, 1983).

It was observed that the utilization of amines such as methylamine and ethylamine caused increased cell biomass production when the microorganisms used had the appropriate enzymatic tools to metabolize formaldehyde and acetaldehyde, respectively. However, in the case of methylotrophic yeasts, the enzymes of the dissimilation pathway can still be expressed and oxidize the toxic formaldehyde with the concomitant generation of energy (Zwart & Harder, 1983).

In a similar fashion, yeasts which are able to grow on ethanol have the ability to utilize ethylamine to meet their nitrogen needs. Ethanol is initially oxidized to acetaldehyde, with the simultaneous generation of a molecule of NADH by an alcohol dehydrogenase while the aforementioned product (acetaldehyde) is also produced by the conversion of ethylamine by an amine oxidase but with hydrogen peroxide and ammonium as by-products. Then, acetaldehyde is converted to acetyl – CoA and can be used for both energy production and carbon assimilation (Zwart & Harder, 1983).

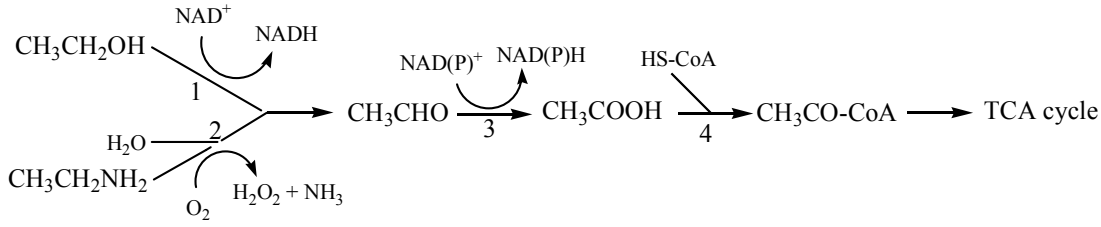

Figure 1.4. The pathway for the utilisation of ethanol and ethylamine.
The enzymes involved are: 1. alcohol dehydrogenase, 2. amine oxidase, 3. aldehyde dehydrogenase, 4. acetyl-CoA synthetase.

When it comes to the metabolism of methylated amines such as dimethylamine and trimethylamine, it was observed in *C. utilis* cells that NADH dependent mono – oxygenases perform the de-methylation of these compounds (Green & Large, 1983)

and subsequently the produced methylamine and formaldehyde molecules are further utilized as mentioned above. Correspondingly, the ethylated amines are presumed to be de-ethylated and the produced ethylamine and acetaldehyde to be further oxidized (Zwart & Harder, 1983).

Yeasts have the ability to grow in the presence of various amine compounds as the nitrogen source, provided that ammonia salts are absent. Unexpectedly, the utilization of methylamine and ethylamine by yeast as a sole energy and carbon source is not possible (Dijken & Bos, 1981). Zwart and Harder (1983) explained this phenomenon by the high nitrogen to carbon ratio in these compounds. Oxidation of amines by amine oxidases provides the necessary carbon source, but there is also an excess of ammonium which represses the expression of the amine oxidase. Therefore, it is not unreasonable to expect that some microorganisms may grow only on longer carbon chain alkylamines (in which the proportion of carbon to nitrogen is lower than the aforementioned amines) as a sole carbon and nitrogen source. Indeed, limited growth of *Aspergillus versicolor* culture on pentylamine and hexylamine as the sole carbon and nitrogen source was observed (Lindley, 1987).

An exception to the inability of yeasts and fungi in general to grow on short chain amines as carbon, energy and nitrogen source was found in the case of strains isolated from soil of the species *Trichosporon adeninovorans*, which was able to grow on propylamine, butylamine and pentylamine (Middelhoven et al, 1984). Additionally, another species, *Trichosporon terrestre*, has also been reported previously to be able to utilize n-butylamine as carbon and nitrogen source (van der Walt & Johannsen, 1975).

As mentioned above, amine oxidase enzymes in yeasts are located in the peroxisomes. However, Fredbort et al. (1996) found also a copper containing amine oxidase located in the cell wall of an *A.niger* strain. The same group reported that the hydrogen peroxide is possibly released to the extracellular space where it is degraded by extracellular catalase. The presumed fate of the long chained linear amines is shown in figure 1.5.

The n – amine is converted to the respective aldehyde with the concomitant ammonium and hydrogen peroxide release, then the aldehyde is oxidized by an aldehyde dehydrogenase to the corresponding carboxylic acid, which subsequently undergoes β – oxidation (figure 1.5.). Therefore, the degradation of C 3 amines and above proceeds through the energy generating route of β – oxidation and higher biomass yields are expected, however, there are no experimental data to confirm this.

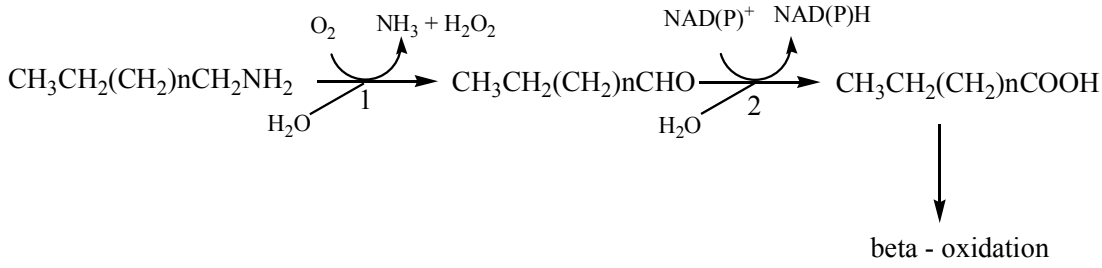

Figure 1.5. Utilisation of long carbon chain amines. The enzymes involved are 1. amine oxidase, 2. aldehyde dehydrogenase

1.1.1.5. Bacterial metabolism of amines and amine oxidase

Unlike fungi, bacteria follow different routes for utilizing methylamine. The enzymes formaldehyde and formate dehydrogenase are said to be absent from cultures of *Arthrobacter* P1 (Levering et al, 1981) as well as at some methylotrophic bacteria (Colby & Zatman, 1975).

Both methylamine and ethylamine enter the cell by an inducible amine-specific transport system (Dijkhuizen et al, 1982). The former is fixed in the ribulose monophosphate (RUMP) cycle, with the aid of the enzyme hexulose-6-

phosphate synthase. This pathway can be used for both energy generation and carbon assimilation. When it comes to ethylamine utilization, initial oxidation by an amine oxidase, is followed by the conversion of the resultant acetaldehyde to acetic acid, and then oxidation via the TCA cycle. As is common for growth on C 2 substrates (as well as fatty acids, hydrocarbons and any other substrate which is degraded into C_2 units) as sole carbon source, the synthesis of the enzymes isocitrate lyase and malate synthetase of the glyoxylate cycle is essential (Ratledge, 2006).

In contrast to many fungi, the bacteria examined so far have the ability of utilizing amines as the sole carbon and nitrogen source (Boer et al, 1989; Hacisalihoglu et al, 1997). Moreover, the conversion of amines to aldehyde in the Gram negative methylotroph *P. versutus,* and the pseudomonads, is carried out by the action of an amine dehydrogenase instead of the amine oxidase (Hacisalihoglu et al, 1997). Additionally, there is no polyamine oxidase activity found in any of the bacterial species which have been examined so far (Murooka et al, 1979).

1.1.1.6. Cytotoxicity of amines

Even though not well defined, aliphatic amines damage the cytoplasmic membranes, and their toxicity is increased with the increase in their hydrophobicity in neutral solution. Additionally, increased pH also increases the toxicity of these compounds, as their 'molecular' form penetrates the cell membrane with comparative ease (Albert, 1985). Subsequently, the molecules may damage many important cell organelles when they enter the cytosolic environment. Therefore, the amine oxidase expression can potentially serve the double role of both amine toxicity removal and use of amines as carbon, energy and nitrogen source.

1.1.2. *Aspergillus niger* as a host organism

1.1.2.1. Introduction : Aspergillus niger

Aspergillus niger is a filamentous fungus, with very simple nutritional needs, growing aerobically on organic matter. In nature, it can occupy a great variety of different environments such as soil, organic waste, or decaying plant material. *A.niger* has been the subject of research and industrial use for several decades due to its use as the major world source of citric acid and high value native enzymes. It first gained practical importance as early as in 1919, when its ability to produce citric acid was industrially exploited. Other acids such as gluconic, itaconic and fumaric acids have also been produced with *A. niger*, even though they are of less economic importance. The main advantages for the industrial use of *A.niger* are its ease of handling, its ability to ferment a wide variety of cheap raw materials and that has been repeatedly assessed as 'generally regarded as safe' by the World Health Organization and US Food and Drug Administration (Jones, 2007; Schuster et al, 2002).

1.1.2.2. Homologous expression of proteins in Aspergillus niger and applications

A.niger due to the absorptive means of obtaining its nutrients has the ability to secrete a wide range of enzymes. Therefore, it has long been used for the homologous production and isolation of higher-value enzyme products including pectinases, proteases, amyloglucosidases, cellulases, and hemicellulases. Glycoamylase and amylases are used for starch processing and the production of high maltose syrups (Murphy & Horgan, 2005). Pectin esterases, endo- and exo-polygalacturonidases and pectin lyases, produced from *A. niger* degrade pectin and they are used in wine and fruit juice production to reduce the juice viscosity, increase its filterability, improve clarification and make the concentration easier. Apart from pectinases, various hemicellulases and cellulases (which can also be provided by *A.niger*) are needed, all working together with the pectinases for the complete cell wall degradation (Grassin & Fauguenbergue, 1999). Hemicellulases are also used to improve the baking process by modifying the rheological properties of the dough and give higher loaf volume and better crumb structure of bread and pastry. Glucose

oxidase and catalase from *A. niger* are used for the enzymatic determination of glucose. Moreover the same enzymes can be exploited for the removal of either glucose or oxygen from foods and beverages and for the production of gluconic acid from glucose (Berka et al, 1992). Another biotechnological useful product of *A.niger* are β-glycosidases which can be used to enhance fruit and floral flavour in juices and wine. β-glycosidases hydrolyse glycosides (the glycosylated aroma precursors) releasing the aglycones which can be terpenes, norisoprenoids, aliphatics and phenolic compounds from the sugar moieties (Cheetham, 1999).

1.1.2.3. Heterologous expression of proteins in Aspergillus niger

Protein production by fungi is very efficient and can reach the levels of grams per litre. In the case of *A.niger* native glucoamylase production can reach the level of 30g/l (Ward et al, 2005). However, comparably high production levels have not been realized in the case of heterologous proteins (especially non – fungal protein production) (Lubertozzi & Keasling, 2009).

There are several factors influencing the production of heterologous proteins by the host organism. Limitations can take place during transcription, translation, translocation and secretion, and extracellular degradation may also occur (Gouka et al, 1997). With regard to transcription, increasing copies of the target gene increases mRNA up to a certain level above which there is no further increase, which is possibly the result of the inadequate specific regulatory protein concentration (Verdoes et al, 1993). In the case of the translational and post-translational levels, problems can take place at translation initiation and elongation. The correct glycosylation of proteins during their passage through the secretory system is also an important aspect of authenticity, particularly when it comes to therapeutic proteins (Archer et al, 1994). Besides glycosylation another important post-translational modification step is protein folding. Particularly the yield of the active protein is adversely affected by incorrect oligomerisation and inefficient ligand (metal-ion/heme) incorporation (Punt et al, 2002).

Moreover, limitations can occur after the secretion of the proteins, as they can be rapidly degraded by extracellular proteases (Van den Hombergh et al., 1995). It has been reported that there was very high production of proteolytic enzymes by the host cells which targeted heterologous proteins, and that even when this problem had been addressed by the deletion of the endogenous protease genes from the host genome, extracellular degradation still occurred in these protease deficient host strains (van den Hombergh et al, 1997).

1.1.2.4. Strategies for improving the heterologous protein production

As mentioned earlier, heterologous protein production from *A.niger* faces some limitation and for that reason different strategies have been adopted to improve protein yields. First of all, the introduction of the heterologous gene under control of an efficient fungal promoter, in a high copy number in order to maximize the transcription of the gene of interest was carried out (Verdoes et al, 1994). Initially the coding region of a non-fungal gene was fused to an efficient fungal expression signal. Later the heterologous gene expressing the product of interest was fused to the 3' end of a highly expressed gene, of which the gene product is efficiently secreted. It is believed that the amino-terminal fusion partner stabilizes the recombinant mRNA, serves as a carrier which improves the translocation of the protein into the endoplasmic reticulum (ER), helps the folding process and protects the heterologous portion from degradation. Fortunately, native protease activity cleaves the carrier from the target protein allowing recovery of pure product without an enzymatic post-treatment. For this gene-fusion strategy mainly the *A. niger* or *A. awamori* glucoamylase genes have been used (Gouka et al, 1997).

A problem often encountered in the heterologous expression is when proteins require codons that are rarely used in the desired host come from organisms that use non canonical code or contain expression-limiting regulatory elements within their coding sequence (Gustafsson et al, 2004). Studies of heterologous gene expression through codon optimization have reported improved heterologous production of the *Solanum tuberosum* α-glucan phosphorylase (Koda et al, 2005) and the *Aequorea victoria* aequorin (Nelson et al, 2004) from *A.niger*.

The high protease secretion problem was addressed by the development of acid protease-deficient host strains which led to reduction in proteolytic activity to approximately 20% of that of the wild types (van den Hombergh et al, 1997). However, even though the development of knock outs leads to improvement of the yields, the filamentous fungi are characterised by a very large number of different proteases which makes the disruption of all of them practically impossible (i.e. *A. nidulans* contains about 80 protease genes (Machida, 2002).

Apart from genetic strategies, process related strategies have been implemented with the aim of reducing the detrimental protease activities. A pH control strategy was adopted to reduce protease activity and increase recombinant yield of a glucoamylase –green fluorescent fusion protein by *A.niger* (O'Donnell et al, 2001). While Ahamed et al. (2005) linked a reduction of protease activity with a reduction of peptide nitrogen concentration in the medium. The same group found that the replacement of a yeast nitrogen base medium with a defined salts medium further reduced protease activity in culture supernatants. According to Wang et al. (2003) relatively low dissolved oxygen levels and high glucose concentration minimised protease activity and led to higher glucoamylase–green fluorescent fusion protein yields. In another study it was found that an effective way to significantly reduce the extracellular protease activity in *A.niger* shake flasks is the addition in the culture of the protease inhibitors chymostatin and pepstatin (serine and aspartic protease inhibitors respectively) (Ahamed et al, 2007).

1.1.3. *Escherichia coli* fermentations

1.1.3.1. Recombinant expression of products

Man has been altering the genetic makeup of many domestic animals from the ancient times by selective breeding. However, it was not until the early 1970's (thanks to the revolutionary work of Paul Berg, Herbert Boyer, and Stanley Cohen) when the direct genetic manipulation of the DNA of animals, plants and microorganisms became possible. The first stage of the DNA recombination is the identification and isolation of the gene – DNA fragment which is responsible for the particular phenotype. The next stage involves the purification of the fragment and the fusion of other DNA molecules in order to form a recombinant molecule. In the last stage, the recombinant molecule is inserted to an appropriate host organism (gene cloning) where the recombinant molecules are replicated by the DNA-synthesizing machinery of the host. Therefore, recombinant DNA technology enables us to clone the gene of interest in a foreign host and produce its product in large amounts.

1.1.3.2. Recombinant expression of proteins in Escherichia coli

Escherichia coli is the most common inhabitant of the gastrointestinal tract of mammals including humans. It was first isolated from the faeces of a child in 1885 by the Austrian pediatrician Theodor Escherich from whom it took its genus name. Some *E.coli* strains are harmless, while others are major animal pathogens. The pathogenic *E.coli* are categorised into those strains causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites. *E.coli* are Gram-negative, facultative anaerobe, non-sporeforming bacilli. They are approximately 0.5 m in diameter and 1.0–3.0 m in length (Welch, 2006).

1.1.3.3. Advantages of the expression of heterologous proteins in E.coli

Undoubtedly, *E.coli* is one of the most widely used host organisms for the expression of heterologous proteins. The preference of the *E.coli* use can be attributed to the well studied genetics of this bacterium (which are far better understood than those of

any other microorganism), the ease of handling and the ability to grow at very high cell densities on relatively cheap substrates, while reaching very high specific growth rates resulting in reduction of the fermentation time and therefore the cost of the process. In addition, the availability of an increasing large number of cloning vectors and mutant host strains, and the generally high yield levels of recombinant proteins have also contributed to the predominance of *E.coli* expression systems (Baneyx, 1999 ; Demain & Vaishnav, 2009). A very wide range of heterologous proteins have been produced from *E.coli* strains which is the standard method for the massive production of many commercialized proteins despite the significant progress in new expression systems, such as methylotrophic yeast strains *Pichia pastoris*, *Candida boidinii*, *Hansenula polymorpha* (now called *Pichia angusta*) (Mack et al, 2009; Yurimoto, 2009), and bacterial strains *Streptomyces lividans* (Ayadi et al, 2007; Pimienta et al, 2007), *Bacillus subtilis* (Tobe et al, 2006), and *Pseudomonas fluorescens* (Chew et al, 2005).

1.1.3.4. Disadvantages of the expression of heterologous proteins in E.coli

Despite the many advantages the *E.coli* expression systems present, there are also some flaws especially when proteins of eukaryotic origin are expressed. The most important weaknesses of *E. coli* as an expression system include the inability to perform many of the post-translational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, and the limited ability to facilitate extensive disulphide bond formation (Makrides, 1996). In addition a disadvantage for therapeutic use of produced recombinant proteins in *E. coli* is the accumulation of lipopolysaccharide (LPS), generally referred as endotoxins, which are pyrogenic in humans as well as other mammals. Proteins intended to be used for this kind of applications must be purified in a second step to become endotoxin-free (Petsch & Anspach, 2000).

1.1.3.5. Recombinant protein production induced stress

E.coli cells as the host for the production of recombinant proteins have long been used with good results; however, they are not designed by nature for the

overproduction of recombinant proteins and as a result cells are faced with various kinds of stresses which often resemble environmental stresses such as heat shock, amino acid depletion and starvation. Additionally, the expression of proteins from species very distinct from *E.coli* causes problems in the correct folding of the proteins especially when it comes to the expression of eukaryotic protein as *E.coli* lacks the machinery for the post translational modifications. The main problems faced in the recombinant expression of proteins in *E.coli* are discussed below.

The metabolic burden imposed on the host cells can be attributed not only to the expression of the genes of the target proteins but also in some cases to the constitutive expression of the antibiotic resistance genes (Hoffmann & Rinas, 2001). However, the main perturbation of the cellular metabolism is always caused when the target protein, usually controlled by strong promoters, is synthesized at high rates. An additional perturbation takes place in the cases where the target product directly influences host cell metabolism. Regarding the latter problem, several strategies have been explored to deal with the toxicity in production processes such as generating properly engineered protein fusions (Wan et al, 1995) and promoting periplasmic secretion (Korant & Rizzo, 1991). This technology also faces some problems, with the most prominent being the basal level of expression of the target protein in uninduced cells especially when the target protein is toxic to the cell.

1.1.3.6. Effects on growth

As mentioned earlier, the cells carrying recombinant plasmids face a metabolic burden. This burden can be defined as the amount of resources, both in the form of energy such as ATP or GTP, and raw materials such as amino acids - that is required to maintain and express foreign DNA in the cell (Glick, 1995). Therefore, the expression of the foreign proteins diverts the use of resources towards heterologous protein synthesis and as a result fewer resources are directed towards growth and for the production of housekeeping proteins. This has some clear consequences. A) Not surprisingly, the specific growth rate of producing cells has been shown to be inversely proportional with the recombinant protein synthesis rate (Jensen & Carlsen, 1990). Moreover, growth completely ceases when only 30% of the normal proteins

are replaced by the target protein, while there is an inverse correlation between growth rate and accumulation level when varied as a function of inducer concentration (Dong et al, 1995). B) The biomass levels are lowered and the respiratory activity is elevated after the induction of the culture due to the energy demand for the extra synthesis of plasmid-encoded and heat-shock proteins (Hoffmann & Rinas, 2001). C) It has also been observed that after induction cells gradually lose the capability to divide, while they remain metabolically active and the specific productivity remains unaffected, which results in an increasing fraction of viable but non culturable cells in the culture (Andersson et al, 1996). However, after induction, there can be an increase in the dry cell weight levels, which flow cytometry studies have shown that this increase is not caused by cell proliferation but by an increase in cell size (Borth et al, 1998; Soriano et al, 1999).

It is well documented that the most energy consuming process in the cell is the synthesis of proteins. Therefore, in the recombinant protein overproducing cells, regeneration of ATP is of critical importance for the maintenance of the normal physiological state of the cell. According to Da Silva and Bailey (1986), the growth rates and biomass yields are not critically affected by the recombinant protein production when the recombinant protein production equals the accumulation rate; however, if there is a simultaneous degradation of the target protein then the energy demand for the target protein synthesis can increase the need for ATP regeneration, through enhanced respiration, at the cost of the biomass formation even with negligible or low accumulation of the target protein (Hoffmann & Rinas, 2001; Schmidt et al, 1999; Weber et al, 2002). In addition, other cellular functions are enhanced during the recombinant protein overproduction such as RNA turnover, protein turnover and energy driven non-equilibrium processes like transport of nutrients can also contribute to the high maintenance requirements upon induction (Bhattacharya & Dubey, 1995). Moreover, the activation of stress responses, such heat shock protein production increases the energy demand for protein synthesis (Hoffmann & Rinas, 2001).

It has been observed that during the recombinant protein production there is a reduction in the synthesis and levels of housekeeping proteins (Vind et al, 1993), including the components of the protein producing system (Dong et al, 1995; Hoffmann et al, 2002; Jürgen et al, 2000). Additionally, increase of the recombinant protein synthesis rate by, for example, manipulating the efficiency of the ribosome binding site (Vind et al, 1993) or by increasing inducer's concentrations (Hartsock et al, 1995) results in a stronger inhibition of the synthesis of the housekeeping proteins. An explanation of the aforementioned paradoxical phenomenon was given by Vind et al. (1993) who suggested that the decreased concentrations of free ribosomal subunits leads to an increased competition among the individual ribosome binding sites for ribosome, and as a consequence the cellular capacity for synthesizing housekeeping proteins (including proteins of the protein producing system) is reduced. Moreover, stronger affinities of the target mRNA for the ribosome comparing to that of the naturally expressed proteins may also add to the phenomenon (Kurland & Dong, 1996). From the above it can be concluded that the synthesis rate and not the accumulation of the recombinant product determines the extent of the inhibition (Hoffmann & Rinas, 2004).

Another reason for the decreased biosynthetic capacity of the induced cells may be an accelerated degradation of the rRNA. This degradation of rRNA is cumulative and contributes to the progressive loss of the protein synthesis capacity (Dong et al, 1995).

A phenomenon called stringent response or stringent control has been observed in cells in amino acid limited cultures where disassembly and degradation of ribosome takes place. In the same manner, recombinant protein overproducing cells face an internal nutritional downshift and subsequently destroy their ribosomes and the rRNA. In contrast to the externally starved cells, recombinant protein overproducing cells cannot return to their previous state and remain inactivated. This behaviour does not allow continuous growth of bacteria while overproducing the recombinant protein. Generally, protein synthesis and growth cease as the expression levels of the target proteins reach 30% (Kurland & Dong, 1996).

1.1.3.7. Inclusion bodies

The most common problem encountered during the recombinant protein production in *E.coli* cells is the formation of insoluble protein aggregates known as inclusion bodies (Ventura & Villaverde, 2006). It is commonly reported that these aggregated proteins are biologically inactive; however, there have been reported cases where they exhibit important enzymatic activity (Tokatlidis et al, 1991).

Even though the recovery of functional proteins by *in vitro* preparatory refolding from inclusion bodies is possible (Middelberg, 2002), it involves optimisation of the process for a particular protein species which results in time consuming efforts and inconsistent outcomes. Additionally, another disadvantage may be poor recovery yields of the soluble protein (Sørensen & Mortensen, 2005b). Therefore, soluble protein production is preferable; however, the limited knowledge of the molecular basis of protein aggregation as well as the protein species variability does not enable us to drive the soluble protein production with the appropriate metabolic or genetic engineering tools (Villaverde & Carrió, 2003).

The formation of the inclusion bodies can be attributed to several reasons. First of all, the cytosolic concentration of macromolecules such as ribosomes, nucleic acids and proteins, is very high especially in the case of the recombinant high-level expression. The protein content in the cytoplasm of transformed *E.coli* cells can reach 200-300mg/ml (Hammarström et al, 2002). In this crowded macromolecular environment exposed hydrophobic residues of nascent polypeptides and folding intermediates may interact inappropriately leading to mis-folding and aggregation (Mogk et al, 2002). In addition, the accumulation of unstructured protein fragments as a result of proteolytic attack contributes to the increased propensity for aggregation (Cabrita & Bottomley, 2004). Another very important causative factor may be the lack of the post-translational modifications needed for the solubility of some eukaryotic polypeptides (Zhang et al, 1998). Improper folding and consequently aggregation can take place if the target protein requires disulphide pairing, which in the reducing cytoplasmic environment is unfavourable (Lilie et al, 1998). It is worth noting that even correctly folded proteins are in danger of

spontaneously unfolding and losing their active structure due to Brownian motion and thermal vibrations (Mogk et al, 2002).

E.coli cells are equipped with an elaborate protection system against the protein aggregates. This system is comprised of a set of molecular chaperones which fight protein aggregation at various stages in the protein's life. To begin with, during the translation process at the ribosomes, the newly synthesized polypeptide chains expose hydrophobic patches which are prone to aggregation. Specific chaperones named trigger factors are associated with the big subunit of the ribosomes and protect these patches from unintended inter or intramolecular interactions and prevent premature folding until a complete domain has emerged from the ribosomal exit site (Bukau et al, 2000; Sørensen & Mortensen, 2005a). Apart from the co-translational level chaperones play a significant role in the post translational level as well. Folder chaperones which belong to the chaperone family HSP70 like the DnaK and GroE systems interact with unfolded proteins and mediate their refolding in an ATP dependent process. In the case of proteins which are not correctly folded (including heat denatured proteins), chaperones of the sHSP family also named holder chaperones (such as IbpA and IbpB) bind to these proteins and prevent their aggregation (Kuczynska-Wisnik et al, 2002). The chaperone SecB binds to the proteins destined for secretion and keeps the secretory proteins in an unfolded state suitable for translocation (Bukau et al, 2000). At a subsequent level, when proteins have already formed aggregates, a bi – chaperone system consisting of the DnaK and ClpB has the capability of disaggregating and mediating refolding of the mis-folded proteins. Moreover, there are also chaperones which possess direct proteolytic activities such as the ClpA which acts together with the ClpxP. It can be also stated that chaperones such as the holders exhibit an indirect proteolytic activity as they stabilise and keep in soluble state mis-folded proteins and thus paving the way for the proteases to degrade them (Schlieker et al, 2002).

Many strategies have been applied to improve recombinant protein solubility in *E. coli.* One very effective way is by lowering the culture temperature. This strategy has been implemented with success in improving the solubility of a number of difficult

proteins including human interferon α-2, subtilisin E, ricin A chain, bacterial luciferase, Fab fragments, β-lactamase, rice lipoxygenase L-2, soybean lipoxygenase L-1, kanamycin nuclotidyltransferase and rabbit muscle glycogen phosphorylase (Vasina & Baneyx, 1997).

Other strategies include simultaneous over-expression of chaperone encoding genes and recombinant target protein genes. According to Ikura et al. (2002) co-overexpression of a molecular chaperone system (DnaK-DnaJ-GrpE) or a folding catalyst (trigger factor) significantly improved the solubility of the recombinant transglutaminase produced in *E.coli* cells. Similarly aggregation and degradation was effectively reduced in the case of the aggregation-prone mouse endostatin, human oxygen-regulated protein ORP150 and human lysozyme produced in *E.coli* when trigger factor was co-expressed especially when the GroEL-GroES chaperone system was simultaneously overproduced (Nishihara et al, 2000). The chaperone systems are co-operative in nature, and the most effective approaches usually involve co-expression of combinations of chaperones belonging to the GroEL, DnaK, ClpB and the ribosome associated trigger factor families of chaperones (Sørensen & Mortensen, 2005b).

Other strategies include the substitution of selected amino acid residues responsible for the protein aggregation (Rinas et al, 1992), cultivation and induction of the cells under osmotic stress in the presence of sorbitol and glycyl betaine (Blackwell & Horgan, 1991), and the co-expression of the eukaryotic protein disulphide isomerases in *E. coli* can also favour the formation of disulfide bonds especially in the less reducing environment of the periplasmic space (Zhan et al, 1999). Moreover, significant progress has been made recently in the field concerning the *in vitro* inclusion bodies solubilisation and refolding in order to get functional and active products (Mayer & Buchner, 2006).

1.1.3.8. Stringent response

Overproduction of a recombinant protein (in *E.coli* cells) with an amino acid composition significantly different from that of the "average" *E. coli* protein can lead

to low protein yields. This event can be attributed to a phenomenon called stringent response and it can be triggered by sudden amino acid depletion. Naturally a shortage of amino acids takes place during the transition of a culture from the exponential to stationary phase where the ratio of aminoacylated-tRNA to uncharged tRNA molecules is significantly reduced due to the lack of amino acids in the intracellular amino acid pool (Chang et al, 2002). Then an alarmone (guanosine tetra-phosphate) is produced, by ribosome-bound RelA upon binding of uncharged tRNA to ribosomes, and mediates the stringent response (Cashel et al, 1996). As a result cells inhibit rRNA synthesis (and indirectly synthesis of the translation and transcription apparatus), induce and repress metabolic pathways in accordance with their physiological needs, and induce many stationary phase survival genes (Cashel et al, 1996; Hengge-Aronis, 1999). Moreover, it has been assumed that an increase in the protease activity after the induction of recombinant protein, with unusual amino acid composition, is linked to stringent response and in particular the need of cells to use the required amino acids (Harcum & Bentley, 1999). A simple and effective way to alleviate this problem is the addition of the appropriate amino acids which will consequently reduce the degradation of the recombinant product (Harcum, 2002).

1.1.3.9. SOS response

The SOS response is a regulatory network that is induced when cells are exposed to a variety of physical and chemical agents that result in DNA damage or interference with DNA replication (Lee et al, 2002b). It has been found that SOS response-inducing events can occur in recombinant systems, when a heterologous protein is overproduced, however it is not yet clear what exactly the causative agent is. There are examples where SOS induction is observed in cultures producing a heterologous protein using a temperature-inducible expression system (Hoffmann et al, 1999) while no SOS induction was recorded using an IPTG-inducible expression system even at elevated temperature (Arís et al, 1998). It has been suggested that transcription of plasmid-encoded sequences provokes induction of the SOS response indirectly through its impact on DNA replication and/or DNA topology (Hoffmann & Rinas, 2004).

1.1.3.10. Heat shock response

In nature the heat shock response is triggered when cells are exposed to a sudden temperature upshift, and comprises a regulon that is controlled by the sigma factor σ^{32} (Hoffmann & Rinas, 2004). The expressed heat shock proteins are molecular chaperones, including DnaK, DnaJ and GrpE, and GroEL and GroES, and proteases with the aim of enhancing protein-folding, degradation, and repair. It has been reported that the accumulation of heterologous or abnormal proteins causes an increase in the expression of gene products belonging to the heat shock family of proteins such as GroEL and DnaK (Dong et al, 1995). Additionally, under these conditions, the production of the *lon* gene product as well as the expression of other proteases is enhanced (Goff & Goldberg, 1987). During the production of recombinant protein inclusion bodies, the concentration of the small heat shock proteins IbpA and IbpB was also found to be increased (Jürgen et al, 2000).

1.1.3.11. The pET expression system

The expression system used in the present study is the pET expression which was developed by Dubendorff & Studier (1991) and Studier et al. (1990), for a variety of expression applications. This system comprises hybrid promoters, multiple cloning sites for the incorporation of different fusion partners and protease cleavage sites, and a high number of genetic backgrounds modified for various expression purposes (Sørensen & Mortensen, 2005a). Target genes are incorporated into the plasmids under the control of a strong bacteriophage T7 promoter. The expression of the target genes is induced by providing a source of T7 RNA polymerase from the host strain

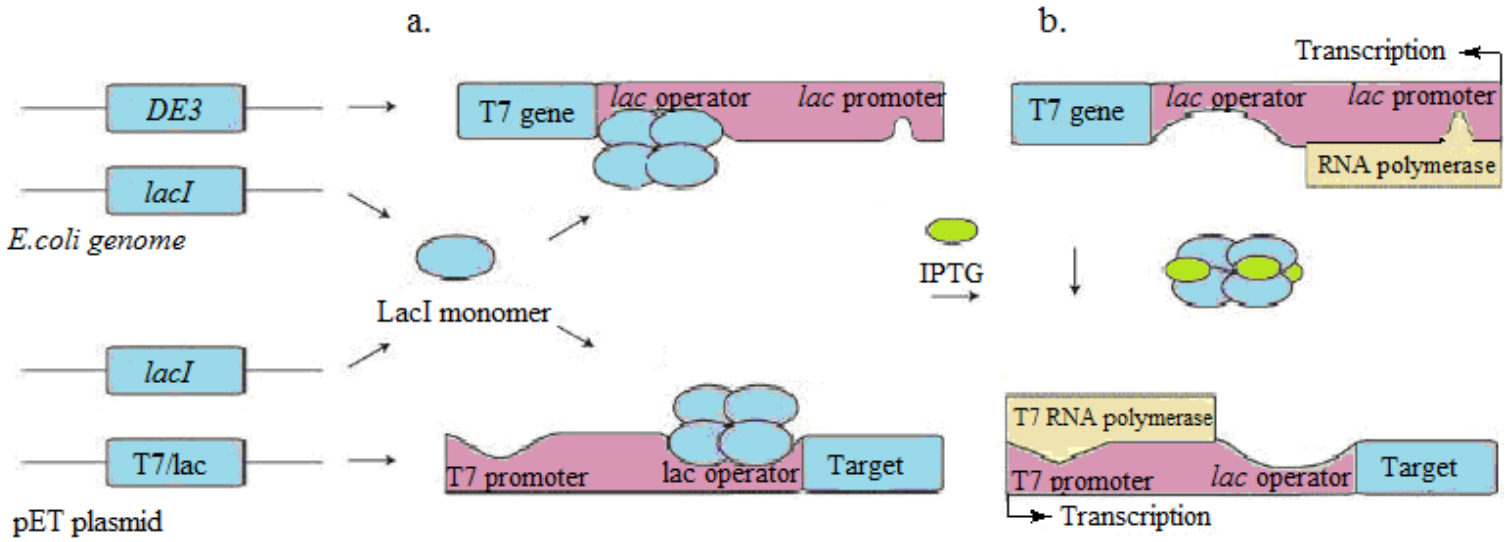

Figure 1.6. The pET expression system. From left to right are shown a general pET plasmid configuration, the macromolecular situations prior to (a) and after induction (b). The lacI gene product functions as a tetramer that binds to the *lac* operator and inhibits transcription of both the genomic lysogenised T7 RNA polymerase and the target protein of the plasmid. When allolactose or IPTG is present bind and allosterically alter the repressor which subsequently looses its affinity for the operator. As a result the native RNA polymerase transcribes the T7 RNA polymerase which subsequently transcribes the target gene (Dubendorff & Studier, 1991; Studier et al, 1990).

which is lysogenized by a DE3 phage fragment, and therefore, encoding the T7 RNA polymerase (Mierendorf et al, 1998). The T7 RNA polymerase is under the transcriptional control of the IPTG inducible *lacUV5* promoter. *LacI* is a weakly expressed gene which encodes for the lac repressor which binds both at the *lacUV5* promoter in the bacterial genome and the T7/lac hybrid promoter located in the plasmid.

When an inducer, for example, IPTG is added, it will bind the tetrameric LacI and trigger its release from the *lac* operator and subsequently T7 RNA polymerase can be transcribed by the RNA polymerase of the host. Simultaneously with the release of the LacI from the genomic *lac* operator, the plasmid's *lac* operator is also freed from the LacI and therefore the target genes can be transcribed by the T7 RNA polymerase.

T7 RNA polymerase transcribes only from the *T7* promoter and its efficiency, specificity, and speed of transcription (230 nucleotides per second comparing to only 50 nucleotides per second for the *E. coli* RNA polymerase) results in the production of the target protein at levels of up to 30% of the total *E.coli* protein (Dong et al, 1995).

1.1.3.12. Other promoters

Even though the choice of the appropriate promoter for the expression of recombinant proteins is highly dependent on the nature of the target protein and how it affects the host physiology, there are some general features which make a promoter suitable.

The promoter should generally be strong, and tightly regulated (having a low basal expression level). A highly repressible promoter is particularly important in cases where the expressed protein is toxic or detrimental to the growth of the host cell. Moreover, an ideal promoter should be easily transferable to other *E. coli* strains, and its induction should be simple and cost-effective. Finally the frequently used ingredients of culture media should be compatible with the promoter (Hannig &

Makrides, 1998). Most of the promoters used in *E.coli* systems are derived from the *lac* promoter which is responsible for the utilization of lactose as a carbon source. Characteristic examples are the *trc* and *tac* which are strong promoters and allow the accumulation of up to 15-30% of total cell protein. Induction of all these promoters including lac promoter is achieved by adding either lactose or the non-hydrolysable lactose analog isopropyl-β-D-1-thiolgalactopyranoside (IPTG). All three promoter systems are regulated by catabolite repression and the metabolic state, which is represented by the cyclic AMP level (Terpe, 2006).

However, when scaling up, IPTG is not ideal because of its high cost and toxicity, while lactose requires constant monitoring during the induction period. Moreover, negatively regulated promoters are not tightly regulated and can show high basal expression levels under noninduced conditions, as observed, for example, with the *tac* promoter, leading to instability and plasmid loss in the culture due to the high metabolic burden. The introduction of a temperature-sensitive mutant *lacI* gene that encodes a thermosensitive *lac* repressor offers a suitable method to induce *lac*-based promoters (Andrews et al, 1996). A disadvantage of thermally inducible gene expression is the induction of the heat-shock response and the concomitant upregulation of proteases. This problem is alleviated by the use of host strains with a deficient *rpoH* (*htpR*) locus. Another option for the heterologous protein production in *E.coli* is the use of the *araBAD* promoter of the arabinose operon. L-arabinose acts as inducer with the activator AraC in the positive control of the arabinose regulon while since arabinose catabolism in *E.coli* is facilitated through the phosphoenolpyruvate: sugar phosphotransferase system, glucose represses the expression of the genes placed downstream the *araBAD* promoter (Schleif 1996). This promoter has also been successfully cloned in other Gram negative species such as *Pseudomonas putida* and *Burkholderia cepacia* for the production of recombinant proteins (Prior et al, 2010). The *araBAD* promoter has been reported to function under very high cell- density conditions; however, the recombinant product quality was shown to be lower than in experiments with lower cell densities (DeLisa et al, 1999).

Another positively regulated promoter used for the recombinant expression of proteins is the *rhaBAD*. In principle, L-rhamnose acts as an inducer with the activator RhaR for synthesis of RhaS, which in turn acts as an activator in the positive control of the rhamnose regulon (Brautaset et al, 2009). The L-rhamnose regulons are also regulated by catabolite repression. A drawback of the particular system is the high cost of the inducer. However, Wilms et al. (2001) reduced the need for L-rhamnose by inhibiting its consumption through the inactivation of L-rhamnulose kinase encoding gene *rhaB*.

1.1.4. *Pichia pastoris* expression systems

1.1.4.1. Background

A survey into the literature shows that *P.pastoris* has emerged as a very successful system for the production of a great variety of heterologous proteins. However, in the beginning of its biotechnological history, *P.pastoris* along with other methylotrophic yeasts belonging to the genera *Hansenula*, *Candida*, *Torulopsis* were employed as potential sources of single-cell protein (SCP) to be marketed primarily as high-protein animal feed. The low cost of methane (the source of the methanol) during the 1970's motivated Phillips Petroleum Company to develop media and protocols for growing *P.pastoris* on methanol in continuous culture at high cell densities (Cereghino & Cregg, 2000). However, the oil crisis of the 1970's caused a dramatic increase in the cost of methane (the source of the methanol). At the same time, the price of soybeans, the major alternative source of animal feed, fell. As a consequence, the economics of single cell protein (SCP) production from methanol became highly unfavourable (Macauley-Patrick et al, 2005). Then, experimentation with the strong and tightly regulated alcohol oxidase (AOX) promoter led to the conclusion that *P.pastoris* could serve as an exceptional system for heterologous protein expression. Moreover, the specific cell compartments which are abundantly present in methanol-grown cells, namely peroxisomes became an object of investigation (Faber et al, 1995).

1.1.4.2. Methanol metabolism

A limited number of yeast species has been found to be capable of growing on methanol as sole carbon and energy source. The so-called methylotrophic yeasts belong to the genera *Hansenula*, *Pichia*, *Candida* and *Torulopsis.* The most prominent representatives which have been used in biotechnology are *Pichia pastoris*, *Hansenula polymorpha* (*Pichia angusta*), *Candida boidinii* and *Pichia methanolica* (Houard et al, 2002).

Yeasts able to grow on methanol share a unique methanol utilisation pathway whose expression is tightly regulated at the level of transcription (Hartner & Glieder, 2006). During growth on methanol, peroxisomes play an indispensable role as they harbour the three key enzymes for methanol metabolism, alcohol oxidase, catalase and dihydroxyacetone synthase.

As can be seen in figure 1.7., initially methanol is oxidised to formaldehyde, generating hydrogen peroxide in the process, by the enzyme alcohol oxidase (AOX). Then the toxic hydrogen peroxide is broken down to oxygen and water by the action of catalase which can act catalatically or peroxidatively. Formaldehyde is either oxidised by two subsequent dehydrogenase reactions (dissimilation pathway) or assimilated in the cell metabolism by condensation with xylulose 5-phosphate (assimilation pathway). The condensation is facilitated by the dihydroxyacetone synthase which catalyses the formation of dihydroxyacetone and glyceraldehyde-3-phosphate from formaldehyde and xylulose-5-P. These C_3 compounds are further assimilated within the cytosol. Fructose -1-6-biphosphate is formed by an aldolase reaction of dihydroxyacetone and glyceraldehyde-3-phosphate, which is then converted to fructose – 6 – phosphate by a phosphatase. Xylulose-5-phosphate is regenerated by the xylulose monophosphate cycle involving transaldolase, transketolase, pentose isomerase and epimerase reactions. One-third of the glyceraldehyde-3-phosphate generated is used for biomass and energy generation (Jahic, 2003).

In the dissimilation pathway (figure 1.7), formaldehyde diffuses from the peroxisomes and spontaneously reacts with glutathione to *S*-hydroxymethylglutathione which is then oxidised in two consecutive reactions to carbon dioxide by the action of a glutathione (GSH)- and NAD^+-dependent formaldehyde dehydrogenase and a NAD^+-dependent formate dehydrogenase, both located in the cytosol. It is believed that the NADH, generated in both dehydrogenase reactions is used in energy production for growth on methanol (Sakai et al, 1997). Apart from the role in energy production, the dissimilation pathway enzymes play a role in the detoxification of formaldehyde and formate, respectively (Patel et al, 1983). A third enzyme involved in this pathway is *S*-formylglutathione hydrolase which hydrolyses the S-formylglutathione to formate and glutathione. It participates in the detoxification of formaldehyde and regenerates glutathione (Lee et al, 2002a; Patel et al, 1983).

In all methylotrophic yeasts glucose and ethanol strongly repress the expression of the methanol pathway genes. In the case of glycerol the extend of repression depends on the yeast species, with *P.pastoris* showing strong repression, whereas use of methanol invariably results in induction (van der Klei et al, 2006).

1.1.4.3. Methanol utilisation phenotype

The cultivation of cells in a medium with methanol as the sole carbon source results in the positive regulation of the expression of *AOX1* and *AOX2* genes. The major alcohol oxidase isozyme is encoded by the *AOX1* gene, responsible for approximately 85% of the utilization of methanol by the alcohol oxidase enzyme. Even though the exact physiological role of the *AOX2* gene in *P. pastoris* is not known, it has been found that for *P. methanolica* and *C. boidinii* its expression is an advantage under high methanol concentrations (Hartner & Glieder, 2006).

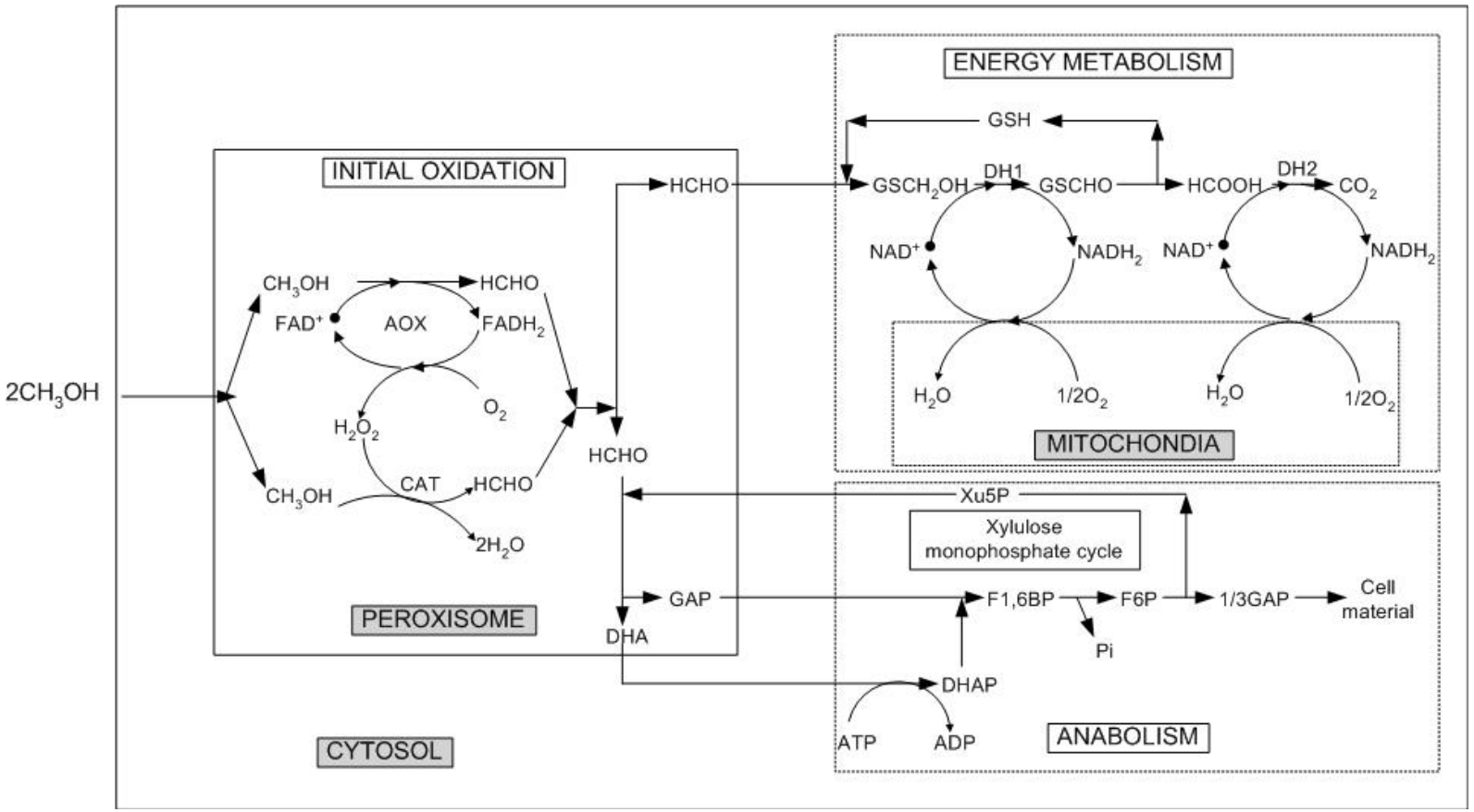

Figure 1.7. Methanol metabolism in *P. pastoris*. AOX: alcohol oxidase; CAT: catalase; DH1: formaldehyde dehydrogenase; DH2: formate dehydrogenase; GSCH2OH S-hydroxymethylglutathione; GSCHO, S-formylglutathione;GAP: glyceroldehyde-3-phosphate; DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose-1,6-bisphosphate; F6P: fructose-6-phosphate; Xu5P: xylulose-5-phosphate. Adapted from Charoenrat et al. (2005).

The alcohol oxidase of the *AOX1* gene has a poor affinity for oxygen and for that reason, *P.pastoris* counterbalances this deficiency by up-regulating the *AOX1* promoter to drive expression of the *AOX1* gene and produce larger amounts of the AOX1 enzyme. In the presence of methanol as the sole carbon source in the culture the AOX enzyme can reach 30% of total cellular protein (Gellissen, 2000). Therefore, the strong *AOX1* promoter can effectively be used to drive the expression of recombinant proteins to high levels even with a single integrated copy of the expression cassette (Daly & Hearn, 2005). Depending on the method of transformation the resulting constructs may have three different phenotypes, Mut^+, mut^s and mut^-. In the first case the expression cassette is inserted into the *HIS4*, and the growth of this transformant on methanol is indistinguishable from wild-type *P. pastoris* cells (Chiruvolu et al, 1997). In the case of mut^s strains the expression cassette is replacing or inserted into the *AOX1* gene, resulting in inactivation of the gene and cells rely on the alcohol oxidase enzyme being produced from the alternative gene (*AOX2*). The AOX2 enzyme has the same specific activity as AOX1 but has a much lower expression level (its transcription is controlled by a weaker promoter) and as a result the methanol consumption is much lower, hence the phenotype of these strains are termed 'methanol utilization slow' (Mut^s). The third host strain used for heterologous protein expression is the mut^- (methanol utilization negative) strain in which both the *AOX1 and AOX2* genes are disrupted. In this case, this inability to grow on methanol requires the use of an alternate carbon source, such as glycerol, for growth and recombinant protein production. However non-limiting glycerol concentrations in the culture causes repression of the *AOX1* promoter (Chiruvolu et al, 1997; Inan & Meagher, 2001). All the phenotypes described, have both advantages and disadvantages. The mut^+ phenotype may give higher growth rates during the induction period; however, the consumption of large amount of methanol and also the storage of these amounts in explosion-proof facilities increases further the cost (Cereghino & Cregg, 1999). Since the alcohol oxidase enzyme needs oxygen for the oxidation of methanol, an important disadvantage of the mut^+ against both mut^s and especially mut^- phenotype is the very high oxygen requirement that can result in oxygen-deficient conditions within the bioreactor (Files et al, 2001).

This is the reason for the lower amount of recombinant proteins produced in mut^+ than mut^s in shake flask cultures (Cregg et al, 1993). A disadvantage of the use of mut^s construct though, is the very long induction times needed for maximal protein expression which can reach 100h (Chen et al, 1997). Nevertheless, as can be seen in section 1.1.4.5. this problem can be alleviated with the mixed feed of methanol and an alternative carbon source which can reduce the induction time, increase cell density and increase the volumetric productivity of protein (Celik et al, 2009; Chiruvolu et al, 1997; Loewen et al, 1997).

1.1.4.4. Alternative promoters

Even though, the AOX1 promoter has been successfully used for the expression of numerous foreign genes, there are circumstances in which this promoter may not be suitable. The most prominent example is the use of methanol to induce gene expression for the production of food products since methane, a petroleum-related compound, is one source of methanol (Ilgen et al, 2005). Alternative promoters to the AOX1 promoter are the *P. pastoris* GAP, FLD1, PEX8, and YPT1 promoters.

The *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) promoter provides strong constitutive expression on glucose, glycerol, methanol or oleic acid at levels high enough to be compared to that seen with the AOX1 promoter (Waterham et al, 1997). The advantages of using this promoter is that methanol is not necessary for induction, and there is no need for shifting cultures from one carbon source to another. Some authors have reported that *GAP* promoter is more efficient that *AOX1* promoter for heterologous protein expression (Delroisse et al, 2005; Döring et al, 1998; Menendez et al, 2003; Waterham et al, 1997). However, in some of these cases the difference in the expression can be attributed to the hypoxic conditions prevailing in the methanol induced cultures. On the other hand, the constitutively expressing nature of the GAP promoter renders it inappropriate for the production of proteins that are toxic to the yeast cells (Cereghino & Cregg, 2000).

Another strong expression promoter is that of the glutathione-dependent formaldehyde dehydrogenase (*FLD1*) which can be induced by either methanol as a

sole carbon source (and ammonium sulphate as a nitrogen source) or methylamine as a sole nitrogen source (and glucose as a carbon source) (Shen et al, 1998). An alternative to the strong expression promoters described so far is the *P. pastoris* PEX8 and YPT1 promoters. The first one promotes the encoding of a peroxisomal matrix protein that is essential for peroxisome biogenesis and it promotes the expression at a low but signicant level on glucose and is induced modestly when cells are shifted to methanol. (Liu et al, 1995) while the second one promotes the encoding of a GTPase involved in secretion and it provides a low but constitutive level of expression in media containing either glucose, methanol, or mannitol as carbon sources (Sears et al, 1998). A recently used promoter for heterologous protein expression is that of the *P.pastoris* enzyme isocytrate lysase (ICL1) which is repressed in the presence of glucose and induced in its absence, or in presence of ethanol (Menendez et al, 2003).

1.1.4.5. Operational strategies

As in every expression system, for the *P.pastoris* systems the optimum conditions (medium, feeding profile, pH, temperature etc) for the recombinant protein production depend on the kind of the strain used and the nature of the foreign protein expressed. However, there are some general guidelines which allow improvement of the productivity.

When high productivity of recombinant proteins is required then cell growth is important for the protein production in bioreactors, since the concentration of the product is roughly proportional to the concentration of cells in the culture (Macauley-Patrick et al, 2005). Therefore, the use of fed-batch bioprocessing is the best way to achieve this objective. *P.pastoris* fed-batch fermentation protocols often include three different stages after the initial batch. A glycerol batch stage is followed by a feeding stage where glycerol is added to increase cell growth to the appropriate levels followed by a transition phase where the induction medium starts to be fed, and finally a methanol induction stage (figure 1.8.).

The objective of the first stage is to get an appropriate biomass level before the onset of the exponential feeding. Cos et al. (2005) reported a specific growth rate of $0.18h^{-1}$ for both mut^s and mut^+ phenotypes of a *P.pastoris* strain during batch growth phase on glycerol. According to other reports , the specific growth rate on methanol of mut^+ phenotype is slower, reaching only $0.14h^{-1}$ (Brierley et al, 1990) while a constitutively flavin expressing *P.pastoris* strain exhibited a specific growth rate reaching approximately $0.13h^{-1}$ (Marx et al, 2008). The presence of four genes encoding putative H^+/glycerol symporters explains the higher specific growth rates on glycerol as a substrate than on glucose and methanol (Lages et al, 1999). Moreover, respiratory (Crabtree-negative) yeasts limit glucose uptake, as they contain few hexose transporter genes, encoding energy dependent symporters with high affinity to glucose. Therefore, Crabtree-negative yeasts can restrict the entry of glucose by their regulated H^+-symport systems and hence prevent the occurrence of overflow metabolism (Van Urk et al, 1989). Consequently, glycerol has been the carbon source of choice in the majority of the works noted in the literature so far. The concentration of glycerol in the batch medium is less than 40 g/l since it has been reported that at higher concentration growth inhibition may take place (Cos et al, 2006). Additionally, in glycerol concentrations above 70 g/l, ethanol formation has been reported (Chiruvolu et al, 1998).

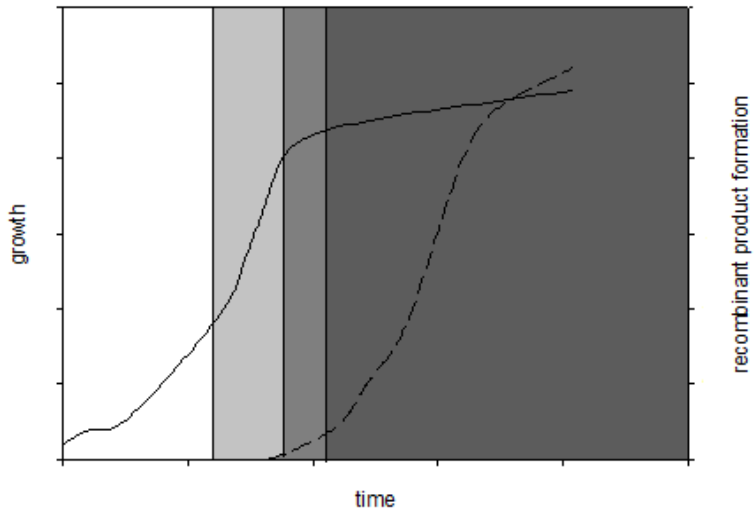

Figure 1.8. Stages of a typical methanol induced *P.pastoris* bioprocess. The different stages are represented with different colours, batch phase (□), feeding phase (■), transition phase (■), methanol feed phase (■). Biomass formation (-) and recombinant protein production (---)..

The feeding stage starts when glycerol is fully consumed and a concomitant increase of the dissolved oxygen tension takes place. The objectives of the feeding stage is firstly, to increase further the biomass level to the appropriate cell density and secondly to gradually introduce the culture to the methanol feeding medium. This takes place through a gradual decrease of the glycerol feeding medium and then gradual slow introduction of the induction medium (containing methanol) which allows the derepression of *AOX1* promoter due to the absence of excess glycerol prior to the beginning of the production stage (Zhang et al, 2000a). In some protocols the decrease of the first and the introduction of the latter happen simultaneously (Potvin et al, 2010). The purpose of the transition is to de-repress the *AOX1* and to allow enough time for the cells express the enzymes of the methanol utilisation pathway so that methanol build-ups and loss of viability will be avoided. It usually takes 2-5 h for cells to adapt to methanol after switching from glycerol when running the standard Invitrogen feed protocol (Stratton et al, 1998). The overall length of the glycerol fed-batch stage depends on the desired biomass concentration prior to methanol induction (Peng et al, 2004).

The last stage is the production stage where methanol induces the production of the recombinant product under the control of the *AOX1* promoter. The methanol feeding strategy determines to a great extend how successful the heterologous protein production can be. The rate of the methanol addition to the culture dictates the specific growth rate of the cells and as a result it has a direct effect on the recombinant protein production (Zhang et al, 2005). Therefore, experimenting with the different growth rates is of vital importance in order to establish the best feeding conditions. However, the slow methanol utilisation nature of the mut^{s} strains and the fact that methanol can be toxic to the cells when accumulated in the culture, while low methanol concentration may not be enough to initiate the AOX transcription (Cereghino & Cregg, 2000), restricts the amount of different feeding rates that can be tested.

From the above, it is obvious that methanol monitoring is crucial for the efficient control of the bioprocess. Traditional off-line techniques for methanol determination

such as GC and HPLC require long waiting time and they are therefore inappropriate. Near infrared spectroscopy (NIRS) was used to model key analytes including methanol and enabled the efficient at-line monitor and control of the methanol concentration in the culture (Crowley et al, 2005). Another rapid at-line method is the determination of methanol using YSI instruments (section 2.8.). However, the alcohol oxidase used in the membrane to produce electron flow oxidises all kind of alcohols including ethanol jeopardising the accuracy of the method when samples with low methanol concentration are analysed. Other techniques were also developed such as an on-line automated sequential injection analysis (SIA) system using stop-flow technique to determine methanol concentration by means of the enzymatic reactions of alcohol oxidase and peroxidase (Surribas et al, 2003). Zhou et al. (2002) developed a probe, which works by the diffusion of the dissolved methanol across a silicone tubing and the vapour phase of the methanol is carried to SnO_2 sensor sensitive to alcohols by resistance drop which is measured by an avometer. A similar technique was used to directly measure the methanol vapour in the stream gas outlet (Ramon et al, 2004).

Control schemes

Another way to control the methanol concentration in the culture is the implementation of either a DO stat or stat strategy which can also maximize protein production and enhance process reproducibility.

The DO-stat strategy is based on the principle that *P. pastoris* uses methanol by the oxidative pathway only in the presence of sufficient amounts of oxygen (Couderc & Baratti, 1980) and consumes methanol as a carbon source and an inducer simultaneously. Therefore, the oxygen level in the culture and the rate of methanol feeding are interrelated. DO-stat processes control the substrate feed to maintain DO concentration at a constant optimal level in the culture medium. While cells are actively growing with adequate carbon sources available, there is a strong demand for oxygen and as a result the DO levels become very low. When the carbon source is depleted, the DO tension begins to rise. Then the carbon source is supplied, the DO levels start to fall because the metabolic pathways become active again (Lee et

al, 2003). This control strategy allows a culture to reach high cell densities while minimizing the accumulation of incompletely oxidized substrates, maintain the culture in a prolonged highly productive state during induction, and prevent substrate accumulation (Chung, 2000).

A drawback of this control method is that high cell density *P.pastoris* cultures may exhibit oscillatory behaviour when methanol is fed under DO-stat operation. This, if left unattended, leads to irreversible loss of culture productivity and hence lower yields, and proper controller tuning is therefore necessary (Chung, 2000). The DO-stat method has an additional problem, if an inhibitory methanol level is reached, a sharp increase in dissolved oxygen will be observed, the response of the system will be then to increase the methanol feeding rate and subsequently, a higher methanol accumulation in the bioreactor will take place. As a result, methanol concentration will increase and hinder the heterologous protein production (Sreekrishna & Flickinger, 2009).

Another common methanol feeding strategy is that based on the control of the specific growth rate. The so called -stat control adjusts the methanol feeding rate based on mass balance equations to theoretically maintain a constant specific growth rate (Potvin et al, 2010). This approach is based on simple cell growth models with no on-line information about the system. Specific growth rate control is considered to be an effective strategy for process optimization as most biochemical processes, including protein production, are either directly or indirectly associated with cell growth. It has been found that the setpoint control strategy is superior to that of constant feeding rate (which resulted in a decreased specific growth rate) in maximizing productivity (Ren & Yuan, 2005). On the other hand, the -stat control strategy as an open loop control strategy presents difficulties in terms of process stability and reproducibility since changes in initial conditions or disturbances in process variables may lead to over- accumulation of methanol and hence reduced productivity (Ren et al, 2003).

Mixed feeds

These techniques enable the monitoring and control of the methanol concentration, however, the increase of the growth rate is impossible with methanol being the only carbon source fed to the culture. Even though during the early years of *P.pastoris* experimentations, researchers used methanol as the only carbon and energy source as well as for the induction of the recombinant proteins by mut^s and mut^+ phenotypes, more recently the use of a multicarbon substrate in addition to methanol is the method of choice. The use of mixed substrates enables the increase of both the energy and carbon supply to recombinant cells in the culture broth (Zhang et al, 2003).

The mixed feeding strategy was initially employed for the mut^- phenotype strains as the product of both the *AOX1* and *AOX2* genes are disrupted and an alternate carbon source, such as glycerol, for growth and recombinant protein production is required (Inan & Meagher, 2001). Then it was applied to the slow methanol utilisation phenotype since it was extensively reported that the disruption of the *AOX1* gene leads to higher induction time for maximal protein expression (above 100h), (Chen et al, 1997; Zhang et al, 2005) and significantly lower biomass concentrations and product yields compared to the mut^+ phenotypes (Orman et al, 2009). This happens because the growth rate on methanol is approximately three times higher for the mut^+ than the mut^s phenotype (Brierley et al, 1990; Cregg & Madden, 1988). Moreover, Brierly et al. (1990) linked the increase of the growth rate with the decrease of the post-induction time needed for the maximal protein expression. Therefore, it is obvious that mixed feed protocols can potentiate a great level of optimization of the bioprocess. Glycerol was the first carbon source to be used in addition to the methanol feed as early as in 1990 by Brierly et al. It is worth noting that a more recent report shows that glucose is a good alternative to glycerol as only few native proteins and no proteases are actually secreted on glucose growth (Mattanovich et al, 2009). However, the use of carbon sources that cause repression of the *AOX1* promoter such as glycerol and glucose imposes an additional monitoring problem. For that reason, the addition of non-repressive sugars such as sorbitol and mannitol has been extensively investigated (Inan & Meagher, 2001).

Thorpe et al. (1999) showed that even though a mut^{s} *P. pastoris* strain does have a lower cell yield on sorbitol than on glycerol, the specific rate of recombinant product formation is increased by 33% relative when run on sorbitol. The volumetric recombinant production of a lipase by a mut^{s} *P. pastoris* was increased 2.2 fold with the appropriate methanol-sorbitol feeding strategy (Ramon et al, 2007).

The volumetric productivity of the recombinant *Rhizopus oryzae* lipase by a mut^{s} *P.pastoris* strain was 1.35 fold higher when a mixed feed of methanol and sorbitol was applied than methanol feed alone (Arnau et al, 2010). Moreover, the same group noted that sorbitol allowed sustained cell growth and recombinant protein production on limiting conditions, but different specific growth rates did not have significant influence on specific production rate of the bioprocess, possibly because the use of a co-substrate improved the energetic state of the cells overcoming partially the unfolded protein response (UPR) and secretion problems. UPR occurs when cells encounter conditions that increase the levels of unfolded proteins and this mechanism is activated in order to compensate for the elevated levels of stress in the endoplasmic reticulum (Ron & Walter, 2007).

Recently, mixed feed strategies have been successfully implemented for mut^{+} phenotypes as well. The highest volumetric recombinant avidin productivity achieved with a mixed feed of methanol and sorbitol was 1.3-fold higher than that of the culture induced with methanol alone (Jungo et al, 2007b). Sorbitol's non-repressive nature allows the batch-wise addition of it rather than incorporating it into the feed as demonstrated by Celik et al. (2009) leading to an 1.6 fold increase of the volumetric productivity of recombinant human erythropoietin. Apart from an 1.85 fold increase of the volumetric productivity of an alkaline polygalacturonate lyase produced by a mut^{+} *P.pastoris* there was also a significant reduction of cell mortality to 8.8% (Wang et al, 2010).

Mixed feeds of sorbitol (or the structurally similar mannitol) and methanol also presents other advantages, such as lower heat production rate and lower oxygen

consumption rate for growth on sorbitol than for growth on glycerol and methanol, or methanol alone (for a given growth rate), since the enthalpy of combustion of sorbitol is much less than glycerol and methanol (Jungo et al., 2007). The reduction in oxygen consumption rate and heat production rate is advantageous in high cell density cultures of recombinant *P.pastoris*, especially during large-scale production.

1.1.4.6. Advantages of P.pastoris over other commonly used expression systems

In the recent years *P.pastoris* has become one of the most widely studied expression systems. Some of the reasons of this trend are lying on the advantages of *P.pastoris* over the more well studied and used organisms in biotechnology such as *Escherichia coli* and *Saccharomyces cerevisiae*. Undoubtedly *E.coli* has been the most extensively used microorganism as a cellular host for recombinant protein expression in the biotechnology industry. However, the increasing need for the expression of proteins derived from eukaryotic genomes that require post-translational modifications has been difficult because this prokaryotic organism lacks the intracellular machinery to achieve these outcomes. Moreover, the lack of a secretion mechanism for the efficient release of proteins into the culture medium, and the limited ability to facilitate extensive disulphide bond formation troubles further the production of eukaryotic proteins (Makrides, 1996).

It has been found that *S. cerevisiae* systems are able to carry out post-translational modifications of expressed proteins – essential features of many heterologous proteins that *E. coli* is unable to provide. Moreover, it has been long used in the food industry and is regarded as safe for use in commercial fermentation processes and, unlike *E.coli*, *S. cerevisiae* does not produce pyrogens or endotoxins (Curran & Bugeja, 2005). Despite its ability to express a wide variety of proteins, *S. cerevisiae* has limitations the most important of which is its very primitive glycosylation system that frequently hyperglycosylates heterologous proteins. Other drawbacks are the very low product yields, with a maximum of 1–5% of total protein, and the fact that presents high plasmid instability (Müller et al, 1998). On the other hand, *P.pastoris* has the advantages of *S.cerevisiae* plus it does not hyperglycosylate proteins and has no highly immunogenic cell wall oligosaccharides (Mousdale, 2007). Moreover, as

in the case of *S. cerevisiae*, it is regarded as safe for use in fermentation. This is in agreement with Miller et al. (2005) who compared the production potentials of the two yeasts *S.cerevisiae* and *P.pastoris* with *E. coli* for eight different single chain antibodies finding that in the case of *E.coli* the antibodies were collected in the undesired form of inclusion bodies in the periplasm, while antibodies produced by *P.pastoris* presented on average higher activities than those produced by *S.cerevisiae* possibly due to hyperglycosylation. Another characteristic example of inappropriate folding resulting in inclusion bodies is the expression of the glycol-protein erythropoietin which when expressed in *E.coli*, the un-glycosylated form is less resistant to unfolding than its native glycosylated counterpart (Narhi et al, 1991).

Nevertheless, the most important advantage of *P.pastoris* over *S. cerevisiae* is the fact that it is a Crabtree-negative yeast meaning that physiologically, *P. pastoris* prefers a respiratory rather than a fermentative mode of growth. Fermentation products such as ethanol, which quickly reach toxic levels in the high cell density environment of a fermenter with Crabtree-positive organisms such as *S.cerevisiae* can cause both growth and recombinant product inhibition (Cereghino et al, 2002). The importance of aerobic metabolism is very well illustrated in the results of Ferndahl et al. (2010) where a genetically modified respiratory *S.cerevisiae* grew to 2.4-fold higher biomass levels and doubled the volumetric productivity of four different recombinant products over the wild type *S.cerevisiae* strain. Due to its respiratory metabolism *P.pastoris* has been reported to grow to biomass levels higher than 160 g/l of dry cell weight (Jungo et al, 2007b) and produce recombinant protein reaching more than 40% of the total expressed proteins and 70% of the secreted (Cregg et al, 1993).

Despite the many benefits, the use of *P.pastoris* for the secretion of heterologous proteins has also limitations. It has been reported that the over-expression of a heterologous protein intended for secretion can potentially overload the secretory pathway and can pose major hindrance to product secretion (Mattanovich et al, 2004). High transcription and translation rates of heterologous genes often overburden the secretory capacity which result in the intracellular accumulation of

high concentrations of misfolded heterologous proteins and ultimately lead to stress reactions as well as direct loss of product (Gasser & Mattanovich, 2007). Moreover, even if *P.pastoris* presents better glycosylation patterns than other microbial systems, its ability to glycosylate proteins derived from mammalian genomes is far less effective than if these compounds are expressed in their natural hosts. This is due to the fact that *P.pastoris* produced proteins usually have high mannose content in the carbohydrate moiety which can result in a short *in vivo* half-life and may make the protein less effective or even immunogenic. Lately, however, new tools have been established for *P.pastoris* to produce glyco-proteins that exhibit a more humanised glycosylation pattern or that secrete core glycosylated proteins (Werner et al, 2007).

1.1.5. Biocatalysis and Biotransformations

1.1.5.1. Introduction

Biocatalysis can be defined as the use of a biocatalyst to achieve a desired conversion under controlled conditions in a bioreactor. A biocatalyst can be an isolated enzyme, an enzyme complex, a cell organelle or a whole cell. In the case of whole cell biocatalysts, the cell can be viable growing or non-growing, or non-viable (Buchholz & Poulsen, 2000). In the last few years there has been a rapid increase in the production of chemicals using biocatalysts to catalyze either a single-step transformation or to catalyze multi-step reactions. The reason for the increasing use and interest in biocatalysts are:

- Enzymes are environmentally friendly. Concerns over climate change and pollution have sensitized both people and industry, and everything that can help alleviate environmental issues is welcome. Enzymes are environmentally benign and easily degradable (Bommarius & Riebel, 2004).
- The fact that enzymes are active mostly at mild, near-ambient conditions of temperature and pH without the need for high pressure and extreme conditions, thus saving money and process energy (Vasic-Racki, 2000). This also eliminates problems faced in traditional chemical synthesis such as decomposition, isomerization, racemization, epimerization, and rearrangement.

- Possibly, the greatest advantage of enzymes is their often unsurpassed selectivity. An enzyme acts on a single type of functional group leaving other sensitive groups untouched. Therefore, there are no unwanted side – reactions as may happen in chemical catalysis. Enzymes present superior enantioselectivity compared with other catalysts and enable the synthesis of enantiomerically pure compounds (Bommarius & Riebel, 2004).

However, the use of enzymes in catalysis present some drawbacks as well which limit their industrial application:

- Some enzymes are co-enzyme dependent (such as oxidoreductases) and therefore they are active only in their holoenzyme form. Co-enzymes such as the pyridine dinucleotides (for example, NAD(P)H) or phosphorylated trinucleotides (such as ATP) are expensive, relatively unstable and must be recycled (Schmid et al, 2001). Unfortunately, it is not possible to replace them by more economical man-made compounds.
- Enzyme behaviour is very well tuned by natural evolution to support the efficient function of the organism that uses them. This however, can be an important drawback, as many enzymes are prone to substrate or product inhibition, a factor which limits the efficiency of the process. While substrate inhibition can be easily avoided by continuous substrate addition, product inhibition is a more complicated problem.
- Enzymes are quite unstable molecules, the catalytic activity of which can be easily negatively affected by the presence of salts, high ionic strength process fluids, and the use of solvents other than water. Moreover, the narrow operation parameters required for enzyme stability can cause limitations in the overall process (Faber, 2000).

1.1.5.2. Whole cells vs. purified enzymes

Whole cells are often used for reactions that require co-factor regeneration, because even though co-factor regeneration *in vitro* is possible, it is generally easier and less expensive to regenerate co-factors in metabolically active cells. However, the use of

whole cells for bioconversions does not come without drawbacks. First of all, the enzymatic activity per unit dry weight is reduced compared to isolated enzyme biotransformation. This problem is even greater when resting cells are used. There might also be substrate or product degradation or other side reaction caused by unwanted cellular metabolism. In the case of the use of growing cultures the purification of the product can be troublesome due to impurities and byproducts introduced by the growing cells. Another problem may arise if solvents must be used as cells may exhibit low tolerance levels. Generally, the decision as to whether whole cells or more or less purified enzymes are used depends on many factors. A characteristic example where the use of whole cells instead of isolated enzymes is advantageous is in the case of dehydrogenases where co-factor regeneration is required. Growing or resting cells can recycle the co-factor internally so that no external addition is necessary (Bommarius & Riebel, 2004). On the other hand, hydrolytic enzymes are the most widely used enzymes in industry because they have the advantage of not needing co-factors and therefore can be used in a purified form leading to higher yields.

1.1.5.3. Chirality

Chiral molecules are molecules whose mirror images are not superimposable upon one another. Chiral compounds rotate the plane of polarized light and they are said to be optically active. Conversely, achiral compounds have superimposable mirror images and they are optically inactive (Challener, 2004). Usually, enantiomers have exactly the same chemical and physical properties such as melting point, boiling point and they can also show the same reactivity in an achiral environment. However, they are in principle totally different compounds when they interact with chiral molecules. Chiral molecules may be receptors or other proteins of the body (Anthonsen, 2000). Characteristic examples of the different characteristics between enantiomers are: carvone, which in its *(L)*-enantiomer form tastes of caraway while the *(R)*-enantiomer tastes of spearmint, and asparagine where the *(L)*- enantiomer has a bitter taste and the *(R)*-enantiomer a sweet taste. A rather tragic example of the importance of chirality in the pharmaceutical industry is the administration of thalidomide as a racemate in the 1960s. The *(R)*- enantiomer of this compound is a

sedative and hypnotic drug while the *(L)*- enantiomer exhibits teratogenic activity. The drug was given to pregnant women to fight morning sickness and caused very serious birth defects and miscarriages (Challener, 2004).

1.1.5.4. Flavoenzymes

Flavoenzymes consist of a very interesting and versatile group of enzymes which can catalyse reaction involved in energy production, oxidation-reduction, oxygenation, light sensing and emission, biodegradation, DNA repair, detoxification, biosynthesis, protein folding, chromatin remodelling and apoptosis. They have a central role in aerobic metabolism through their ability to catalyze both one- and two-electron transfer reactions (Mattevi, 2006). The most common flavin cofactors are FMN and FAD, which are synthesized *in vivo* from riboflavin (vitamin B1) by the action of riboflavin kinase (Karthikeyan et al, 2003; Spencer et al, 1976) and FAD synthetase (Manstein & Pai, 1986). They play a fundamental role in aerobic metabolism through their ability to catalyze both one- and two-electron transfer reactions. Flavins are the only organic co-factors that are able to utilise molecular oxygen for oxygenation reactions since the other known oxygenating co-factors always depend on a metal ion for their reactivity (Fraaije & van Berkel, 2006). Enantio- and regioselective oxygenations and oxidations are reactions leading to valuable fine chemicals, however, they are difficult to achieve by chemical synthesis routes as oxygen insertion or oxidation reactions often involve tedious and costly blocking and de-blocking steps and are catalyzed by heavy metals. Hence, flavin containing oxidases and monooxygenase are very attractive alternatives as these biocatalysts are able to catalyze a huge variety of oxidation and monooxygenation reactions while exhibiting significant selectivity. Other, remarkable advantages of the use of flavin-dependent enzymes in biocatalysis are the renewability of the catalyst, the possibility to use mild process conditions and the reduced formation of by-products due to fewer side reactions (Fraaije & van Berkel, 2006). It has been found that many flavin-containing oxidases are active with amines and amino acids, and their high enantio- and regio-selectivity make them well suited for chiral organic synthesis while some of them have narrow substrate specificity, others are more relaxed (Fotheringham et al, 2006; Joosten & van Berkel, 2007).

1.1.5.5. Amines

Optically pure amines are widely used as resolving agents (Nieuwenhuijzen et al, 2002), chiral auxiliaries (Henderson et al, 2000) and can also be used as building blocks for the preparation of valuable pharmaceutical and agrochemical end products (Berger et al, 2001). Some characteristic examples of important pharmaceuticals containing chiral amine building blocks are (*S*)-rivastigmine for the treatment of Alzheimer's and dementia of Parkinson patients, (*S*)-repaglinide for the treatment of type II diabetes, (*S*)-DMP 777 (Phase II) for the treatment of cystic fibrosis and rheumatoid arthritis, (*S*)-solifenacin (Phase III) for the treatment of overactive bladder (*R*)-garenoxacin for the treatment of bacterial infections and (*R*)-levocetirizine used as histamine antagonist (Turner & Carr, 2007).

Therefore, non-racemic chiral amines are an important, but not easily prepared class of organic molecules. Most of the methods used to prepare optically pure amines are largely based on the resolution of racemates either by recrystallisation of diastereomeric salts formed after adding one or a mixture of chiral carboxylic acids as resolving agents (Höhne & Bornscheuer, 2009) or by enzyme catalysed kinetic resolution of racemic substrates using lipases, acylases and transaminases. These methods give low overall enantiomeric excess (ee) of the product and are generally viewed as uneconomic and non-competitive (Turner et al, 2005).

1.1.5.6. Biotransformations for the production of chiral amines using Amine Oxidases

As described earlier, amine oxidases have been classified into two groups, the Cu/TOPA-dependent and flavin-dependent amine oxidases. The reactions catalysed by these two enzymes can be seen in figure 1.9. In the catalytic cycle of the first the intermediate imine remains covalently bound to the protein and hence these types of enzymes are unsuitable for specific biotransformation use (Alexeeva et al, 2002). On the other hand, the FAD amine oxidases generate free imines which can then be reduced back to racemic amines by the action of a reducing catalyst. To be more

specific the *A.niger* monoamine oxidase selectively oxidizes only the (*S*)-enantiomer of the amine to the corresponding imine, which subsequently dissociates from the enzyme and further reacts to a ketone and ammonia in the aqueous environment. Rather than allow the imine to simply hydrolyze to the corresponding ketone the addition of abiotic reductant such as amine borane and sodium cyanoborohydride reduces the "waste" imine back, to yield the racemic amine that then undergoes another round of enantioselective oxidation to the imine. Starting from a racemic mixture, the slow-reacting (*R*)-enantiomer is left behind in optically pure form after the fast reacting enantiomer has been completely converted. For a highly enantioselective MAO such as the MAO-5N used in this work, this would resemble a typical kinetic resolution. Repeated cycles of chemical reduction and selective enzymatic oxidation lead to a reaction mixture consisting of 100% optically pure (*R*)-enantiomer amine. An advantage of this model is that both reactions take place simultaneously and hence can be carried out in a one-pot reaction (Turner & Truppo, 2010).

The Turner group decided to modify the MAO-N from *A.niger* in order to optimise both enantioselectivity and catalytic activity against chiral compounds which are important pharmaceutical or intermediates. The *A.niger* MAO-N was reported to have high activity towards simple aliphatic amines (for example, amylamine, butylamine) but was also active, even though at a lower rate, towards benzylamine. The same group selected a-methylbenzylamine as a model system for study in view of its importance as a chiral amine. Since the *A.niger* MAO-N enzyme possessed very low, but measurable, activity towards *S*-a-methylbenzylamine and even slower oxidation of the (*R*)-enantiomer it was attempted to increase the activity of the enzyme towards *S*-a methylbenzylamine through a directed evolution experiment. The enzyme was subjected to several rounds of random mutagenesis coupled with screening using methylbenzylamine as the substrate resulting in a mutant, Asn336Ser, for which the catalytic turnover, as measured by the kcat, was improved by approximately 50-fold (Alexeeva et al, 2002). In addition, this variant had generally good activity toward a wide range of chiral primary amines. Further rounds of directed evolution resulted in a triple-point mutant variant, Asn336Ser/Met348Lys/Ile246Met (MAO-3N) which, in addition to accepting *S*-α-

methylbenzylamine as substrate, displayed improved activity toward chiral secondary amines such as methyltetrahydroisoquinolone (Carr et al, 2005). Further work led to the MAO-5N mutant, which was identified on the basis of its ability to oxidise cyclic tertiary amines, such as N-methyl-2-phenylpyrollidine (Dunsmore et al, 2006).

The latter variant shows very high activity and enantioselectivity towards a broad range of primary, secondary and tertiary amines (Bailey et al, 2007). The very successful results of the directed evolution experiments were followed by a development of a "desktop" model which can be used to predict the suitability of particular substrate for oxidation by MAO-N variants. Different industrially important chiral amines were deracemised with enantiomeric excesses reaching 100% (Turner & Truppo, 2010).

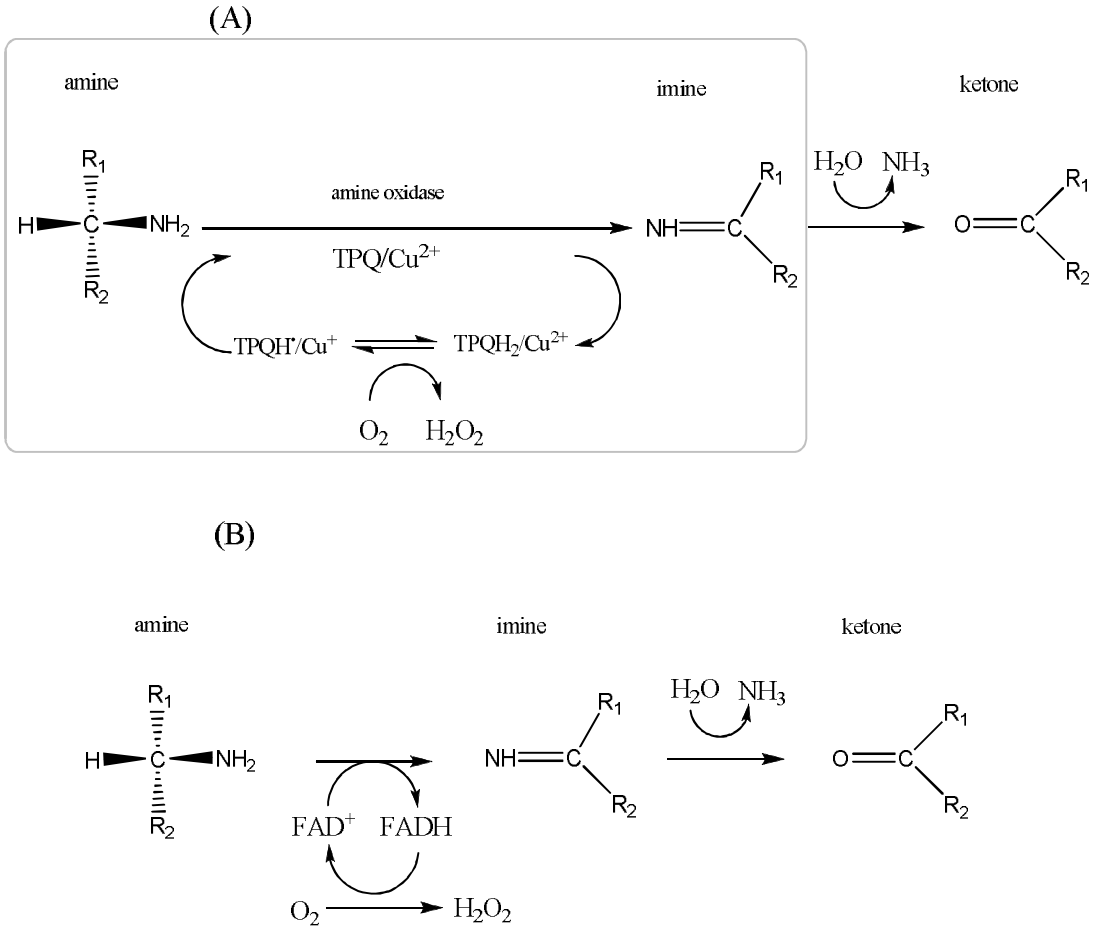

Figure 1.9. Reaction mechanism of the (A) Cu/TOPA-dependent and (B) flavin-dependent amine oxidases. The intermediate imine in the reaction (A) remains covalently attached to the enzyme.

1.2. Aims

The aims of this work can be summarised as follows:

- To gain a better understanding of the homologous expression of the amine oxidase in its natural host and its capacity for high level production (chapter 3). Moreover, to increase the productivity of the industrially important *A.niger* FAD amine oxidase if necessary at the expense of the Cu amine oxidase.

- To establish the appropriate conditions for the over-expression of the MAO-5N enzyme in the heterologous hosts *E.coli* and *P.pastoris*. The basic criterion for the successful expression of the enzyme was not the gravimetric amount of enzyme produced but the volumetric (total) activity of the enzyme in the cultures. The industrial goal was the use of whole cells or cell free extracts of the produced culture for deracemisation of non optically pure amines. Therefore the enzymatic activity was the most suitable way to measure the enzyme's value as a biocatalyst. It is worth noting that the specific activity (expressed as the amount of substrate converted per unit of time per biomass) is also of particular interest, as the higher the specific activity, the lower the loading needed for the subsequent biotransformation step. In other words smaller quantity of cells would be needed for loading the biotransformation reactor. Consequently, there was a focus upon steps to both increase the total activity of the bioprocess but also to retain the specific activity at high enough levels.

- Even though the primary aim was to get the highest possible activities, this must happen in a cost effective way, without using techniques or materials which significantly increase the cost and/or limit the scalability of the process.

- The overall aim was a critical assessment of microbial expression systems for the production of an industrial important oxidase. Moreover, an increase of the productivity of the process along with a reduction of cost was attempted. This was achieved by choosing the most effective microbial system and developing the most suitable fermentation conditions for the expression of the monoamine oxidase enzyme.

Chapter 2

Materials & Methods

2.1. Bioreactor

The fermentations were carried out using a stainless steel bioreactor BIOSTAT C.-DCU, (B.Braun Biotech International, Switzerland), which has a total volume of 22L and maximum working volume of 15L. The stainless steel culture vessel (C15-3) has a double wall heat exchanger and side wall viewing window. The internal height to diameter ratio of the vessel is the standard 3:1, and the diameter of the vessel is 21cm. There are four internal baffles with 1.5cm width and 57cm length. Three adjustable six-bladed Rushton turbines were located on the stirrer shaft. The diameter of each turbine is 8.5cm. The fermenter was connected to the main laboratory lines for air and water. The filtered sterile air was sparged into the vessel via a circular annular sparger fitted in the bottom of the vessel for even distribution of bubbles.

The vessel was heated and cooled using a water jacket. Accurate temperature control to within ±0.1°C of the setpoint was achieved through the control loops of the DCU.The pH (Mettler Toledo Ltd., Leicester, UK) was controlled to within ±0.05 of the setpoint by automatic operation of two peristaltic pumps supplying acid and base solutions from reservoirs to the reactor through silicone rubber tubing. Dissolved oxygen tension (DO) was monitored by a pO_2 electrode (Mettler Toledo Ltd., Leicester, UK). The probe was calibrated at the fermentation temperature and agitation speed by introduction of oxygen free nitrogen (OFN) (0% saturation value) and air (100% saturation value). Foam was controlled manually using a peristaltic pump which was linked to a reservoir containing sterile polypropylene glycol 2025 (PPG) as the antifoam agent.

.

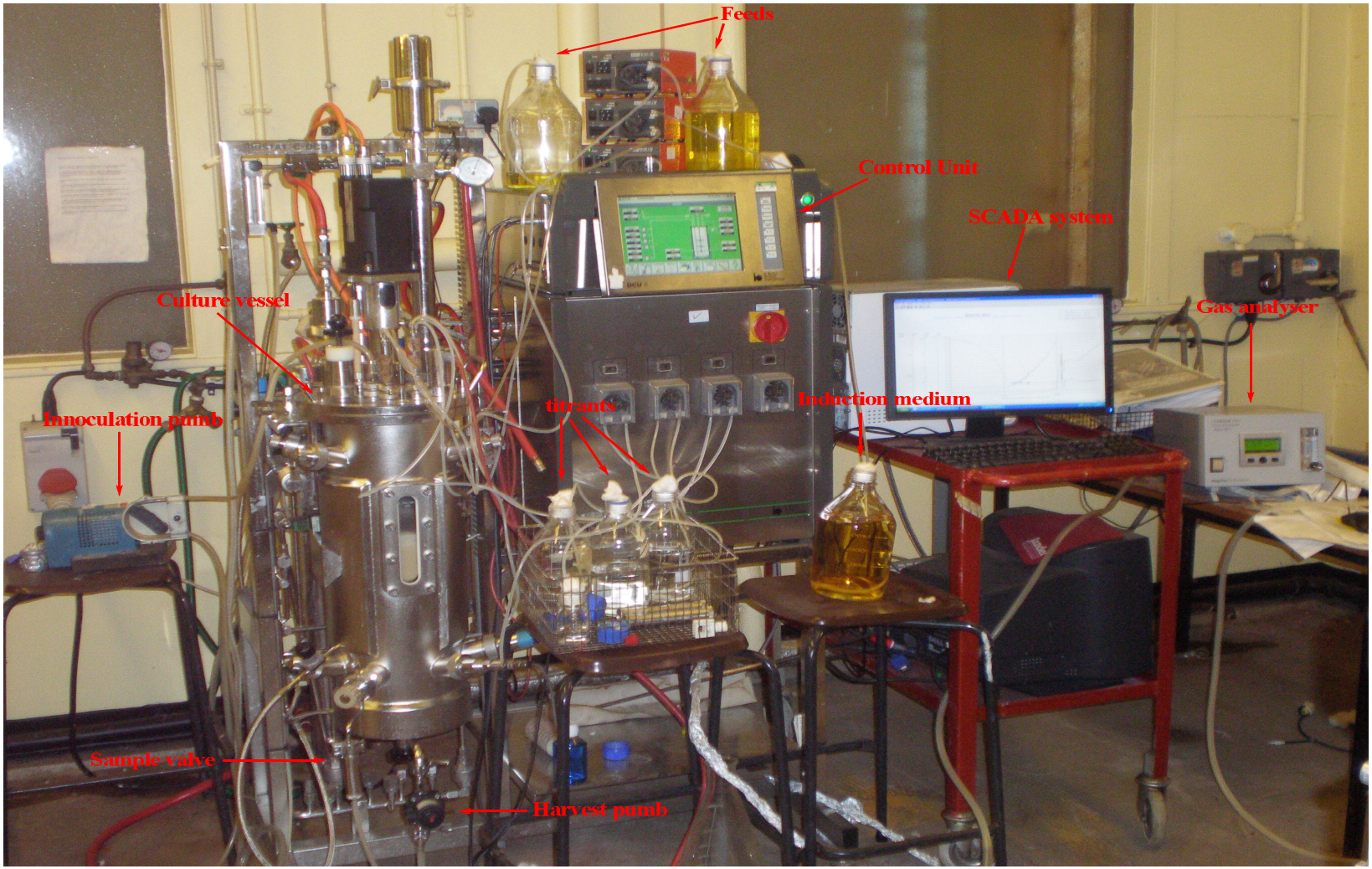

Figure 2.1. Operational configuration of the biostat C bioreactor during a *P.pastoris* fed batch bioprocess

2.2. Analytical Equipment

2.2.1. Spectrophotometer

A dual-light path UV-Vis spectrophotometer with thermostatted rotary 7-cell changer in sample position (BioMate 5, Thermo Scientific, Hemel Hempstead, Hertfordshire, UK) was used for the determination of optical density, amine oxidase activity, galactose, glucose, acetate concentration.

2.2.2. Gas analyser

The exit gas composition was measured using a digital gas analyser TANDEM PRO (Applikon Biotechnology Ltd, Tewkesbury, Gloucestershire, UK).Two gases were used for calibration oxygen 19.0% (v/v) and carbon dioxide 1.75% (v/v). The CO_2 produced (carbon dioxide evolution rate, CER) and the O_2 consumed (oxygen consumption rate, OUR) were expressed in mM/l/min and then used to calculate the respiratory quotient (RQ):

$$RQ = \frac{CO_2 generated}{O_2 consumed}$$

2.2.3. Incubator

Cultivations in 1L Erlenmeyer flasks were carried out in a rotary shaker (New Brunswick Scientific, Edison, USA).

2.2.4. YSI 2700 analyser

YSI's enzyme sensor technology employs one or more enzyme catalysed reactions to ultimately produce hydrogen peroxide. Hydrogen peroxide is electrochemically oxidised at the platinum anode of an electrochemical probe. This produced a probe signal current.

A substrate enters the sample chamber and is stirred and diluted. The substrate then diffuses through a thin polycarbonate membrane material. Once past the

polycarbonate membrane, the substrate encounters an extremely thin layer of the appropriate oxidase enzyme. There the following reaction occurs:

$$\text{Substrate} + O_2 \xrightarrow{\text{Oxidase}} H_2O_2 + \text{Byproduct}$$

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

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

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 (calibrator) and gives the final concentration result of the unknown.

2.2.5. High Pressure Cell Homogeniser

Prior to assaying intracellular protein content and the activities of amine oxidase, cell disruption was necessary. This was performed using a high-pressure cell homogeniser (Model 4000, Constant Systems Ltd., Warwick, UK). Samples (5 mL) were pipetted into the piston chamber of the homogeniser, and the collection cup assembly was fitted. The cells were passed twice through the homogeniser at a pressure of 30kpsi, after which the homogenate was collected and centrifuged to obtain a clear supernatant. Distilled water was passed twice through the homogeniser between samples to remove any remaining artefacts and prevent false results.

2.3. Microorganisms and Culture Conditions

2.3.1. *Aspergillus niger* strains

A. niger B-1D

A. niger strain B-1D, contained the hen egg white lysozyme (HEWL) cDNA gene fused to a *Aspergillus awamori* glucoamulase (*glaA*) promoter. The HEWL cDNA gene was inserted at the *Bgl*II cloning site between the *glaA* promoter and terminator in the plasmid pGPT-*pyr*G1. pGPT-*pyr*G1 complemented the *pyr*G mutation in *A. niger* AB4-1. All transformations were performed with protoplasted *A. niger*.

A. niger 337690

This strain was isolated from soil of an Iraqi desert.

A. niger 149007ii (ATCC 16404)

This strain was isolated from a Blueberry in North Carolina. This strain was recently re-named as *Aspergillus brasiliensis* according to the reference Varga et al. (2007).

A. niger 320725

This strain was isolated from a diesel tank in Aukland, New Zealand.
A.niger B-1D was donated by Prof. David Archer (University of Nottingham). The rest of the *Aspergillus* strains were bought from CABI (www.cabi.org).

Master cultures were produced by plating from potato dextrose agar (PDA) slopes, stored at 4°C, onto fresh PDA plates. Spore suspensions were then produced from these plates, and aliquoted to a final volume of 200mL in Eppendorf tubes. These spore suspensions were then stored at -80°C, although cultures were also maintained on PDA slopes refrigerated at 4°C. The master culture spore suspensions were produced by washing a 7-day old plate with DMSO as a cryoprotectant. Spore suspensions for inoculation purposes were produced by washing 7-day old plate cultures with sterile water containing 0.1% Tween-80.

2.3.2. *Escherichia coli* strains

The following *E.coli* systems were used:
E.coli BL21(DE3) / pET16b His MAO-5N
E.coli BL21(DE3) / pET28b His MAO-5N

The cDNA of the gene expressing the monoamine oxidase from *A.niger* (mao-N gene: 2193bp, mao-N mRNA 1488b encoding for a 495 amino acid long protein) obtained from Schilling and Lerch (1995b) and cloned in an *E.coli* BL21 pET16b vector and then undergone a directed evolution experiment so that the substrate specificity was improved towards *S*-a-methylbenzylamine (Alexeeva et al, 2002) and then the resulted MAO-5N was inserted into vectors mentioned above carrying a gene for kanamycin resistance *E.coli* BL21(DE3) / pET28b and ampicillin resistance *E.coli* BL21(DE3) / pET16b.

Master cultures were produced by carrying out flask fermentations with LB medium carrying 50 g/ml of the appropriate antibiotic. At OD_{600} = 0.5 abs when cells are at the exponential phase of growth, aliquots of 0.8ml culture are poured into 2ml freezing vial (Sigma-Aldrich, Dorset, UK) containing 0.2ml glycerol and stored in -80 °C. Cultures are revived by streaking them on LB agar plates (containing 50 g/ml of antibiotic) and put into an incubator at 30°C for 48h. A single colony was used to inoculate a flask.

2.3.3. *Pichia pastoris* strain

The strain is Mut^S CBS 7435 and it harbours linear cassettes amplified from the pPpB1 vector carrying the histidine tagged MAO-5N to enable the purification of the enzyme.

2.4. Media Composition

2.4.1. Medium Composition for *A.niger* bioprocesses

The medium used in this series of experiments was as follows: 25g glucose, 2.5g ammonium chloride, 10mL salts solution, 5mL vitamin solution, and water to 1L. The salts solution contained 0.35M potassium chloride, 0.22M magnesium sulphate heptahydrate, 0.56M potassium phosphate monobasic, 50mL trace elements solution, and water to 1L.

The trace elements solution was made to the following specifications: 0.1mM sodium borate decahydrate, 2mM cupric sulphate pentahydrate, 4mM ferric phosphate tetrahydrate, 5mM manganese sulphate dihydrate, 3mM molybdic acid sodium salt dihydrate, 28mM zinc sulphate heptahydrate, and water to 1L.

The vitamin solution contained 0.2mM *p*-aminobenzoic acid, 0.2mM thiamine hydrochloride, 40nM biotin, 0.8mM nicotinic acid, 0.4mM calcium D-pantothenic acid, 0.2mM pyridoxine monohydrochloride, 0.3mM riboflavin, and water to 1L. The vitamin solution was filter sterilised through a syringe using 0.2 m cellulose acetate filters.

Unless stated otherwise, all chemicals used in the work were purchased from Sigma-Aldrich, Poole, Dorset, UK.

2.4.2. Medium Composition for *E.coli* bioprocesses

Inoculum : 60ml Salt solution , 6ml 50% (w/v) Glucose, 0.6 ml Magnesium Sulphate 1M, 0.6 ml Trace Elements solution, 0.3 ml antibiotic 50mg/ml, distilled water to 300 ml volume

Batch Medium: 2g/l Ammonium sulphate, 14.6 g/l Potassium phosphate dibasic, 3.6g/l Sodium phosphate monobasic dihydrate**,**0.5 g/l $(NH_4)_2$H-citrate, 0.1g/l polypropylene glycol (1ml),

11.9 g/l Glucose, 2ml Magnesium Sulphate 1M, 2ml Trace Elements, 10ml antibiotic 50 g/ml, water up to 1l, autoclaved separately while the antibiotic was filter sterilised through a syringe using 0.2 m cellulose acetate filter and added post-autoclave to the bioreactor.

Glucose Feed: 50ml Magnesium Sulphate 1M, 10ml Trace Elements, 550.9g Glucose, 5g yeast extract (Oxoid, Basingstoke, Hampshire, England), distilled water to 1l

Salt solution: 10g/l Ammonium Sulphate, 73g/l Potassium phosphate dibasic, 18g/l Sodium phosphate monobasic dihydrate, 2.5 g/l Ammonium citrate dibasic.

Trace Element solution : 0.5 g/l Calcium Chloride dihydrate, 10.03g/l Ferric chloride 0.18 g/l Zinc Sulphate heptahydrate, 0.16 g/l Copper Sulphate pentahydrate, 0.15 g/l Manganese sulphate monohydrate, 0.18 g/l Cobalt Chloride hexahydrate, 22.3 g/l disodium EDTA dihydrate.

The antibiotic was carbenicillin (Apollo scientific Bredbury, Stockport, UK) and kanamycin for the *E.coli* BL21(DE3)/pET16b and the *E.coli* BL21(DE3)/pET28b, respectively.

The recombinant protein expression was induced either by 10mM IPTG (Apollo scientific Bredbury, Stockport, UK) or 200g/l lactose solutions.

2.4.3. Medium Composition for *P.pastoris* bioprocesses

Inoculum: 2g yeast extract, 4g peptone (Oxoid, Basingstoke, Hampshire, England), 20ml potassium phosphate buffer 1M at pH 6, 20ml Yeast Nitrogen Base with amino acids 134g/l, 0.4ml Biotin 0.2 g/l, 0.2 ml Zeocin 100mg/ml (InvivoGen, San Diego, California, USA), sterile water to 200 ml volume.

The **batch medium** used for *P.pastoris* bioprocesses was:
95ml Phosphoric Acid (85%), 1.36g Calcium sulphate, 28.59g Potassium sulphate, 23.17g Magnesium sulfate heptahydrate, 12.8g Potassium hydroxide, 6g EDTA disodium salt dihydrate, 2.2g Sodium chloride, 400g Glycerol, 1ml polypropylene glycol 2025, water to a final volume of 8.5l

After sterilisation in the fermenter the following were added:
43.5ml PTM_1 Trace Elements, 134g yeast nitrogen base water to a final volume of 1.5 liter.

Glycerol Feed
500ml Glycerol, 12ml PTM_1 Trace Elements, Water up to 1000ml

Methanol Feed
1000ml Methanol, 12ml PTM_1 Trace Elements

Sorbitol feed
2.5M sorbitol, 12ml/L PTM_1 Trace elements

PTM_1 Trace Elements
6.0 g Cupric sulphate pentahydrate, 0.08 g Sodium iodide, 3.0 g Manganese sulfate monohydrate, 0.2 g Sodium molybdate dihydrate, 0.02 g Boric Acid, 0.92g Cobalt chloride hexahydrate, 20.0 g Zinc chloride, 65.0 g Ferrous sulphate- heptahydrate, 0.2 g Biotin, 5.0 ml Sulfuric Acid, 0.4g Calcium Sulfate, water to a final volume of 1 liter.

2.5. Shake Flask Cultivation

For the investigation of the effect of ammonia and nitrate on the induction of the *A.niger* 337690 culture by butylamine, the following flasks were prepared:

1) Control with ammonia (without inducer)
25 g/l glucose
2.5 g/l ammonia chloride
10 ml/l salt solution
5m/l vitamin solution

2) Control with nitrate (without inducer)
25 g/l glucose
3. 97 g/l sodium nitrate
10 ml/l salt solution
5m/l vitamin solution

3) Induced by butylamine with ammonia
25 g/l glucose
1.38 g/l ammonia chloride
2.08 ml/l butylamine
10 ml/l salt solution
5m/l vitamin solution

4) Induced by butylamine with nitrate
25 g/l glucose
2.19 g/l sodium nitrate
2.08 ml/l butylamine
10 ml/l salt solution
5m/l vitamin solution

The salt and vitamin solution is described in section 2.4.1. the final volume of the broth is 0.4l and the flask volume is 1l.

2.6. Bioreactor Batch Cultivation

2.6.1. *A.niger*

The reactor was inoculated with 500ml of a 48h -old shake flask culture grown at 25°C and 200rpm. The pH was kept constant at 4.0 by automatic addition of 2.0M H_2SO_4 and 2.0M NaOH. Temperature was maintained at 25°C throughout. The agitation speed was set at 400rpm, and air flow rate was controlled at 1 volume of air per volume of culture per minute (vvm) automatically.

2.6.2. *E.coli*

Inoculum preparation for the *E.coli* BL21(DE3) pET16b: It was necessary to wash the cells before using them as an inoculum because of the build up of β-lactamase which can cause plasmid loss. The washing steps have to be done aseptically. Cells were spun down at 4000rpm for 15 minutes at 4^{o}C, the supernatant was thrown away and the pellet was re-suspended with LBcarb(50μg/ml) to the same volume as the original culture. This procedure was done twice and then the washed and re-suspended cells are used as the inoculum for the bioreactor.

The reactor was inoculated with a LB – flask grown until the Optical density of the culture at 600nm reached 0.5 – 1 Abs. The shake flask culture grown at 30°C and 250rpm. The temperature in the bioreactor was kept constant at 30°C, the speed was set at 350rpm, and air flow rate was controlled at 2 volume of air per volume of culture per minute (vvm) automatically, while the pH was not controlled.

2.7. Bioreactor Fed - Batch Cultivation

2.7.1. *E.coli*

The bioreactor was filled with 10l of medium and inoculated with 300ml of culture grown at 30°C to an OD_{600nm} = 0.5-1 Abs. The temperature of the bioreactor was set at 30 ^{o}C throughout the runs. The pH was kept at 7.0 by automatic addition of titrants (25% (v/v) NH_4OH serving as both base and nitrogen source and 2M H_2SO_4). The

dissolved oxygen probe was calibrated with air and oxygen free nitrogen at 300rpm. The DO_2 setpoint was 30% and for the aerated cultures it was maintained at the preset levels by cascading to stirring rate from 300 – 900 rpm and then from 1vvm – 3vvm of air while in the oxygen enriched cultures it was cascaded to stirring rate from 300 – 900 rpm and then from 0 – 100% oxygen enrichment. When glucose was fully consumed (OD_{600nm} at this time is normally around 12) the glucose feeding was turned on. For the IPTG induced cultures, when the cells reached an OD_{600nm} = 40 then IPTG was added to a final concentration of 1mM. In the case of the lactose induced cultures, lactose feed (300g/l) was started at OD_{600nm} = 40, 55 or 70. The lactose concentration of the culture was kept close to 2g/l.

Feed profile

When the glucose was fully consumed the glucose feeding was turned on.
The glucose feeding profile is described below:
0 minutes - 30minutes ➔ start feed at 0.17ml/min
30 minutes - 1h 30mins ➔change to 0.34ml/min
1h 30mins - 2h 30mins ➔change to 0.67ml/min
2h 30mins - 3h 30mins ➔change to 1.07ml/min
3h 30mins – induction time ➔change to 1.34 ml/min. This rate is kept at this set point up until the OD=40 and induction starts.
At point of induction the rate is changed to 1.07ml/min

Optimised feed

The optimized glucose feeding profile is described below:
0 minutes – 15minutes ➔ start feed at 0.66 ml/min
15 minutes – 30 minutes ➔ change to 1.05 ml/min
30 minutes – 45 minutes ➔ change to 2.15ml/min
45 minutes – 1hour ➔ change to 2.51 ml/min
1hour – 3hour 30min ➔ change to 3.24 ml/min
3hour 30min - induction time ➔ change to 3.61 ml/min

When the cells reach the desired OD_{600nm} then IPTG is added to a final concentration of 1mM or lactose is added in a fashion that its concentration in the culture broth is maintained at 2g/l.

2.7.2. *P.pastoris*

The bioreactor was filled with 10l of medium and inoculated with 300ml of culture grown at 30°C to an OD_{600nm} = 6 - 10 Abs. The temperature of the bioreactor was set at 30 ^{o}C throughout the runs. The pH was kept at 5.8 by automatic addition of titrants (28% 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 300rpm. The DO setpoint was 30% and for all cultures it was maintained at the preset levels by cascading to stirring rate from 300 – 900 rpm and then from 1vvm – 3vvm of aeration rate. When glycerol was fully consumed the glycerol feeding was turned on. The feeding profiles for all the *P.pastoris* fermentation carried out can be seen in table 2.1.

When the methanol feed started, it was incrementally increasing; while the glycerol feed was gradually decreasing. This phase of the process also called adaptation phase (section 1.1.4.5.) is used to enable the pre-adaptation of the *P.pastoris* cells metabolism to the carbon and nitrogen sources used in the induction phase.

The methanol feeding profile was kept constant in all experiments. In the case of methanol - sorbitol co-feed, the separate sorbitol feed (2.5M) was adjusted so that the ratio of the carbon content from methanol and sorbitol solutions would be 2:1, 1:1 and 1:2.

Table 2.1. Feeding profile of *P.pastoris* fermentations for 10l volume. The methanol or methanol-sorbitol feed begins at 6.33h of the glycerol feed (grey colour).

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

2.8. Analytical Procedures

2.8.1. Biomass estimation

2.8.1.1. Dry cell weight for A.niger

Biomass was estimated according to the method of Wongwicharn *et al.* (1999b). 5mL aliquots of fungal culture were withdrawn and were filtered through a 4.25cm diameter GF/C filter, which has a particle retention size of approximately 1.2µm (Whatman Ltd., Maidstone, UK). The filter cake was washed twice with 5mL of distilled water, dried for 20 minutes in a microwave oven (650W) on medium-low power, and cooled in a desiccator before weighing. All samples were analysed in triplicate.

2.8.1.2. Dry cell weight for E.coli and P.pastoris

1ml aliquots of culture were withdrawn and loaded in pre-weighed Eppendorf tubes and centrifuged for 5 min at 10.000 rpm in a benchtop microcentrifuge (Eppendorf Microcentrifuge 5415D, Eppendorf AG, Hamburg, Germany). The supernatant was removed and cells were re-suspended in water and centrifuged again, after which the Eppendorf with the cell pellet was dried in an oven at 105°C for 24 h then was weighed after being cooled in a desiccator for 2 h.

2.8.1.3. Wet cell weight for E.coli and P.pastoris

1ml aliquots of culture were withdrawn and loaded in pre-weighed Eppendorf tubes and centrifuged for 5 min at 10.000 rpm in a benchtop microcentrifuge (Eppendorf Microcentrifuge 5415D, Eppendorf AG, Hamburg, Germany). The supernatant was removed with the aid of a micropipette and the tubes were centrifuged again and supernatant was carefully removed with a micropipette taking caution to avoid removing any pellet. Then the Eppendorf tubes with the cell pellets were weighed.

2.8.1.4. Optical density

Appropriately diluted samples were used to measure the optical density of the culture at 600nm.

2.8.2. Monoamine oxidase activity

The monoamine oxidase activity was assayed in the supernatant by measuring the release of H_2O_2 during the conversion of a substrate to product (n-amylamine in the current experiments). Hydrogen peroxide is measured by a colorimetric reaction which occurs with the addition of horseradish peroxidase (HRP), serving as catalyst and a substrate, 4-aminoantipyrine/ 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA) oxidised to a coloured product, the absorbance of which is measured at 510nm (figure 2.2).

The assay mixture contained:
10 ml 1M Potassium phosphate buffer pH7.8
39 ml water
20 mg TBHBA in 1ml DMSO
15.24 mg 4-AAP

The reaction took place in 1ml cuvettes where 50 l of appropriately diluted sample was added to:
500 l assay mix stock solution from above
100 l of 100 mM substrate (amylamine unless otherwise stated) at same pH as assay mix stock solution
10 l HRP solution at 5 mg/ml
340 l water

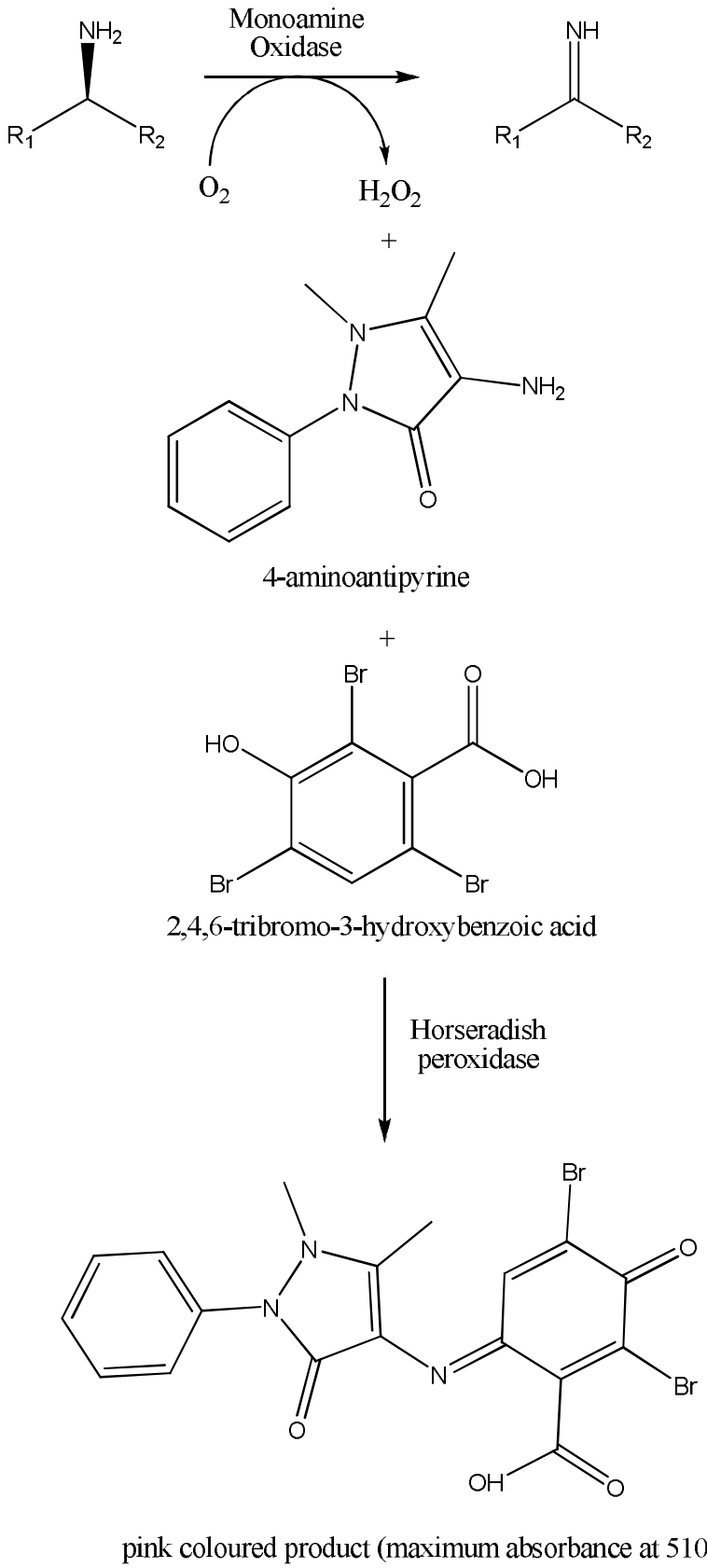

Figure 2.2. The reaction sequence of the amine oxidase activity determination.

Then the cuvettes were immediately transferred to the spectrophotometer set at 30 ^{o}C and the rate of change of absorbance at 510 nm was monitored. In addition negative controls were run with i) no substrate and ii) no oxidase to give the background rate of absorbance change. This value was subtracted from all observed rates.

The rate of change of 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 1 ml cuvette of the spectophotometer is 1 cm. In the case of the plate reader the path length is 0.53cm. Using the Beer-Lambert law the change in concentration of colorimetric product and therefore the change in concentration of substrate is:

Concentration change ($M\ s^{-1}$) = AbsUnits (s^{-1}) / [29,400 ($M^{-1}cm^{-1}$) x 1(cm)]

2.8.3. Glucose concentration

Enzymatic kit determination

Glucose concentrations in culture supernatants were measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 716 251 035). 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).

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

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

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

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 340nm.

YSI determination

The YSI 2007 was used for this determination (section 2.4.4.). This was a direct reading of dextrose in solution at the enzyme sensor. The enzyme glucose oxidase is immobilised in the YSI Dextrose membrane.

$$\text{D - glucose} + O_2 \xrightarrow{\text{Glu Oxidase}} H_2O_2 + \text{D - glucono-}\delta\text{-lactone}$$

2.8.4. Acetate concentration

Acetate concentrations in culture supernatants were measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 148 261 035). The test principle is as follows: Acetic acid (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).

$$\text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\text{CS}} \text{citrate} + \text{CoA}$$

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

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

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 340nm. Because of the equilibrium of the preceding indicator reaction, the amount of NADH formed is not linearly (directly) proportional to the acetic acid concentration.

2.8.5. Methanol concentration

YSI determination

The YSI 2007 was used for this determination (section 2.4.4.). This was a direct reading of methanol in solution at the enzyme sensor. The enzyme alcohol oxidase is immobilised in the enzyme membrane,

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

2.8.6. Lactose and galactose concentration

Enzymatic kit determination

Lactose and galactose concentrations in culture supernatants were measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 176 303 035). The test principle is as follows:

Lactose is hydrolyzed to D-glucose and D-galactose at pH 6.6 in the presence of the enzyme β-galactosidase and water

$$\text{Lactose} + H_2O \xrightarrow{\text{beta galactosidase}} \text{D-glucose} + \text{D-galactose}$$

D-Galactose is oxidized at pH 8.6 by nicotinamide-adenine dinucleotide (NAD) to D-galactonic acid in the presence of the enzyme β -galactose dehydrogenase (Gal-DH)

$$\text{D-galactose} + NAD^+ \xrightarrow{\text{Gal-DH}} \text{D-galactonic acid} + NADH + H^+$$

The amount of NADH formed in the second reaction is stoichiometric to the amount of lactose, and D-galactose, respectively. The increase in NADH is measured by means of its light absorbance at 340nm.

YSI determination of lactose

The YSI 2007 was used for this determination (section 2.4.4.). This was a direct reading of lactose in solution at the enzyme sensor. The enzyme galactose oxidase is immobilised in the enzyme membrane,

$$\text{Lactose} + O_2 \xrightarrow{\text{Gal oxidase}} H_2O_2 + \text{Galactose Dialdehyde derivative}$$

2.8.7. Ammonia concentration

Ammonia concentrations in culture supernatants or filtrates were measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 11 112 732 035). The test principle is as follows: In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide - adenine dinucleotide (NADH), ammonia reacts with 2-oxoglutarate to L-glutamate, whereby NADH is oxidized.

$$\text{2-Oxoglutarate} + \text{NADH} + NH_4^+ \xrightarrow{\text{GIDH}} \text{L-glutamate} + NAD^+ + H_2O$$

The amount of NADH oxidized in the above reaction is stoichiometric to the amount of ammonia. NADH is determined by means of its light absorbance at 340nm.

2.8.8. Nitrate

Nitrate concentrations in culture filtrates were measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 905 658 035). The test principle is as follows: Nitrate is reduced by reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to nitrite in the presence of the enzyme nitrate reductase (NR).

$$\text{Nitrate} + \text{NADPH} + H^+ \xrightarrow{\text{NR}} \text{nitrite} + \text{NADP+} + H_2O$$

The amount of NADPH oxidized during the reaction is stoichiometric to the amount of nitrate. The decrease in NADPH is measured by means of its light absorbance at 340nm.

2.8.9. Sorbitol

Sorbitol in culture supernatants was measured using a commercial kit (RBiopharm Rhone Ltd., Glasgow, UK. ART number 10 670 057 035). The test principle is as follows: D-Sorbitol is oxidized 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).

$$\text{D-Sorbitol} + NAD^+ \xleftarrow{\text{SDH}} \text{D-Fructose} + NADH + H^+$$

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 diaphorase

$$NADH + INT + H^+ \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan}$$

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

2.8.10. Replica plating for plasmid retention determination

Plasmid retention was measured according to the method described by Lederberg & Lederberg (1952). Appropriately diluted samples from the cultures were first dispersed on a non selective agar with a glass spreading rod. After incubation, replica plating permits the copying of a pattern of microbial growth from the initial agar plate to a series of others which have the appropriate antibiotic. The method uses fabrics to make the transfer without disturbing spatial relationships. The number of colony forming units (cfu) on the selective agar to the number of cfu on the non-

selective agar gives the proportion of the cells retaining the plasmid or the cassette for *E.coli* and *P.pastoris* cells respectively.

2.9. Biotransformations

The ability of the produced cells to efficiently convert substrate to product was evaluated by whole cell biotransformations. Cells harvested from fermentation were centrifuged and the supernatant was discarded. Then the cell pellets were freeze-dried and stored at 4 ^{0}C until use. Chemical reactors with working volume 1l were filled with 0.5l of medium containing 200mM of a-methyl-benzylamine buffered at pH 7 using phosphate buffer and 2M sulphuric acid. Biotransformation started with the addition of 0.6g/l freeze dried cells. The stirring rate during the process was maintained at 330rpm, the airflow 1vvm, while the temperature was set at 30 ^{0}C. The concentration of a-methyl-benzylamine was determined by an HPLC method developed by Ingenza ltd.

2.10. Statistical analysis

Calculation of error

The errors for all measurements given in all the results chapters are standard deviations. The standard deviation, *s*, describes how closely the individual values cluster around the mean value and it can be expressed mathematically by the following equation:

$$s = \sqrt{\frac{\sum (x - \bar{x})}{n-1}}$$

Where $\bar{x}$ is the mean value, χ is the individual values and n the sample size.

Two-sample independent t test

When needed, 2 sample t test was performed to determine if the means of data between two sample groups differ at the 95% confidence level, or if the mean for one is smaller or larger than that for the other. In all cases the variances s^2 as well as the standard deviation are assumed different or at least it is not claimed that they are the same. The mathematical expression of the test is the following:

$$t = \frac{\bar{x}_\alpha - \bar{x}_\beta}{\sqrt{\frac{s_\alpha^{\,2}}{n_\alpha} + \frac{s_\beta^{\,2}}{n_\beta}}}$$

Where $\bar{x}_\alpha$, $\bar{x}_\beta$ are the means of sample groups α and β, s_α^2 s_β^2 are the variances of samples group α and β.

Chapter 3

Aspergillus niger process for homologous production of Amine Oxidase

3.1. Introduction and Aims

In this chapter, fermentations with the filamentous fungus *A.niger* were carried out. As discussed in chapter 1, *A.niger* is a very popular host for both the heterologous and homologous production of many industrially important enzymes due to its ability to secrete bioactive proteins with efficient post-translational processing (Wang et al, 2003). The objective of the overall project is the optimization of the production of the enzyme amine oxidase from microorganisms. In this chapter, a method for the correct determination of the industrially useful FAD monoamine oxidase was established. Moreover, the appropriate nitrogen source for the production of the AO was investigated and then various strains of *A.niger* were tested regarding their ability to produce amine oxidase and the industrially useful FAD monoamine oxidase (section 1.1.5.6).

When it comes to the production of a native enzyme as is the case of this part of the research a thorough understanding of the physiology of the microorganism is required. Towards that direction the present work sought to elucidate the effects of two different nitrogen sources on the physiology of the *A.niger* cells, and to be more specific, on the biomass production and most importantly, on amine oxidase activity. Additionally, the levels of activity which correspond to the flavin adenine dinucleotide (FAD) amine oxidase was studied, and the relationship between the nitrogen source and the percentage of the FAD amine oxidase activity was examined. Furthermore, maximization of the amine oxidase production was attempted through incorporation of an FAD-AO inhibitor in the media or the testing of different media pH's. Finally fermentations were carried out in a fermenter vessel where the butylamine induction took place when biomass reached its highest value.

3.2. Determination of the FAD amine oxidase activity, use of appropriate enzymatic inhibitor

3.2.1. Introduction

Schilling & Lerch (1995b) have observed the inhibitory effect of semicarbazide hydrochloride on the copper containing amine oxidase. However, using this compound in order to determine the amount of amine oxidase activity which corresponds to the FAD – amine oxidase entails the risk that this compound could cause inhibition of the FAD amine oxidase activity. To verify this, a recombinant *E.coli* strain was used which expresses only the wild type *A.niger* FAD - amine oxidase (MAOWT). The effect of the semicarbizide on the FAD – amine oxidase activity was measured during a fermentation of *E.coli* BL21(DE3) pET16b (MAOWT). Moreover, the efficacy of the known inhibitors of the copper amine oxidase needs to be tested. Towards this direction the percentage of inhibition of the best known inhibitors of the copper and FAD amine oxidase were tested and compared.

3.2.2. Results and Discussion

In this experiment the concentration recommended by the literature of 10^{-3}M of semicarbazide hydrochloride was used (Schilling & Lerch, 1995b). The results of table 3.1 clearly show that the use of the semicarbazide did not cause any inhibition to the FAD amine oxidase activity from this strain.

From the above we can conclude that the semicarbizide hydrochloride can be used for the inhibition of the copper containing amine oxidase in preparations which contain both the copper and the FAD amine oxidase, as it appeared that it causes no inhibition to the FAD amine oxidase.

The correct determination of the FAD amine oxidase activity could also be jeopardised by the inability of the inhibitor to fully repress the activity of the Cu amine oxidase. Bearing in mind that for the overall amine oxidase activity, responsible are the Cu-amine oxidase and the FAD monoamine oxidase, the inhibition of the one leads to the determination of the activity of the other. Therefore in order to examine the efficacy of the semicarbazide in inhibiting the Cu-AO, the FAD-AO inhibitor pargyline was used to inhibit the FAD-AO of the *A.niger* 337690 strain used in these experiments and the results of the pargyline and semicarbazide inhibitions were compared.

From figures 3.1. we can draw the conclusion that both semicarbazide and pargyline gave the same results regarding the percentage of the activity that belongs to the FAD and the Cu amine oxidase. Therefore both can be used for the correct determination of the proportion of the FAD/Cu amine oxidase activity.

Table 3.1. Total amine oxidase activity before and after the addition of the Cu-AO inhibitor (semicarbazide). The *E.coli* BL21(DE3) pET 16b (MAO-N) was grown in shake flasks until OD =1-2 and then amine oxidase activity was analysed with and without the incorporation of semicarbazide hydrochloride 10^{-3}M.

Repeats	Without inhibitor (µmol/l/min)	With inhibitor (µmol/l/min)	Activity after inhibition (%)
1st	18.27	17.76	97.19
2nd	18.91	18.77	99.29
3rd	18.35	18.94	103.18
Average	18.51	18.49	99.89

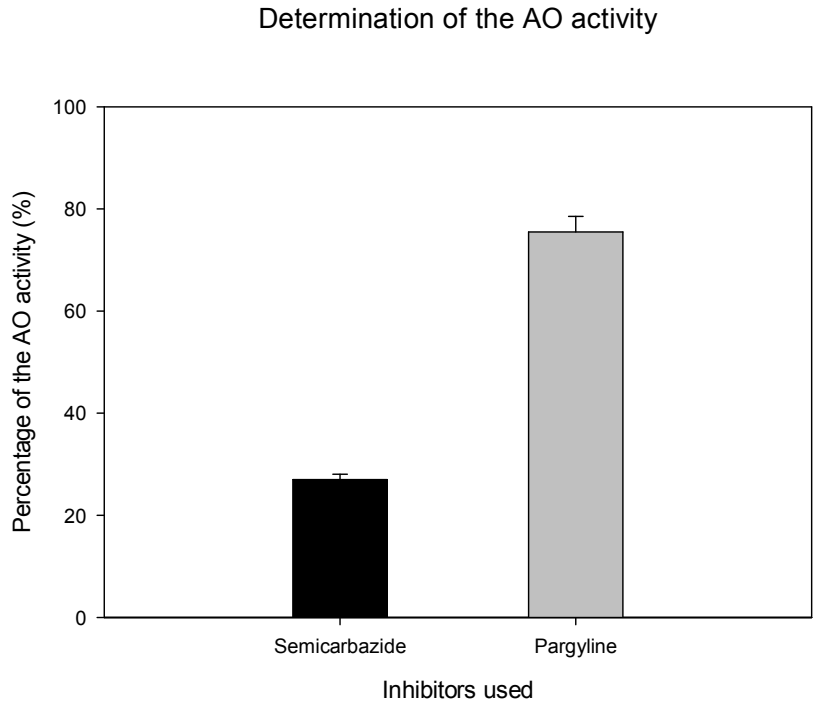

Figure 3.1. The percentage of amine oxidase activity belonging to the FAD – MAO (■) and the Cu –AO (■), determined by the use of a Cu – AO enzyme inhibitor (semicarbazide) and a FAD – MAO enzyme inhibitor (pargyline). *A.niger* 337690 spores were used to inoculate 1l flasks and grown until stationary phase. Samples were withdrawn and analysed in triplicate.

3.3. Selection of the appropriate nitrogen source

3.3.1. Introduction

In this work the compound used to induce the synthesis of the amine oxidase is n-butylamine as it was found that the highest amine oxidase induction was achieved with n-butylamine as inducer (Frébort et al, 1997; Schilling & Lerch, 1995c). The key industrial objective is to obtain the highest possible amine oxidase production and an appropriate balance between the biomass and specific amine oxidase activity needs to be established. It has been found that an amine, in the present case butylamine, cannot be used as both the carbon and the nitrogen source by fungi (Dijken & Bos, 1981; Lindley, 1987) with only very few exceptions (Middelhoven et al, 1984). High concentrations of amines can be toxic to the cells (Albert, 1985) and thus do not enable the use of amines as a the sole nitrogen source, since a sufficiently high concentration of biomass requires a higher nitrogen concentration than the toxicity threshold for butylamine (0,2% - 0,25 (v/v)) allows. Therefore, in order to tackle this problem either:

(i) a fed – batch bioprocess will be followed where a gradual addition of butylamine will take place so as not to exceed the toxicity limits, or

(ii) another nitrogen source will be chosen (in addition to butylamine) whereby a high biomass will be achieved before the induction with butylamine.

In a previous work on amine oxidase production by *A.niger* (Schilling & Lerch, 1995b) the authors used a medium described by Vogel (1956) where the original nitrogen source, ammonium nitrate, was replaced by sodium nitrate. Even though it is known that a primary nitrogen source such as ammonium strongly inhibits the synthesis of amine oxidase, a search in the literature did not generate any information regarding the effect of a secondary nitrogen source such as nitrate on amine oxidase synthesis in the presence of an inducer. The present work attempted to elucidate the behaviour of *A.niger* (the strain number 337690 was used as a model organism) in the presence of butylamine and a secondary nitrogen source (in this case sodium nitrate) and compare it to the respective case where ammonium and butylamine co –

exist. In this experiment, a comparison between the use of ammonium and nitrate salts as additional nitrogen sources used in conjunction with butylamine was carried out.

3.3.2. Results and discussion

Production of Biomass and intracellular protein concentrations

As can be seen in the Figure 3.2.A., the use of sodium nitrate instead of ammonium chloride caused a significant decrease in the biomass concentration of both control and butylamine - induced cultures. Especially in the case of the control this difference in the biomass concentration is vast. At the end of the process (144h) the final biomass concentrations for the induced cultures were 8.66 g/l and 6.52 g/l for the ammonium and nitrate containing cultures respectively, while for the controls were 13.58 g/l and 7.29 g/l for the ammonium and nitrate containing cultures respectively.

In Figure 3.2.B., the intracellular protein concentrations for the control cultures did not have significant differences, while in the case of the induced culture the nitrate containing cultures after the 72h had significantly higher intracellular protein concentrations and reached a peak at 120h (185.2 mg/g DCW), at the same time the amine oxidase activity was significantly higher as well as can be seen in figure 3.3.

Amine oxidase activity and specific activity

The induced culture containing the nitrate appears to have significantly higher specific activity levels. This figure clearly shows that the ammonium ions cause an inhibition of the amine oxidase activity as only when ammonium was fully consumed (figure 3.5.B.) amine oxidase increased to high levels (figure 3.3). This inhibition caused by ammonium ions occurs possibly by inhibiting the synthesis of this enzyme at the level of transcription (Marzluf, 1997). The specific activity of the ammonium containing culture reached 1.8 mol/g DCW/min at 96h and remained constant until the end of the process, in contrast, the nitrate containing cultures

exhibited a very abrupt increase in the amine oxidase activity and reached a peak at 72 hours (3.31 mol/g DCW/min). The amine oxidase level decreased, a fact which might indicate the consumption of butylamine, while the ammonium containing culture remained stable, presumably because there was not enough amine oxidase produced in the initial stages of the process and as a result the butylamine concentration was not significantly reduced as may have happened in the nitrate containing culture.

The total amine oxidase activity appeared to be higher for the culture containing the sodium nitrate as the main nitrogen source (figure 3.3.B). This happened from 48h until 120h. After that time there was an abrupt fall in the activity of amine oxidase for the nitrate containing culture, while the activity in the ammonium containing culture remained relatively stable resulting in a considerably higher activity for the culture containing the ammonium source. The maximum total amine oxidase activity of the nitrate containing culture was 16.69 mol/l/min (120h) and of the ammonium containing culture was 14.51 mol/l/min (144h). The difference between the nitrate and ammonium containing culture even though significant was not as vast as that of the specific activity and this was attributed to the better growth of the ammonium culture which is also reflected in the higher biomass levels.

Proportion of the flavin adenine dinucleotide (FAD) and copper (Cu) containing amine oxidase

Figure 3.4. shows that as the time passed the percentage of the activity that belongs to the FAD containing amine oxidase gradually and slowly increased in both induced cultures (the only exception was the 72h nitrate containing culture where the percentage of the FAD amine oxidase activity is higher than the 96h). Another thing to note is that during the first 72 hours of the bioprocess the percentage of the FAD amine oxidase activity was much higher for the nitrate containing culture while after that time the ammonium containing culture appeared to have slightly higher percentage of the FAD amine oxidase activity.

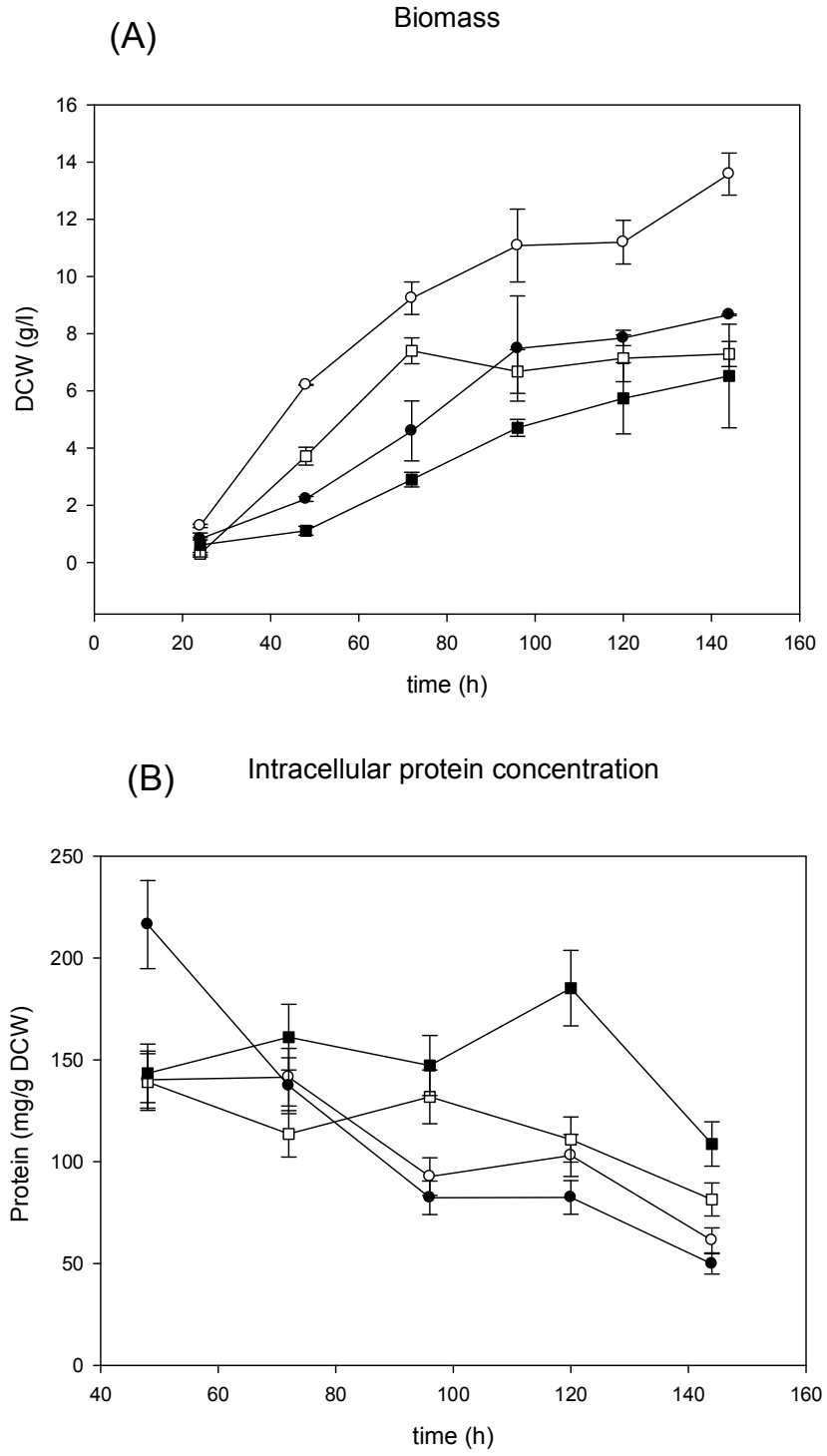

Figure 3.2. Time profiles of the biomass concentrations (A) and intracellular protein concentration (B) of the uninduced (control) cultures with ammonia (○) and nitrate (□) and the induced cultures with ammonia (●) and nitrate (■) as nitrogen source in shake flasks fermentations of *A.niger* 337690. Culture conditions: flasks cultivation, 25°C, 200 rpm.

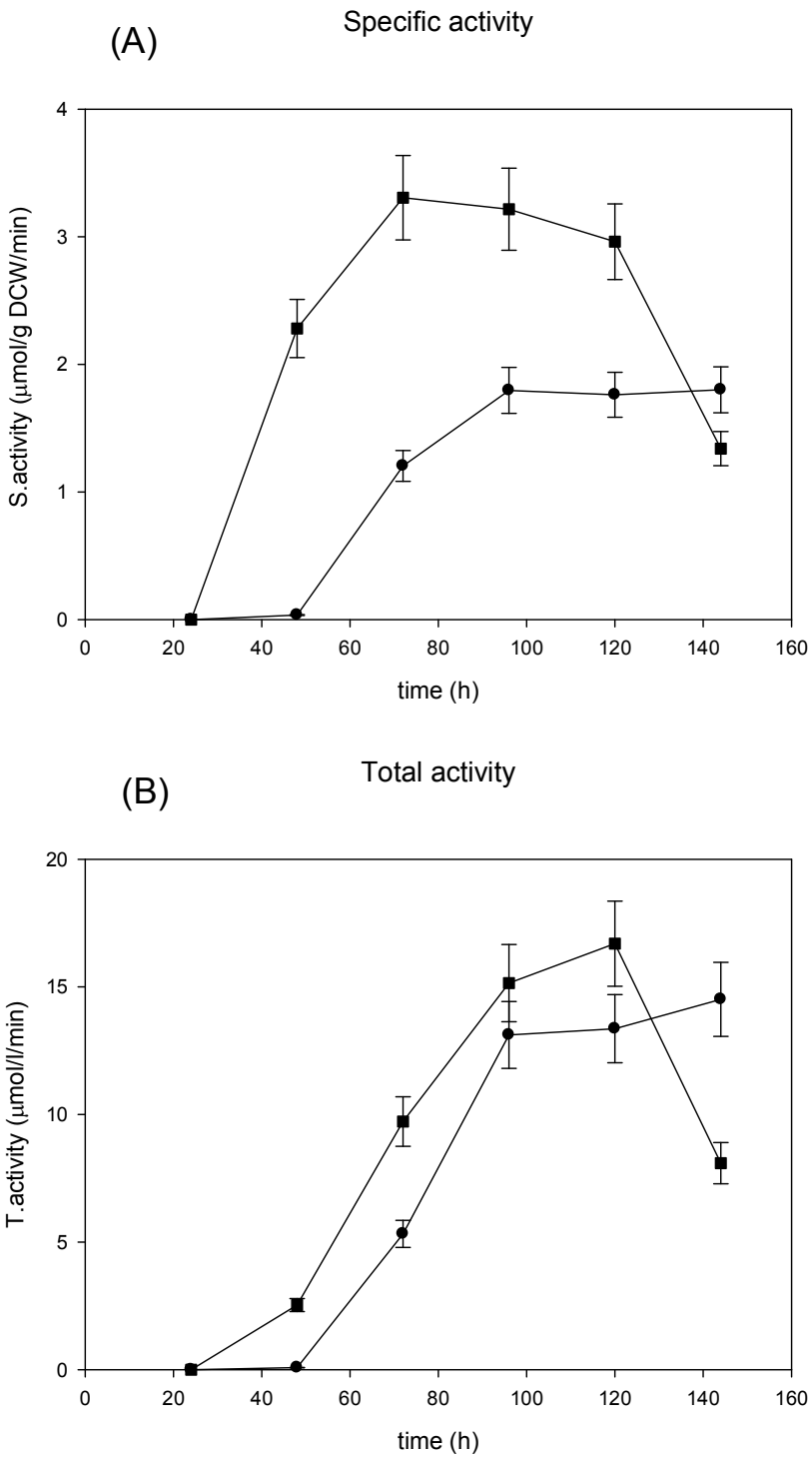

Figure 3.3.Time profiles of the (A) specific amine oxidase activities and (B) total specific amine oxidase activities for the induced cultures containing ammonia (●) and nitrate (■) as nitrogen source in shake flasks fermentations of *A.niger* 337690 . Culture conditions: flasks cultivation, 25 ^{o}C, 200 rpm.

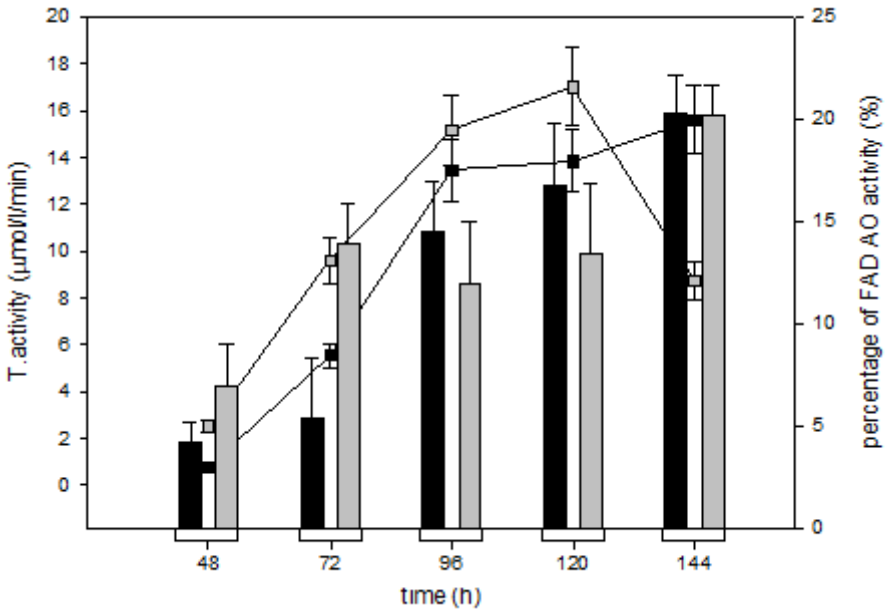

Figure 3.4. Total amine oxidase activity (lines) and percentage of the activity (columns) attributed to the FAD amine oxidase v. time for the ammonium (■) and nitrate (■) containing *A.niger* 337690 cultures. Culture conditions: flasks cultivation, 25 ^{o}C, 200 rpm

It is possible that the percentage of the copper and the FAD containing amine oxidase activity could be linked to the amount of the butylamine present in the broth. It is also possible that the reduction of the butylamine concentration caused the reduction of the Cu - amine oxidase and the increase of the FAD amine oxidase. If this is the case it can also be assumed that the synthesis of the FAD amine oxidase is more easily induced when the organism is in need of a nitrogen source, while the copper containing amine oxidase is predominantly induced when the cells are not under nitrogen limitation or when the cells are stressed by the presence of a toxic amine.

The FAD amine oxidase may be mainly responsible for the utilisation of amines as nitrogen sources, while the copper amine oxidase could be a detoxifying agent (Frébort et al, 1997). The first assumption may be supported by the fact that when the ammonium nitrogen was fully consumed (ammonium is the preferable nitrogen source) the FAD amine oxidase activity was significantly increased. In the case of the nitrate containing culture there were no abrupt increases in the FAD amine oxidase activity percentage, (apart from at 144h, the point where the nitrate nitrogen was already consumed!) as the amine present in the medium was simultaneously used from the beginning of the process along with the nitrate. Another supporting fact is that at the initial stages the ammonium containing culture had lower FAD amine oxidase activity than the nitrate containing culture. This can be explained by the fact that the ammonium is the preferential nitrogen source (Marzluf, 1997) and as a result there is no need for utilisation of butylamine as a nitrogen source, however, nitrate is not such a good nitrogen source and the organism uses at the same time nitrate and butylamine as a nitrogen source.

Residual glucose and primary nitrogen concentration

In Figure 3.5.A, it is obvious that the addition of the inducer caused a delay in the consumption of glucose, by increasing the lag phase, as the glucose was fully consumed 24 hours later in the induced cultures. The glucose consumption rate was higher in the case of the cultures containing ammonium (0.495 g/l/h and 0.437 g/l/h for the control and the induced culture respectively) than the cultures containing

nitrate (0.367 g/l/h and 0.325 g/l/h for the control and the induced culture respectively).

From Figure 3.5.B., we can see that the nitrogen from the ammonium in the ammonium containing cultures was consumed much quicker than that of the nitrate containing cultures. It is worth noting that in the control culture, which contained the sodium nitrate, the organism did not consume the whole available nitrate, in contrast to the rest of the cultures where the nitrate was fully consumed. It is remarkable that the specific growth rate of the control nitrate containing culture is $0.062h^{-1}$ while that of the control ammonia culture $0.056h^{-1}$. Hence, in the exponential phase of growth, *A.niger* appears to grow faster on nitrate but as it seems quickly decelerates and comes to an abrupt halt. At this time the residual nitrate concentration reaches the lowest value and remains there until the end of the process. Hence, it is obvious that *A.niger* has a lower affinity for nitrate as nitrogen source than ammonium the concentration of which reaches 0 g/l at the end of the process.
Another evidence to back up this is that the nitrogen uptake rate in the nitrate containing cultures was very low (0.0078 g/l/h and 0.0044 g/l/h, for the control and induced cultures respectively) compared to the respective ammonium containing cultures (0.0095 g/l/h and 0.0086 g/l/h for the control and induced cultures, respectively). Therefore, we can deduce that the cells show a preference for the utilisation of the ammonium cations at the expense of butylamine since the culture containing ammonium chloride seemed to consume this nitrogen source much quicker than the cultures containing the sodium nitrate. It is also obvious from the comparison of the control cultures that the ammonium chloride was more easily utilised by the cells than the sodium nitrate.

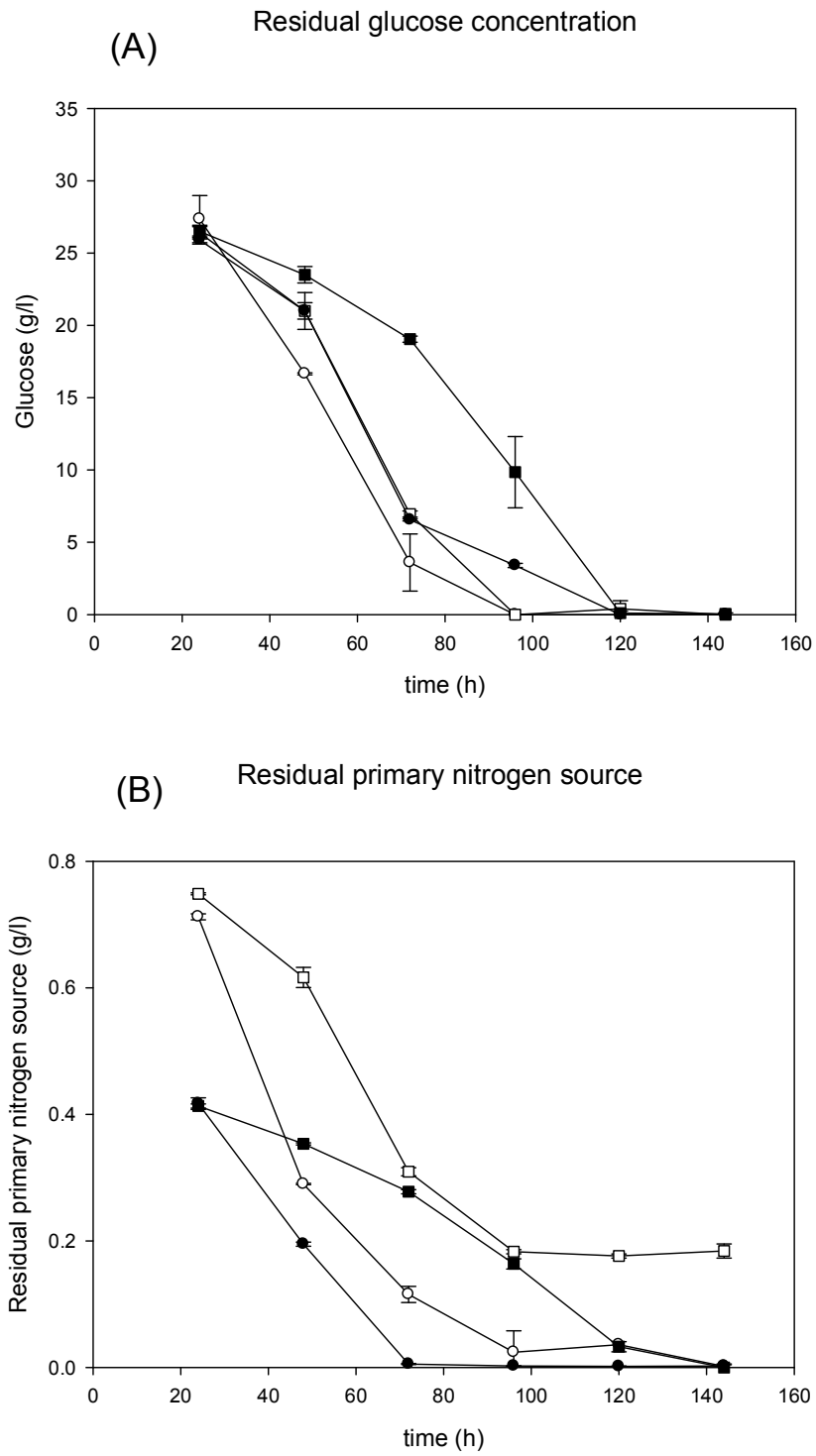

Figure 3.5. Time profiles of the residual glucose (A) and nitrogen source concentrations (B) of the uninduced (blank) cultures with ammonia (○) and nitrate (□) and the induced cultures with ammonia (●) and nitrate (■) as nitrogen source in shake flasks fermentations of *A.niger*. Culture conditions: flasks cultivation, 25°C, 200 rpm

3.3.3. Conclusions

As expected the presence of ammonium cations causes inhibition of the amine oxidase expression, however, there was some detectable activity at 48h, before the exhaustion of the ammonium chloride. The amine oxidase activity soared to very high levels when ammonium was completely depleted; however, the activity was still lower than that of the nitrate containing culture.

Figures 3.3. and 3.5.B, show that the nitrate containing cultures used the nitrogen from the nitrate and butylamine simultaneously for their growth as the nitrate consumption rate was low and the amine oxidase activity relatively high compared to the ammonium containing cultures where the ammonium nitrogen was fully consumed after 72 hours and the specific amine oxidase activity started to rise at this point of the process.

Even though ammonium containing cultures exhibited better growth patterns leading to higher biomass levels, the amine oxidase activity was lower. To conclude nitrate appeared to be more appropriate than ammonium as a primary nitrogen source for the production of amine oxidase. Nitrate containing cultures exhibited significantly higher total amine oxidase activities and during the first hours of the process significantly higher FAD amine oxidase activity which outweighs their reduced biomass production.

3.4. Testing of *A.niger* strains for amine oxidase production

3.4.1. Introduction and objective

Before starting the processes in bioreactors, it is important to identify first the appropriate strains which can be induced and produce the amine oxidase enzyme. It is reported that some strains do not have the capacity to produce this enzyme, or in other cases only one of the two kinds of amine oxidase can be expressed (either FAD or Cu amine oxidase) (Frébort et al, 1997; Frébort et al, 1996). Thus primary

evaluation of some strains and their ability to degrade/ utilize the butylamine is required.

In this experiment 4 different *Aspergillus niger* strains were grown in flasks to compare their ability to grow in a defined medium and to produce amine oxidase when induced by butylamine. All strains are isolated from different environments. A recombinant strain (*A.niger* B1-D) expressing a heterologous protein other than amine oxidase was also used for comparison reason. It was expected that the overproduction of the heterologous protein will occupy the translation machinery of the cells and therefore the expression of amine oxidase would be lower than the wild type strains. A control and an induced culture were run for each of the strains tested. The experiment was done in triplicate to help ensure reproducible results and the most representative of them are presented.

3.4.2. Results

3.4.2.1. A.niger 149007ii

Production of Biomass and intracellular protein concentration

As can be seen in figure 3.6 there were no significant differences ($p>0.05$) in the biomass between the control and the induced cultures of *A.niger* 149007ii. Both cultures exhibited the same patterns and they both reached a peak at 96h (7.4 and 8.23 g/l for the control and the induced culture respectively). After that point there is a slight decrease in the biomass levels for both cultures. The specific growth rates during the exponential phase were $0.037h^{-1}$ and $0.039h^{-1}$ for the control and induced culture respectively. As can be deduced from both figures 3.6.A and 3.7.B the reason for the slightly higher biomass of the induced culture at 96h and higher growth rate was the exhaustion of the nitrogen source of the control culture.

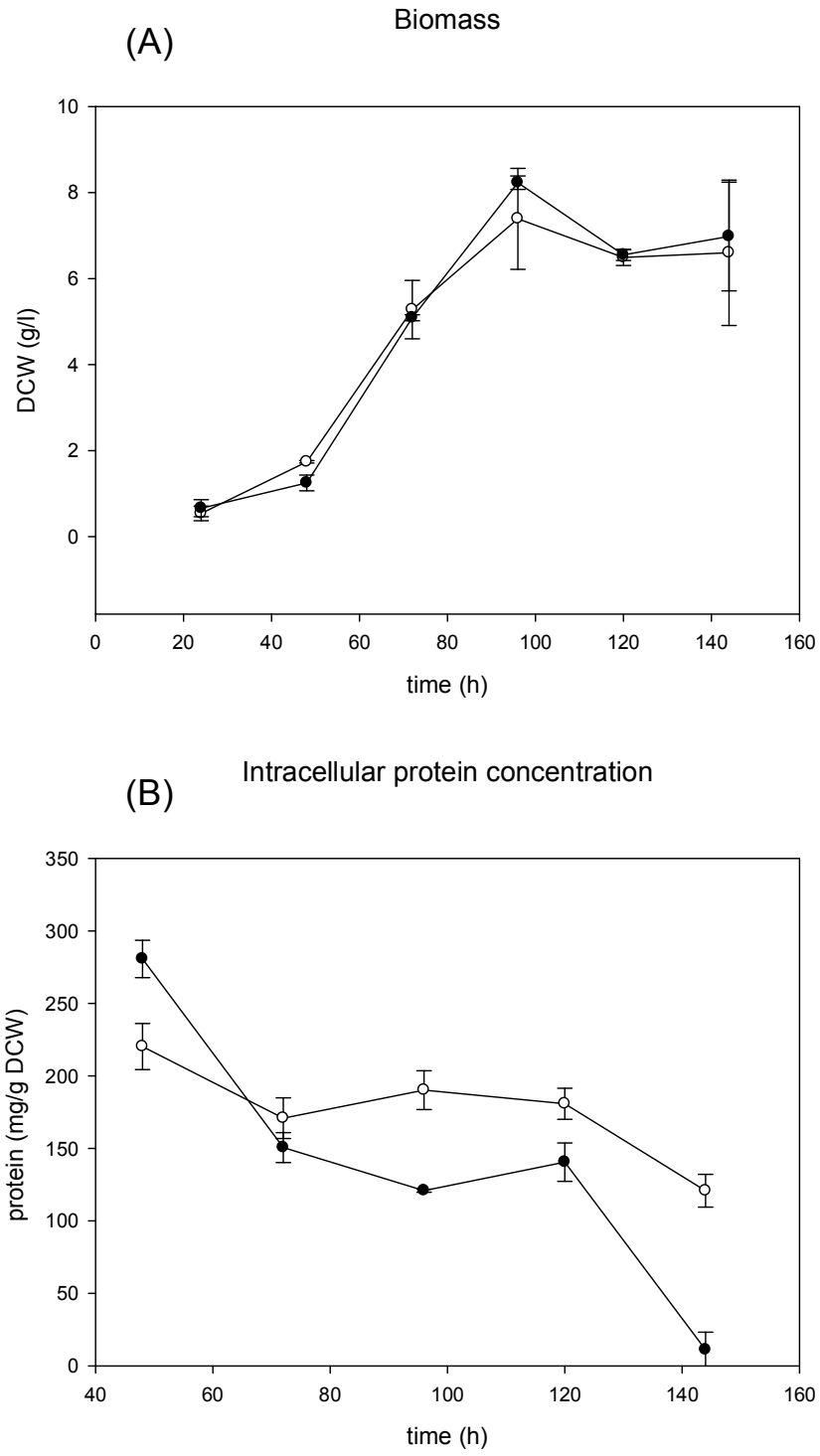

Figure 3.6. Time profiles of the (A) biomass and (B) intracellular protein concentration of the uninduced (○) and induced cultures (●) in shake flasks fermentations of *A.niger* 149007ii. Culture conditions: flasks cultivation, 25^{o}C, 200 rpm.

The intracellular protein concentration for both the control and the induced culture showed a gradual decrease. After 72h the induced culture exhibited lower protein concentration and this difference increased until the end of the processes. This fall in intracellular protein concentration especially for the butylamine induced culture could be a result of culture decay, a fact which agrees with the biomass decrease (figure 3.6).

Amine oxidase activity

Figure 3.7.A. shows an increase of the amine oxidase activity until 96h where it reached a peak (24.1 mol/l/min). After that point, there was a constant decrease in the activity until the end of the process. At 48h the percentage of the FAD amine oxidase activity reached 52% and after that point fell to lower levels ranging from 21-36%. At the time when the total amine oxidase activity peaked (96h), the FAD – amine oxidase accounted for 37% of the total activity.

Residual glucose and nitrate concentration

Figure 3.7.B. shows the residual nitrate concentration for the control and the induced culture. In the case of the induced culture, the consumption of nitrate was much slower, possibly due to the simultaneous utilization of butylamine as a nitrogen source. In the control culture the nitrate was fully consumed at 96h where in the induced culture the nitrate concentration was almost consumed at the end of the process (144h). The nitrate consumption rate of the control culture was 0.035g/l/h while that of the induced culture was 0.016 g/l/h.

The control culture appeared to be more efficient in utilizing the glucose. The glucose consumption rate for the control culture was 0.57 g/l/h while for the induced culture was 0.35 g/l/h. Moreover, the glucose was fully consumed by 96h for the control culture and by 120h for the induced culture. Hence, the addition of the inducer caused a 54.5% decrease of the nitrate consumption rate which could be partially explained by the simultaneous use of the two nitrogen sources and 38.6% decrease of the glucose consumption rate which can be attributed to the toxic effect butylamine exerts on cells.

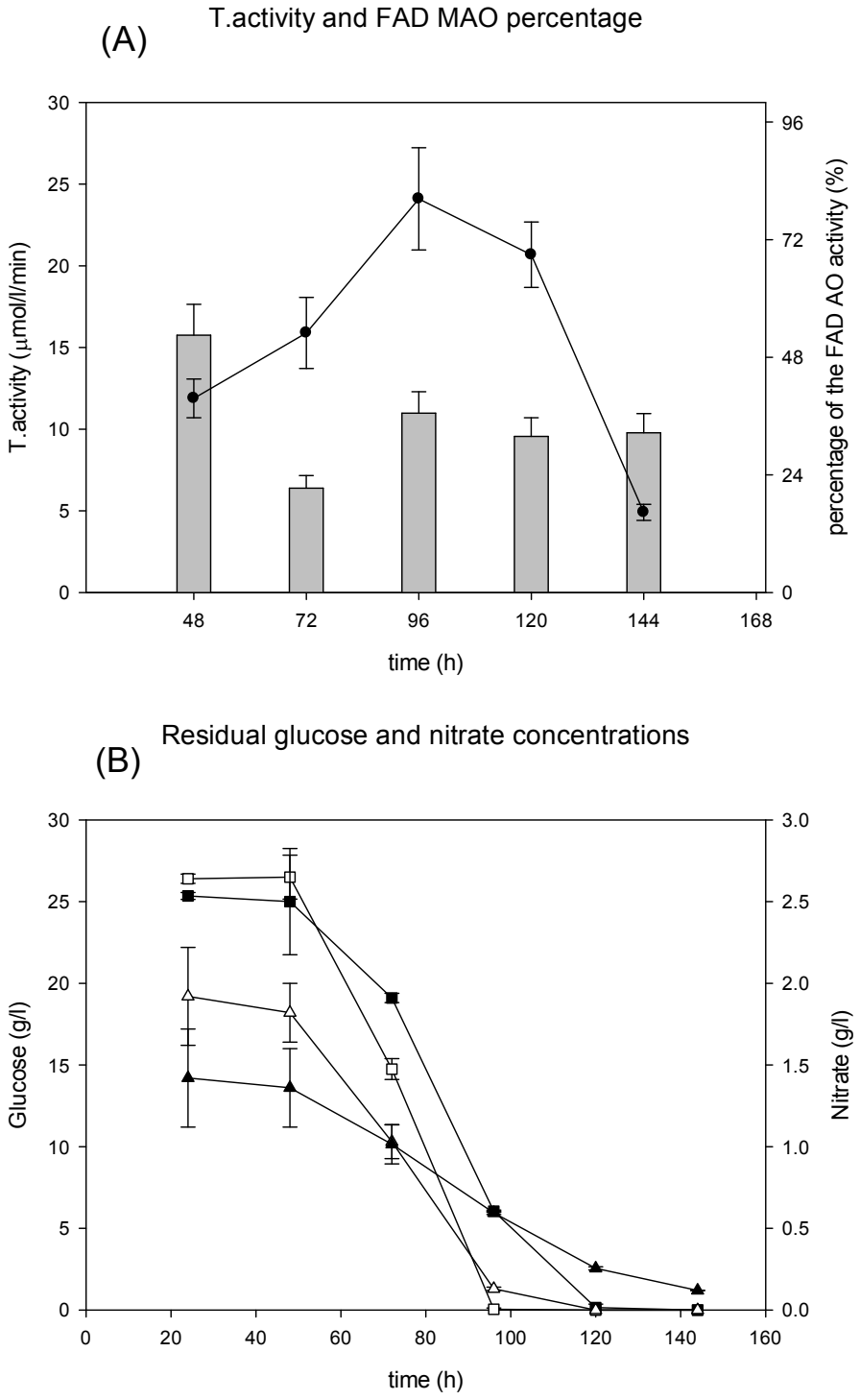

Figure 3.7. (A) Time profiles of the total activity and percentage of the FAD MAO (B) Residual glucose and nitrate concentration of uninduced ((□) and (Δ) for glucose and nitrate respectively) and induced cultures ((■) and (▲) for glucose and nitrate respectively) in shake flasks fermentations of *A.niger* 149007ii. Culture conditions: flasks cultivation, 25^{o}C, 200 rpm.

3.4.2.2. A.niger 337690

Production of Biomass and intracellular protein concentration

In the case of the *A.niger* 337690 the addition of the inducer significantly lowered the biomass concentration (figure 3.8.A). The control culture reached a peak at 96h (11.98 g/l) while the induced culture reached a peak 24 hours later (10.78 g/l). In both cultures after that peak there was a decrease in the biomass levels, possibly due to the depletion of the carbon source (96h for both cultures) as can be seen in figure 3.9.B. Moreover, the specific growth rate of the control culture was significantly higher than that of the induced ($0.035h^{-1}$ and $0.024h^{-1}$ respectively).

Regarding the intracellular protein concentration there was a sharp decrease for both cultures until the 96h after which the protein concentrations were relatively stable until the end of the processes.

Amine oxidase activity

The total activity constantly reduced from 48h onwards and at the end of the process (144h) the activity fell to 1.2 mol/l/min. The highest activity achieved was 14.9 mol/l/min at 48h. FAD amine oxidase activity ranged from 9.5-16.5% of the total activity during the whole process.

Residual glucose and nitrate concentration

Figure 3.9.B. shows that there were no significant differences between the control and the induced culture regarding the consumption of glucose (t-test, $p>0.05$). Both cultures have completely consumed glucose by 96h. The control culture's glucose consumption rate was 0.39g/l/h while the induced culture's was 0.20 g/l/h.

As far as the residual nitrate concentration is concerned the induced culture was unable to fully utilize the available nitrate, however the control culture completely consumed the nitrate by 96h. The nitrate consumption rate for the control culture was 0.036 g/l/h and for the induced culture 0.015 g/l/h.

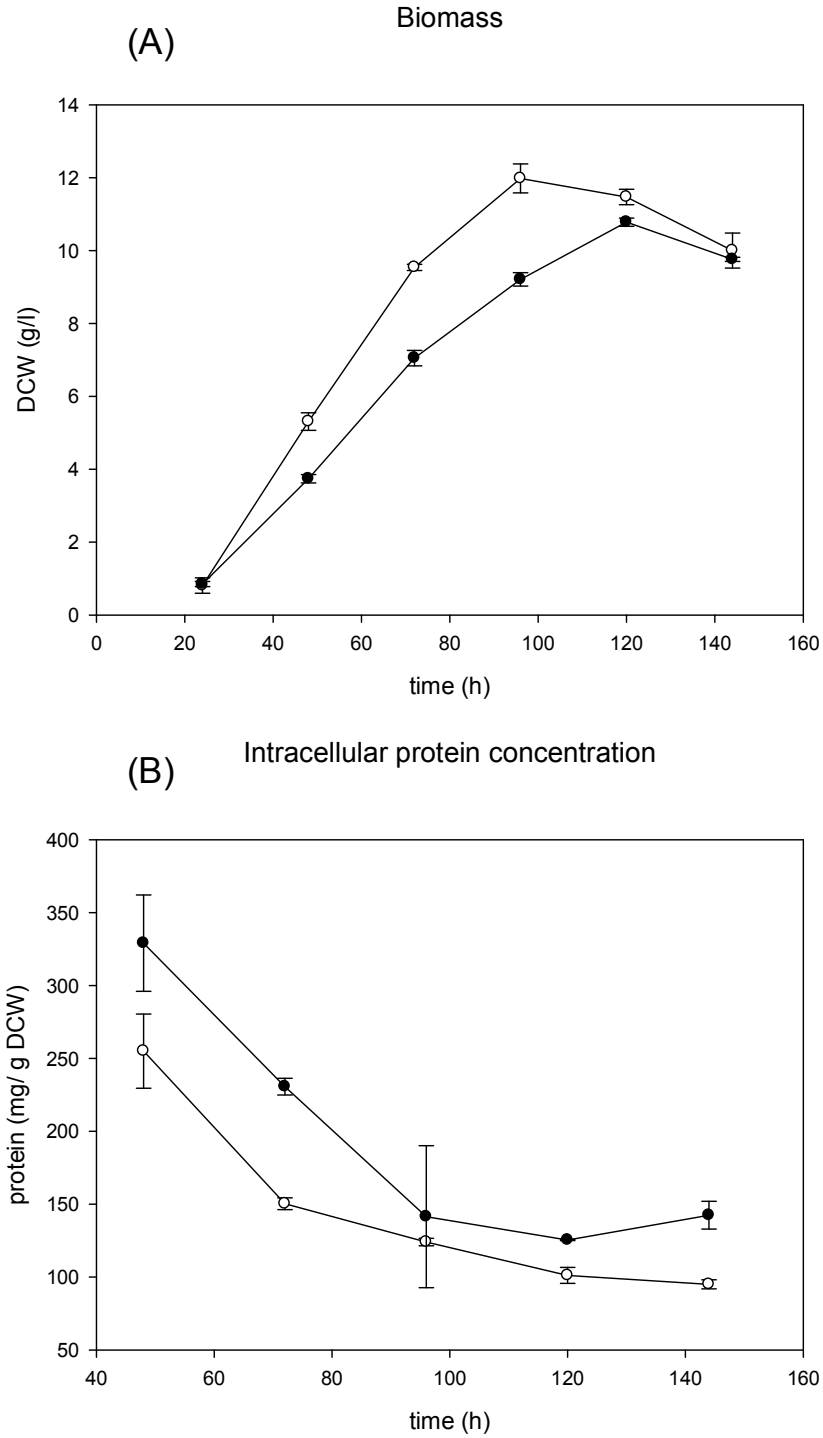

Figure 3.8. Time profiles of the (A) biomass and (B) intracellular protein concentration of the uninduced (○) and induced cultures (●) in shake flasks fermentations of *A.niger* 337690. Culture conditions: flasks cultivation 25^{o}C, 200 rpm.

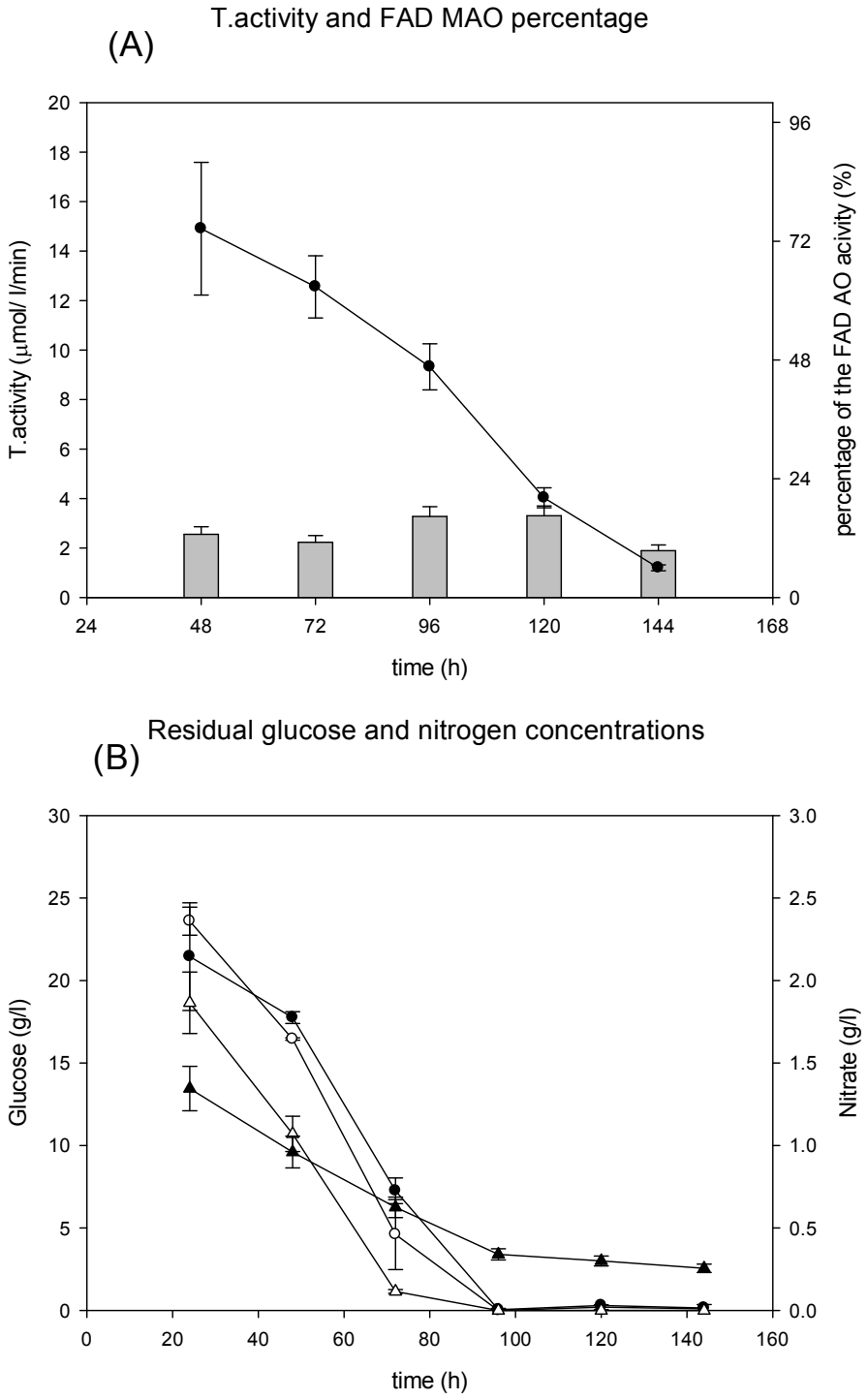

Figure 3.9. (A) Time profiles of the total activity and percentage of the FAD MAO (B) Residual glucose and nitrate concentration of uninduced ((□) and (Δ) for glucose and nitrate respecitively) and induced cultures ((■) and (▲) for glucose and nitrate respectively) in shake flasks fermentations of *A.niger* 337690.
Culture conditions: flasks cultivation 25°C, 200 rpm.

3.4.2.3. A.niger 320725

Production of Biomass and intracellular protein concentration

In the case of the *A.niger* 320725, the biomass concentration appears to be slightly higher for the induced culture than the control; however, this difference was not significant ($p > 0.05$). Both cultures continued growing during the end of the process without showing any apparent signs of stationary and decline phase. The specific growth rates were $0.032h^{-1}$ and $0.034h^{-1}$ for the control and induced cultures respectively.

The induced culture presented stable intracellular concentrations during the first 96h ranging from 233 to 255 mg/g DCW. After that point there was a gradual decrease until the end of the process. The intracellular protein concentration of the control culture was relative stable but with a declining trend from the start until 120h ranging from 190 to 246 mg/g DCW.

Amine oxidase activity

In Figure 3.11.A. the total amine oxidase activity rose abruptly from 48h to 72h and reached a peak of 20.9 mol/l/h. After that point the total activity gradually decreased which led to activity as low as 5.1 mol/l/h at the end of the process. The FAD amine oxidase activity reached the highest value at the 48h (39.6%) but then constantly decreased to a much lower level reaching 10% at the end of the process.

Residual glucose and nitrate concentration

This strain exhibited an apparent difficulty in utilizing sodium nitrate as can be seen in Figure 3.11.B. It is obvious that both the induced and control culture reduced the nitrate levels only very slowly. In the first 48h there was no obvious nitrate consumption, and after that point the consumption rate was 0.017 g/l/h and 0.015 g/l/h for the control and induced culture respectively. The nitrate was not completely depleted at the end for both processes. Apparently this organism is not very efficient in utilizing the nitrate and utilization of other nitrogen sources might be better. Figure 3.11.B. shows that the induced culture consumed the glucose 24h faster (at 120h while in the control culture glucose was exhausted by 144h).

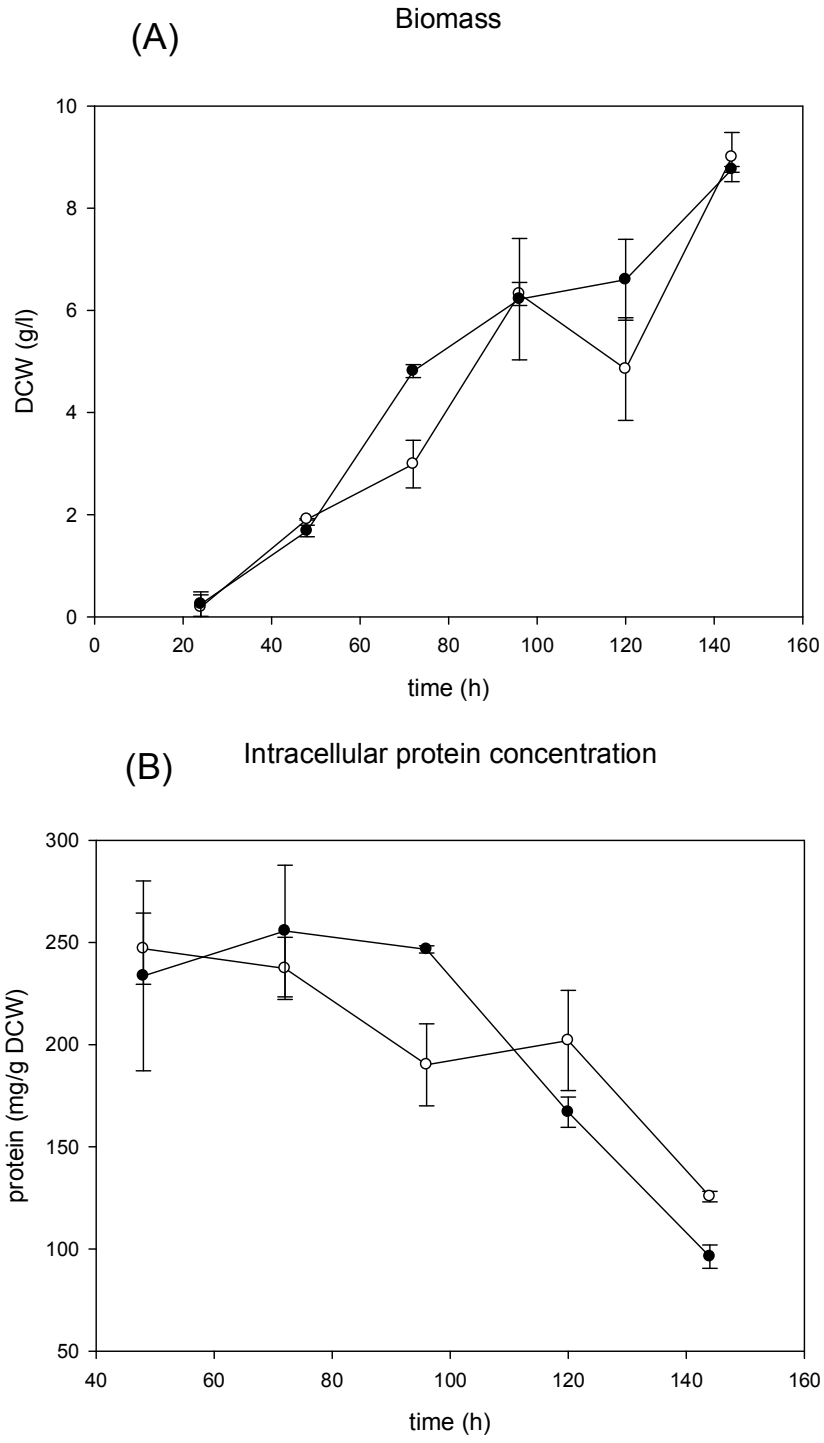

Figure 3.10. Time profiles of the (A) biomass and (B) intracellular protein concentration of the uninduced (○) and induced cultures (●) in shake flasks fermentations of *A.niger* 320725. Culture conditions: flasks cultivations, 25°C, 200 rpm.

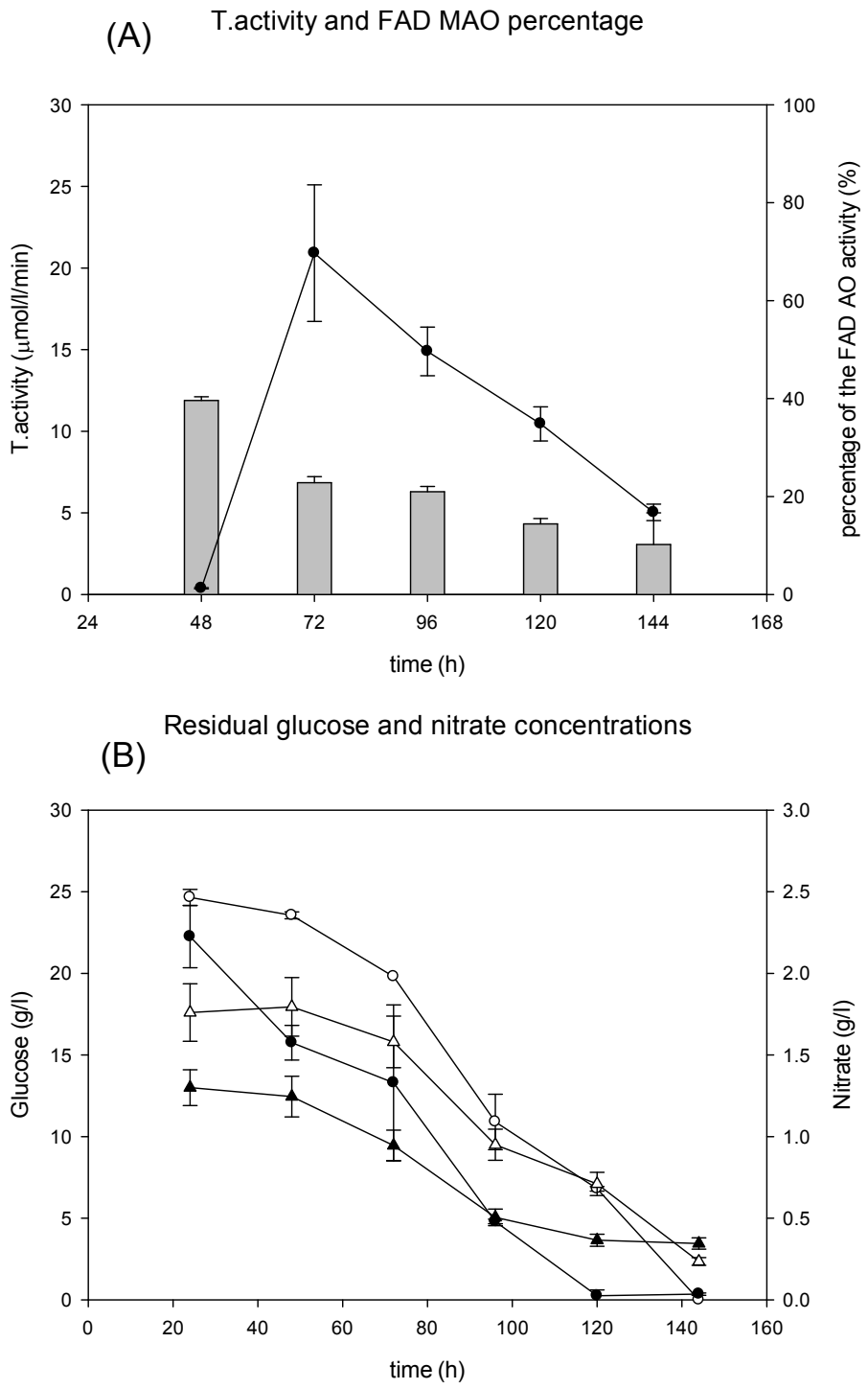

Figure 3.11. (A) Time profiles of the total activity and percentage of the FAD MAO (B) Residual glucose and nitrate concentration of uninduced ((□) and (Δ) for glucose and nitrate respecitively) and induced cultures ((■) and (▲) for glucose and nitrate respectively) in shake flasks fermentations of *A.niger* 320725. Culture conditions: flasks cultivations, 25°C, 200 rpm.

The glucose consumption rate was 0.25 g/l/h and 0.23 g/l/h for the control and induced culture respectively.

3.4.2.4. A.niger B1-D

Production of Biomass

A first look in figure 3.12.A. shows that there were no noteworthy differences between the control and the induced culture in terms of biomass production and that the addition of the butylamine was not enough to cause changes in the biomass levels. However, the maximum biomass concentration was 6.8g/l (120h) for the control and 5.98g/l (144h) for the induced culture. The specific growth rate was also higher for the control culture reaching $0.014h^{-1}$ while that of the induced culture was only $0.008h^{-1}$. The intracellular protein concentrations for both cultures followed the same trend and decreased from 245 to 85 mg/g DCW at the end of the processes.

Amine oxidase activity

The total amine oxidase activity like the intracellular protein concentration reached a peak at 48h (21.2 mol/l/min) and then it very rapidly fell to lower, nearly non-detectable levels. The FAD amine oxidase activity was very low during the whole process and ranged from 11.2 % to 16.5 % of the overall activity.

Residual glucose and nitrate concentration

Both cultures were very inefficient in utilising the nitrate as only a small portion of the available nitrate was consumed. The residual nitrate concentration at the end of the processes was 0.65 g/l and 0.62 g/l for the control and induced culture respectively. The control consumed glucose faster than the induced culture; the glucose uptake rate was 0, 22 g/l/h for the control culture and 0, 19 g/l/h for the induced culture. Glucose was almost completely consumed at 120h in the control culture while in the butylamine – induced culture glucose was completely depleted in the end of the process (144h).

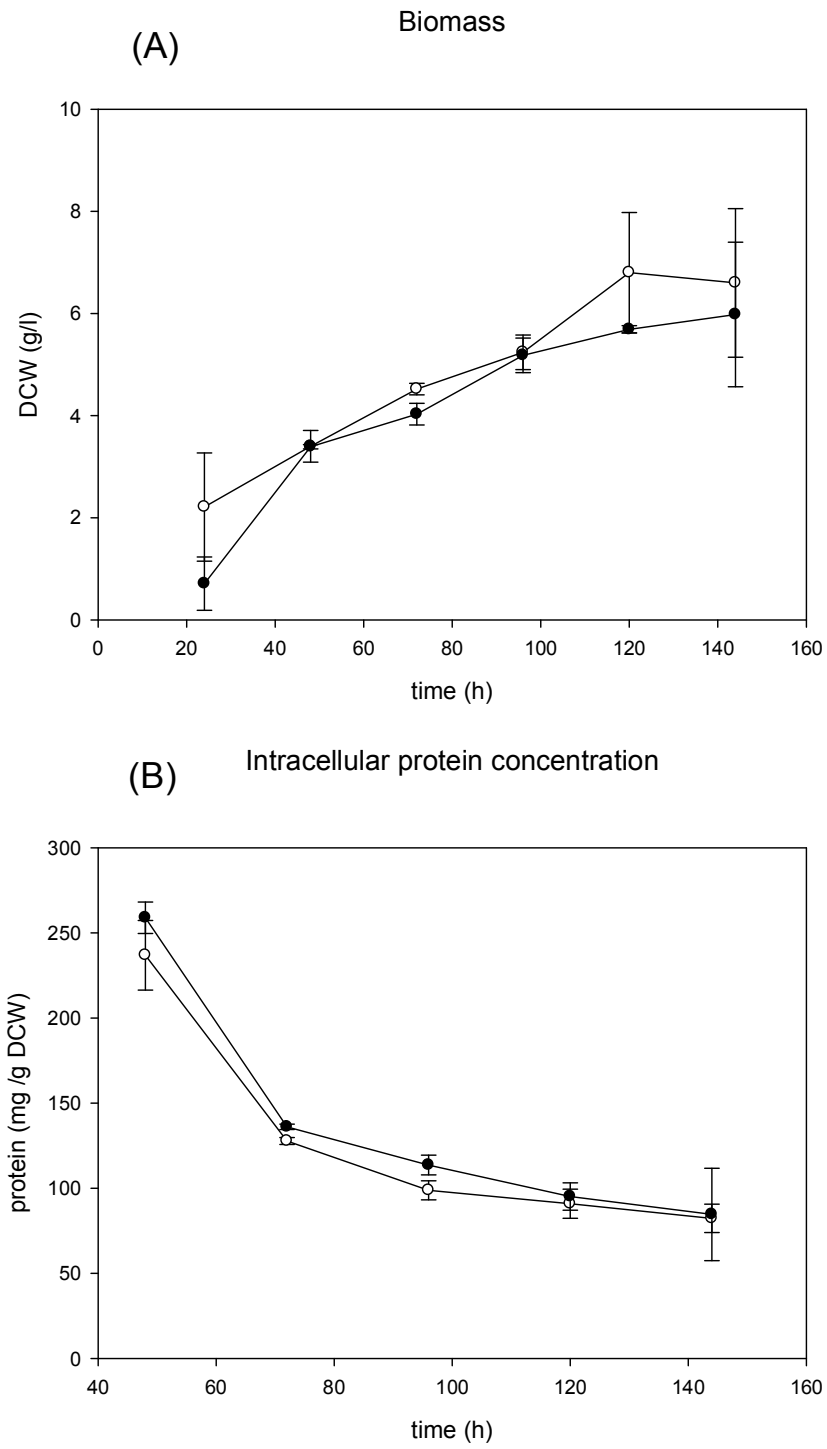

Figure 3.12. Time profiles of the (A) biomass and (B) intracellular protein concentration of the uninduced (○) and induced cultures (●) in shake flasks fermentations of *A.niger* B1-D. Culture conditions: flasks cultivations 25 ^{o}C, 200 rpm.

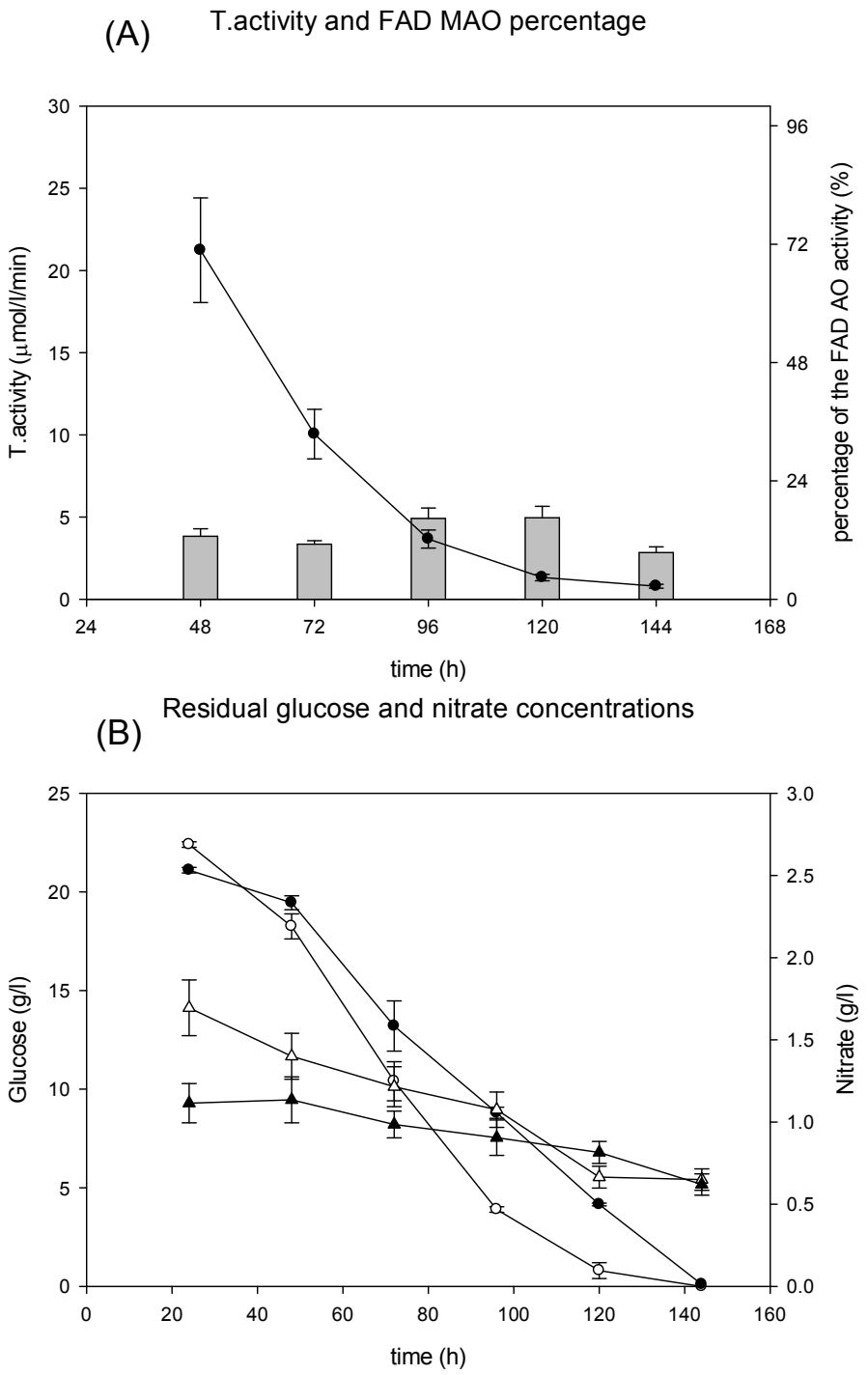

Figure 3.13. (A) Time profiles of the total activity and percentage of the FAD MAO (B) Residual glucose and nitrate concentration of uninduced ((□) and (Δ) for glucose and nitrate respecitively) and induced cultures ((■) and (▲) for glucose and nitrate respectively) in shake flasks fermentations of *A.niger* B1-D. Culture conditions: 25 ^{o}C, 200 rpm.

3.4.3. Discussion

A comparison between the strains is very important as it may draw some clues regarding which is the most appropriate strain for the production of amine oxidase. The dry cell weight figures show that all of the strains, apart from the *A.niger* 337690, were not significantly negatively affected by the presence of the butylamine. However, it is worth noting that even though this strain had significantly lower biomass levels in the induced culture by comparison to the control, the average biomass levels were still higher than that achieved by the other strains. From the activity figures it can be seen that the highest activity (expressed as total activity) was achieved by the strain *A.niger* 149007ii followed by the *A.niger* B1-D, *A.niger* 320725 (all of these strains had activities above 20 mol/l/min) while the lowest activity was achieved by the strain *A.niger* 337690 (14.9 mol/l/h). All of the *A.niger* strains tested had the ability to produce both the copper and the FAD containing amine oxidase. The FAD amine oxidase activity level does not seem to follow a specific pattern in all of the strain used. In the case *A.niger* 320725 the ratio of the FAD amine oxidase activity seems to reduce as time passes while in the *A.niger* 337690 strain this ratio seems to increase with the time (although not significantly apart from the last measurement). The residual nitrate figures show that some organisms were not able to utilize the whole available amount of sodium nitrate (*A.niger* B1-D, *A.niger* 320725) both in the control and the induced cultures. The *A.niger* strain 337690 while consumed the nitrate in the control culture it was not able to fully consume it in the induced culture. It is worth noting that the *A.niger* 320725 strain was consuming faster the nitrate in the induced culture than in the control culture. In the glucose consumption figures, it is remarkable that the only strain which appeared to consume the glucose faster after the addition of butylamine was the strain *A.niger* 320725. From all the above, the *A.niger* 149007ii seemed to be more appropriate for the purpose of the overall project as it exhibits the highest productivity and seems to retain high amine oxidase activity levels for longer time than the cultures of the other strains. Another important advantage of the *A.niger* 149007ii is that it exhibits the highest proportion of FAD – amine oxidase activity among all other strains. Characteristically, the highest total FAD amine oxidase

activity for the strains tested were 8.82 mol/l/min for the *A.niger* 149007ii, 4.77 mol/l/min for the *A.niger* 320725, 2.71 mol/l/min for the *A.niger* B1-D , 1.90 mol/l/min for the *A.niger* 337690. Moreover, *A.niger* 14900ii shows a good ability to utilise sodium nitrate as a nitrogen source and grow, in the presence of butylamine, at satisfactorily high biomass concentrations (maximum biomass 8.23g/l at 96h).

3.5. The effect of the inducer's form on the amine oxidase activity

3.5.1. Introduction and hypothesis

In the case of aliphatic amines, such as butylamine which is the inducer used, the fungitoxicity increases with the pH of the solution because the molecular form (non-ionic) of these molecules penetrates the cell membrane with comparative ease (Albert, 1985). However, higher amine oxidase activities were observed in the initial experiments (not shown) than the later ones. In the initial experiments the pH was not controlled while in the later ones the pH was adjusted at 4, and lower amine oxidase activity levels occurred in these experiments. It could well be that the differences in the activities may be due to the fact that in the unadjusted cultures the pH was very high due to the addition of butylamine, and as a result a higher amount of the inducer was in the molecular form, resulting in more butylamine entering the cytosolic environment of the cell. As a response the cells may direct the production of amine oxidase towards the intracellular environment. In other words, at higher pH's the same concentration of butylamine will be more effective as greater amounts of it (which is in the molecular form) will cross the cell membrane and cause induction of the amine oxidase expression. It can be assumed that the cytoplasmic enzymes might be adversely affected by a consequent increase of the internal pH (pHi). However, fungal cells have a strong buffering capacity and they can regulate the internal pH by the action of the vacuolar H^+-ATPase (Bencina et al, 2009). The vacuoles are acidic and they can regulate the internal pH by pumping protons across the vacuolar membrane maintaining the cytosolic pH stable over a wide range of external pH's. It has been found that alkaline pH caused an enhanced expression of

the vacuolar H^+-ATPase and enabled sufficient growth of *A.oryae* in medium at pH 10 (Kuroki et al, 2002).

Frédbort et al. (2000) found a copper containing amine oxidase located in the cell wall of an *A.niger* strain. The copper containing amine oxidase which is found in cell homogenates is probably an improperly folded precursor of the cell wall copper amine oxidase. There is also a single gene for both forms of the copper amine oxidase (Frébort et al, 1996). It is possible to speculate that the cells are able to regulate the location of the copper amine oxidase enzymes dependent on where the problem is located. By increasing the pH, the problem becomes higher intracellularly where the butylamine concentration will increase and cause damage to the organelles and correspondingly the amine oxidase levels will be higher intracellularly in order to counter the problem.

Therefore, the following experiment was designed to examine the above hypothesis, and to clarify whether or not the form of butylamine determines the expression of the amine oxidase enzyme intracellularly or on the cell wall. This can be achieved by adjusting the pH of the medium to different levels. The pKa of butylamine is 10.8, (Gamer et al, 2002) for that reason the pHs chosen are 4, 6 and 8 where the ratio of the cationic to molecular form of butylamine will be: 1.58×10^{-7} 1.58×10^{-5} and 1.58×10^{-3} respectively.
In this experiment the use of the bioreactor enables the control of the pH at the predetermined levels.

3.5.2. Results

Biomass production and intracellular protein production

In the case of the culture grown at pH 4, after 48h the rapid growth phase started and lasted until the 96h after which we can observe a gradual decrease in the biomass concentration probably due to cell autolysis (figure 3.14.A). The maximum concentration was 8.98 g/l at 96h. The specific growth rate was 0.03 h^{-1}. Both the pH 6 and pH 8 culture exhibited very low biomass levels. The biomass of the pH 6

culture increased very slowly and reached a peak at 120h which was 1.59 g/l. In the case of the pH 8 culture the biomass reached a maximum very early (at 48h) and it was 1.21 g/l. After that point there was a gradual and slow decrease in the biomass.

From Figure 3.14.B. it can be seen that the intracellular protein concentration of the pH 4 culture increased slowly from 24h to 72h where it reached a peak of 173.3 mg /g DCW. After that point the protein concentration decreased to much lower levels (94.8 mg/g DCW) and after 120h the intracellular protein concentration further decreased. In the case of the pH 6 culture the intracellular protein concentration increased sharply from 24h to 48h where it reached a peak of 149.3 mg/g DCW after that point the intracellular protein concentration was gradually decreased. The culture grown at pH 8 showed lower intracellular protein concentration than the two other cultures. There were no significant changes during the whole process with the highest protein concentration reached at 72h (82.6 mg/g DCW). The culture grown at pH 4 exhibited higher intracellular protein level than the culture of pH 6 and significantly higher than the culture of pH 8. Therefore, it is obvious that the increase of the culture pH resulted in a profound decrease of the intracellular protein concentration.

Total and specific amine oxidase activity

As can be seen in Figure 3.15.A, the total activity of the culture grown in pH 4 increased sharply after 24h and reached a peak at 48h of 53.87 mol/l/min. The activity then dropped to 35.13 mol/l/min (72h) after which it continued declining even more rapidly and at 144h reached very low, nearly non detectable, activity levels. Similarly to pH 4 culture, the total amine oxidase activity of the pH 6 and pH 8 cultures increased rapidly from 24h and 48h and reached peaks of 39.53 mol/l/min and 27.25 mol/l/min respectively. After that point both the amine oxidase activity of pH 6 and pH 8 decreased with the pH 6 culture showing a more abrupt decrease than the pH 8 culture.

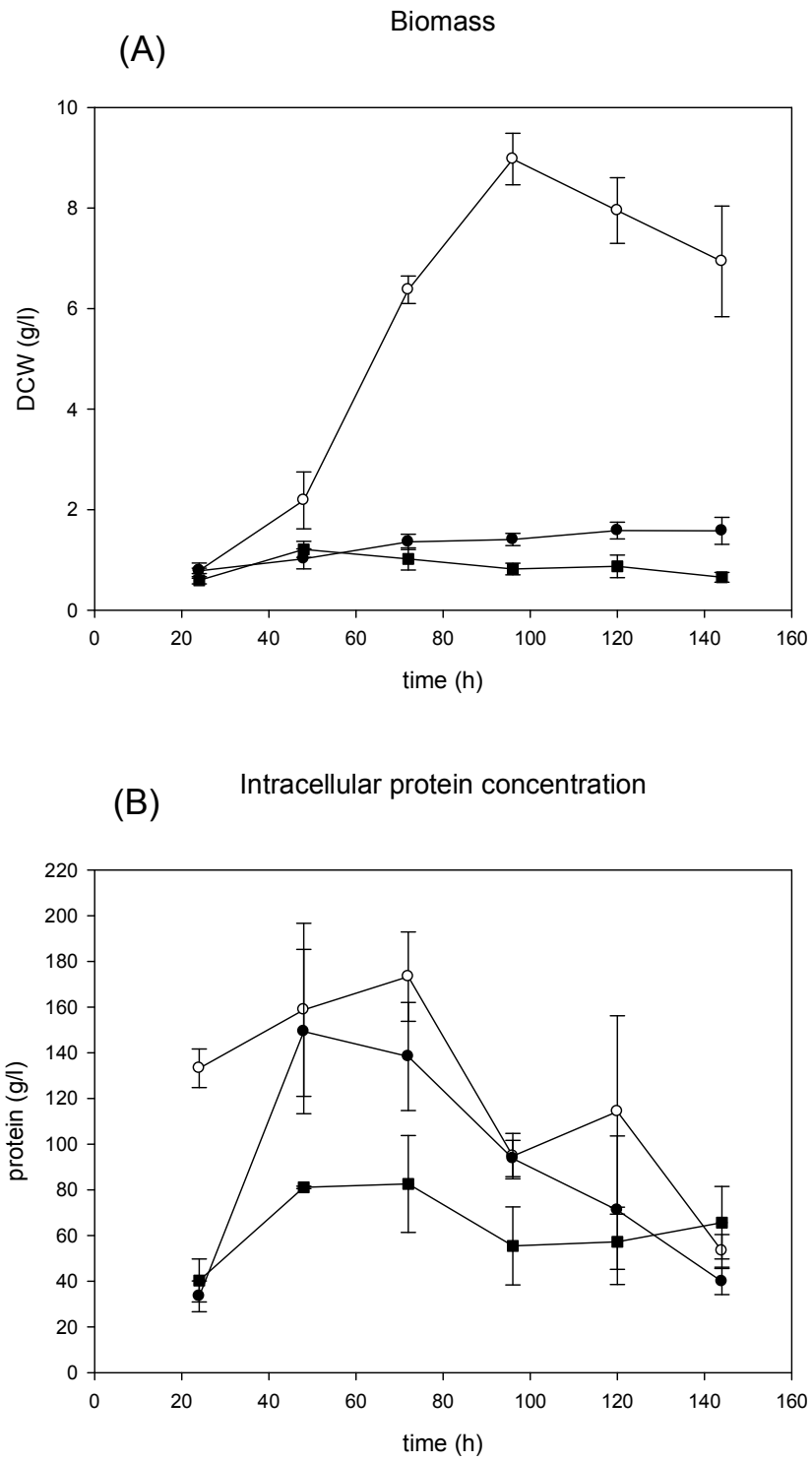

Figure 3.14. Time profiles of the (A) biomass and (B) intracellular protein concentration of the induced *A.niger* 14900ii cultures grown at pH 4 (○) pH 6 (●) and pH 8 (■). Culture conditions: bioreactor cultivation, 25^0C, 400rpm, 1vvm

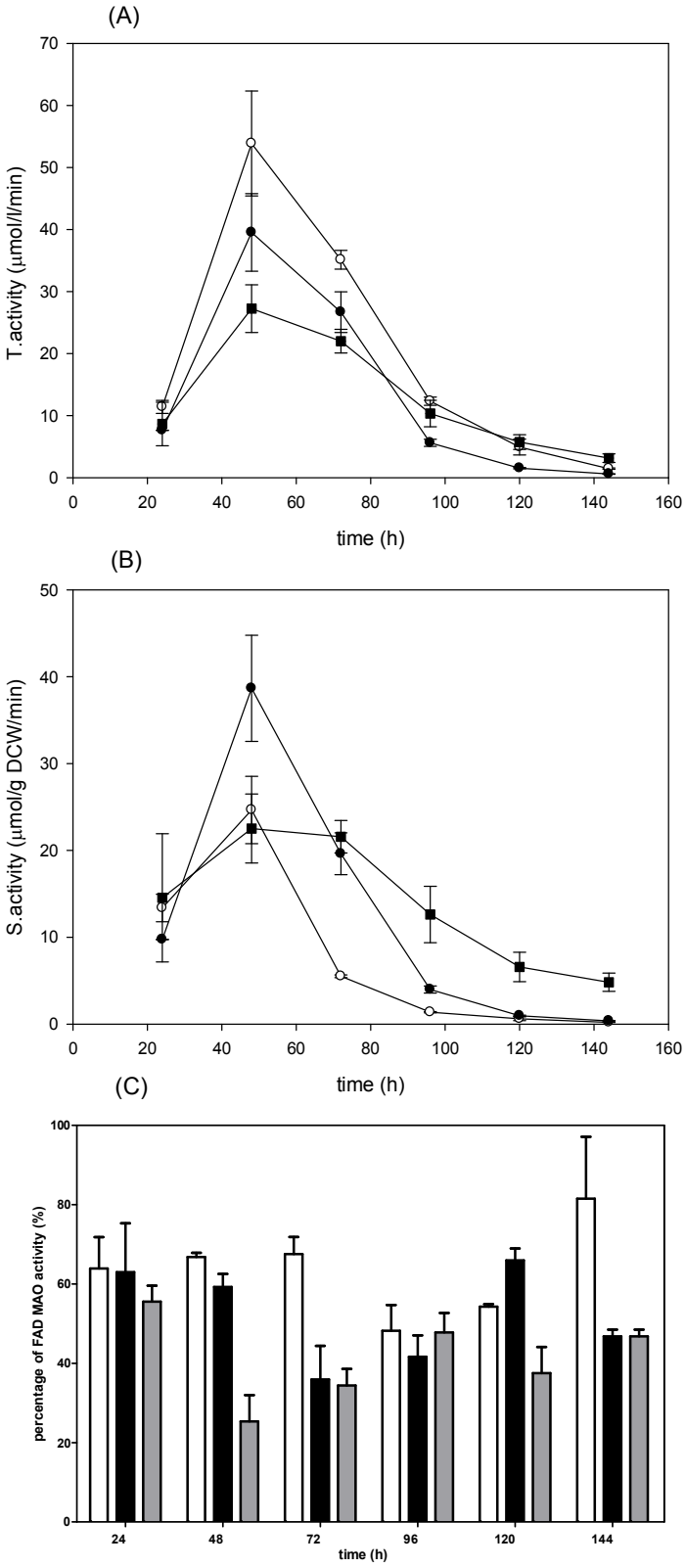

Figure 3.15. Time profiles of the (A) total and (B) specific amine oxidase activity of the induced cultures grown at pH 4 (○) pH 6 (●) and pH 8 (■).(C) time profiles of the percentage of FAD MAO activity of *A.niger* 14900ii cultures grown at pH 4 (□) pH 6 (■) and pH 8 (■). Culture conditions: bioreactor cultivation, 25^0C, 400rpm, 1vvm

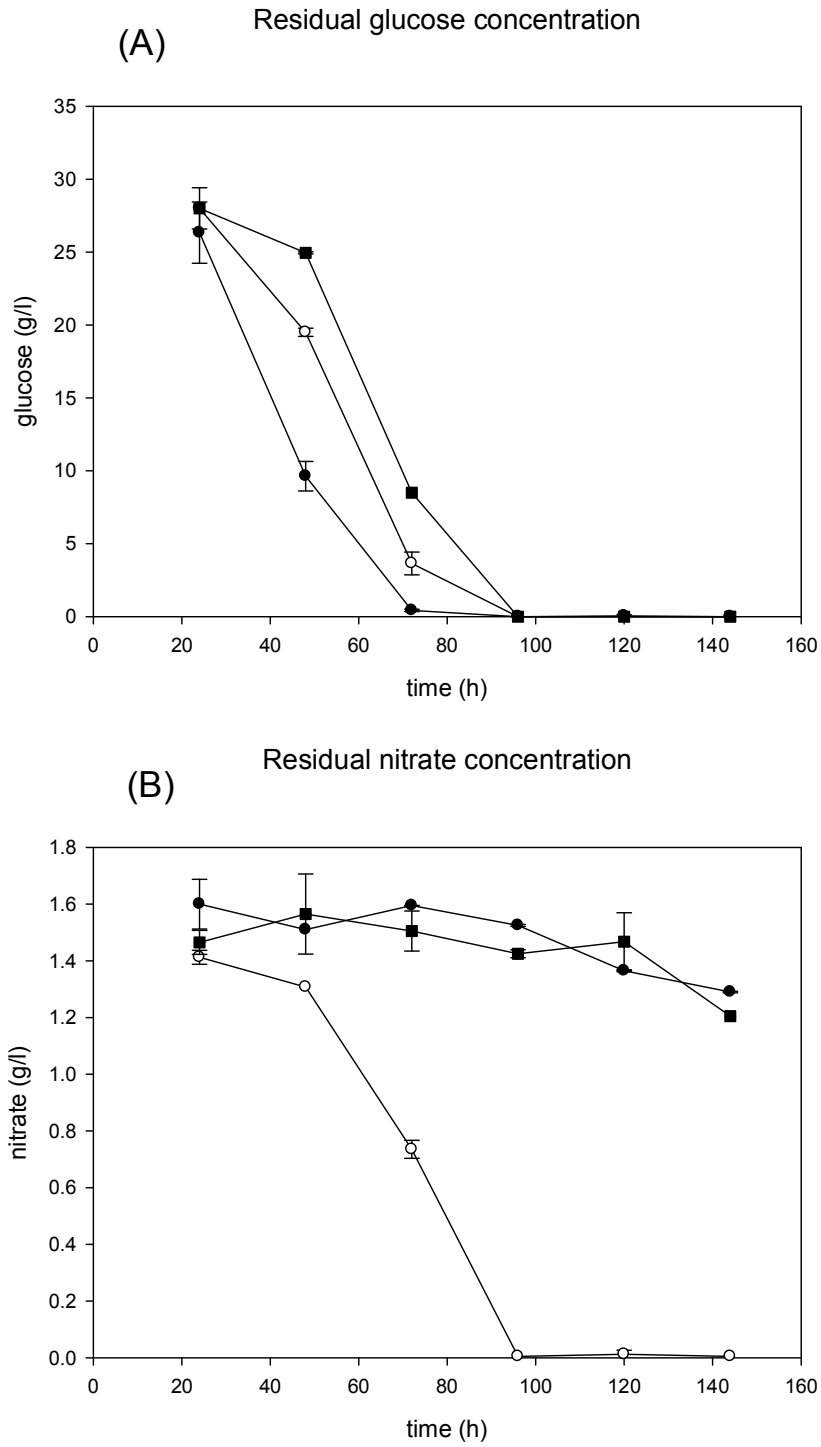

Figure 3.16. Time profiles of the residual (A) glucose and (B) nitrate concentrations of the induced cultures grown at pH 4 (○) pH 6 (●) and pH 8 (■).Culture conditions: bioreactor cultivation, 25^0C, 400rpm, 1vvm

In all the cultures there was an abrupt increase in the specific activity from the 24h to 48h where all the cultures reached their highest peaks (24.66, 38.66 and 22.52 mol/g DCW/min for the pH 4, pH 6 and for the pH 8 respectively). After that point the specific amine oxidase levels decreased. The decrease in the specific amine oxidase activity was not so sharp in the pH 8 culture as it was in the other two cultures.

The statistical analysis shows that the culture grown at pH 4 has significantly lower specific amine oxidase activity than the culture grown at pH 6 and 8 (t-test, $p < 0.05$). Moreover, the culture grown in pH 8 appears to have higher average specific activity; however, this difference is not significant (t-test, $p > 0.05$).

FAD amine oxidase activity

In Figure 3.15.C., we can see that the activity that corresponds to the FAD amine oxidase activity did not follow a specific pattern for any of the cultures. The pH 4 had higher FAD amine oxidase activity than the culture grown at pH 6 and significantly higher than pH 8. Moreover the culture grown at pH 8 had higher FAD amine oxidase activity than the culture grown at pH 6. Therefore the following order of activity was followed:

FADAO pH 4 > FADAO pH 8 > FADAO pH 6.

Residual glucose and nitrate concentration

As can be seen in figure 3.16.A., glucose was completely consumed by 72h for the pH 6 culture while for the other two cultures it was consumed at 96h. Moreover, the mean glucose consumption rate was 0.50 g/l/h 0.54g/l/h and 0.40 g/l/h for the pH 4, pH 6 and pH 8 cultures respectively.

In figure 3.16.B., the culture grown in pH 4 could efficiently consume the sodium nitrate (nitrate was completely consumed in this culture by 96h), while the other two cultures showed little ability to consume the nitrate at those pHs, as the residual concentrations remained relatively stable throughout the process. The mean nitrate consumption rate of the pH 4 culture was 0.02 g/l/h.

3.5.3. Discussion

From the above results it is important to focus on the inability of the organism to utilise the sodium nitrate at some pH's, and on the specific amine oxidase figures, as the very limited growth in the cultures grown in pH 6 and pH 8 does not allow us to draw any direct conclusions from the total amine oxidase figure (figure 3.15.A).

In *A.niger*, nitrogen metabolism is regulated by the nitrogen metabolite repression system which leads to the repression of enzymes and permeases involved in the utilisation of nitrogen sources other than ammonium or L-glutamine (Wiame et al, 1985).

According to Arst & MacDonald (1975) the catabolism of nitrate by fungi is facilitated by the enzymes nitrate permease, nitrate reductase and nitrite reductase. In the case of the current processes there is no ammonium or L-glutamine in the medium. The only nitrogen sources which are present are sodium nitrate and butylamine, therefore, theoretically, nitrate consumption should occur in all the above processes and not only in the process with pH 4. Taking into consideration all the above, it can be assumed that the production of the nitrate utilising enzymes must be carried out in all the bioprocesses, but nitrate consumption may be stopped perhaps by the function of the nitrate permease being inhibited possibly due to the high extracellular pH in the processes with the pH 6 and pH 8.

A search into the literature backs the above hypothesis. The presence of an H^+/nitrate symport activity was shown in the plasma membrane of cells of *Candida utilis* (Eddy & Hopkins, 1985) *Aspergillus nidulans* (Downey & AGedeon, 1994) and *Neurospora crassa* (Blatt et al, 1997). In the latter case it was observed that 2 H+ are transported for every NO_3^- and 1 H^+ is secreted back to the environment via the activity of an H^+ – ATPase , an activity which alkalinizes the surrounding environment (Blatt et al, 1997) while there is an intracellular charge balance. The same group showed that a decrease in pH simultaneously increases the affinity of the carrier for the NO_3^- and NO_2^- transport across the membrane (δINO_3^-). Therefore, nitrate transportation across the cell membrane is facilitated by the electrochemical

gradient (negative inside, positive outside the cell) which enables the co-transport of $2H^+$ (down the gradient) and NO_3^- (up the gradient). The maintenance of a high pH in the medium acts as an uncoupler which disrupts the proton motive force and does not allow the acidification of the medium.

Unlike nitrate, glucose was however, consumed as the glucose uptake is taking place through a constitutive facilitated diffusion system (Carlile et al, 2001) and no energy input or proton gradient is required. The oxidation of glucose leads to the generation of energy in the mitochondria in the form of ATP which is then used by the H^+-ATPase to expel H^+, create a proton motive force and consequently acidify the medium, however, the constant pumping of alkali does not allow it and as a result the nitrate uptake is not possible. Therefore, cells are relying only on butylamine as a source of nitrogen which is confirmed by the high specific amine oxidase activity of the cultures grown on pH 6 and 8. Under these conditions, growth is not possible as all the energy produced in the form of ATP is consumed by H^+-ATPase and the very limited amount of nitrogen uptake cannot sustain decent growth (Figure 3.17).

Even though there was no nitrate consumption in the cultures at pH 6 and pH 8, there was some limited growth which can be explained by the utilisation of butylamine as a nitrogen source as explained above. As reported in the literature, butylamine cannot be easily utilised as the sole carbon and nitrogen source because of the relatively high nitrogen to carbon ratio: oxidation of butylamine by amine oxidase provides the necessary carbon source, but there is also an excess of ammonium which represses the expression of the amine oxidase (Zwart & Harder, 1983). Maybe that is the reason why after the 72h there is still reasonable specific amine oxidase activity. This might also be the reason why there was no significant difference in the specific amine oxidase activity of the two processes, that is to say, the cells could not produce more amine oxidase, as the higher activities result in higher intracellular ammonium levels which inhibit the amine oxidase production.

Regarding the levels of the intracellular amine oxidase enzymes, it can be concluded from the specific amine oxidase figure that even when the growth is limited the cells

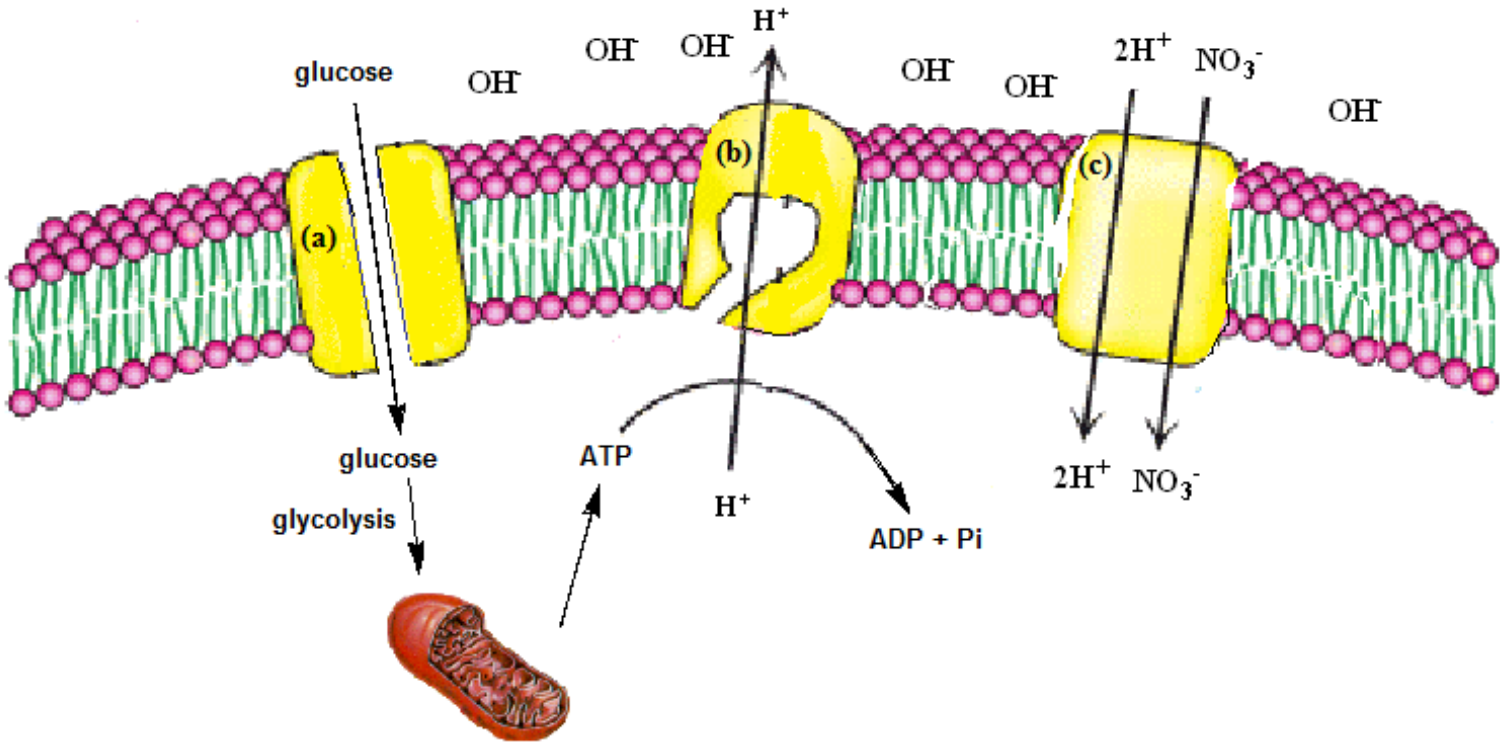

Figure.3.17. The speculative effect of higher pH in energy generation and growth, when nitrate is the only nitrogen source. Glucose is passively transported across the cell membrane through a facilitated diffusion permease (a) and oxidised (initially through glycolysis in cytosol and then through the citric acid cycle in mitochondria) leading to the formation of reducing equivalents (FADH and NADH) which are further oxidised in the mitochondrial ETC to produce energy in the form of ATP. Nitrate uptake is based on a coupled process where protons being transported from inside to outside the cell membrane by the plasma membrane H^+-ATPase (b) via hydrolysis of ATP and re-entry of the protons driven by the protonmotive force, down their electrochemical gradient, via the nitrate permease protein (c), every two protons accompanied by a nitrate anion. When the protonmotive force is disrupted, cells are continuously utilising glucose for the production of ATP which is consumed, however, by the H^+-ATPase, and therefore no energy molecules remain for the construction of new cell constituents and, hence, growth.

are still able to produce amine oxidase at high levels. Additionally, as mentioned in the results section the culture grown in pH 6 exhibits significantly higher specific amine oxidase activity which can be explained by the higher ratio of butylamine to cells that is to say fewer cells have to confront more butylamine than in the case of the culture grown in pH 4 where there is sufficient growth and there is biomass and therefore less amine oxidase can be produce from the cells to efficiently catabolise the butylamine. A second explanation can be the confirmation of the hypothesis made in the introduction; however, the difference in the specific amine oxidase activity is not high enough to safely support this hypothesis.

3.6. *In vivo* addition of the amine oxidase inhibitor semicarbazide hydrochloride

3.6.1. Introduction

A possible way to increase the production of amine oxidase would be to keep the amount of inducer constant throughout the process. Therefore, the cells would constantly be under the same pressure for the production of the enzyme. A way to achieve this can be a fed-batch process where the inducer will be added gradually so as to retain it at a certain concentration. Another way will be the addition of an inhibitor which will hinder the inducer's breakdown and therefore its concentration will not be significantly reduced. In our case, there are two amine oxidase enzymes produced by the *A.niger* (Frébort et al, 2000). The exact physiological role of each amine oxidase is still elusive. Moreover, an interesting experiment would be to measure the amount of total amine oxidase when one of the two amine oxidases is inhibited. In order to satisfy this question the semicarbazide was added to the broth which is an irreversible inhibitor of the copper containing amine oxidase. The amine oxidase activity of the *A.niger* cells results from the copper containing amine oxidase located in the cell wall, as well as from the copper containing and FAD containing amine oxidases which are found in the cytosol. Therefore we can expect inhibition of the cell wall amine oxidase but we cannot be sure whether this inhibitor can penetrate the cell membrane and also inhibit the intracellular copper containing

amine oxidase. Flask fermentations were carried out with and without the inhibitor semicarbazide as can be seen in section 3.6.2.

3.6.2. Results

Biomass, total amine oxidase activity and FAD amine oxidase activity

After 48h and before 144h, the biomass concentrations of the inhibited culture appeared slightly lower than the control, however, this difference is not statistically significant. The maximum biomasses achieved were 8.99 g/l and 8.80 g/l for the control and inhibited culture respectively. The total amine oxidase activity followed a similar pattern for the two cultures and no significant differences were recorded at any time in the processes. The maximum total amine oxidase activity was 28.9 and 30.7 mol/l/min for the control and induced culture respectively. On the contrary, the percentage of the activity belonging to the FAD-amine oxidase is significantly higher for the inhibited culture reaching 82.4% at 48h. After that point there is a reduction to lower percentages ranging from 51-63%. The control culture presented percentages of the FAD-AO fluctuating between 24.2% and 55%.

3.6.3. Discussion and conclusions

The addition of the semicarbazide did not seem to significantly influence the growth of the organism. Moreover, the overall total amine oxidase activity seemed to be the same for both cultures. However, it is apparent that there was a significant difference in the FAD amine oxidase activity level between the two cultures. This difference can be either explained by the inhibition of the copper containing amine oxidase and therefore the production of more enzyme (both FAD and Cu – amine oxidase) in order to counterbalance the inactivation of the Cu amine oxidase, and as a result the overall amine oxidase activity remains unaffected, and it is just the ratio of the activity of the two enzymes that is changed. A question would be how semicarbazide behaves in the fermentation broth, if and at what degree it is degraded either by the organism or by the conditions (since it is a chemically sensitive molecule). Moreover, the reduction of the Cu amine oxidase activity and the consequent increase in the FAD amine oxidase activity which take place in the cytosol

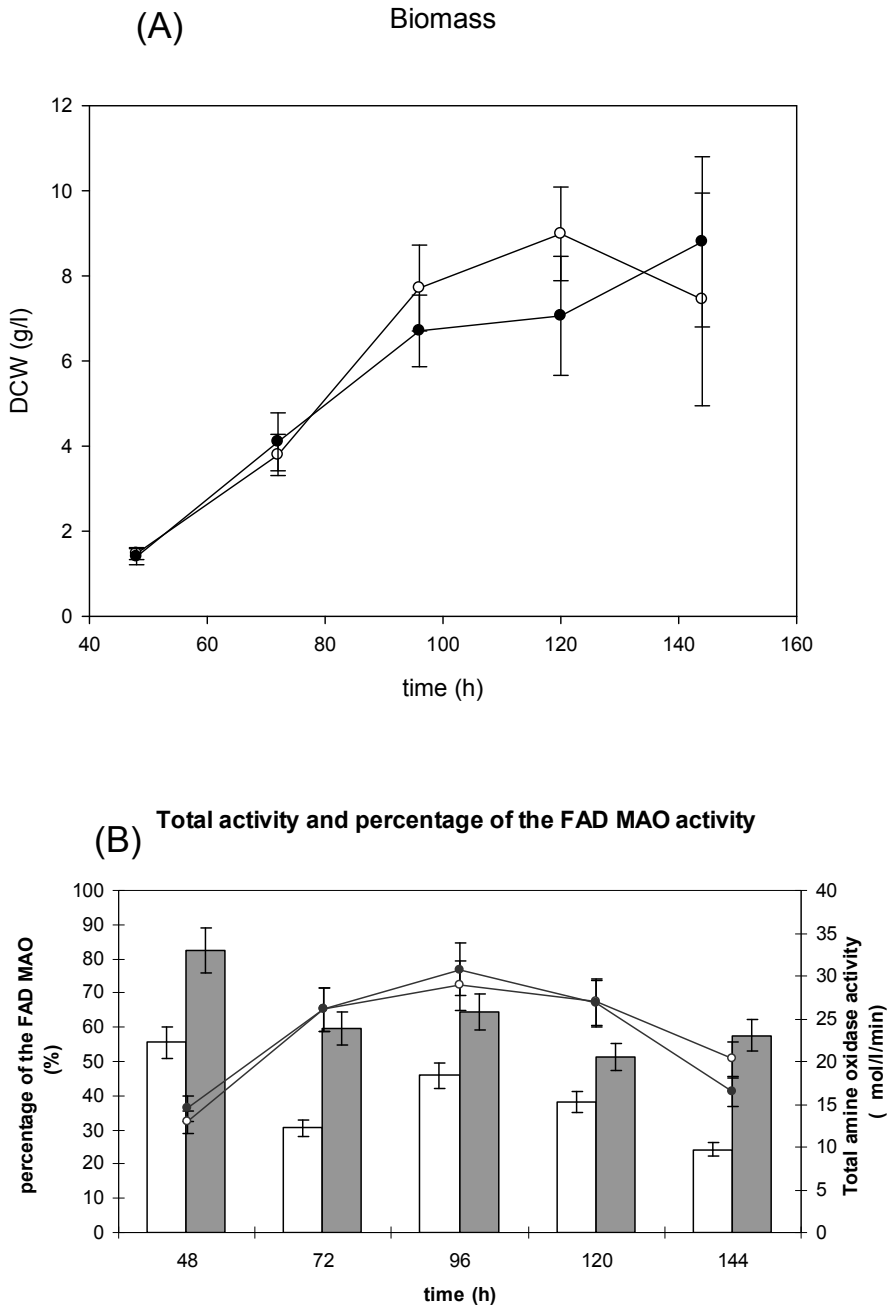

Figure.3.18. Time profiles of the (A) biomass concentrations, and (B) total MAO activity along with the percentage belonging to FAD MAO for the control (○) and the inhibited *A.niger* 14900ii culture (●). Culture conditions: flasks cultivations, 25^0C, 200rmp

gives us a sign that the molecule is able to pass from the extracellular to the intracellular space. It is worth mentioning that the decrease in the percentage of the FAD amine oxidase activity after the 48h could be attributed to the binding of the semicarbazide to the enzyme and as a result the inhibitor concentration is reduced and it is not enough to inactivate the new enzyme produced by the organism.

3.7. *A.niger* fermentations and amine oxidase induction

3.7.1. Introduction

The most important step in the production of an enzyme is the scaling up to bioreactor cultivation conditions. As discussed earlier butylamine is a very potent inducer of the amine oxidase, however, the toxicity to the growing organism can cause growth inhibition resulting in lower biomasses and hence lower yields. Moreover, the incorporation of the butylamine from the start of the culture causes high initial expression of the amine oxidase enzymes. Therefore, there is early degradation of the inducer, so when the biomass reaches the optimum levels the inducer has been almost completely depleted and thus the yield is again negatively affected. Furthermore the addition of butylamine in the beginning of the process can be extremely stressful for the culture as the transfer of the inocula from a relatively friendly to a very toxic environment and bearing in mind that the amount of cells in the inoculation is very low, the ratio of butylamine/cells is very high resulting in higher toxicity. Consequently, the next obvious stage to follow in this work was the addition of the inducer at later stages of the fermentation and more specifically at the time when biomass has reached its highest concentration. This can only happen in bioreactor cultivation as in the case of flask culture, pH control is not possible since the addition of butylamine would cause a dramatic increase of the pH. The only possible flaw of a later addition of butylamine is that the cells would not have the time to pre-adapt to the harsh conditions created by the inducer. In this experiment two fermentations were carried out, a control where no butylamine was added in order to establish the optimum time for the amine oxidase induction, in other words, the time when biomass reaches the highest concentration and then a second culture where induction takes place at the point of maximum biomass concentration.

3.7.2. Results

The addition of butylamine in the induced culture took place at the time when the maximum biomass concentration was observed in the control culture just before the start of the stationary phase (69h process time).

Biomass production and intracellular protein production

In Figure 3.19.A., the biomass concentrations for the two cultures followed a similar pattern until the time induction took place. The induced culture reached a higher biomass concentration (peaked at 96h) of 8.04 g/l while the control culture reached 7.22 g/l at the same time. After 96h, there was a rapid decrease of the biomass in the induced culture, whereas the control culture maintained stable biomass levels until the end of the process.

The intracellular protein concentration (Figure 3.19.B.) of the non-induced culture reached a peak of 182 mg/g DCW at 48h, while the induced culture reached a peak of 172 mg/g DCW 26 hours later (after the induction).

Residual glucose and nitrate concentration

The residual glucose concentration for both the control and induced culture showed a similar pattern. Glucose was depleted for both cultures at about 96h. The glucose consumption rate was 0.50 g/l/h and 0.45 g/l/h for the control and the induced culture respectively.

As can be seen in Figure 3.20.B., the nitrate in the control culture was completely consumed 24 hours earlier than that in the induced culture. The nitrate consumption rate of the control culture was 0.051 g/l/h and 0.041 g/l/h for the induced culture.

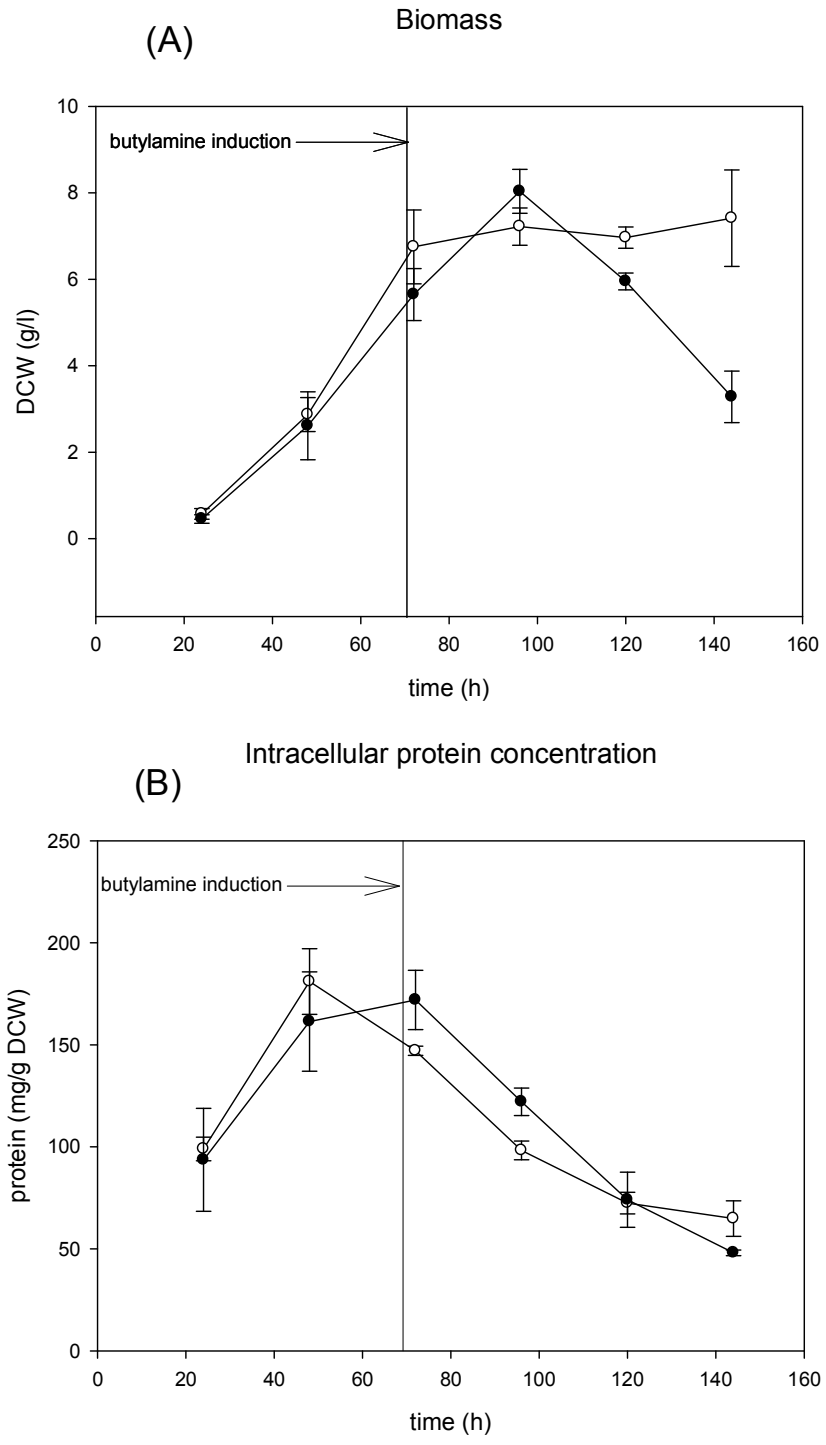

Figure.3.19. Time profiles of the (A) biomass concentrations, and (B) the intracellular protein concentrations for the non induced (○) and induced cultures (●) of *A.niger* 149007ii. Culture conditions: bioreactor cultivation, 25^0C, 400rpm, 1vvm

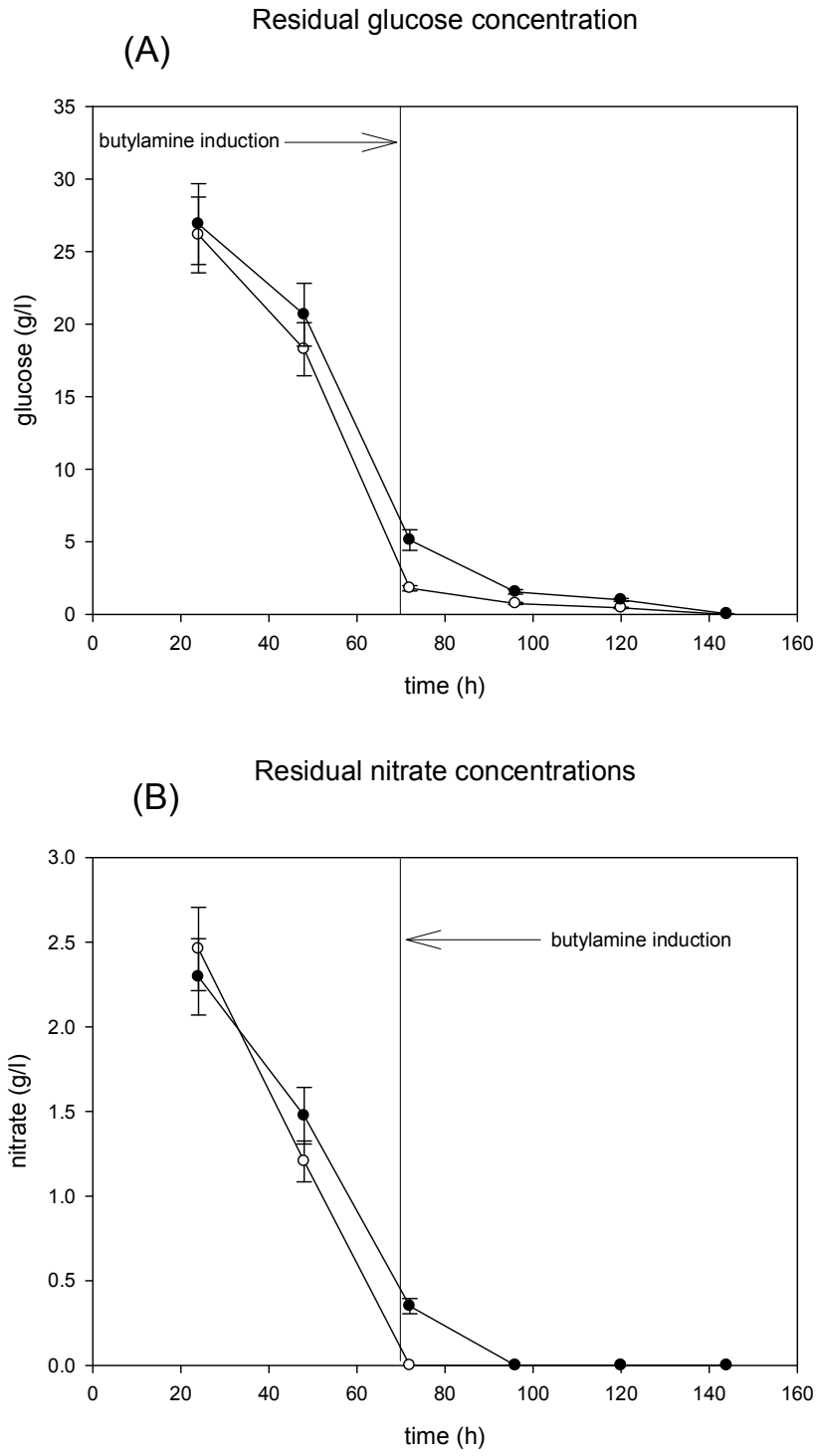

Figure.3.20. Time profiles of the (A) residual glucose and (B) nitrate concentrations for the non induced (○) and induced cultures (●) of *A.niger* 149007ii. Culture conditions: bioreactor cultivation, 25^0C, 400rpm, 1vvm

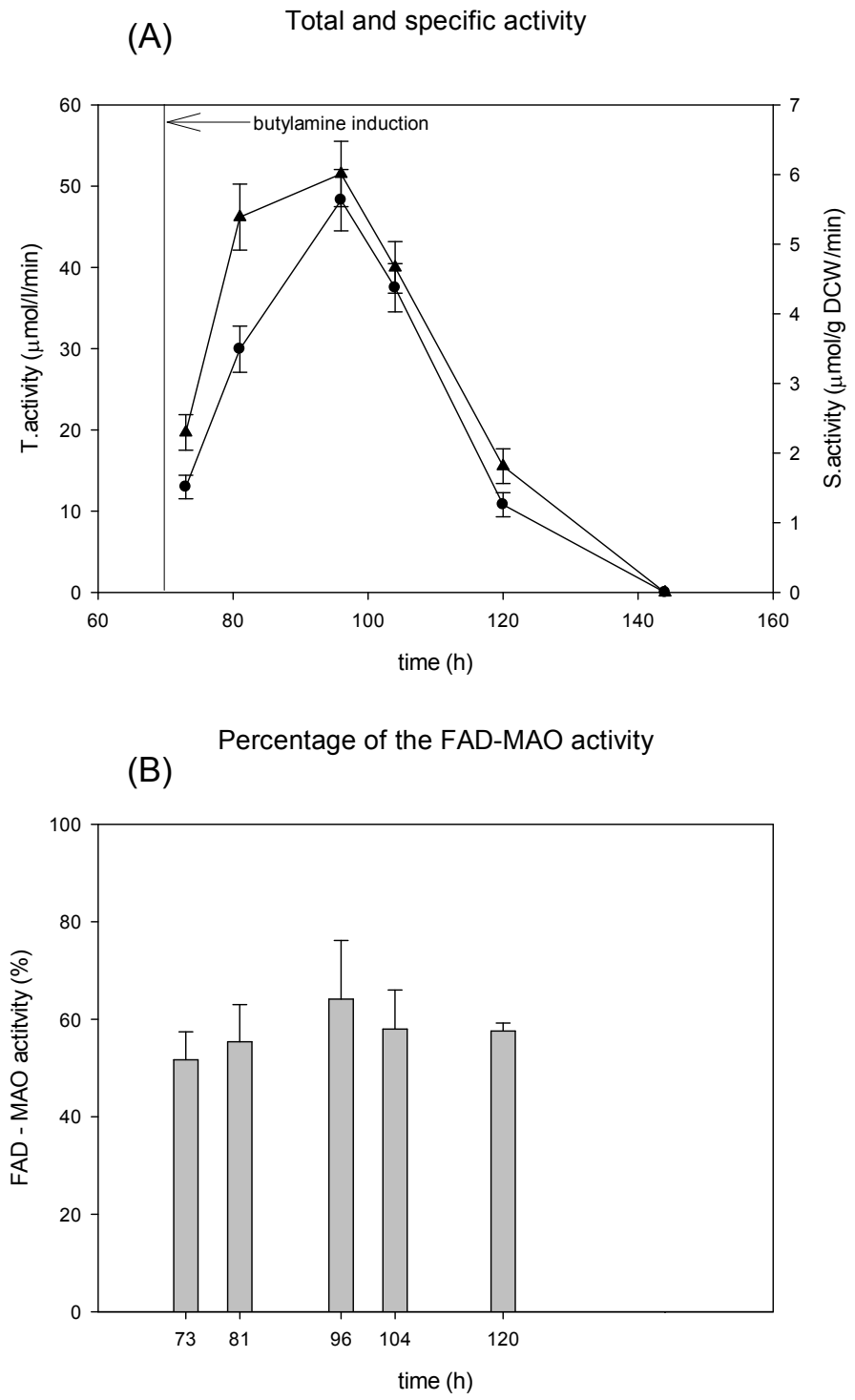

Figure.3.21 A.Time profiles of the total (●) and specific amine oxidase acivity (▲) of the induced *A.niger* 14900ii culture. B. Percentage of the amine oxidase activity belonging to the FAD-MAO enzyme. Culture conditions: bioreactor cultivation, 25^0C, 400rpm, 1vvm

Total and specific amine oxidase activity

As can be seen in Figure 3.21.A, the total amine oxidase activity of the induced culture quickly increased and 26h after the induction reached its highest value of 48.28 mol/l/min (96h). After that point there was a rapid decrease of the activity. The specific amine oxidase activity, followed a very similar pattern. The specific activity climbed initially to 5.39 mol/g DCW/min at 81h and then the highest value was achieved at 96h which was 6.01 mol/g DCW/min, and then it followed a downward trend until the end of the induced process.

The proportion of the amine oxidase activity attributed to the FAD containing enzyme was always very high, fluctuating between 51 to 64% (Figure 3.21 B). Almost immediately after the onset of the butylamine induction the proportion of the FAD amine oxidase activity was 51% and at 96h reached the peak at 64%. Then there was slight decrease to 58% until the end of the process.

3.7.3. Discussion and Conclusions

As has been previously observed in flask cultivations (section 3.4.2.1), the addition of butylamine can cause an increase of the growth phase compared to the control culture, and ultimately lead to higher maximum biomass concentrations. This can be attributed to the exhaustion of carbon and nitrogen source in the control cultures. Indeed, figure 3.20.B. shows that at the point the stationary phase begins in the control culture there was no glucose and sodium nitrate left in the culture, while, in the induced culture there was both carbon and nitrogen source left. This delayed exhaustion of nutrients could be either a result of the toxicity of butylamine which slows down the metabolism or a result of the fact that butylamine is consumed simultaneously with glucose and nitrate and hence, delays their consumption without reducing the carbon and nitrogen uptake rate though. Butylamine is obviously causing significant changes to the culture even if it is added at later stage of the culture as in this experiment. The decrease in the biomass concentration of the induced culture was very steep and signifies its toxic effect on the fungal cells, however, the changes caused to the intracellular protein levels are not striking, as

only between 73h and 96h (4h and 27h after induction) there was a significant increase of the protein concentration of the induced culture which may be attributed to a peak in amine oxidase production (especially for the 96h sample as can be seen in the specific activities of figure 3.21.A.).

A comparison between the culture with the inducer added from the beginning (section 3.5. pH 4) and the culture where the addition took place at a later stage is of particular interest. In the latter case, the biomass seemed to decrease more rapidly after the peak which was reached for both cultures at the same time (96h), however, the overall biomass differences were not significant. As expected the maximum amine oxidase activity was recorded as early as the 48h of the culture for the first culture and at 96h (27h post induction) for the latter. It would be expected as discussed in the introduction that the addition of the inducer at later stage to result in higher total amine oxidase activity, however, this is not the case as the butylamine pretreated culture exhibited higher (even though not significantly) total activity. Generally, we can conclude that the total activity levels had the same average, whereas this is not the case for the specific amine oxidase activities. The butylamine pre-treated culture exhibited significantly higher specific amine oxidase activities during the whole process. This phenomenon can be explained by the very high ratio of butylamine concentration / cells in the butylamine pre-treated culture which results in higher expression of the amine oxidase enzymes to combat the elevated toxicity of butylamine. Therefore, a possible way to increase the amine oxidase expression in the culture induced at 69h would be to increase the concentration of butylamine so that the ratio butylamine concentration / cells would be similar to that of pre-treated culture when the specific amine oxidase activity was soared to high levels. Theoretically, if cells have the ability to reach as high specific amine oxidase activities as 20 mol/g DCW/min in the pre-treated culture when the biomass was only 2.2 g/l then it would be prudent to expect similar high specific activities in the culture induced at a later stage when the biomass concentrations are much higher (above 5.5 g/l) which will be translated to about 2.5 times higher volumetric (total) amine oxidase activity.

It is worth noting that such an addition may cause a very high increase of the copper containing amine oxidase which has been assumed to play more a detoxifying role (Frébort et al, 1997) rather than the FAD containing amine oxidase. A comparison between the culture induced from the beginning and the one induced at 69h shows that even not significantly the copper amine oxidase activity was higher at the time when butylamine was introduced to the culture (the beginning of the process for the first and 69h for the latter culture). Here it is also important to take into consideration the fact that there are two copper amine oxidases found in *A.niger*, encoded by the same gene (Frébort & Adachi, 1995), one of which (AO-I) is located on the cell wall while the other one which is less active (AO-II, which is a monomer of AO-I) is located in the cytosol. During the analysis of the amine oxidase activity as can be seen in chapter 2 the cytosolic content is analysed, therefore it is highly possible that the true copper amine oxidase activity of the fungal cells to be much higher than the observed. However, the scope of the project was not the determination of the copper but of the industrially important FAD – amine oxidase activity, hence, the copper amine oxidase expression was not further investigated.

3.8. Synopsis and conclusions

Throughout the course of these experiments the capability of the homologous amine oxidase production by *A.niger* was examined. The industrial need for the FAD – amine oxidase led to the establishment of a method to correctly determine the activity belonging to the FAD containing enzyme. In the appendix, a list of compounds tested to be used as substrates of the FAD and Cu containing amine oxidase of the *A.niger* 149007ii is presented. The appropriate conditions for the examination of the homologous amine oxidase expression was established by initially first examining the effect of the ammonia and nitrate as main nitrogen source on the physiology of the cells and the enzyme production. The results clearly showed the negative effect of ammonia repression of the amine oxidase expression as the delayed expression led to lower yields than in the cultures where nitrate and butylamine were simultaneously used. The replacement of ammonium chloride with

sodium nitrate in the medium led to a 25% increase in the total amine oxidase activity. A way to improve the productivity was to test the efficiency with which different *A.niger* strains express the enzyme under the same conditions. This test led to a selection of a strain with increased amine oxidase expression capability and the yield was increased 1.6 fold (with the selections of the new strain). Further optimisation was attempted with scaling up to bioreactor cultivation which resulted in a further 2 fold increase in yield (section 3.7.2.). This was achieved by the addition of the inducer at the late exponential phase of the culture. Finally, a successful way to increase the ratio of the industrially useful FAD/ Cu containing amine oxidase is the *in vivo* addition of the Cu amine oxidase inhibitor which results in a 1.5 fold increase of the FAD amine oxidase activity while the overall amine oxidase activity remains stable.

Chapter 4

E.coli: heterologous production of Amine Oxidase

Abstract

In this chapter, a monoamine oxidase (MAO-N) enzyme derived from *A.niger* (the DNA of which was manipulated through directed evolution to increase the enzyme's specificity towards α- methylbenzylamine resulting in the MAO-5N) was expressed in different mode fermentations of the gram negative bacterium *E.coli*. Initially, batch fermentation was carried out where the target enzyme was auto-induced (based on diauxic growth). Then fixed volume fed batch fermentations were used to enhance the enzyme production by increasing cell concentrations and thereby, improving productivity. Different variables where changed in order to investigate the appropriate conditions for the maximal monoamine oxidase production. Different *E.coli* constructs, inducers, feeding profiles were tested in the first section, while in the second section induction biomass and aeration-oxygenation methods were examined.

4.1. *E.coli* MAO bioprocess optimisation

4.1.1. Introduction

The advent of recombinant DNA technology extended the range of potential fermentation products and enabled us with the overproduction of important commodities which otherwise would be very limited in their natural hosts. A vital step towards the overexpression of an industrial important product such as the MAO enzyme in the particular work is the selection of the appropriate expression system and the proper fermentation conditions. In this section, an initial investigation of the appropriate conditions for the expression of the MAO-5N in the most popular host bacterium so far, the gram negative *E.coli*, was carried out. The purpose is to establish an efficient production scheme with the use of inexpensive components and scaling up friendly conditions. The successful protein production from a recombinant construct is strongly determined by factors such as host cell-vector interactions, plasmid segregational instability and the extent of cellular stress responses (Andersson et al, 1996). Towards that direction, the suitable fermentation mode was examined, and the use of a construct which can efficiently overexpress the target

enzyme in a high cell density culture was investigated. In literature, there are several studies examining the replacement of the ubiquitous lac promoter inducer IPTG by the cheaper, by-product of the dairy industry, lactose (Hoffman et al, 1995; Kilikian et al, 2000). However, its metabolisable nature, and therefore, the dual role of lactose as both carbon-energy source and inducer poses further challenges in the fermentation procedures and frequently results in low volumetric yields and difficulties in process control (Altenbuchner & Mattes, 2005; Gombert & Kilikian, 1998). These problems were addressed in the first section (4.1) of this chapter.

4.1.2. Results

4.1.2.1. LB batch fermentation of E.coli BL21 (DE3) pET16b

In Figure 4.1., we can clearly see that soon after the inoculation the cells started respiring and at 6h the dissolved oxygen reached the lowest level (60%). After that point there was an abrupt increase of the DO_2 indicating a decrease in the respiratory activity of the cells. The pH was not controlled and after 5h it started rising reaching pH 8.5 at about 12h and it remained stable until the end of the process. A possible explanation for this rise can be the deamination of proteins and peptides of the LB medium which results in the release of ammonia. The biomass levels were increased soon after the inoculation and at 6h reached 2 g/l. After this point there was a slight increase which led to the maximum biomass of 2.43 g/l at the end of the process (24h).

The maximum total MAO activity was reached at 15h and it was 71.53 mol/l/min. The total activity exhibited a very slight decrease until the end of the process at 24h where it was 65.8 mol/l/min. The specific MAO activity followed a similar trend and reached a peak at 15h of 32.5 mol/g DCW/min, while, afterwards it was decreased reaching 27.1 mol/g DCW/min at the end of the process.

The plasmid retention appeared to be relatively high at 12h (90%), however, 5h later there was a reduction to about 60% signifying segregational instability of the construct.

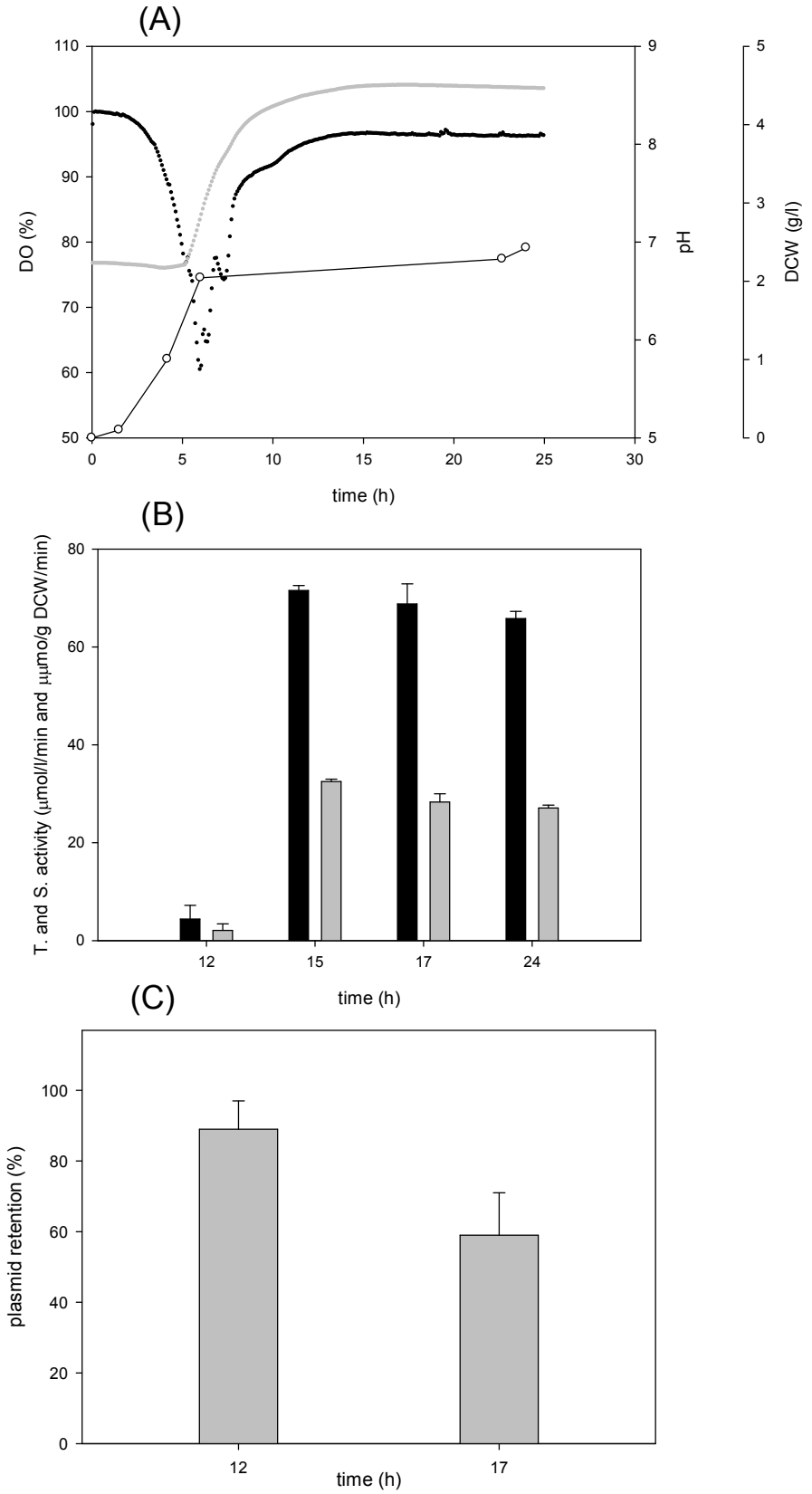

Figure 4.1. Time profiles of the (A) dissolved oxygen (●), pH (●), biomass (○) ,(B) total (■) and specific (■) MAO activities and (C) plasmid retention of the *E.coli* BL21 (DE3) pET16b culture. Culture conditions: 30°C, 350 rpm, 2 vmm

4.1.2.2. Fed Batch E.coli BL21 (DE3) pET16b

A fixed volume fed batch fermentation was carried out afterwards. Fed batch is the fermentation of choice in industry as described previously. The bioreactor is inoculated and left to run until glucose is consumed and DO_2 start to rise. Then a concentrated nutrients solution is added to enable satisfactory increase of the biomass levels until OD_{600nm} = 40, when IPTG induction takes place. During the whole fed batch period, no product is withdrawn which results in the medium volume being slightly increased.

As can be seen in figure 4.2.A., the start of feed took place at about 15.6h process time when glucose was consumed, and it lasted for about 7h until the optical density reached 40. IPTG was added and as it is obvious there was a decrease in the growth rate after the induction. The maximum recorded dry cell weight was 13.88 g/l at 28.5h process time. The specific growth rate during the feeding stage of the process and before the induction was 0.175 h^{-1}.

The MAO activity was detectable; however, it was ranging to very low levels (4-6 mol/l/min). The reason behind the low amine oxidase expression is attributed to the loss of the MAO harbouring plasmid as can be seen in figure 4.2.B. The plasmid retention at the onset of the induction was already very low (15.8%) while after 9h reached a percentage of only 2%. Not surprisingly then, the total MAO activity ranged to nearly not detectable levels.

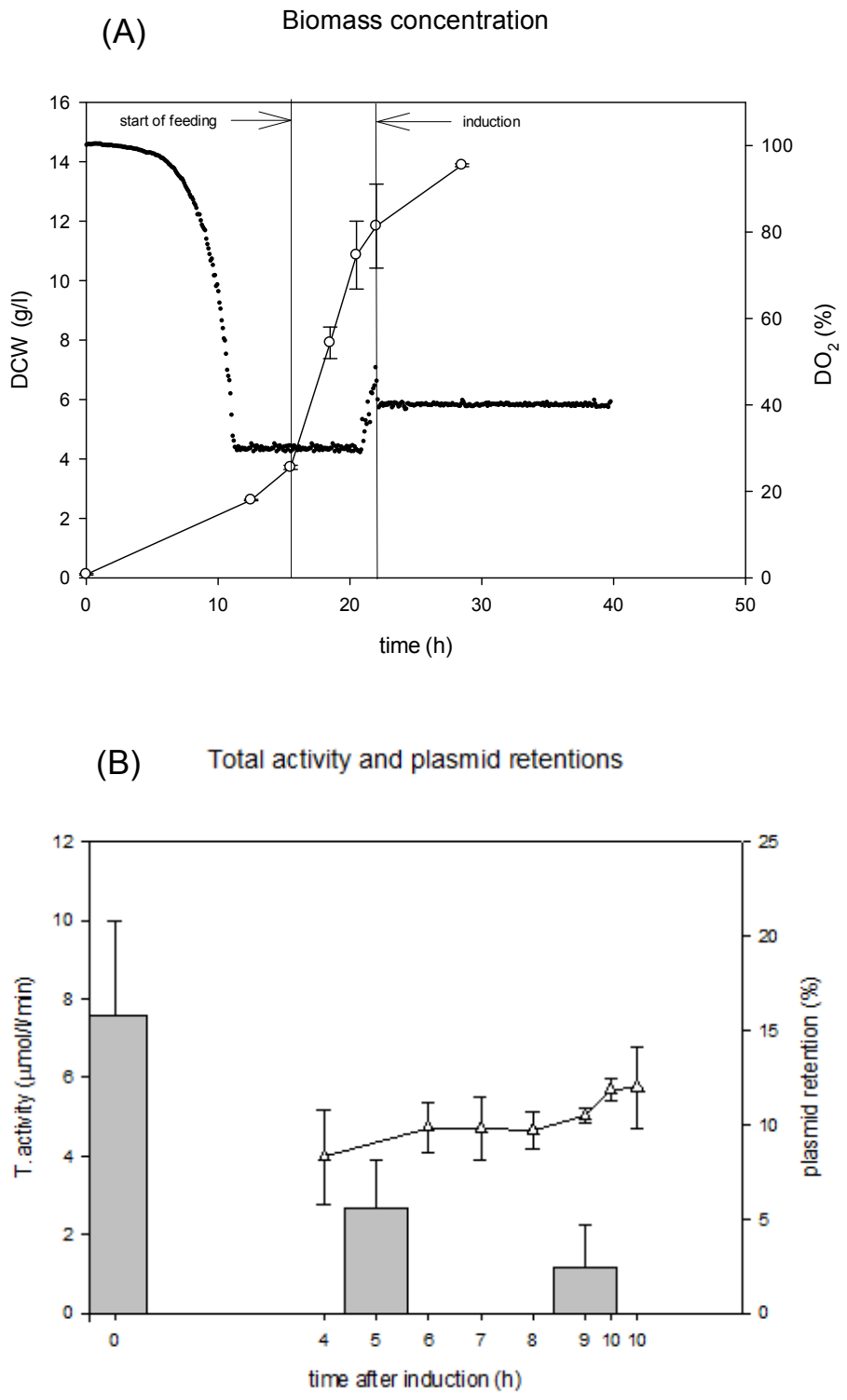

Figure 4.2. Time profiles of the (A) dissolved oxygen (●), biomass (○) and (B) total MAO activities (Δ) and percentage of plasmid retention (■) of *E.coli* BL21 (DE3) pET16b. Culture conditions: 30°C, 300-900 rpm, 1-3 vmm cascaded to 40% and 30% DO_2 before and after induction, respectively.

4.1.2.3. Fed Batch E.coli BL21 (DE3) pET28b

The very low levels of the expressed monoamine oxidase caused by the loss of the plasmid posed serious questions about the efficiency of the construct under the demanding conditions of a fed-batch culture. For that reason, it was replaced by a kanamycin resistant construct reputed to be more stable.

The start of the exponential feed took place at about 16h process time when glucose was consumed, and it lasted for about 8h until the optical density reached 40 and IPTG induction started.

The maximum recorded dry cell weight was 18.13 g/l at 35.15h process time. The specific growth rate between the start of feed and induction was 0.14 h^{-1}. After the IPTG induction there was a decrease in the growth rate.

Both the total and specific activity did not present significant fluctuations between 5h and 10h after induction. The total MAO activity followed a slightly upward pattern and reached a peak of 119.8 mol/l/min 8h post induction and after that point was decreased to 94 mol/l/min. The specific MAO activity was ranging between 6.52 to 7.56 mol/g DCW/min the first 8h after induction, while after this point it was reduced to lower levels (5.9-5.1 mol/g DCW/min). Figure 4.3.B. shows that there was no significant plasmid loss during the induction phase of the fermentation.

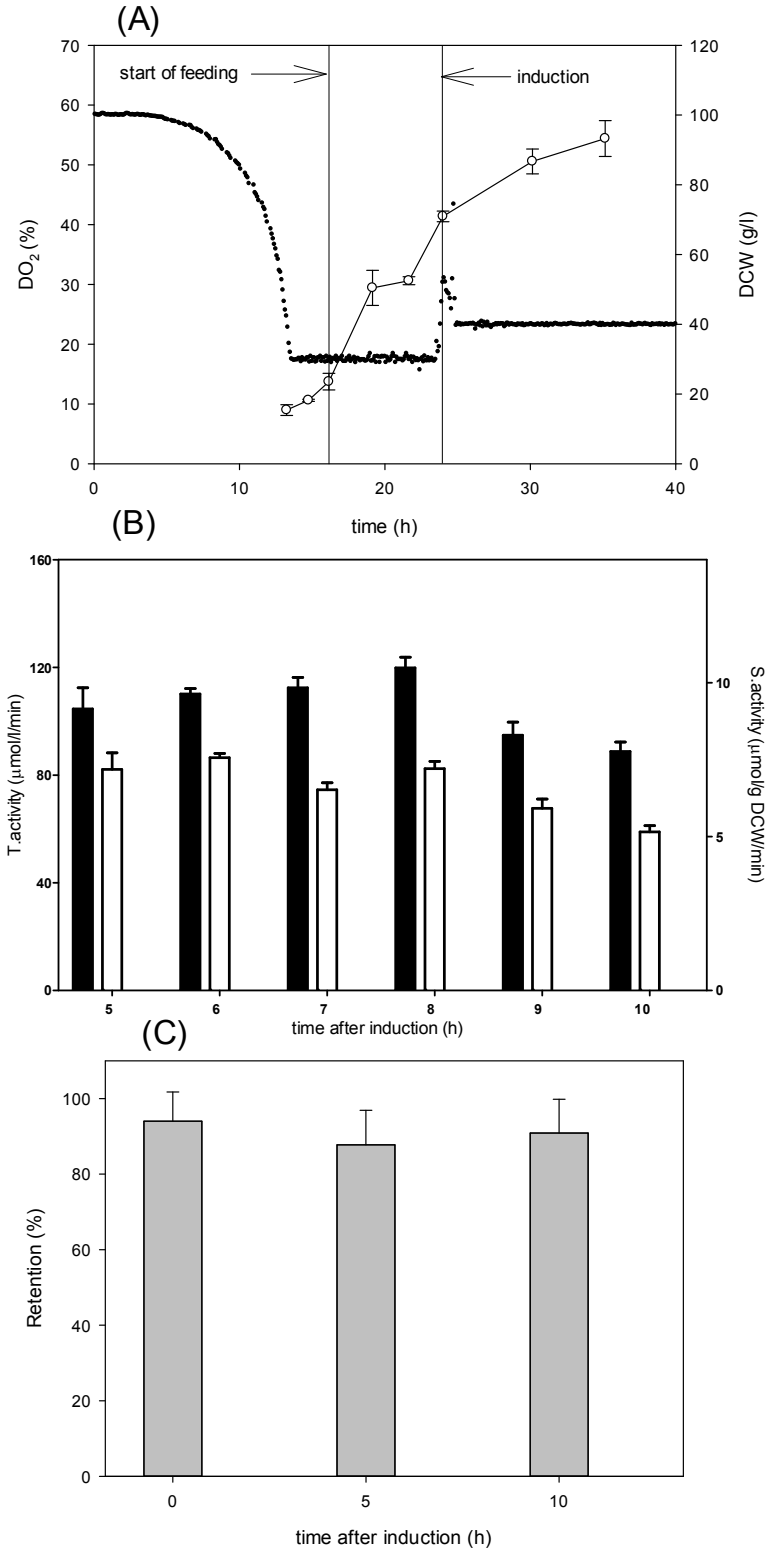

Figure 4.3. Time profiles of the (A) dissolved oxygen (●), biomass (○) and (B) total (■) and specific MAO activities (□), and (C) percentage of plasmid retention (■) of *E.coli* BL21 (DE3) pET28b. Culture conditions: 30°C, 300-900 rpm, 1 -3 vmm cascaded to 40% and 30% DO2 before and after induction respectively.

4.1.2.4. Fed Batch E.coli BL21 (DE3) pET28b (optimised feed)

The exponential feed of this culture was started at 17.6h and it lasted until OD = 40 at about 21.38h. The increase in the feeding rate necessitates the surveillance of the acetate concentration as described earlier in this chapter. The maximum acetate concentration was reached at 22.21h process time, shortly after the onset of induction. After that point, there was a rapid decrease to about 0.53 g/l at 25.38h process time and it remained at these levels until the end of the process. The specific growth rate during the feeding stage was $0.34h^{-1}$ and the biomass reached 12g/l at the end of it. After 26h the biomass concentration was almost stabilised and reached maximum concentration of 26.47g/l at 35.5h process time.

In figure 4.4.B. it can be seen that there was a moderate gradual increase of the total monoamine oxidase activity from 3h to 7h post induction where it reached a peak of 112.67 mol/l/min. The total activity remained stable until 8h post induction and afterwards started dropping and reaching 66.85 mol/l/min at the end of the process. Regarding the specific MAO activity, there was an increase from 4h to 7h post induction reaching a peak of 6.41 mol/g DCW/min . After 8h post induction there was a rapid decrease of the specific activity which reached 2.53 mol/g DCW/min at the end of the process. Once again there was no significant plasmid loss during the induction phase of the fermentation of this strain containing the pET28b plasmid.

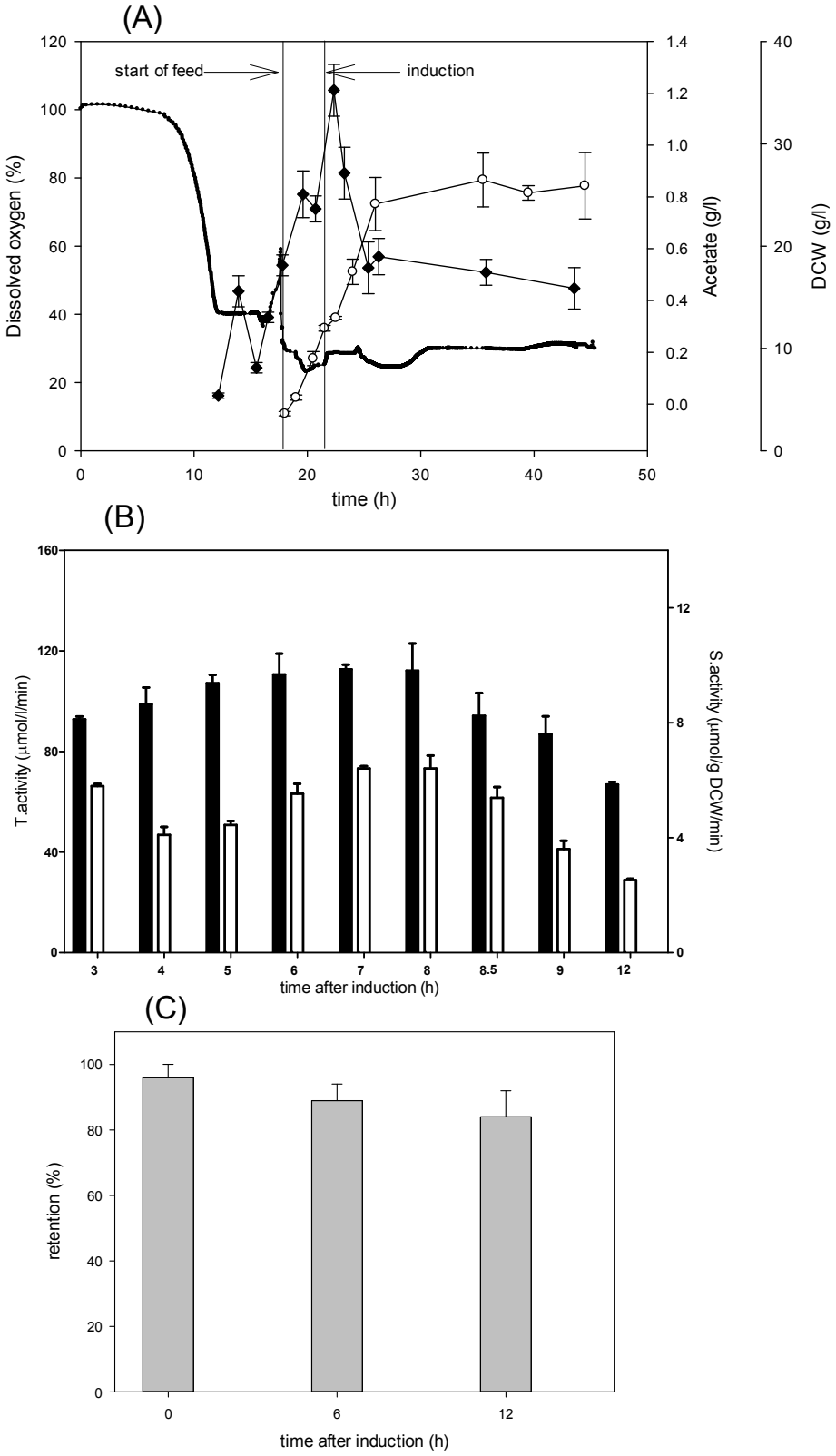

Figure 4.4. Time profiles of the (A) dissolved oxygen (●), biomass (○), acetate concentration (♦) and (B) total (■) and specific MAO activities (□), and (C) percentage of plasmid retention (■) of *E.coli* BL21 (DE3) pET28b. Culture conditions: 30°C, 300-900 rpm, 1 -3 vmm cascaded to 40% and 30% DO_2 before and after induction, respectively

4.1.2.5. Fed Batch E.coli BL21 (DE3) pET28b with lactose as inducer instead of IPTG

The exponential feed of this culture was started at 17.56h and it lasted until OD = 40 at about 21.24h process time when lactose induction took place. The specific growth rate during the feeding stage was 0.34 h^{-1}. After induction the culture continued growing in exponential mode until 28h process time when it plateaued for 9.5h. The maximum biomass concentration was reached at 37.5h process time (27.5g/l) and after that point there was a decrease in biomass signifying culture decay. Regarding the acetate concentration, there was a rapid increase from 18h to 22.5h when it reached a peak of 2.63 g/l. Afterwards, there was an initial drop followed by a new increase of the acetate levels to concentrations reaching 4.6g/l at the end of the process (45.5h). The use of lactose as an inducer and the fact that the specific *E.coli* construct is lac positive necessitated the use of a continuous feed of lactose. However, it is imperative that the concentration of the residual lactose must be monitored. In figure 4.5.A. it is obvious there is a constant increase in lactose concentration soaring to levels which might be growth limiting – toxic to the cells (Dykhuizen & Hartl, 1978). The accumulation rate was 0.56 g/l/h and at the end of the process the lactose concentration was 15.13 g/l (45.5h).

The total MAO activity reached the highest value 3.5h after the onset of the lactose induction (107.4 mol/l/min). Later on at 5.5h post induction, there was a rapid decrease in the total activity (79.1 mol/l/min). After that point and until the end of the process, the total activity was fluctuating to lower levels, between 45-51 mol/l/min. The specific MAO activity followed a similar pattern, reaching a peak at 3.5h post induction of 6.72 mol/g DCW/min. Afterwards the specific activity was significantly reduced initially to 3.86 mol/g DCW/min and then at level ranging between 1.86 and 2.28 mol/g DCW/min. Figure 4.5.C.shows no significant loss of plasmids at any point in the process.

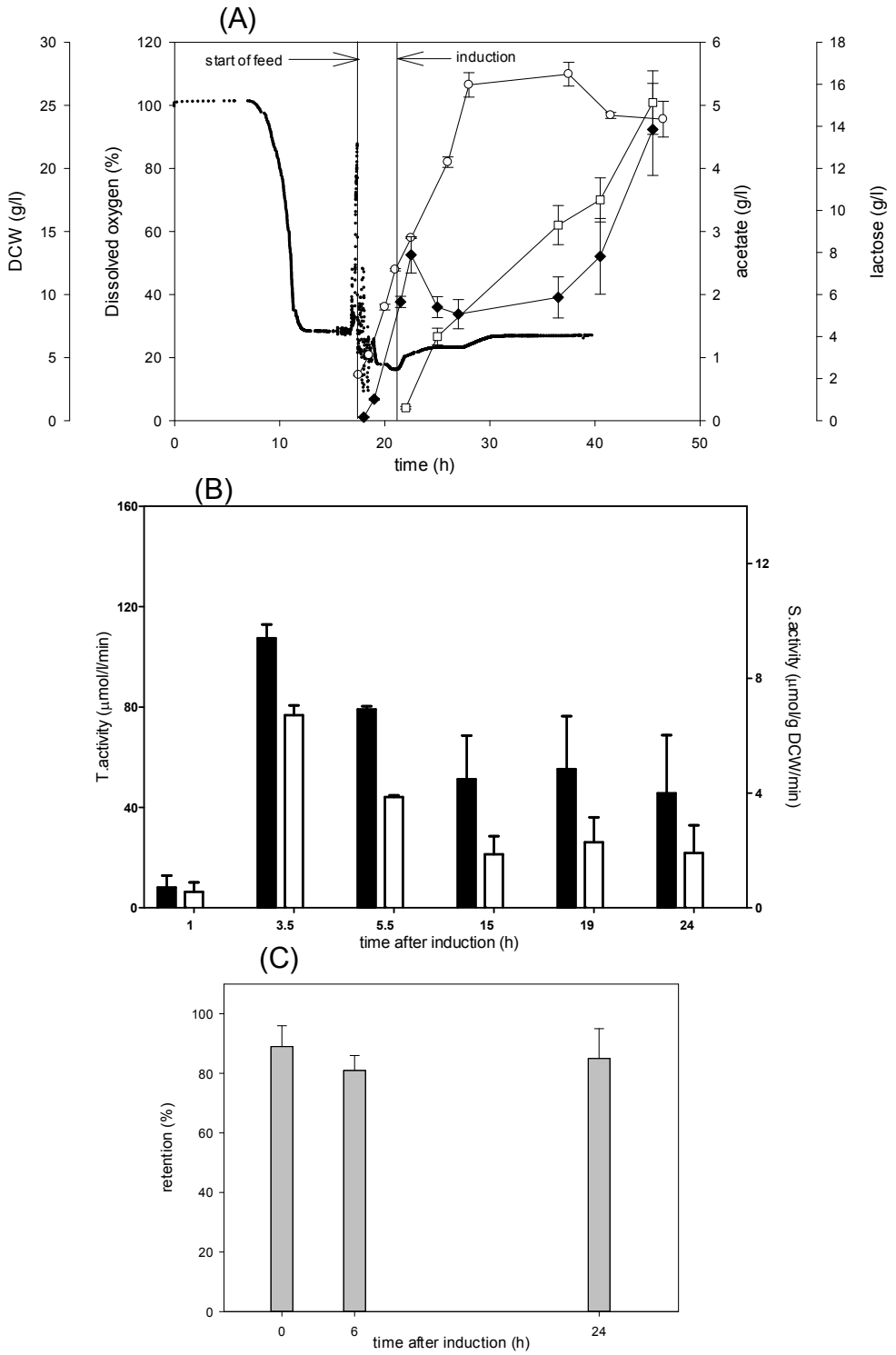

Figure 4.5. Time profiles of the (A) dissolved oxygen (●), biomass (○), acetate (♦) and lactose concentration (□) (B) total (■) and specific MAO activities (□), and (C) percentage of plasmid retention (■) of *E.coli* BL21 (DE3) pET28b. Culture conditions: 30°C, 300-900 rpm, 1 -3 vmm cascaded to 40% and 30% DO_2 before and after induction respectively

4.1.2.6. Fed Batch E.coli BL21 (DE3) pET28b with controlled addition of lactose as inducer.

The exponential feed of this culture was started at 15.54h and it lasted (for 4.4h) until OD = 40 at about 19.98h process time when lactose induction took place. The specific growth rate during the feeding stage was 0.33 h^{-1}. After the lactose induction the specific growth rate was decreased, and the maximum biomass concentration was reached at the end of the process (41.5h) and it was 28.53 g/l. The acetate concentration reached its highest peak of 1.89 g/l at 22.5h process time (2.5h after the lactose induction). Then there was a rapid drop and it led to lower levels ranging between 0.8 to 0.9 g/l (from 15h to 24h post induction). The lactose concentration was ranging between 2 to 5 g/l (not shown).

In this process the highest total MAO activity was reached 2h later than that of the uncontrolled lactose addition and it was 132.8 mol/l/min (5.5h post induction). The total activity remained stably at high levels until 24h after induction when it was decreased to 99.89 mol/l/min. Likewise, specific MAO activity reached its maximum at the same time as the total MAO activity and it was 7.18 mol/g DCW/min. Then there was a gradual decrease initially to 5.26 mol/g DCW/min (15h-19h post induction) and at the end of the process to 3.73 mol/g DCW/min (24h post induction). Figure 4.6.C. shows that there was no significant plasmid loss during the induction phase of the fermentation of this strain containing the pET28b plasmid.

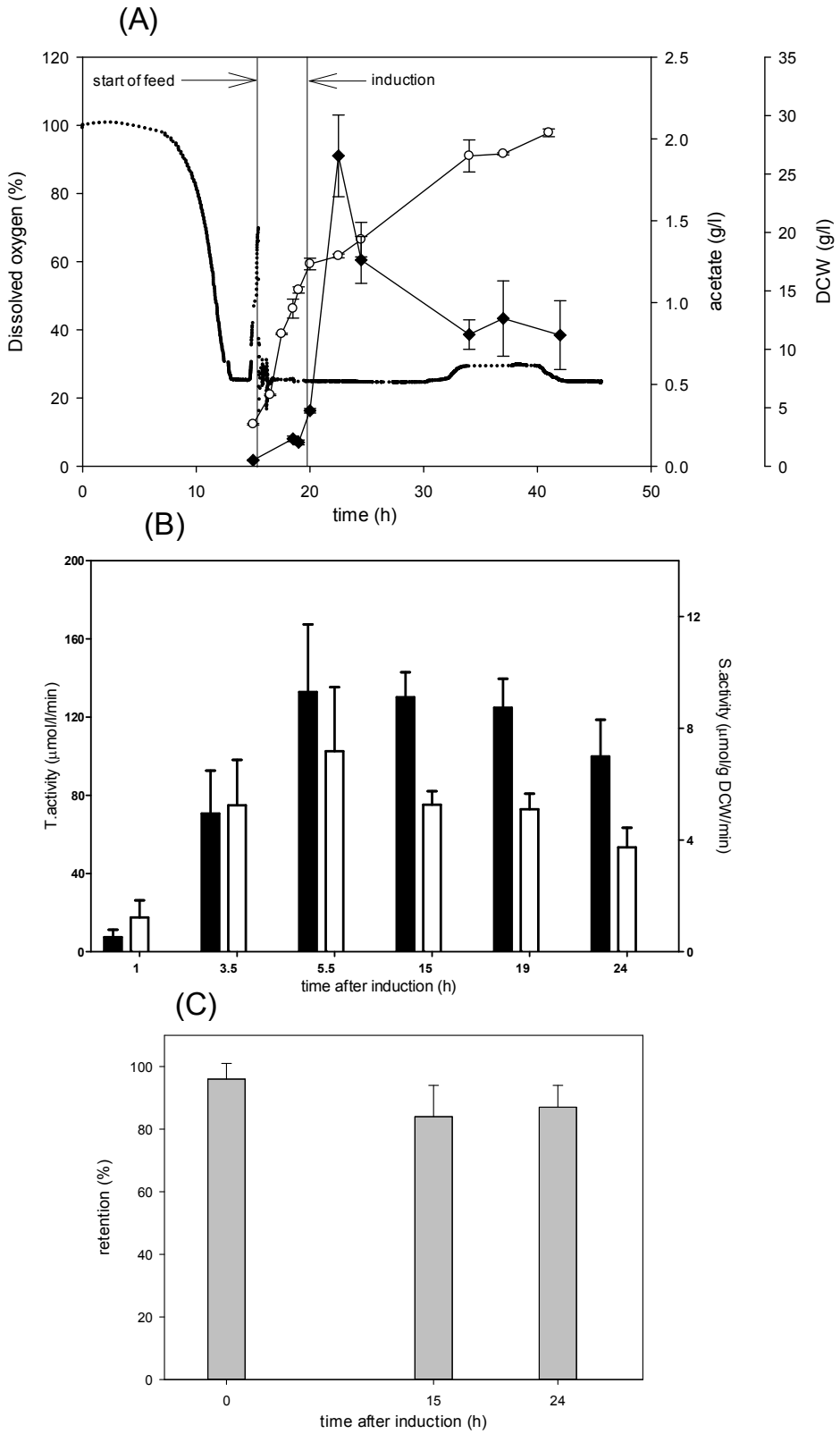

Figure 4.6.Time profiles of the (A) dissolved oxygen (●), biomass (○), acetate concentration (♦) and (B) total (■) and specific MAO activities (□), and (C) percentage of plasmid retention (■). Culture conditions: 30°C, 300-900 rpm, 1 -3 vmm cascaded to 40% and 30% DO_2 before and after induction respectively

4.1.3. Discussion

Regarding the autoinduced batch fermentation, it has been reported that growth on amino acids prevents induction of target proteins by lactose in the early stages of growth (Studier, 2005). When these repressors have been depleted during growth, a shift in cellular metabolism toward the import and consumption of lactose occurs and hence induction by lactose takes place (Blommel et al, 2007). As can be seen in figure 4.1.B. at 12h there was hardly any monoamine oxidase activity detected, while later at 15h there was an abrupt increase signifying derepression of the lac operon. This method of auto-induction does not require any complex process controls and reaches high yield of product in a very short process time. However, as it will be discussed later, the product yields are significantly increased when other mode of fermentations are used.

4.1.3.1. Plasmid instabilities

The construct used in the first two fermentations exhibited surprisingly low plasmid retention leading to very low expression of the recombinant product. Not surprisingly the fed-batch process presented much higher plasmid instability than the batch process. The prolongation of the fermentation time in the fed – batch culture and the very fast growth of the cells during the feeding process increases the risk of cells losing the plasmid, since the number of generations is significantly increased. Theoretically, the earlier a single cell loses the plasmid, the earlier the culture will be dominated by plasmid free cells, a phenomenon called internal contamination. This is obvious when the batch and the fed batch cultures are compared (figures 4.1.C. and 4.2.B). In the batch culture the plasmid retention is reduced as time passes but remains at levels which enable a very satisfactory monoamine oxidase expression. On the other hand this strain performs completely different under the severe conditions of the fed batch culture when the fermentation is prolonged and the generation time is reduced increasing the probability of plasmid loss. Plasmids are always a metabolic burden for the cells, imposed by plasmid replication and recombinant gene expression (Glick, 1995), which leads to lower growth rates, and

as a result there is a natural preference of plasmid free cells during cultivations (Yazdani & Mukherjee, 2002). Therefore when the first plasmid – free daughter cell appears then gradually the culture is being taken over by the plasmid free cells. For that reason, it is of vital importance to retain selective pressure in the culture until the end of the process.

The ampicillin resistant construct contains a gene in the plasmid expressing the beta-lactamase enzyme which is responsible for the resistance to the penicillin derivatives, such as ampicillin and carbenicillin. Ampicillin and carbenicillin belong to the beta-lactam group of antibiotics. The beta-lactams cause acylation of the active site serine of the DD-transpeptidase that cross-link the glycan molecules thereby interrupting the synthesis of cell wall peptidoglycan (Summers, 1996). Beta-lactamase catalyses the hydrolysis and inactivation of the antibiotic which then undergoes rapid breakdown to small fragments (Frère, 1995). The degradation of the antibiotic means loss of the selective pressure and the cells are more than willing to get rid of the energy consuming plasmids. A number of studies have shown that ampicillin has already been considered unfavoured in fermentation as it degrades very fast (Korpimäki et al, 2003; Wülfing & Plückthun, 1993). Even though, carbenicillin (which is the antibiotic used in the first two fermentation) is supposed to be less susceptible to degradation by beta-lactamases (Basker et al, 1977), it is reported to be degraded fast leaving cells without selection pressure.

Now the question ‘what can be done?’ arises. There are ways to increase the segregational stability of the cells. The most obvious way will be the gradual addition of the antibiotic from time to time in order to maintain a relatively stable concentration over time and therefore maintain selection pressure (Fleming & Patching, 2008). However, this strategy requires the antibiotic concentration in the culture to be assayed periodically so that the degradation rate can be calculated and then the appropriate amount of antibiotic to be added. Korpimäki et al (2003) showed that higher concentration of the antibiotic would slow the growth of plasmid-containing cells. Therefore, it is of vital importance to maintain the concentration of the antibiotic below certain levels causing inhibition and above levels which are not

enough to cause selection pressure. It is obvious that this strategy increases both the complexity and the cost of the process by far and it is almost prohibitive when scaling up to industrial levels. Therefore, it was decided a new construct to be used with a plasmid carrying a kanamycin resistance gene.

Kanamycin belongs to the aminoglycoside group of antibiotics which exert their effects through interaction with ribosomes in gram negative bacteria. Kanamycin inhibits bacterial cell growth through interactions with at least three ribosomal proteins causing inhibition of protein synthesis and introduction of translation errors (Pestka & Kivie Moldave, 1974). The *kan* gene on plasmid offers resistance to the bearing cells by the expressed phosphotransferase which phosphorylates the hydroxyl moieties in the kanamycin molecules (Davies & Wright, 1997). Even though, the mechanism of resistance still relies on the degradation of the antibiotic, the plasmid carrying the kanamycin resistance appears to be much more segragationally stable with plasmid retention always above 80% as can be seen in figures from 4.3.C. to 4.6.C.

4.1.3.2. Feed optimisation

As can be seen in figure 4.3.C. the use of the *E.coli* BL21(DE3) pET 28b solved the segregational stability problem and led to much higher total MAO activities. The maximum total MAO activity was 1.82 times higher than the one reached in the batch culture of *E.coli* BL21(DE3) pET16.

The transfer of the fermentation protocol from *E.coli* BL21(DE3) pET16 to *E.coli* BL21(DE3) pET 28b had a remarkable effect on the time needed to reach the desirable biomass levels for the beginning of induction (OD_{600nm} = 40). The ampicillin resistant construct needed 6.5h from the start of the feed to reach the induction biomass while the kanamycin resistant strain needed approximately 8h. This difference can be explained by the much lower plasmid retention in the ampicillin resistant construct which had as a result the culture to be dominated by the fast growing plasmid free cells. In contrast, the second construct maintained the plasmid (plasmid retention 94% at the induction point) which led to lower growth rates than the ampicillin resistant construct (0.14 h^{-1} and 0.175 h^{-1} for the ampicillin

and kanamycin resistant construct respectively). From the industrial point of view it is of vital importance the fermentation time to be as short as possible without compromising the product quality of course. Therefore, the next obvious optimisation step was the modification of the feeding profile in a way that the desirable induction biomass will be reached earlier.

The change of feed led to a significant increase of the specific growth rate from $0.175h^{-1}$ to $0.34h^{-1}$ and reduced the time between the start of feed and the induction by almost 50%. Regarding the productivity of the culture, there were no noteworthy differences between the two cultures. The optimised-feed culture presented higher biomass levels after the induction; however, the overall total MAO activity was only slightly higher than that of the non optimised feed culture. Interestingly, the specific MAO activity of the optimised feed culture was lower than that of the non-optimised (statistically significant at 4h, 5h and after 8.5h post induction, $p<0.05$). This phenomenon can be attributed to the high acetate production of the feed-optimised culture. The faster growth rates achieved in higher feeding profiles are associated with higher by-product secretion (Eiteman & Altman, 2006). Figure 4.4.A. shows that the acetate concentration was above 1g/l short time after the IPTG induction. This is not only a waste of substrate which could be otherwise used for the production of biomass and recombinant product, but also acetate concentrations above certain limits are exerting negative effects on both biomass and recombinant product formation (De Mey et al, 2007). Overall, the higher biomass levels of the optimised-feed culture seem to counteract the negative effect of acetate and lead to slightly higher total MAO activities about 4h faster than the non-optimised feed culture.

4.1.3.3. Change of inducer

To further reduce the cost of the industrial production of MAO-5N, we can replace expensive chemicals with cheaper ones. A potential candidate is the inducer, IPTG which has a very high cost for large scale production of recombinant protein and presents toxicity to humans so that its presence as a contaminant of the final purified protein product is unacceptable (Donovan et al, 1996; Gombert & Kilikian, 1998).

Figure 4.4.A. and Figure 4.5.A. show that the DO and biomass profiles are similar with the lactose induced culture exhibiting slightly higher biomass levels the first hours after the onset of the lactose induction. This can be obviously explained by the fact that lactose can be used as a carbon source. However, during the last hours of the process, there was a decrease of the biomass for the lactose induced culture. Moreover, a striking difference between the two cultures is the acetate concentrations which appear to be much higher for the lactose induced culture. Again, lactose can be blamed for this discrepancy as the higher substrate concentration (both glucose and lactose in the feeding medium) increases the growth rate and consequently the acetate production due to carbon overflux (El-Mansi, 2004). In addition in figure 4.5.A. it is obvious that lactose concentration builds up imposing further stress on the culture as explained below.

The uptake of lactose by *E.coli* is mediated by the lactose transporter (lactose permease), a proton-driven co-transporter belonging to the major facilitator superfamily, encoded by the *lacY* gene. This symporter uses the proton gradient across the *E. coli* membrane generated by the oxidation of fuel molecules to drive the uptake of lactose against a concentration gradient (Nicholls & Ferguson, 2001). In other words, the lactose permease links lactose transport to the proton motive force created by either the electron transport chain or, under anaerobic conditions by the ATP hydrolysis catalyzed by the F_1F_0-proton-translocating ATPase. The result is the transport of one proton and one lactose molecule into the cell, with the net accumulation of lactose (Figure 4.7) (Sahin-Tóth & Kaback, 2001).

Therefore, lactose can act as an energy uncoupler, by breaking the energetic coupling between the primary pump and the ATP synthase. The build up of lactose concentration in the culture medium can then produce a significant reduction or a collapse in the driving force for ATP synthesis, and subsequently the ATP pool within the cell is reduced (Ahmed & Booth, 1981; Straight et al, 1989). This reduction is expected to have as a consequence growth inhibition (Dykhuizen & Hartl, 1978; Studier, 2005) and of course less available energy for the recombinant protein synthesis. This is very obvious in figure 4.5.B. where after 3.5h post

induction there was a decrease of the total MAO to very low levels. A comparison of the specific MAO activities between the cultures of figure 4.4.B. and 4.5.B. shows that the difference is enormous, clearly indicating that the cells were completely incompetent to produce the recombinant product after 3.5h post induction, time which coincides with an accumulation of lactose above 5 g/l. To conclude, it is imperative to efficiently control the lactose concentration in the culture in order to avoid the negative impact of its accumulation which leads to lower MAO activities, higher acetate concentration and cell autolysis during the later stages of the process.

One way to avoid the lactose accumulation in the culture would be to change the post-induction nutrient feeding strategy and use lactose as the sole carbon and energy source which at the same time keep the *lac* operon constantly induced. This would, theoretically, make the lactose monitoring and control much easier as the rate of addition can be linked to the dissolved oxygen readings (exhaustion of lactose will cause increase of DO, while lactose overfeed will lead to DO reduction under preset limits). It is known that the volumetric yield of the product depends on both the biomass concentration and the specific cellular product yield. However, the nongenetic population heterogenecity of the amount of the lactose permease (LacY) in the cell envelope, which varies from none to a few molecules in each cell of the culture, leads to insufficient growth and hence lower volumetric yields (Menzella et al, 2003). Therefore, the use of lactose as both inducer and carbon-energy source would give lower total MAO activities. For that reason, it was decided to insist on co-feeding during the induction period but this time effective control of the lactose concentration is achieved by monitoring using the YSI analyser.

A comparison between the uncontrolled and controlled lactose cultures show that the average biomass concentrations ranged at the same levels. However, as expected, the first one exhibited slightly higher biomass concentrations in the first hours after induction while the latter continued increasing until the end of the process leading to higher values in the end of the process. This comes as no surprise if we bear in mind that the lactose concentration increased linearly and in the first hours after induction the lactose levels were optimal while as time passed increased to cell-damaging

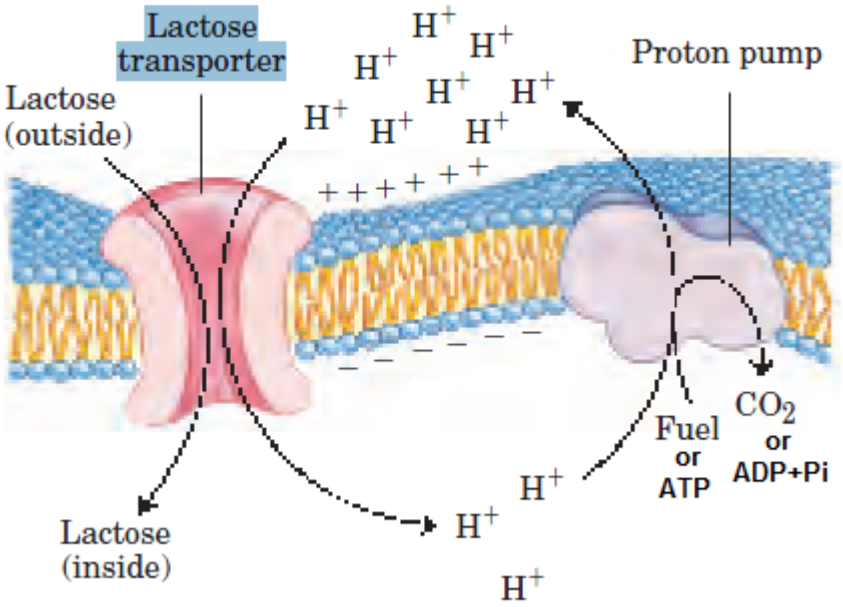

Figure 4.7 Lactose uptake in *E. coli.* The primary transport of H^+ out of the cell, driven by the oxidation of substrate (fuels) or ATP hydrolysis by F_1F_0-ATPases, establishes both a proton gradient and an electrical potential (inside negative, outside positive) across the membrane. Secondary active transport of lactose into the cell involves symport of H^+ and lactose by the lactose permease. The uptake of lactose against its concentration gradient is entirely dependent on this inflow of H^+, driven by the electrochemical gradient (adapted from Nelson & Cox (2004).

levels as described above. The same holds true for the MAO activities, where in the first 3.5h after induction the uncontrolled lactose culture presented higher activities while after that point there was a rapid decrease leading to significant lower levels than the lactose controlled culture. It is noteworthy that in both lactose induced cultures the acetate concentration followed a similar pattern until 5.5h post induction, while after that point the uncontrolled culture presented significantly higher concentrations. Therefore, all the indices show that the control of the lactose concentration in the culture led to a healthier culture which had as a consequence a significant increase of the overall productivity.

The change from IPTG to lactose as inducer, led to a slight but not statistically significant increase of the biomass concentration, increased the acetate secretion in the culture. Lactose increased even though not significantly the productivity, as the maximum total MAO activity was 132.88 mol/l/min comparing to 119.82 mol/l/min of the IPTG induced culture. The most important gain from the transition from IPTG to lactose is the time at which the maximum MAO activity was achieved, which was achieved 2.5h earlier than the IPTG induced culture. All in all, lactose as an inducer appears to be much more suitable for the large scale fermentations of the MAO enzyme as it is much more cost effective and leads to better yields at an earlier stage of the process.

4.1.4. Conclusions

To conclude, in this section a considerable optimisation of the heterologous MAO expression from *E.coli* was achieved. In the first place, an easy to prepare and run batch fermentation was carried out where the expression was auto-induced by the lactose of the medium. Then the productivity was attempted to be increased by the use of a fed batch culture, however, the particular construct was unable to retain the plasmid under the harsh conditions of the fed-batch process and it was replaced by a

more robust construct. The new construct showed remarkable plasmid stability and the total MAO activity was improved by 67.5% compared to the batch auto-induced culture. The industrial need to scale up lead us to the optimisation of the feeding profile so that the fermentation time will be reduced. An almost 50% reduction of the time between the onset of feed and induction was achieved with the MAO remaining to the same levels. In the end the replacement of the IPTG with lactose, further reduced the cost and caused an increase of the total MAO by 10.9%.

4.2. Effects of dissolved oxygen availability and induction culture biomass on MAO activities

4.2.1. Introduction

A potential problem for the successful overexpression of the MAO enzyme in the previous section was the high acetate concentrations. In this section this problem is attempted to be overcome by the use of oxygen enriched air so that the dissolved oxygen tension will be maintained at the predetermined levels. The advantages of aerobic respiration are unquestionable as more energy is produced (in the form of ATP), and reducing equivalents are recycled preventing the accumulation of wasteful and toxic by-products (e.g acetate), allowing higher biomass and specific growth rates to be achieved (Ratledge, 2006). Thus, the general aim is to maximise cell mass and protein production by imposing highly aerobic conditions on the culture such that C flux to metabolites such as acetate is minimised. Nevertheless, this highly aerobic metabolism has also an associated risk, in terms of formation of free radical species (Cabiscol et al, 2000; Iuchi & Weiner, 1996; Touati, 2000). Aerobic and facultative aerobic organisms including *E.coli* have an elaborate defence system containing superoxide dismutases and catalase to minimise cellular damage from free radicals. However, when the concentration of oxidising species exceeds the capacity of the cell's defence system then a condition known as oxidative stress takes place, which can severely damage cell components such as DNA, lipids, and proteins (including recombinant proteins) (Dunster et al, 1997; Farr & Kogoma, 1991). Therefore, "foreign" proteins expressed in highly aerated systems may be subject to

oxidative damage, such as carbonylation, which could affect their subsequent activity (Li et al, 2008). This is an area which has received relatively little attention to date.

The vulnerability of a protein to oxidative stress depends on the number of the oxidation prone residues that it contains and the location of these sites in the protein sequence. It has been reported that the common sites for the oxidative reactions are in tryptophan, tyrosine, histidine, cysteine and methionine residues (Berlett et al, 1996; Kim et al, 2001; Stadtman & Levine, 2003). Oxidation in cysteine and methionine residues are of great importance in this context, as it has been reported to be directly linked with significant activity deterioration in many proteins (Manning et al, 2010; Thomas & Mallis, 2001; Vogt, 1995).

4.2.2. Results

Cultures were induced at differing optical densities to examine the effects of cell mass at induction point upon subsequent enzyme activity levels. A range of OD induction points were examined from OD 40 up to OD 70. For each induction level, there was a normally aerated process and a process where the sparge gas was enriched with oxygen.

4.2.2.1. Induction at OD 40

In these fed-batch processes the cultures were induced when the culture's optical density reached 40 (dry cell weight about 12 g/l). In one process normal air was used as the sparge gas, while in the second oxygen enriched air was used to help maintain the appropriate DO setting. Although neither of these cultures became oxygen limited, the dissolved oxygen levels in the aerated process were significantly lower than those of the oxygen enriched process (figure 4.8. A and B). Specifically, after induction by the addition of lactose, there was a slow decrease of the dissolved oxygen from 25% to about 16%, while in the oxygen enriched process the dissolved oxygen remained stable at 30% from induction until the end of the process. The aerated culture showed a slight increase of the dissolved oxygen level after 28h which can be explained by the decrease in the lactose feed rate. The acetate

concentration in the aerated culture reached its highest value (1.21 g/l) at about 25h when the dissolved oxygen stood at its lowest level of 16% of air saturation. By contrast, in the oxygen enriched culture the highest value of acetate (0.51g/l) was recorded at about 19hours (before lactose induction), which coincides with an increase of the feeding rate at that point, suggesting that acetate accumulation is a result of cells exceeding their respiratory capacity. However, in both processes after induction the concentrations of acetate were reduced and remained significantly lower than the acetate levels which have been reported to negatively affect both cell growth and protein production (Han et al, 1992; Koh et al, 1992; Luli & Strohl, 1990). The aerated culture reached a maximum biomass concentration of 27.5 g/l while that of the oxygen enriched culture was 34.9 g/l. The specific growth rates during the fed batch phase before the induction were $0.33h^{-1}$ and $0.32h^{-1}$, and after induction, $0.20\ h^{-1}$ and $0.21h^{-1}$ for the aerated and oxygen enriched processes, respectively.

From figure 4.9. A and B it can be seen that both the total and specific monoamine oxidase activity are higher for the oxygen enriched culture in the first 3.5 hours after the induction, and after that point there is an increase of the activities for the aerated culture while the activities of the oxygen enriched culture started to fall. This trend is more readily apparent in the specific activity figures (Figures 4.9.B.) where the differences between the activities of the two cultures were even more prominent.

To summarise, under oxygen enrichment cells reach their maximum MAO activity much more rapidly than in the aerated process, but the MAO activity thereafter drops rapidly from its maximum. By contrast, the optimum harvest time in terms of enzyme specific activity for the aerated culture is much later at about 15h after induction, but is relatively stable. This is the first report of enhanced productivity and activity of MAO type enzymes using oxygen enrichment. Despite the protective environment which the intracellular environment of the cells offers, these findings may also indicate a specific effect of oxygen upon the enzyme activity.

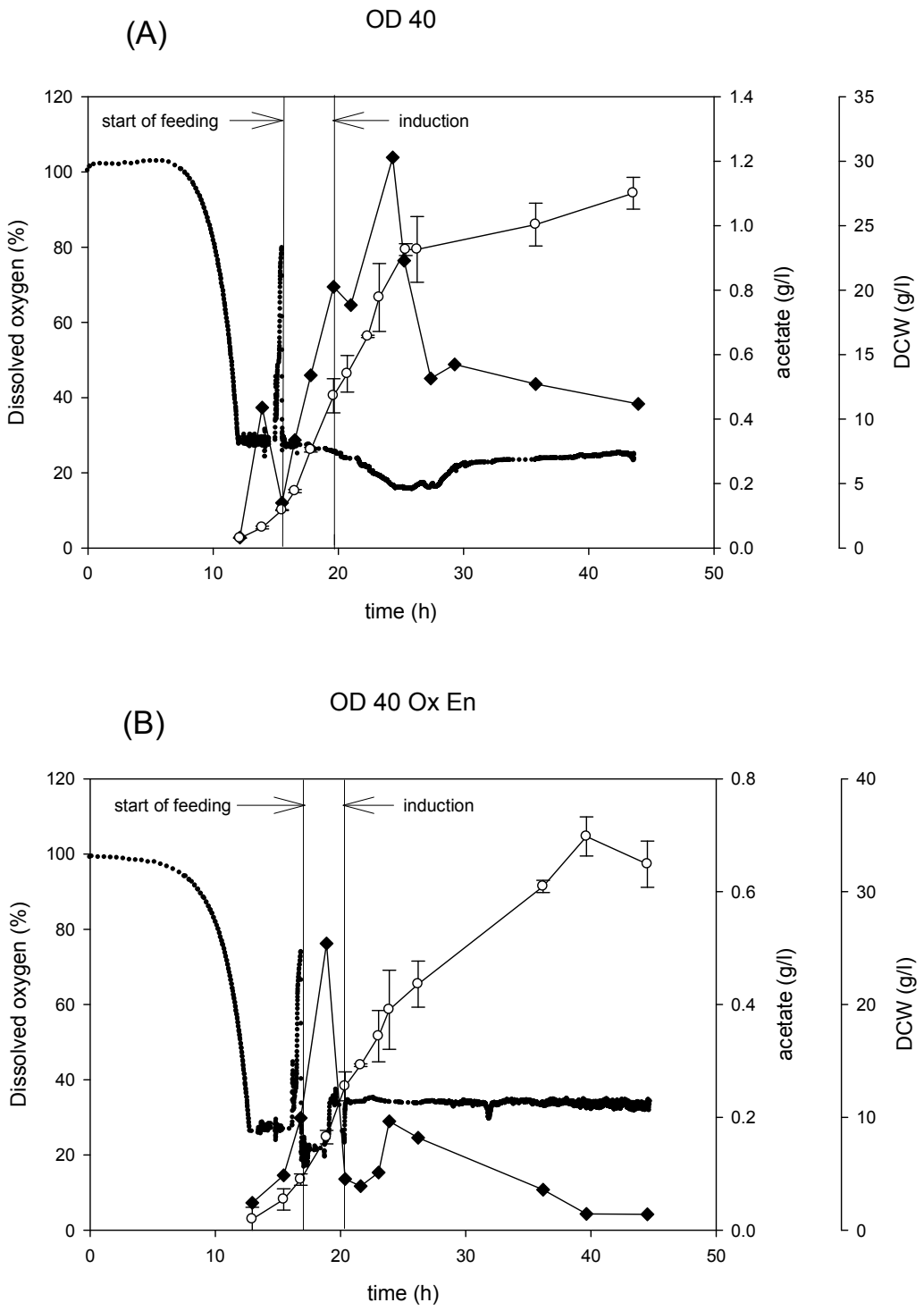

Figure 4.8. Time profiles of dissolved oxygen (●), acetate concentration (♦) and dry cell weight (○) of the aerated (A) and oxygen enriched *E.coli* BL21(DE3) culture (B). Cells were induced with lactose controlled addition when OD 40.

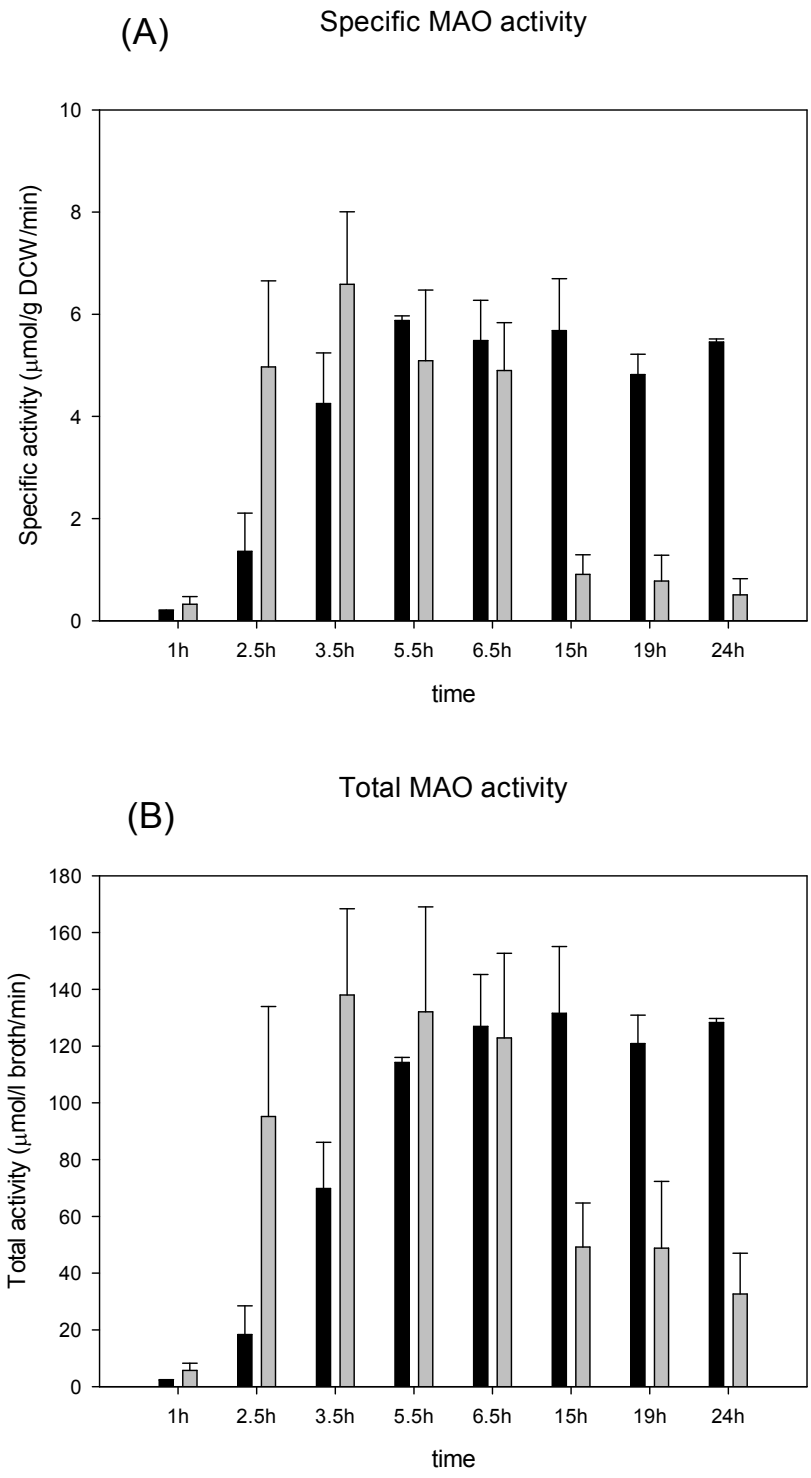

Figure 4.9. A. Specific MAO activity (mol/g DCW/min) of the aerated (■) and oxygen enriched (■) *E.coli* BL21(DE3) culture v. time after the lactose induction. B. Total MAO activity (mol/L broth/min) of the aerated (■) and oxygen enriched (■) *E.coli* BL21(DE3) culture v. time after the lactose induction. Cells were induced with lactose controlled addition when OD 40.

4.2.2.2. Induction at OD 55

One potentially useful strategy investigated to enhance protein formation in these cultures, was to maximise cell mass before lactose induction since in the case of many heterologous proteins there is a close relationship between cell mass of the expression system, and maximal levels of the desired protein. Accordingly, induction at higher OD's was examined using aerated and oxygen enriched cultures. In the normally aerated process it became impossible to maintain the dissolved oxygen tension at the preset level following induction, and by about 2.5h after induction the process became oxygen limited (Fig 4.10.A.). On the contrary, the oxygen enriched culture was not oxygen limited at any stage. As can be seen in figure 4. 10. (A and B), the acetate concentration in the aerated cultures reached very high levels immediately after the culture became oxygen limited, and kept increasing until it reached a peak of 8.24g/l at about 19h after the start of induction. It is clear that there was a change in the metabolic state of the cells from respiration to fermentation caused by the depletion of oxygen due to the very high cell mass present. According to Contiero et al. (2000), acetate levels above 1g/l caused growth inhibition and negatively affected protein production. By contrast, the acetate concentration of the oxygen enriched culture never exceeded 0.31 g/l, and generally was well below the critical levels where growth and protein production have been reported to be affected. The maximum biomass concentration for the aerated culture was 30.3 g/l at 6.5h after the onset of induction and after that point there was a gradual slow decrease of the biomass levels until the end of the process. The biomass concentration of the oxygen enriched culture was continuously increasing and it reached its highest value at the end of the process (35.8 g/l).

The activity results in figure 4.11. show that the aerated culture exhibited significantly higher MAO activities during the whole process after the induction of the *lac* promoter. Once again, the difference in the activities is slightly clearer when expressed as units of activity per unit biomass (specific activity), and during the last hours of the process. This shows that cells produce less functional enzyme in the oxygen enriched culture and this is especially obvious during the last hours of the process from 15h hours to 24h after induction.

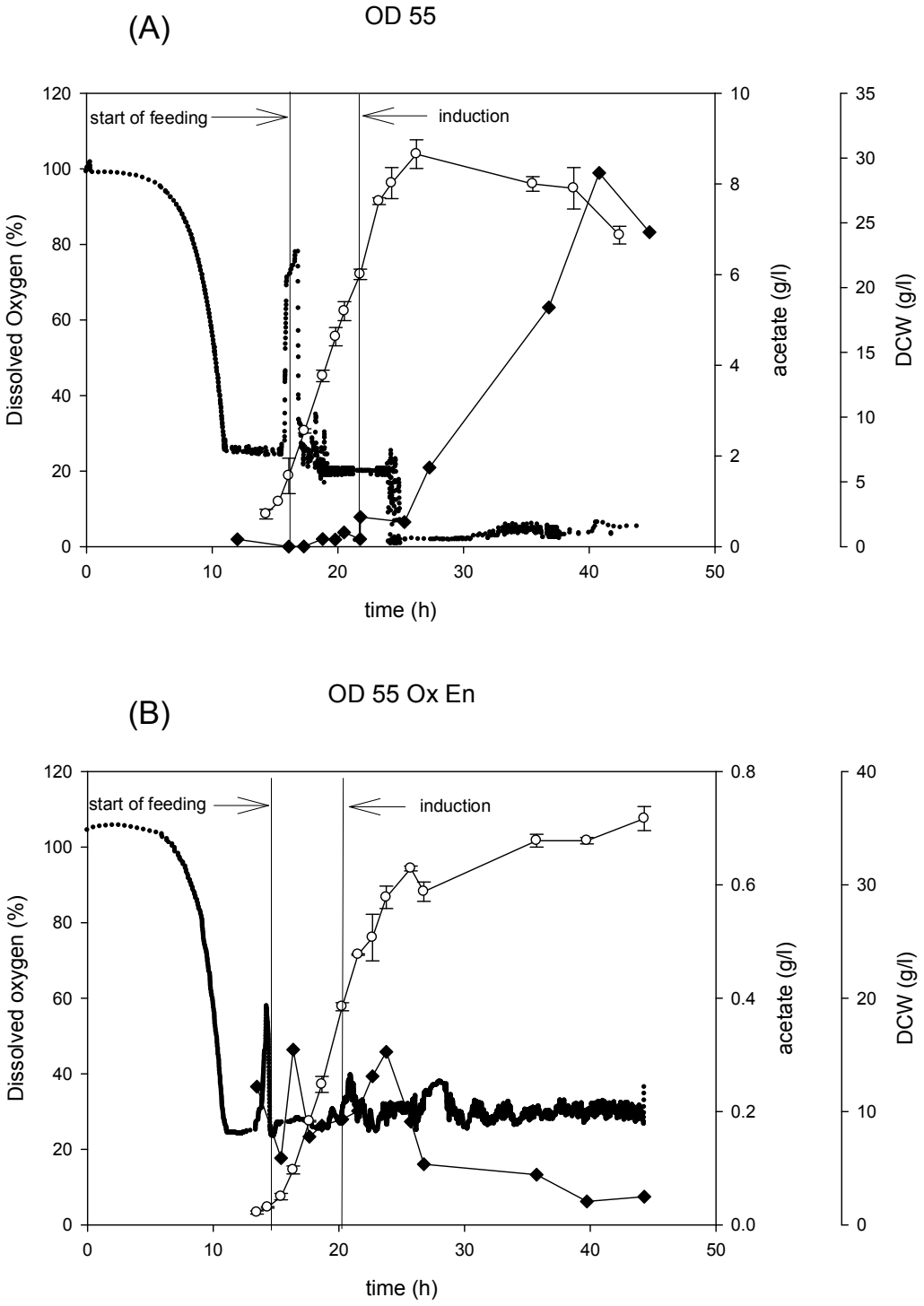

Figure 4.10. Time profiles of dissolved oxygen (●), acetate concentration (♦) and dry cell weight (○) of the aerated (A) and oxygen enriched *E.coli* BL21(DE3) culture (B). Cells were induced with lactose controlled addition when OD 55.

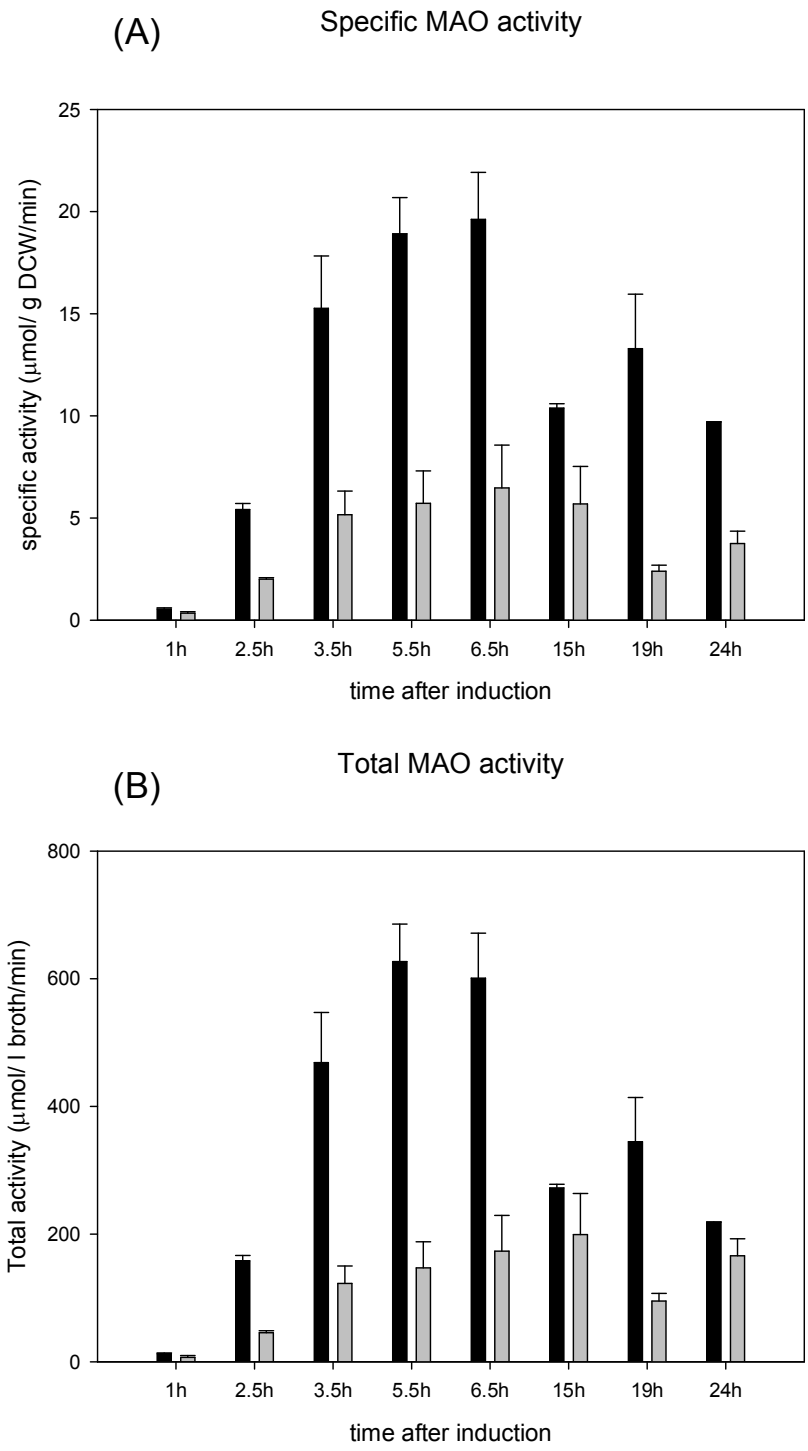

Figure 4.11. A. Specific MAO activity (mol/g DCW/min) of the aerated (■) and oxygen enriched (■) *E.coli* BL21(DE3) culture v. time after the lactose induction. B. Total MAO activity (mol/L broth/min) of the aerated (■) and oxygen enriched (■) *E.coli* BL21(DE3) culture v. time after the lactose induction. Cells were induced with controlled lactose addition when OD 55.

This once again points to the highly dynamic nature of these fed-batch cultures. Namely, the need to keep oxygen supply high enough to maximise cell mass and the specific growth rate in order to achieve high protein production, and to avoid C flux to waste products, such as acetate, but to avoid oxygen levels which are so high they may lead to reduced enzyme activity as noted in the oxygenated cultures.

4.2.2.3. Induction at OD 70

In order to examine the effects of oxygen supply further, cultures were induced at high OD levels of 70 (equivalent to DCW of about 20 g/L) When the cultures were induced at OD 70, the effect of oxygen limitation was even more pronounced in the aerated process. The DO reached 0% well before the induction of the culture due to the higher biomass levels present at this stage of the process relative to earlier processes induced at lower OD's. Oxygen limitation was associated with a switch to fermentative metabolism resulting in a rapid increase in acetate to almost 30g/L, levels likely to cause growth inhibition and product damage (Dittrich et al, 2005). On the other hand, the oxygen enriched culture did not become oxygen limited at any stage of the process, and acetate reached a maximum concentration of only 0.22 g/l indicating a highly aerobic metabolism in this culture. The difference in oxygen availability of the two cultures also resulted in very dramatic impact on cell biomass with the oxygen enriched culture reaching a maximum biomass of 40.0g/l and the aerated culture a maximum biomass of only 26 g/l at 6.5h after the induction, while there was a reduction of the dry cell weight after that point for the aerated culture.

The specific activity (figure 4.12.A.) of the aerated culture was higher between 3.5h and 6.5h after induction, while after that point there were no statistical differences between the two cultures. Figure 6 B shows that the volumetric MAO activity of the aerated and oxygen enriched culture followed a similar pattern, gradually increasing and reaching a peak at 5.5h after the induction, with the oxygenated culture exhibiting higher total MAO activities after 6.5h. Moreover, both total and specific MAO activities of the aerated and oxygen enriched cultures were drastically lower when cultures induced at OD_{600nm} 70.

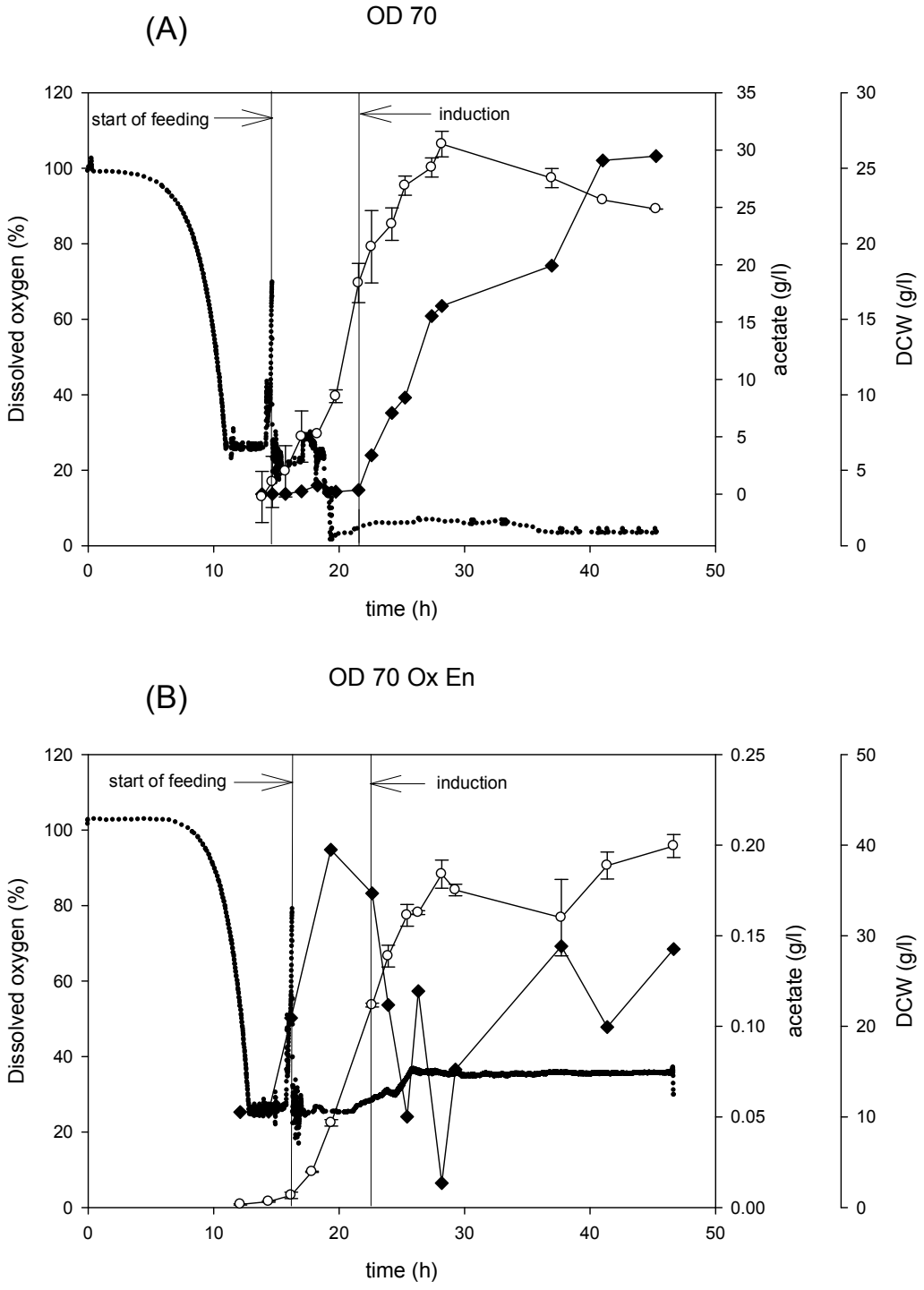

Figure 4.12. Time profiles of dissolved oxygen (●), acetate concentration (♦) and dry cell weight (○) of the aerated (A) and oxygen enriched *E.coli* BL21(DE3) culture (B). Cells were induced with controlled lactose addition when OD 70.

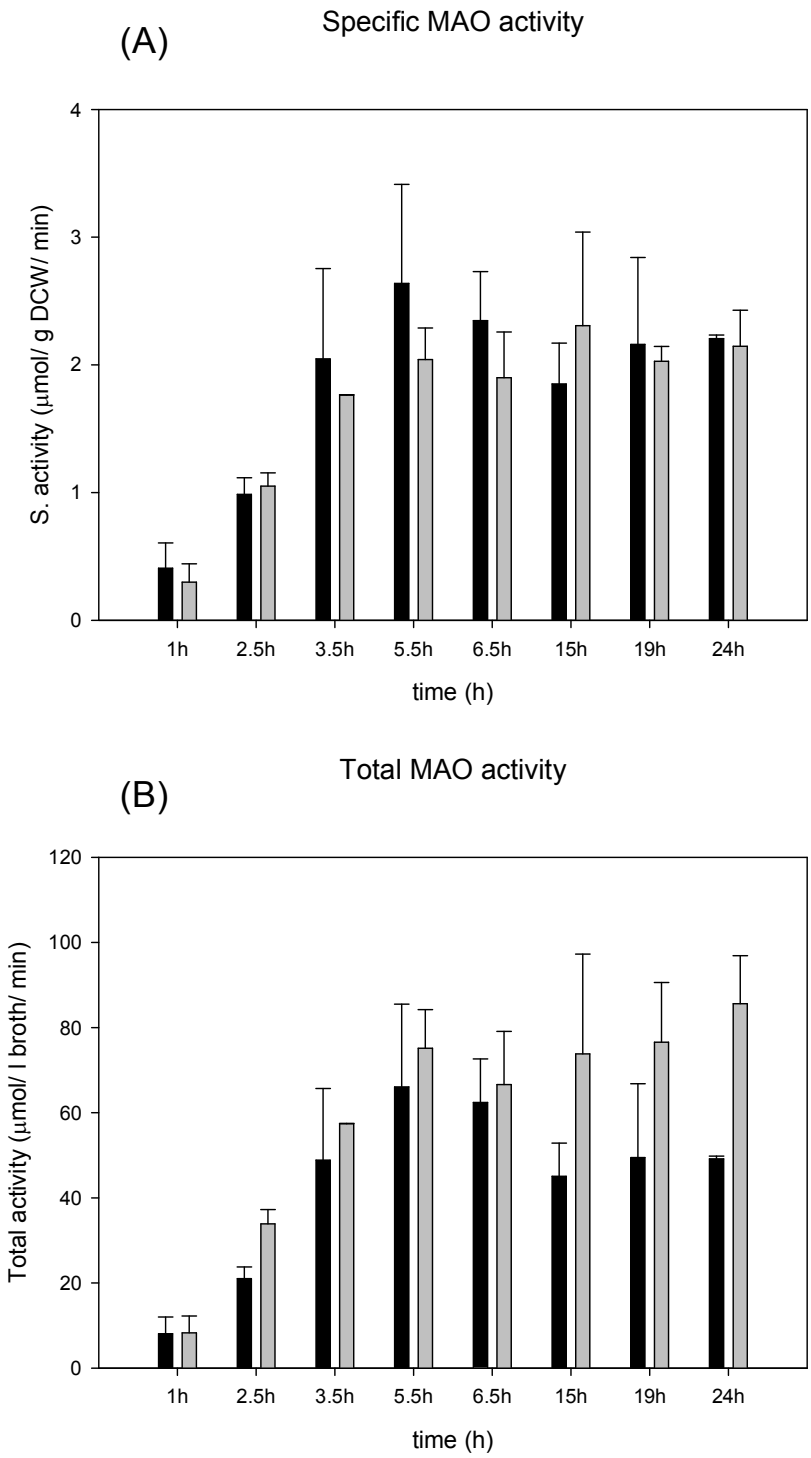

Figure 4.13.A. Specific MAO activity (mol/g DCW/min) of the aerated (■) and oxygen enriched (■) *E.coli* BL21(DE3) culture v. time after the lactose induction. B. Total MAO activity (mol/L broth/min) of the aerated (■) and oxygen enriched(■) *E.coli* BL21(DE3) culture v. time after the lactose induction. Cells were induced with lactose controlled addition when OD 70.

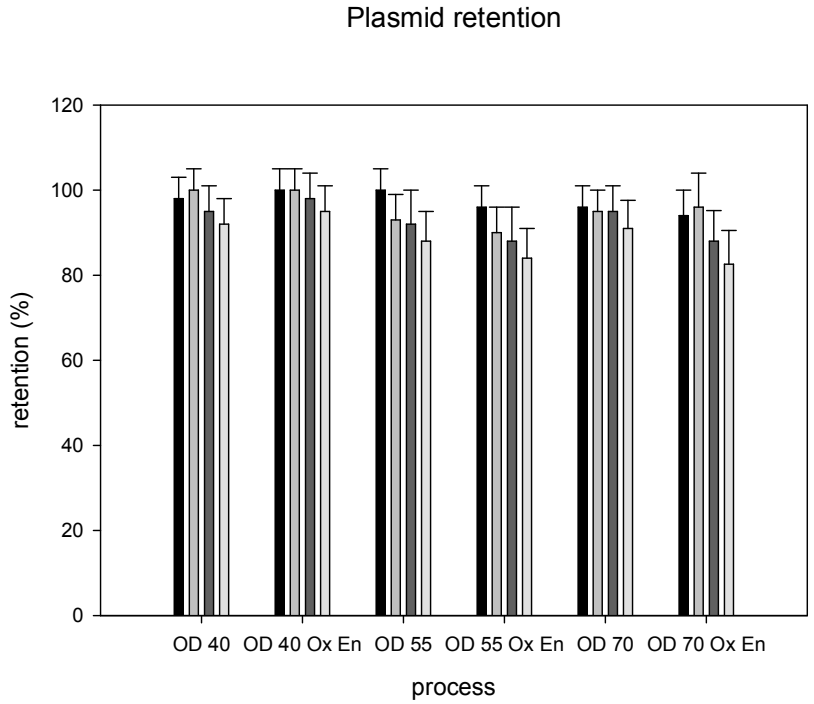

Figure 4.14. Plasmid retention percentages at 0h (■) , 6.5h (■), 15h (■) and 24h (■)after the lactose induction of the *E.coli* BL21(DE3) processes. Samples withdrawn from the bioreactor were appropriately diluted and streaked on LB-agar plated incubated at 30 ^{0}C for 48h and then replica plated on LBkan plate and incubated again at 30 ^{0}C for 48h.

Table 4.1. Specific growth rate during exponential feeding, maximum total and specific MAO activity, peak dry cell weight and acetate concentrations achieved in the bioprocesses of the *E.coli* BL21(DE3).

Processes	**(h^{-1})**	**Maximum specific MAO activity (mol/g DCW/min)**	**Maximum total MAO activity (mol/L broth/min)**	**Peak DCW (%)**	**Peak acetate (g/l)**
OD 40	0.33	5.68	131.54	27.52	1.21
OD 40 Ox En	0.32	6.58	138.02	34.89	0.51
OD 55	0.31	19.6	600.9	30.3	8.24
OD 55 Ox En	0.33	6.47	199.2	35.8	0.31
OD 70	0.27	2.64	66.08	26	29.5
OD 70 Ox En	0.31	2.31	85.61	39.9	0.22

4.2.2.4. Plasmid retention

As can be seen in Figure 4.14., retention of the plasmid in these cultures was between 85 and 100%, and no significant differences between the processes were recorded. Therefore, the presence of the antibiotic through the whole process ensures satisfactory plasmid segregation stability.

4.2.3. Discussion

4.2.3.1. Effects of dissolved oxygen levels on cell mass and enzyme activities

According to the literature the effect of the dissolved oxygen levels on recombinant proteins production is strongly dependent on the strain and plasmid used. Earlier works showed that comparatively unstable plasmids, such as those which contain the ampicillin resistance gene, are negatively affected by the absence of oxygen, resulting in lower plasmid retention levels (Hopkins et al, 1987; Li et al, 1992). This happens because dissolved oxygen is essential both for the growth of the aerobic host cells and also for providing energy for the replication and segregation of recombinant plasmids and subsequent expression of the plasmid-borne genes (Zabriskie & Arcuri, 1986).

Obviously plasmid loss potentially has a direct effect on the amount of recombinant protein that is produced in a process. In the present study, by contrast, the construct used in this work did not exhibit statistically significant plasmid loss due to the selective pressure imposed (figure 4.14). So any decreases in MAO activity such as those noted here must be due to other, processing related, factors.

In the case of the fermentations induced at OD 40, the differences in the oxygenation state of the cultures were not as great as for the cultures induced at OD 55 and 70. However, the oxygen enriched culture exhibited a significantly higher maximum biomass concentration and specific growth rates (as shown in the results section, table 4.1.). These effects were even clearer in the cultures induced at OD 55 and 70. Therefore, as expected, oxygen enrichment seems to boost the growth by increasing both the growth rate (more oxygen available to the Electron Transport Chain) and the maximum biomass values. This is in agreement with the studies of Gill et al. (2008)

where in *E.coli* batch cultures, oxygen enrichment increased the specific growth rate and biomass from 0.68 h^{-1} 3.8g/L to 0.93 h^{-1} and 8.1 g/L, respectively. In the recombinant production of penicillin g acylase by *E.coli*, Liu et al. (1999) reported an increase of both biomass and recombinant product concentration of 2.1 and 1.4 times respectively when oxygen enrichment was used.

It is clear from the results presented above that increasing recombinant protein yield by the use of oxygen enrichment is highly complex. Oxygen seems to very effectively enhance the biomass levels, and so increase the volumetric activity of the protein by avoiding fermentative metabolism, therefore, eliminating the formation of the wasteful and damaging acetate and taking advantage of the higher ATP generation. This effect is clear in figure 4.13. A and B where the total MAO activity is higher for the oxygen enriched culture while the aerated culture presented higher specific activity. There are contradictory reports, both positive (Belo & Mota, 1998; Bhattacharya & Dubey, 1997; Goyal et al, 2009) and negative (Castan et al, 2002; De Leon et al, 2003; Losen et al, 2004; Mohammadian-Mosaabadi et al, 2005; Tomazetto et al, 2007), of high oxygen availability on the specific activity of the recombinant proteins described in the literature. This would suggest that the optimum oxygen levels are dependent on the target protein (Qoronfleh, 1999) and the host strain (Li et al, 1992). A characteristic negative example is the production of recombinant human interferon-γ by an *E.coli* BL21(DE3) culture, where an increase of the dissolved oxygen of the culture from 30% to 60% of air saturation, resulted a 10-fold increase in the extent of carbonylation of the recombinant protein (an important quality factor indicating significant oxidative damage and deterioration in the enzyme product) (Mohammadian-Mosaabadi et al, 2005). In this case the effect of oxygen was direct on the enzyme, however in other works, the lower activities are attributed to increased maintenance functions during the oxygen enriched recombinant protein production. Indicative of this phenomenon can be a drop of the biomass yield on glucose and a simultaneous increase of the oxygen consumption per glucose (Castan et al, 2002). Some authors propose that protein oxidation can lead to very high energy and resources consumption for the synthesis of molecular chaperones to repair or degrade the inappropriately folded oxidatively damaged

proteins, either native or heterologous (Hartl et al, 1994; Szabo et al, 1994). Our results clearly show that the function of the recombinant MAO-5N in the current study is detrimentally affected by the high availability of oxygen in these cultures.

Thus, it is clear that oxygen enrichment or other methods of enhancing oxygen supply to recombinant *E.coli* strains overproducing proteins or enzymes can lead to significant increases in cell mass, and product levels, but that there is already some evidence of product damage at heightened levels of oxygen availability in the present study as shown by the rapid decline in specific MAO activity in the oxygen enriched OD 40 cultures compared to aerated cultures.

4.2.3.2. Acetate effects on culture physiology and MAO activity

Acetate is an undesirable by-product in *E.coli* fed batch fermentation for many reasons. Most importantly it has been reported that acetate concentrations as low as 0.5g/l slows down the growth rate of the culture (Nakano et al, 1997) and inhibits protein formation (Jensen & Carlsen, 1990; Koh et al, 1992). Moreover, the formation of acetate, as well as other byproduct formation, constitutes a waste of carbon source (Gokarn et al, 2001) which could otherwise be used for the construction of new cells and protein synthesis (El-Mansi, 1997). It has also been reported that acetate acts as an uncoupler of the proton motive force, since its non-dissociated form can re-enter the cell, dissociate due to the higher internal pH, reducing the intracellular pH (Axe & Bailey, 1995; Repaske & Adler, 1981) and therefore leading to lower ATP synthesis.

The induction of the culture at higher optical densities makes the oxygenation of the culture more difficult as in higher biomass concentrations there is a much increased oxygen demand. One method of meeting this higher demand is to use oxygen enriched air to maintain the dissolved oxygen at the predetermined levels. As might be expected from the above, a comparison between aerated processes shows that the very high levels of acetate concentration in these processes induced at OD55 and 70 (8.2 and 29.5 g/l respectively) caused significant reduction in the biomass. The inferior biomass levels of the aerated culture can be attributed to the prohibitively

high acetate concentration (March et al, 2002). Both effects are clearly a result of the oxygen limitation of the culture. The impact upon these processes of reducing carbon flux to acetate has been clearly shown: at – line monitoring of acetate concentration using NIR spectroscopy, allowed appropriate adjustment of the feeding rate leading to significant acetate reduction which led in turn to a 2.5 fold improvement in biomass concentration of an oxygen enriched process (Macaloney et al, 1997). This clearly shows that the main obstacle to high biomass production is acetate accumulation, as a result of the so called overflow metabolism (Andersen & von Meyenburg, 1980; El-Mansi & Holms, 1989; Holms, 1996; Xu et al, 1999).

In terms of MAO specific activity, by contrast with biomass, no negative effect of oxygen limitation and acetate concentration was apparent when induction OD rose from 40 to 55, instead there was a massive increase for the aerated culture at OD 55 (compared to the oxygen enriched OD 55). However, when acetate levels reached very high concentrations (high induction OD aerated) overall specific MAO activity was much reduced relative to aerated processes induced at lower OD's. However, oxygenated cultures at OD 70 had similarly low specific MAO activities so the reduced MAO activity here cannot be simply related to acetate levels.

4.2.3.3. Effects of oxygen on oxidase type enzyme production

The oxygen limiting conditions of the aerated cultures in this study at OD 55 are associated with an increased activity of MAO despite the facts that the acetate concentrations reached levels which are reported be inhibitory for both growth and protein production (Contiero et al, 2000), and less energy in the form of ATP and reducing power (Åkesson et al, 1999), as well as less building materials are available for protein production (El-Mansi, 1997; Varma & Palsson, 1994). Since we have demonstrated no significant loss of plasmid, the above findings may indicate that high oxygen availability negatively affects the activity of this enzyme. It has been widely noted that many oxidase type enzymes are subject to damage by the presence of reactive oxygen species in biotransformations (Pilone & Pollegioni, 2002) where the crude enzyme is unprotected in the reaction media in the presence of H_2O_2. However, the effect of oxygen supply during fermentation to produce such oxidase

type enzymes has not been studied before. Whole cells provide a sheltered environment to the cytosolic enzymes, but exposure to high oxygen levels causes oxidative stress leading to the formation of reactive products and damage to many cellular constituents including proteins (Farr & Kogoma, 1991; Sies, 1993). The susceptibility of proteins to those species depends on many factors including the nature of the amino acid residues the protein is made of, the position of these residues in the protein (whether they are part of the active centre of the protein or they are located on less important parts of protein), the presence of disulphide bonds between cysteine groups.

It may also be reasonable to expect that recombinant protein overexpression augments the effects of oxidative stress, as studies have shown that accumulation of foreign proteins in cells represents a considerable stress in itself (Hoffmann & Rinas, 2004). Recombinant proteins are produced in larger amounts than native proteins but do not usually have sufficient appropriate chaperones when compared to native proteins (Straus et al, 1988) and therefore, modification due to oxidations may lead to inappropriate folding, reduced activity (Fucci et al, 1983; Stadtman, 1990) and inclusion bodies (Dean et al, 1997; Shacter, 2000). Another fact supporting this hypothesis is the basal amounts of housekeeping proteins produced during the induction of T7 RNA polymerase of the lysogenic host *E.coli* strains as the phage polymerase is much faster in the transcription than the native RNA polymerase leading to a reduced amount of native protein being produced (Studier et al, 1990).

Regarding other oxidase type enzymes, Kim et al. (2008) found that the optimal DAAO expression in an *E.coli* culture when dissolved oxygen tension was reduced from 50% to 30% after the induction of the culture. The activity of the enzyme was 18.5 mol/ml/min and the OD_{600nm} 81. This group attributed this effect to the reduced amount of oxygen to be used as a substrate of the DAAO, thus, being less toxic by turning over less d-alanine and less hydrogen peroxide being produced. In our work, MAO does not interfere with the cell metabolism and does not cause any toxic effect on the host cells. However, in the study of Kim et al. (2008) the possible inhibitory effects of oxygen and hydrogen peroxide upon DAAO itself when the dissolved

oxygen levels are high after the induction (resulting in lower activity) were not considered.

4.2.3.4. Oxygen toxicity to MAO

Monoamine oxidase from *A.niger* is characterised by a very high number of oxygen sensitive amino acids residues. Most importantly there are 12 methionine groups and 10 cysteine, some of which are located very close to the FAD cofactor (Schilling & Lerch, 1995a). In terms of broad comparability, methionine and cysteine residues in *T.variabilis* DAAO have been shown to be oxidised to -SOH or $-SO_2H$ under oxidative conditions (Keck, 1996), causing significant stereochemical changes which prevent the binding of the FAD cofactor to the apoenzyme, and consequently negatively affecting the activity of the enzyme (Ju et al, 2000; Slavica et al, 2005). Similarly, chemical modifications of the thiol group of a cysteine residue locating in the FAD binding site of *Rhodotorula gracilis* DAAO leads to enzyme inactivation and degradation (Pollegioni et al, 1997). It is worth mentioning in this context that apart from damaging, oxidations resulting in sulphoxide -SOH formation and disulphide bond formation (S—S) which may be reversible, both sulphinic ($-SO_2H$) and sulphonic acids ($-SO_3H$) may also be formed under these conditions by essentially irreversible reactions (Eaton, 2006; Ghezzi & Bonetto, 2003; Thomas & Mallis, 2001). Moreover, the formation of sulphoxide and disulphide derivatives does not merely lead to enzyme inactivation, also leads to the targeted degradation of the affected proteins (Farr & Kogoma, 1991). A tryptophan residue of *Rhodotorula gracilis* DAAO is reported to be oxidised and prevent the dimerization of the protein when under the influence of H_2O_2 (de la Mata et al, 2000). The Trp55 and Trp70 of the same protein could be located at the FAD-binding domain (Mattevi et al, 1996) and constitute another possible site of oxidation. Alonso et al. (1999) found that a decrease in the aeration rate (and hence oxygen supply) in an *E.coli* culture increased the DAAO activity, and reduced the percentage of the apoenzyme form, suggesting poor binding of the FAD cofactor to the apoenzyme.

In figure 4.15. the 3D structure of *Aspergillus niger* MAO-5N which is used in this work is depicted. The apoenzyme is coloured white, the FAD yellow, cysteines

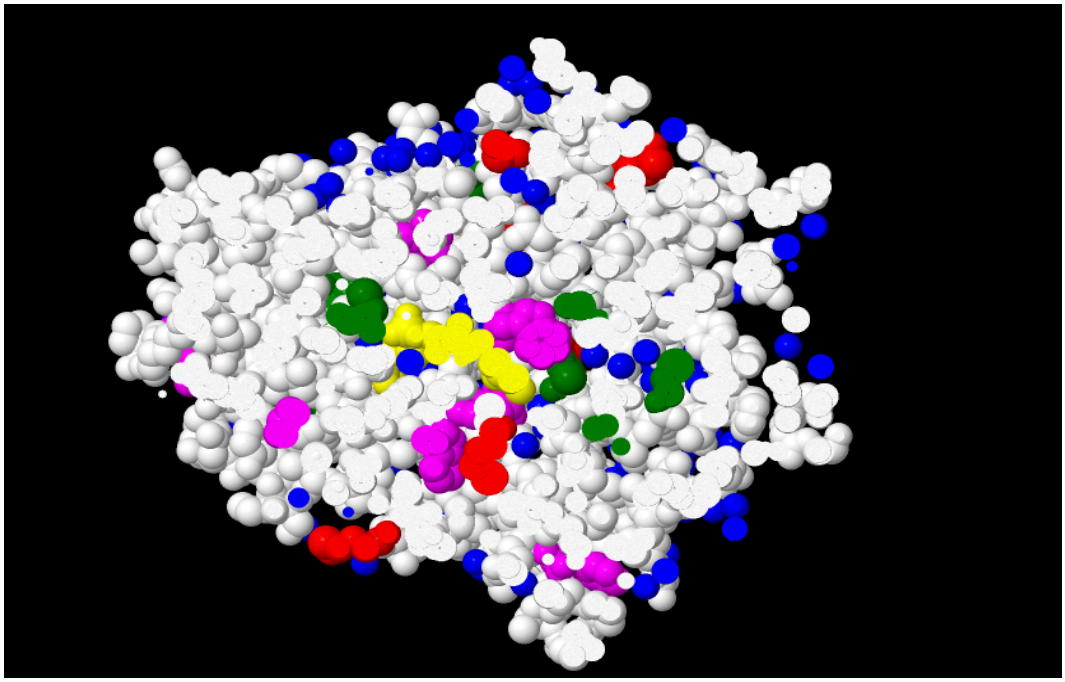

Figure 4.15. The 3-D structure of *Aspergillus niger* MAO-5N cut by 35%. FAD yellow coloured, cysteine green coloured, methionine red coloured, tryptophan magenta coloured and water molecules blue coloured. PDB ID: 2vvm (Atkin et al, 2008).

green, tryptophan magenta and methionine groups red. The molecule is cut by 35% so that the inside of the protein where the FAD cofactor is folded can be seen. As it seems the FAD cofactor of chain A is within very close proximity to oxygen sensitive groups (cys600, trp430, cys277, trp420 and trp94). Therefore, it is highly possible that like DAAO, MAO's sensitive cysteine groups when oxidised cause conformational changes which hinders the proper binding of the FAD cofactor and consequently negatively affecting the activity of the enzyme.

Therefore, the MAO in the present study might be inactivated under highly oxidative conditions due to stereochemical changes in the binding site of the FAD cofactor. Our findings are consistent with oxygen induced damage or inhibition of MAO in the highly aerobic induction phases of the oxygen enriched processes. Broadly speaking, our findings are in agreement with the reports relating to oxidative damage to other classes of oxidase type enzymes in *E.coli*, and based upon careful consideration of the amino acid sequence of these enzymes, entirely reasonable. Thus, our study indicates that process conditions optimal for cell mass propagation at high rates may not be ideal for achieving high levels of active MAO or oxidase type enzymes generally, due to the high levels of oxidative damage to the product in the induction stage.

4.2.4. Conclusions

Our results show that oxygen enrichment can be beneficial to *E.coli* cultures, especially those induced at high optical densities, by improving both the growth rates and the maximum biomass levels. However, the oxygenation of the cultures may adversely affect an oxidative sensitive recombinant product such as MAO. Moderate oxygenation slightly increased the levels of MAO (induction at OD 40), nevertheless, oxygenation at higher OD levels had a dramatic negative effect on activity despite the absence of growth and protein damaging levels of acetate. To conclude, in an ideal process, dissolved oxygen should be maintained at levels low enough to allow satisfactory growth and prevent fermentative metabolism, but not high enough to cause oxidative damage to the recombinant product.

Chapter 5

Heterologous production of Amine Oxidase using *Pichia pastoris*

5.1. *P.pastoris* MAO bioprocess optimisation

5.1.1. Introduction

The increasing popularity of the *P.pastoris* expression system can be attributed to several factors such as: 1) the presence of a strong and tightly regulated promoter derived from the alcohol oxidase I gene *(AOX1)* of *P. pastoris* which is very well suited to facilitate the controlled expression of foreign genes (Cregg et al, 2000). 2) Unlike *S.cerevisiae* there is a strong preference for respiration instead of fermentative growth. The lack of fermentative metabolism results in higher biomass levels (up to 160 g/L dry cell weight) (Jungo et al, 2007b), and no secretion of growth and protein inhibitory overflow metabolites such as acetate and ethanol (Jungo et al, 2007a) as happens in other expression systems such *E.coli* and *S.cerevisiae,* and hence enhanced product yield. 3) The capability of carrying out many eukaryotic post-translational modifications such as,disulphide-bond formation, proteolytic processing and glycosylation without hyperglycosylating the proteins as *S.cerevisiae* does (Cereghino et al, 2002).

The high cell density processes of recombinant *P. pastoris* involve a multi-phase process. The first phase is a batch culture with growth on glycerol to achieve high cell densities rapidly while repressing foreign gene expression. This is followed by a glycerol-limited fed-batch phase to further increase biomass and de-repress methanol dissimilating enzymes. The final stage usually involves the induction of recombinant protein expression through the fed-batch feeding of methanol as sole carbon and energy source. This procedure has led to the successful production of more than 600 enzymes at amounts significantly higher than that achieved with *E.coli* high cell density cultures (Zhang et al., 2009).

Most expression media found in the literature contain the very expensive Yeast Nitrogen Base with or without amino acids (YNB). The use of such an ingredient jeopardises the economic feasibility of the potential scaling up to industrial levels of the enzyme production processes of the present work. Therefore, initially an attempt

to substitute YNB with the widely used yeast extract (YE) was made, and the effects on biomass and the recombinant MAO expression were examined.

The slow methanol utilisation strains (as the one used in these experiments), generated by the disruptive integration of the recombinant gene into the *AOX1* locus, exhibit genetically reduced capacity to assimilate methanol resulting in slower growth rates and long induction times (Thorpe et al, 1999). For that reason, researchers have employed mixed feeding profiles where methanol is co-fed with another carbon source. Initially, glycerol and glucose were used enhancing the product yield (Cereghino & Cregg, 2000; Cos et al, 2006). However, the repressive nature of these compounds (repressing the *AOX1* promoter) led to the exploitation of other carbon sources, such as mannitol and sorbitol. It has been reported that even build-ups of sorbitol in the culture have no negative effects on both growth and recombinant protein production (Celik et al, 2009). Therefore, sorbitol was chosen as the alternative carbon source for the optimisation of the current process. Methanol and sorbitol were continuously added at various preset ratios (section 2.7.2.), while the rate of methanol addition was maintained the same in all the processes presented below.

5.1.2. Standard protocol YNB run

To begin with, a typical *P.pastoris* fed batch protocol was used and was treated as a benchmark run for all the subsequent processes. 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 50.3 g/l at 22.5h, when methanol feeding begun. After that point there was, as expected, a decrease of the growth rate, and at the end of the process the maximum DCW recorded was 86.1g/l at 87.4h. The specific growth rate after the start of glycerol and before the methanol feeding was $0.13h^{-1}$ while after the start of methanol induction the specific growth rate decreased to $0.071\ h^{-1}$. The immediate drop in the dissolved oxygen levels after inoculation shows that the cells started respiring almost immediately and at about 8.8h process time the dissolved oxygen reached its preset level of 20%.

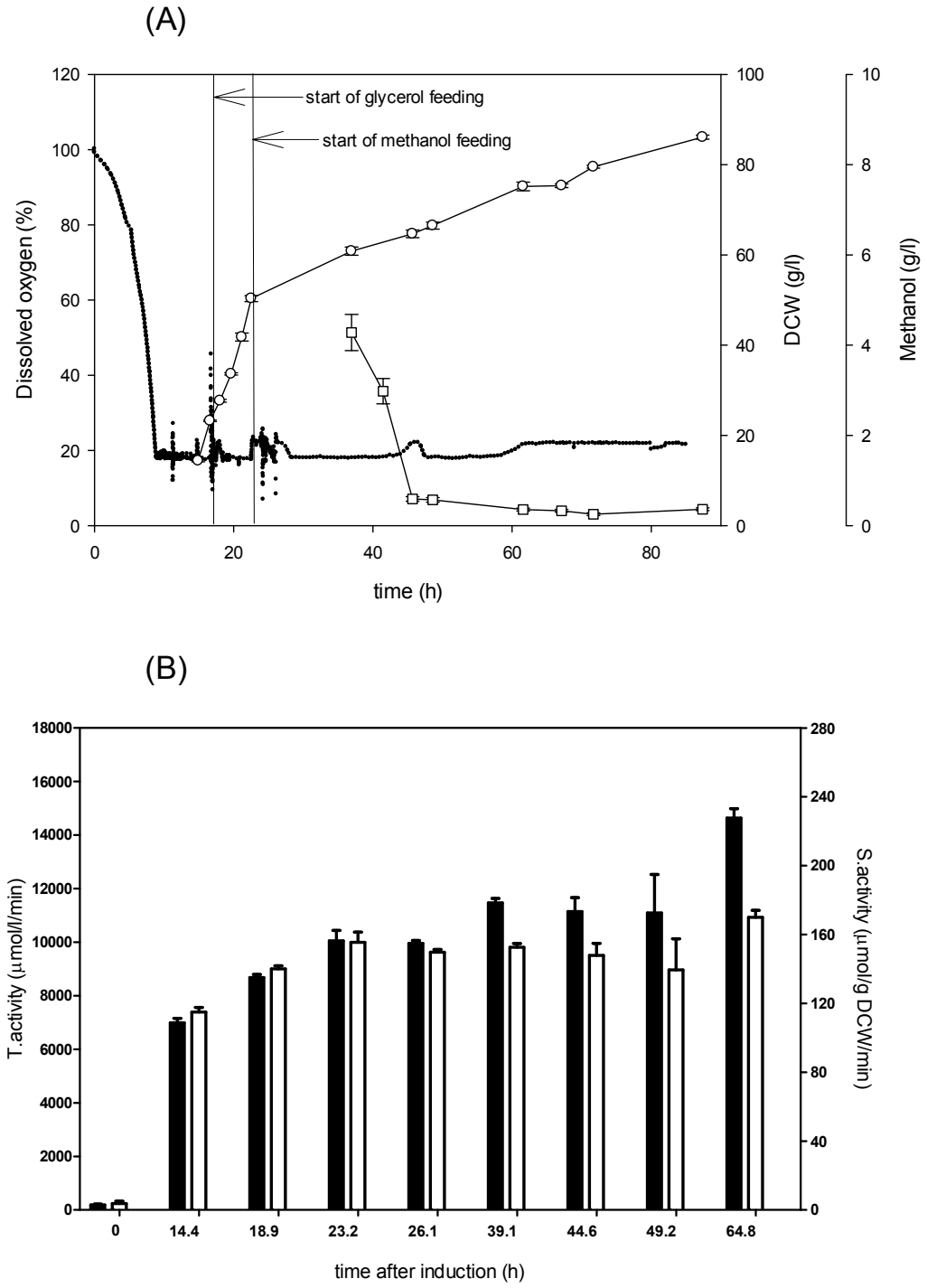

Figure 5.1. Time profiles of the (A) dissolved oxygen (●), biomass (○) and methanol concentration (□) and (B) total (■) and specific MAO activities (□) of the *P.pastoris* cultures.Culture conditions: Bioreactor cultivations, 30^0C, pH 5.8, DO setpoint = 20%, 300-900rpm and 1-3vvm cascaded to DO.

The recorded methanol concentration in the culture never exceeded 4.5g/l and between 45.7h and the end of the process (87.4h) was fluctuating between 0.3 and 0.6g/l. The total monoamine oxidase activity exhibited a gradual increase from the beginning of induction until the end of the process (figure 5.1.B.). At the end of the process (64.8h post induction) the highest total activity was reached (14633.7 mol/l/min). Interestingly enough all the activities measured were very high compared with the activities achieved with *E.coli* as the host organism (chapter 4), with the lowest being recorded at 14.4h after the onset of induction (6992.7 mol/l/min). It is worth noting that even the pre-induction sample exhibited a relatively high activity of 187.1 mol/l/min despite the *AOX* promoter being repressed by glycerol. The specific monoamine oxidase activity increased from 114.9 mol/g DCW/min at 14.4h post-induction to 155.4 mol/g DCW/min at 23.2h post-induction and then it plateaued until the end of the process when it peaked at 169.9 mol/g DCW/min (64.8h post-induction). The pre-induction sample had a specific activity of 3.72 mol/g DCW/min (22.5h process time). Replica plating with and without the antibiotic zeocin was carried out to determine the retention of the expression cassette, and no loss was found.

5.1.3. Substitution of YNB with YE

A first attempt to improve the scaling up of the process is to reduce the cost. Towards that direction the very expensive YNB was replaced by the much cheaper Yeast Extract. Biomass reached a concentration of 57.3g/l shortly before the onset of the methanol induction (22.3h). After that point growth almost ceased as the biomass only slightly increased, reaching a peak at 44.3h of 66.1 g/l. Characteristically, the specific growth rate after the beginning of the glycerol feeding and before methanol induction was $0.10h^{-1}$ while after methanol induction fell to $0.0063h^{-1}$. After the peak was reached in biomass concentration at 44.3h (21.9h post-induction) there was a gradual slow decrease of the biomass to lower levels, reaching a concentration of 61.7 g/l at the end of the process (87.5h). Once again, cells started respiring almost immediately after inoculation which led to a rapid fall of the dissolved oxygen, which reached the preset level of 20% at 8.4h (figure 5.2.A).

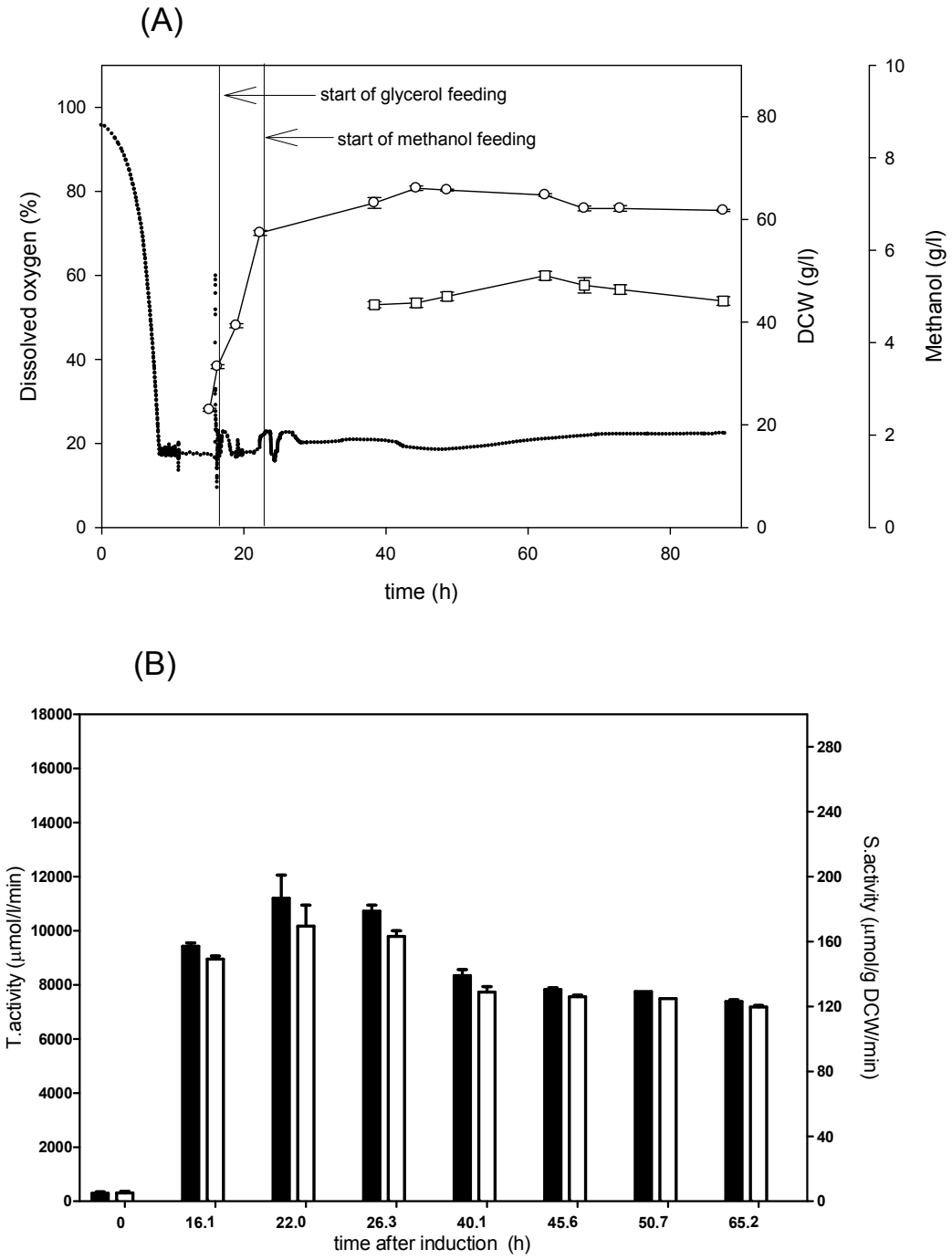

Figure 5.2. Time profiles of the (A) dissolved oxygen (●), biomass (○) and methanol concentration (□) and (B) total (■) and specific MAO activities (□) of the *P.pastoris* cultures.Culture conditions: Bioreactor cultivations, 30^0C, pH 5.8, DO setpoint = 20%, 300-900rpm and 1-3vvm cascaded to DO.

The methanol concentration remained stable after the induction at levels ranging from 4.7g/l to 5.3g/l. The total monoamine oxidase activity reached a peak at 22h post-induction (44.3h process time) of 11197.9 mol/l/min. Afterwards there was a gradual decrease initially to 10722.5 mol/l/min at 26.3h post-induction and then a further decrease which led to a final activity of 7385.7 mol/l/min at the end of the of the process (62.2h post-induction, 87.5 process time). The specific monoamine oxidase activity followed a similar pattern and peaked at 22h post-induction (169.5 mol/g DCW/min). Then it gradually decreased and reached a specific activity of 119.65 mol/g DCW/min by the end of the process. The pre-induction sample gave relatively high total and specific activities 295 mol/l/min and 5.1 mol/g DCW/min respectively.

5.1.4. Mixed feeds, Methanol – Sorbitol feed, (Methanol concentration in the culture uncontrolled)

As described in the literature, one way to reduce the long induction time required for maximal protein expression of slow methanol utilisation strains (as used here) is the addition of an alternative carbon source (Peng et al, 2004). In this process the non-repressive sugar sorbitol was used at a ratio of methanol to sorbitol of 2:1 fed at a constant rate.

In figure 5.3.A., it can be seen that the biomass reached a concentration of 54.4g/l just before the onset of methanol feed. At the end of the process (85.1h), biomass reached its maximum value of 79.8 g/l. The specific growth rate of the culture, after the beginning of the glycerol feeding and before methanol induction was $0.11h^{-1}$ while after methanol induction was reduced to $0.0057h^{-1}$.

In figure 5.3.A., it is obvious that the methanol feeding led to an increase of the residual methanol concentration to very high levels likely to cause growth inhibition to cells (Zhang et al, 2000a). The accumulation rate was approximately 0.44 g/l/h. The sorbitol concentration on the other hand increased without reaching high

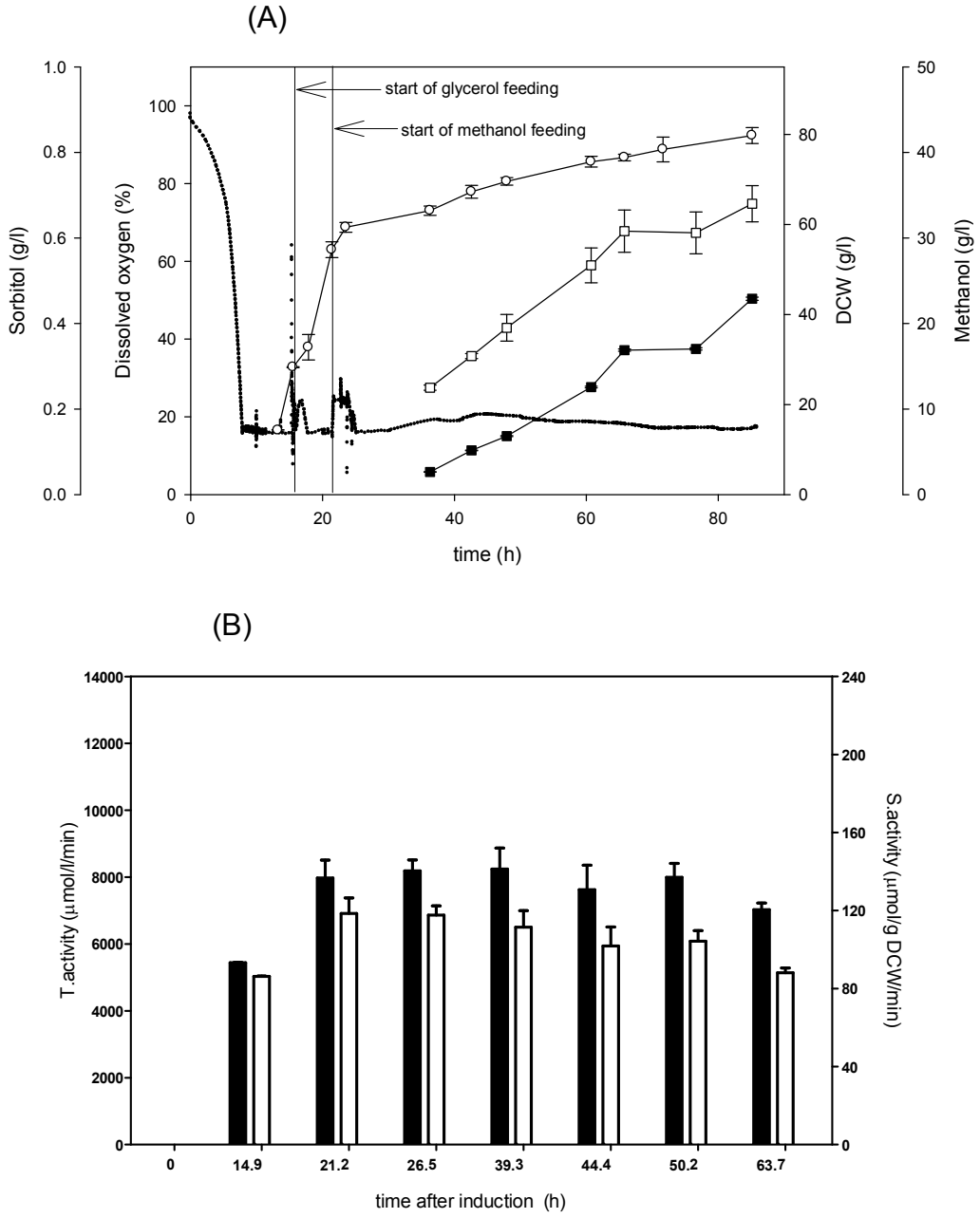

Figure 5.3. Time profiles of the (A) dissolved oxygen (●), biomass (○), methanol (□), sorbitol concentration (■) and (B) total (■) and specific MAO activities (□) of the *P.pastoris* cultures. Culture conditions: Bioreactor cultivations, 30^0C, pH 5.8, DO setpoint = 20%, 300-900rpm and 1-3vvm cascaded to DO.

concentrations. To be more specific, the maximum sorbitol concentration reached at the end of the process (85.1h) was 0.46 g/l.

The total monoamine oxidase activity increased from 5440 mol/l/min to 7975 mol/l/min (from 14.9h to 21.2h post-induction) and then remained relatively stable until 39.3h post-induction when it reached a peak of 8242 mol/l/min. After that point there was a slight decrease and at the end of the process the total activity was 7029 mol/l/min (85.1h process time). The specific activity followed a very similar pattern; however, it reached a peak at 21.2h after induction of 118.6 mol/g DCW/min. Then the specific activity gradually and slowly decreased until it reached 88.1 mol/g DCW/min at the end of the process.

5.1.5. Mixed feeds, Methanol Sorbitol feed, (with the methanol concentration in the culture controlled)

It has been reported that methanol accumulation in the culture can have negative effects on both growth and protein production (Zhang et al, 2000b). For that reason it is imperative to control the methanol concentration at levels which do not become toxic to the cells nor do they disrupt the recombinant protein production. In this run the YSI 2700 analyser was used for the rapid at line determination of the methanol concentration in the culture.

From Figure 5.4 it can be seen that the biomass concentration just before the start of the methanol induction was 58 g/l, while afterwards the culture biomass continued increasing until the end of the process when it reached its highest value of 97.4 g/l (85.6h). The specific growth rate after the onset of the glycerol feeding and before methanol induction was 0.10 h^{-1}, whereas after methanol induction it was reduced to 0.0084h^{-1}.

The efficient control of methanol feeding in this process resulted in methanol concentrations lower than 5g/l and close to limiting levels after 46.3h. The sorbitol

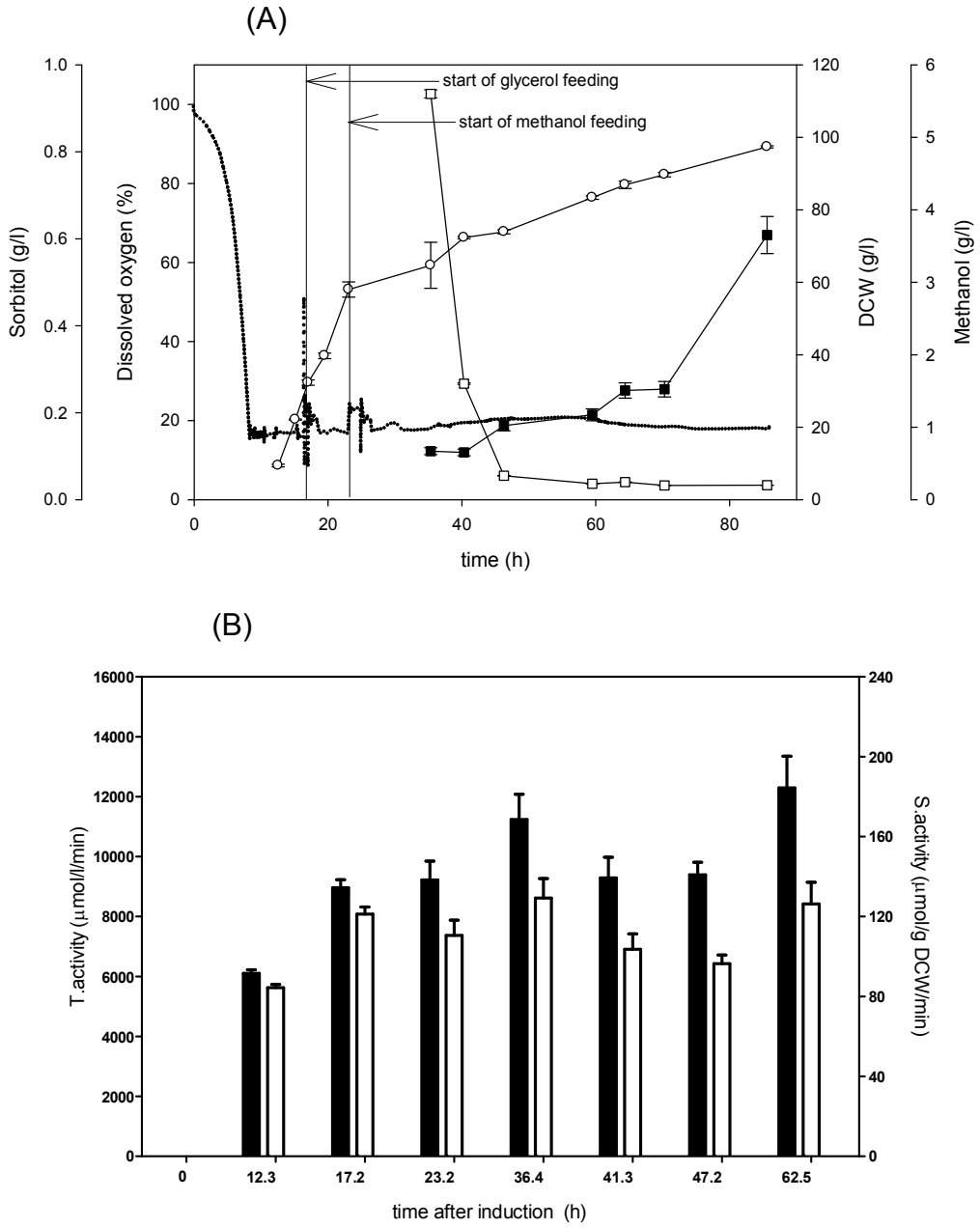

Figure 5.4 Time profiles of the (A) dissolved oxygen (●), biomass (○), methanol (□), sorbitol concentration (■) and (B) total (■) and specific MAO activities (□) of the *P.pastoris* cultures.Culture conditions: Bioreactor cultivations, 30^0C, pH 5.8, DO setpoint = 20%, 300-900rpm and 1-3vvm cascaded to DO.

concentration followed an upward trend, however, the maximum concentration reached at the end of process (85.6) was only 0.61 g/l.

Figure 5.4.B. shows that the total monoamine oxidase activity was steadily increasing from the onset of methanol induction until 36.4h post-induction when it reached a peak of 11236.7 mol/l/min. Afterwards, there was a transient decrease to lower levels (about 9200 mol/l/min) and by the end of the process (62.5h post-induction) a new, higher peak this time was achieved of 12295.1 mol/l/min. The highest specific monoamine oxidase activity was achieved at 36.4h post-induction (129.3 mol/g DCW/min) while at 47.2h post induction it decreased to 96.4 mol/g DCW/min before the end of the process when the specific activity increased again to 126.3 mol/g DCW/min.

5.1.6. Discussion

Interestingly enough, there are no significant differences during the batch phase in the biomass concentration between the culture supplemented with YNB and the one with YE. However, after 48.5h process time, the culture containing the yeast extract ceased to grow and there was a slight decrease in biomass indicating either cell autolysis or cell flocculation. Cell flocculation is a common feature in some yeasts and it takes place through the interaction between cell wall mannose and surface proteins and in particular zymolectins according to the lectin-carbohydrate bonding theory (Stratford, 1993). The flocs are further stabilised by calcium bridging where a calcium ion links two cells via negatively charged cell wall components. However, in the high shear stressed conditions prevailing in the bioreactor, it is rather unlikely that cells can be aggregated into flocs and either sediment or float (Speers et al, 2006). On the contrary, the YNB culture continued growing until the end of the process. This discrepancy in cell growth could be a sign of a nutrient limitation for the culture containing the YE. However, this happened only during the later stages of the process, while the incorporation of the supplements had been done in the batch medium. YNB contains a satisfactory nitrogen source in the form of ammonium

sulphate (5 g/l, which equates to1.06g of nitrogen/l), and the amino acids histidine HCl (10 mg/l), methionine (20 mg/l), tryptophan (20 mg/l). Moreover, it contains small amounts of vitamins (about 2mg/l of each). On the contrary yeast extracts powder composition is not well defined and it presents high batch-batch variability. The total nitrogen is about 10-12% (w/w) while the amino nitrogen is 5.1% (w/w) which gives about 1.34-1.68g/l total nitrogen in solution and 0.68g/l from amino nitrogen in solution. Yeast extract is a good source of amino-nitrogen and vitamins, particularly the water soluble B-complex vitamins. Therefore, the difference in the biomass formation cannot be attributed to nitrogen source limitations, especially if we bear in mind that ammonia is also added as the base in these processes, and B-complex vitamins are also present in the YE containing culture. Another possible reason for the biomass discrepancy could be the shortage of a particular amino acid which may be rarely needed by the host organism, but might be frequently encountered in the sequence of the recombinant product. Such amino acids in the YNB are histidine, methionine and tryptophan. According to Carnicer et al. (2009) the percentage (% mol/mol) of these amino acids in extracts of wild type *P.pastoris* strain growing in a chemostat culture are 1.89%, 0.79% and 1.40% respectively. The percentages of those amino acids in the recombinant product in the present study (MAO-5N) are 3.1%, 2.67% and 2.87% respectively. Bearing also in mind that the protein content of *P.pastoris* can reach 50% of the DCW and that the recombinant protein can occupy up to 40% of the total protein content (Cregg et al, 1993) and that the average concentrations of those amino acids in YE are very low, it could be assumed that the metabolism of the cells might be negatively affected by a lack of these amino acids. To confirm that, we would expect a similar and maybe greater negative effect on the recombinant protein production. However, even though the total monoamine oxidase activity is significantly higher for the YNB culture, the specific monoamine oxidase activity is only slightly higher which shows that the effect is basically deleterious for biomass and not for the specific recombinant protein production. It is worth noting though that the activity of the YE run presents a declining trend from 22h post-induction onwards, while that of the YNB is gradually increasing until the end of the process.

A mineral deficiency in the culture containing the yeast extract may also be considered especially if it is borne in mind the importance of metals such as zinc and magnesium (which are abundant in YNB) for the maintenance of yeast proper physiological conditions (Walker, 2004). However, both the batch medium and the methanol feed medium contain satisfactorily high concentrations of these minerals which makes this deficiency possibility unlikely.

Another possibility for the differences between the two runs, and the lower biomass and activity yields of the YE supplemented culture could be the lack of a vitamin needed as cofactor for the metabolism of one carbon compounds such as methanol (vitamins with such properties are biotin, folic acid and thiamine, which theoretically should be present in sufficient quantities in YE though). This possibility is further supported if the process in figure 5.4 is taken into consideration, as the addition of the sorbitol seemed to cause a steady increase of the biomass, and the activity levels followed a similar pattern with that of the YNB supplemented run. However, a definite conclusion cannot be drawn, as the amount of carbon source added in the MeOH Sorbitol 2:1 fed culture was higher than that of methanol alone fed culture.

As can be seen in figures 5.3. and 5.4., the accumulation of methanol in the culture had detrimental effects for both biomass and recombinant protein yield. The biomass concentration and total monoamine oxidase activity were significantly lower for the culture where methanol accumulation took place. However, the specific MAO activity, even though it was lower for the culture with the methanol accumulation, the difference was not significant, indicating that the high methanol concentration did not cause noteworthy damage to the recombinant protein production. Nevertheless, the lower biomasses observed in this culture, led to an overall reduction of the product yield. According to the literature, the toxicity of methanol is a result of the accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which are the oxidized products of methanol by the alcohol oxidase and are toxic to the cell (Couderc & Baratti, 1980; Zhang et al, 2000a). Under low methanol concentrations this toxicity is negated as this step in methanol metabolism takes place within the peroxisome, which sequesters toxic hydrogen peroxide away from

the rest of the cell and it is dismutated by the peroxisomal catalase to water and oxygen. Formaldehyde then either enters the xylulose-5- phosphate cycle or is degraded to carbon dioxide by the action of a NAD^+-dependent formaldehyde dehydrogenase and a NAD^+-dependent formate dehydrogenase, both located in the cytosol (van der Klei et al, 2006).

5.2. Selection of the appropriate Methanol, Sorbitol ratio induction/feeding for the optimisation of the total monoamine oxidase expression

5.2.1. Introduction

Three different ratios of methanol sorbitol feedings are presented below, the process with methanol sorbitol 2:1 which has been described above is included for comparison reasons. The target of this research is to increase the amount of carbon available to the cells through an increase of the feeding rate. Since an increase in methanol feeding will lead to methanol accumulation due to disruption of the *AOX1* gene of the slow methanol utilisation strain (mut^s) used in these experiments, the non repressible sugar sorbitol was chosen to enhance the carbon flux to *Pichia* cells. As described in the materials and methods (section 2.7.2.) the ratio is based on the amount of carbon in each of these two compounds. The carbon feed rate is manipulated by keeping the feeding rate of the methanol solution stable, while the sorbitol solution, which is co-fed, increases, thus increasing overall carbon flux, without inhibitory effects and so boosts the metabolism.

5.2.2. Results

Biomass concentrations

From figure 5.5.A. it is clear that the increase of the carbon feeding rate after induction results in an increase in biomass concentrations. The biomass concentration of the methanol : sorbitol 2:1 run is significantly higher than that of the methanol fed culture (figure 5.2.A.). The biomass concentration of the methanol : sorbitol 1:1 run is significantly higher than that of the methanol sorbitol 2:1. Finally,

the methanol sorbitol 1:2 feeding run exhibited significantly higher biomass concentrations than all the other cultures. Apart from the biomass levels, the enhancement of growth is also apparent in the specific growth rate values. The methanol fed culture of figure 5.2.A. had a post induction specific growth rate of $0.0063h^{-1}$, while the cultures co-fed with sorbitol showed 0.0084 h^{-1}, 0.0091 h^{-1} and 0.0096 h^{-1} for the methanol sorbitol cultures of 2:1, 1:1 and 1:2 ratios, respectively.

Respiratory quotient

It is clear from figure 5.5.B. that with the addition of sorbitol in the feed during induction, there was a statistically (t-test, $p<0.05$) significant increase in the respiratory quotient of the cultures. The average respiratory quotient of the cultures after methanol induction was 0.552, 0.608 and 0.789 for the methanol sorbitol cultures of 2:1, 1:1 and 1:2 ratios, respectively.

Total and specific monoamine oxidase activity

As can be seen in figure 5.6.A. the specific activity of the methanol sorbitol 1:1 fed culture increased very sharply from 22.4h to 44.4h process time, when it reached a value of 154.6 mol/g DCW/min. Then, it continued increasing and reached its peak of 173.9 mol/g DCW/min at 62.6h process time (40.2h post induction). Afterwards, until the end of the process, the specific activity fluctuated between 148.5 and 171.5 mol/g DCW/min. The specific activity of the methanol sorbitol 1:2 culture increased rapidly from 23.1h to 49.8h process time and then decelerated and it reached a peak of 134.1 mol/g DCW/min at 62.8h process time. After that point there was a gradual decrease in the specific activity to 113.6 mol/g DCW/min at 73.6h process time where it remained until the end of the process. The methanol sorbitol 1:1 fed culture not only achieved the highest specific activity value but also after 40h process time and until the end of the processes exhibited significantly higher specific monoamine oxidase activity than both the methanol sorbitol 2:1 and 1:2 cultures.

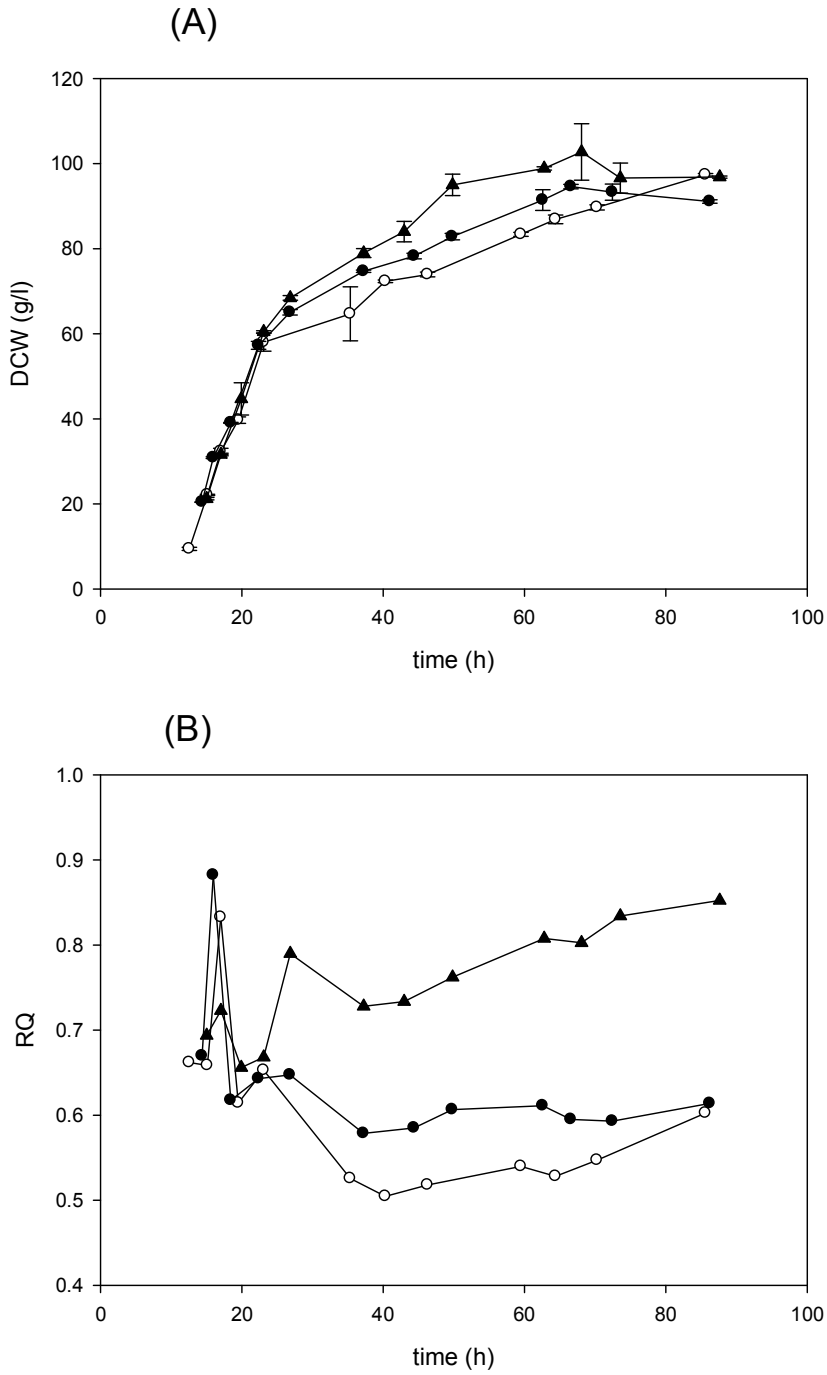

Figure 5.5. Time profiles of (A) biomass concentration and respiratory quotient (B) of the *P.pastoris* cultures with MeOH-Sorbitol feeding ratios 2-1 (○) , 1-1 (●), 1-2(▲).Culture conditions: Bioreactor cultivations, 30^0C, pH 5.8, DO setpoint = 20%, 300-900 rpm and 1-3vvm cascaded to DO.

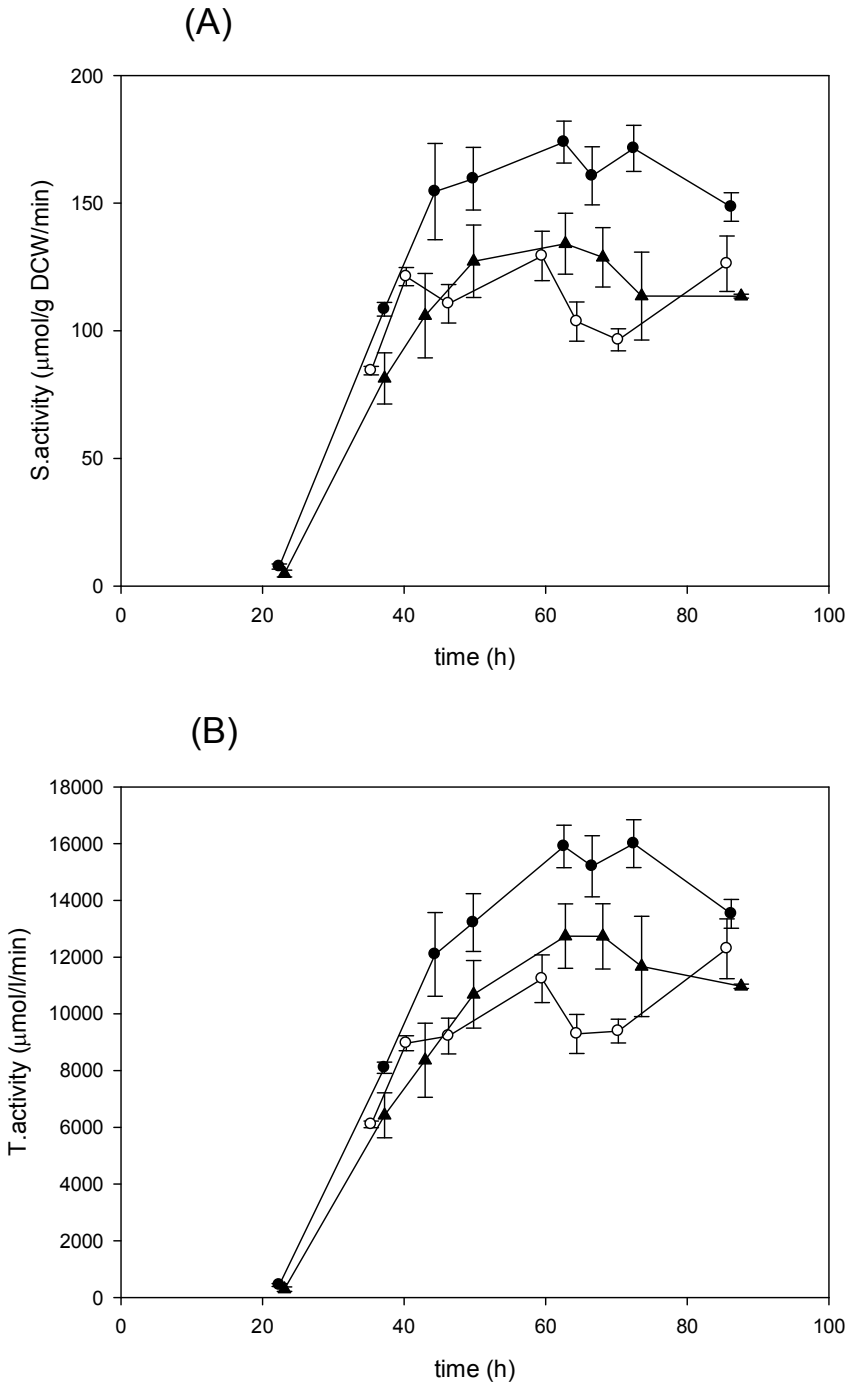

Figure 5.6. Specific (A) and total (B) monoamine oxidase activities of the *P.pastoris* cultures with MeOH-Sorbitol feeding ratios 2-1 (○), 1-1 (●), 1-2(▲). Culture conditions: Bioreactor cultivations, 30^0C, pH 5.8, DO setpoint = 20%, 300-900rpm and 1-3vvm cascaded to DO.

The total monoamine oxidase activity of the methanol sorbitol 1:1 fed culture increased very sharply and reached its peak of 16000 mol/l/min at 50.1h post-induction time (72.4h process time). After that point there was a decrease to 13526 mol/l/min by the end of the process. The total activity of the methanol sorbitol 1:2 fed culture reached its peak of 12740.8 mol/l/min at 62.8h process time where it remained stable for 5.5h after which it decreased to 10971.4 mol/l/min by the end of the process. Like in the specific activity, in total activity as well, the methanol sorbitol 1:1 fed culture reached the highest value, and generally exhibited significantly higher values than the rest of the cultures.

5.2.3. Discussion

The feeding strategy during induction of *P.pastoris* cultures, which also dictates the specific growth rate, is one of the most important factors for maximizing recombinant protein production, since all of the biochemical reactions for product formation are directly or indirectly associated with cell growth (Shioya, 1992). In a typical feeding protocol where methanol is used as the sole carbon and energy source (as well as inducer), the maximum achievable growth rate is very low as *P.pastoris* mut^s grows very slowly on methanol. According to Boze et al. (2001) the rate of the methanol feed is about three times lower with mut^s than with mut^+ strains in order to maintain the methanol concentration below a critical value. Therefore in this series of processes, the effects of increasing the amount of carbon flowing to the cells by increasing the feeding rate through the addition of an alternative non-repressive sugar as sorbitol was examined.

It is clear from the findings of this study that the ratio of methanol sorbitol 1:2 gave the best biomass yields. This was expected, as there was approximately 200% more carbon available than with the methanol feed alone, 150% more than with the methanol sorbitol ratio 2:1 and 100% more than with the methanol sorbitol ratio 1:1, and the additional sorbitol should not be associated with inhibition or repression. Therefore, the increase of the carbon feeding rate has a direct on the growth rates and the biomass concentrations as can be seen in the result section (5.2.2.). When it

comes to the recombinant monoamine oxidase activities though, things are more complex. The process with a methanol sorbitol ratio 1:1 feed presented by far the best activities. To be more specific, if we compare the sorbitol enhanced runs with the methanol only fed run (figure 5.2.) which can serve the role of control then the Methanol Sorbitol 2:1 run presented a 109.8% of the total MAO activity and 76.3% of the specific MAO activity of the control. In the case of Methanol Sorbitol 1:1, 142.9% of the total MAO activity and 102.6% of the specific MAO activity, while the Methanol Sorbitol 1:2 exhibited 113.8% of the total MAO activity and 79.1% of the specific MAO activity of the control. It is therefore, obvious that the increase of feed above certain limits did not have any positive effect on the specific activity but on the contrary it reduced it. The total MAO activity, however, was always higher for the sorbitol co-fed cultures than the control (methanol only fed culture) due to the positive effect of higher growth rates on biomass formation.

In other expression systems, such as *E.coli*, it has been reported that the increase of growth rates above a threshold limit reduces the recombinant protein production (Dong et al, 1995; Hoffmann & Rinas, 2004) as more carbon and energy is directed to biomass formation. It is very possible that the same happens in this expression system.

This is in agreement with the results of Boze et al. (2001) who found the maximum recombinant protein production of a mut^s *P.pastoris* was achieved at specific growth rate of 0.09 h^{-1} while the maximum biomass concentration was recorded at $= 0.1\ h^{-1}$. However, in that case different media components were added to the feeding medium in each experiment (vitamin and trace element in the first run and yeast extract in the latter run). In this work the maximum specific growth rate $=$ $0.096\ h^{-1}$ (Methanol sorbitol ratio 1:2) gave the highest biomass concentrations, however the maximum specific recombinant enzyme activity was achieved at a specific growth rate $= 0.091\ h^{-1}$ (Methanol Sorbitol 1:1).

A comparison between a methanol alone and a methanol sorbitol medium of a mut^s phenotype in batch cultures for the production of a lipase showed an 8.7-fold

increase in lipase productivity for the cells growing in the mixed medium. However, the addition of sorbitol for a mut^+ phenotype only slightly increased the productivity of the same enzyme in the batch culture (1.7-fold). The implementation of a mixed substrate strategy applied to a Mut^s fed-batch culture leading to a 2.2-fold increase in the volumetric heterologous protein productivity and 1.7-fold increase in the specific productivity, compared with the methanol-only feeding strategy (Ramon et al, 2007).

The use of either sorbitol or glycerol at a ratio 1:1 with methanol for the recombinant production of bovine enterokinase light chain by a mut^s *P.pastoris* led to an 1.7 and 2.3-fold increase of the volumetric productivity for the sorbitol and glycerol enhanced feed respectively (Peng et al, 2004). On the contrary, Thorpe et al. (1999) found that in the recombinant production of sea raven antifreeze protein by a mut^s *P.pastoris*, the use of sorbitol/methanol feeding strategy led to 1.3-fold higher specific productivity than that of a glycerol/methanol feeding strategy, even though the biomass yields were lower on sorbitol/methanol.

According to Resina et al. (2007), transcription appears to be the major limiting factor in extracellular *Rhizopus oryzae* lipase production by *P.pastoris* under growth-limiting conditions (0.005 to 0.01 h-1) the recombinant product secretion levels are higher at higher growth rates. However, at very high growth rates, a phenomenon known as unfolded protein response which deals with proteins not properly folded is observed, explaining the decreased recombinant protein production at very high specific growth rates. This is true for other fungal systems such *Trichoderma reesei* where the formation and secretion of the recombinant product is reduced in higher growth rates (Pakula et al, 2005).

To conclude, the increase of energy and carbon supply to recombinant *P.pastoris* cells using a non-repressing C source, seems to enhance both biomass formation and specific product production, however, for every expression system, it needs to be tested as to which specific growth rate is the maximum which can be achieved without overloading the translational machinery of the cells.

As stated in the literature, the adoption of a mixed feed strategy during induction reduces the time required for maximal protein expression (Celik et al, 2009). In this work the increase of sorbitol in the feed had as a direct effect the reduction of the time needed for the maximal monoamine oxidase activity. The maximal volumetric productivities were achieved at 62.5h, 50.1h and 39.7h post induction for the methanol sorbitol 2:1, 1:1 and 1:2 feed ratios. This reduction in induction time and hence in the overall process time can have a considerable reduction in the cost of a scaled up industrial process.

As can be seen in figure 5.5.B , (approximately after 27h process time) an important characteristic of the use of sorbitol in the induction feeding process is the increase of the respiratory quotient. As expected, sorbitol oxidation results in lower oxygen consumption rate for growth than methanol oxidation (since sorbitol is a more oxidised substrate than methanol) and as a result an increase of sorbitol concentration in the feed leads to an increase of the carbon evolution rate oxygen consumption rate ratio (respiratory quotient).

High oxygen requirements are very frequently a very important problem in high cell density cultures. *P.pastoris* culture can reach cell densities up 160 g/l DCW (Jungo et al, 2007b) and the high demand of oxygen due to the methanol reduction state necessitates the use of oxygen enriched air which increases the production costs and may cause difficulties in scale-up (Curvers et al, 2001), while at the same time, the direct contact of cells with oxygen from the oxygen enriched air potentially causes more damage to cell constituents and to the recombinant product than when air with only 21% O_2 comes to contact with the culture.

Since oxygen consumption rates and heat production are closely correlated during aerobic growth, less heat will be produced during mixed substrate growth on sorbitol and methanol than on methanol as sole carbon source or mixed feeds of glycerol and methanol (Wang et al, 2010). Indeed, considering that the enthalpy of combustion of sorbitol is about 8% lower than that of glycerol and about 30% lower than that of methanol, for a given growth rate less heat will be released in cultures with mixed

feeds of sorbitol and methanol than with feeds of methanol alone or mixed feeds of glycerol and methanol (Jungo et al, 2007b). It has been reported that reactor cooling frequently represent a major technical limitation for *P.pastoris* high cell density operations (D'Anjou & Daugulis, 2001; Hoeks et al, 2005).

Therefore, any method which reduces the oxygen consumption rate and the heat production rate without affecting recombinant protein productivity is advantageous. The culture of MeOH Sorbitol 1:1 feed ratio, presents the best option as there is a great increase in biomass, specific product activity and even though sorbitol increases the carbon flux there is only a moderate increase of the oxygen consumption rate and hence only a small increase of the heat evolution which dispenses with the need for oxygen enriched air and reduces the cooling requirements.

5.3. Comparison of *P.pastoris* and *E.coli* MAO producing cells in Biotransformation

5.3.1. Introduction

The overall aim of this work is the optimisation of the MAO-5N production for the subsequent use as a catalyst for the preparation of chiral amines. The simultaneous application of the enantioselective amine oxidase and a non selective reducing catalyst results in optically pure compounds. The enantioselective MAO oxidizes only the *S*-enantiomer to the corresponding imine, which is then reduced *in situ* back to the racemic amine. Repeated cycles result in eventual accumulation of the *R*-enantiomer which can reach 100% yield (Turner et al, 2005).

In order to evaluate the biotransformation capacity of the cells produced, a comparison between cells produced from different fermentation conditions was carried out. The fermentations which gave the highest monoamine oxidase activity yields were repeated and cells were harvested at the optimum time as had been identified from the earlier runs. The cells were initially freeze dried to cause cell permeabilisation and to maintain stability (Matsumoto et al, 2001) and then stored

until they were used for the biotransformation. In particular, the aerated *E.coli* fermentation run induced at OD = 55, the *P.pastoris* with methanol sorbitol induction feed ratio 1:1 and the *P.pastoris* standard protocol with YNB in the batch medium were repeated and tested in terms of the ability to effect biotransformation.

The use of whole cells as biocatalysts has been gaining a lot of attention as they can be much more readily and inexpensively prepared than the isolated enzymes. Moreover, enzymes in cells are protected from the external environment, and hence, they are generally more stable in the long-term than free enzymes (Ishige et al, 2005). This is especially important when the enzymes are oxidatively sensitive as appears to be the case for oxidoreductases such as D-amino acid oxidase, hydroxymethylfurfural oxidoreductase (Koopman et al, 2010; Pilone & Pollegioni, 2002; Slavica et al, 2005) and the MAO-5N used in the present study as proposed in chapter 4. The substrate used was a racemic mixture of a-methylbenzylamine as a model compound which its (*S*)-enantiomer is converted to acetophenone by the action of the recombinant enantioselective MAO-5N (figure 5.7.).

5.3.2. Results

The *E.coli* cells reached a substrate conversion of 19.2% at 1.5h and after that point there was only a very slight increase (reaching a maximum of 20.2% at 2h). The conversion of the *P.pastoris* cells followed a completely different pattern. The *P.pastoris* cells produced with the YNB protocol exhibited a very slow conversion rate in the beginning but finally reached a maximum conversion of 37.6% at 2h process time, while the *P.pastoris* cells of the optimised protocol (Methanol Sorbitol 1:1 ratio) presented a high initial conversion rate which was later reduced and reached the maximum of 40.4% at 2.2h. The dissolved oxygen concentration fell instantly to low levels after the addition of the cells for all three experiments. In particular, *E.coli* cells lowered the dissolved oxygen to 4.3 mg/l while the *P.pastoris*

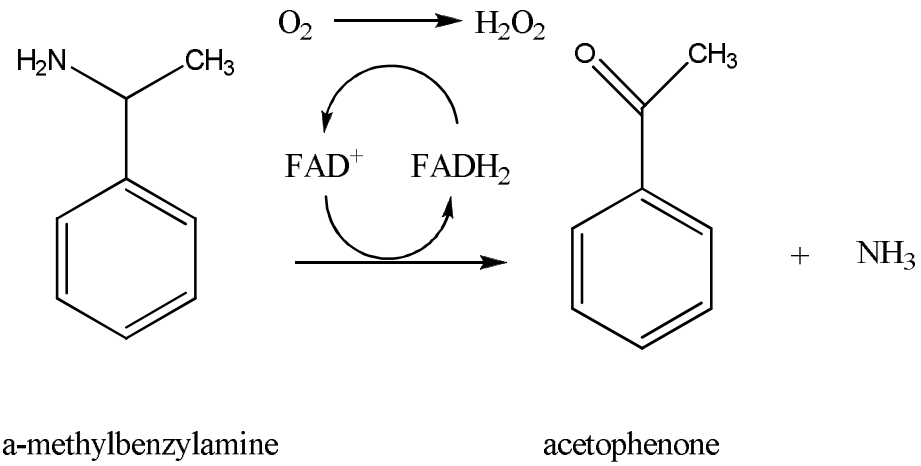

Figure 5.7. Conversion-oxidation of the a-*S*-methylbenzylamine to acetophenone and ammonia with the concomitant reduction of FAD^+ to $FADH_2$. The regeneration of the FAD^+ cofactor is facilitated by the simultaneous reduction of molecular oxygen to hydrogen peroxide.

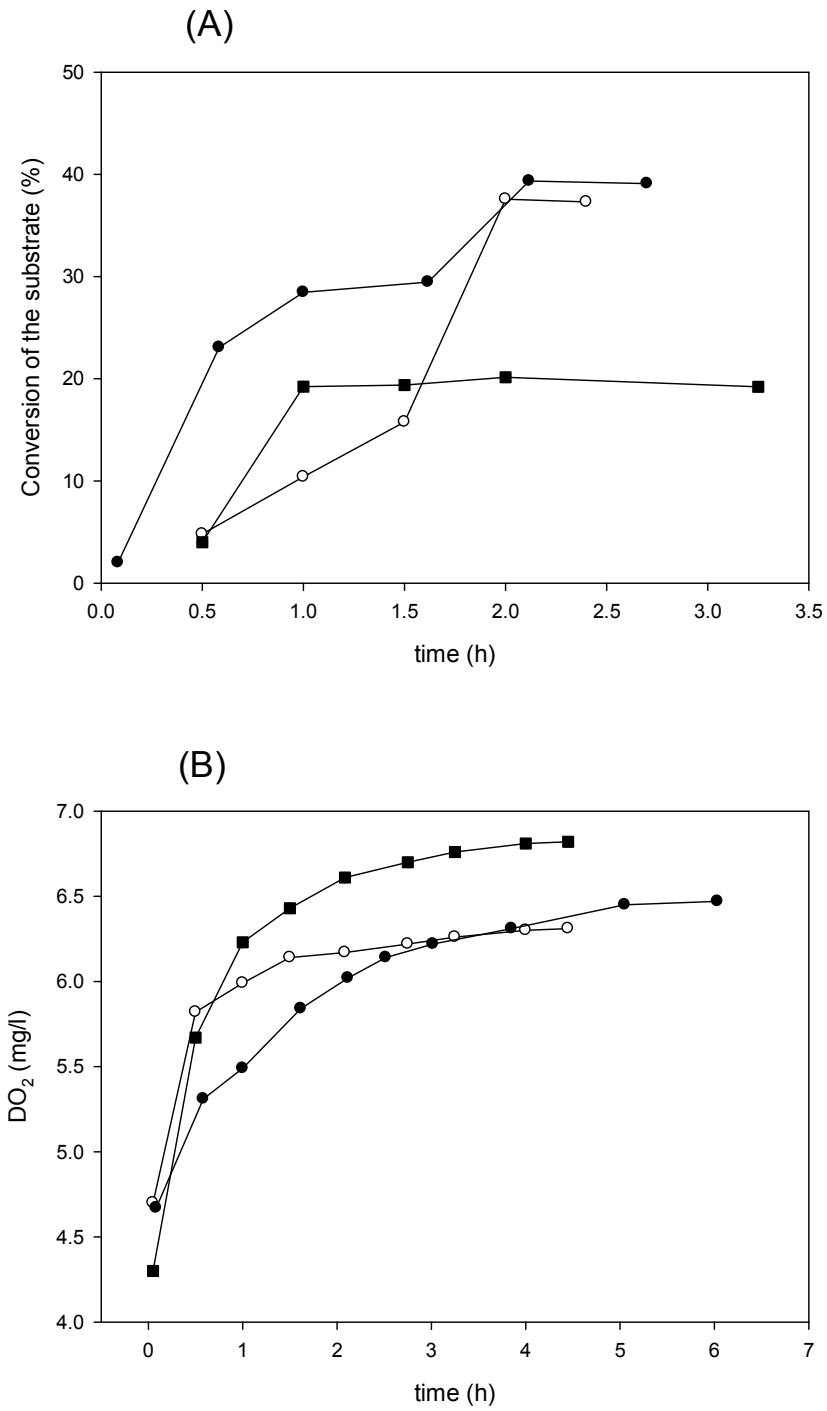

Figure 5.8. A.Conversion of a-*S*-methylbenzylamine to acetophenone and (B) dissolved oxygen concentration for the *E.coli* (■), *P.pastoris* non-optimised (○) and *P.pastoris* optimised (●) cultures. Conditions: cell loading 6g/l, 330rpm, 1vvm, 30^0C

cells to 4.7 mg/l. The dissolved oxygen of *E.coli* run very rapidly increased while the *P.pastoris* cells of the YNB protocol increased less sharply. The dissolved oxygen of the optimised *P.pastoris* fermentation protocol presented the slowest decrease of all three runs.

5.3.3. Discussion

A very important factor influencing biotransformation is the efficient cofactor regeneration (Schmid et al, 2001). From figure 5.7. it is evident that the current bioconversion has the advantage of relying on the provision of oxygen for recycling FAD^+ which simplifies the process. Figure 5.8.B. shows that oxygen was never the limiting factor of the biotransformation, therefore, the differences in conversions between the different processes can be attributed to other features.

It appears that the biotransformation of the a-*S*-methylbenzylamine by the *E.coli* cells proceeds at a slower pace and the enzyme is quickly deactivated as the conversion achieved is approximately half than that of the *P.pastoris* runs. It was expected that the *P.pastoris* cells would present higher conversion rate than the *E.coli* cells as the specific MAO activity of the former was 8.7 times higher. Whole cell biotransformations are severely dependent upon the barrier functions of cell wall and cell membranes. Therefore, a comparison between two different systems as *P.pastoris* and *E.coli* is difficult due to the different nature of those barriers. Gram negative bacteria such as *E.coli* containing 2 membranes and a peptidoglycan layer in-between, present a higher obstacle for the transport of substrate inside the cell than the eukaryotic yeast cell wall and membrane. A fact that may reduce the bioconversion ability of *E.coli* cells significantly.

The difference in the specific MAO activity between the *P.pastoris* cells of the YNB and the optimised protocol run is approximately 3% higher for the latter. As expected, there is barely any difference in the final conversion between the two cell types in the biotransformation. The optimised cells seem to convert the substrate much faster in the beginning; however, there is an abrupt deceleration at about 1h

which may be a result of the intracellular accumulation of the product. The ideal situation would be that once the a-*S*-methylbenzylamine is converted to acetophenone inside the cells and accumulated to an appropriate level, it would be released from cellular confinement and allow the product to be collected outside the cell (Chen, 2007). However, it is possible that acetophenone export may not be able to keep pace with its production rate, and hence product build-up takes place leading to lower substrate conversion rates due to higher competition between substrate and product in the active centre of the enzyme.

Chapter 6

Conclusions and Future work

6.1. Conclusions

- Despite the optimisations achieved in chapter 3, the homologous amine oxidase production from *A.niger* when using the native amine oxidase promoter system will always be limited by the generation of ammonia from the degradation of the amine, used as inducer, which strongly represses the expression of the gene. Therefore, the use of recombinant systems will always be advantageous. This is obvious in chapter 4 and 5 where the volumetric enzymatic activities were more than 10 and 300 fold higher for the *E.coli* and *P.pastoris* culture, respectively. Moreover, the fact that there is more than one gene expressing amine oxidases in the natural host, makes the selection of the appropriate one, in the case of the industrially useful FAD containing AO (MAO-N), more difficult requiring repression of the gene expressing copper containing amine oxidase or purification in a later stage (during downstream process). Both strategies will significantly increase the cost making the industrial use unfavourable.
- Regarding the expression of the mutated MAO-5N in *E.coli*, this work shows that the negative effects of oxygen previously reported in free enzyme preparations, are not limited to these acellular environments but are also discernible in the sheltered environment of the cytosol of *E.coli* cells. Therefore the optimal process for MAO-5N production is aerated, not oxygenated, and induced at moderate cell density, as it clearly represents a compromise between oxygen supply effects on specific growth rate/induction cell density, acetate accumulation, and high specific MAO-5N activity.
- The optimisation of the *P.pastoris* protocol for the production of MAO-5N showed that the most important flaw of the original protocol (similar to the one suggested by Invitrogen) is the inadequate amount of carbon supply to the cells in the feed during the induction phase. The methanol may be enough for the survival of the cells; however, it is not enough for a satisfactorily high production of the recombinant product.
- The use of sorbitol as an additional feed overcame this problem as both growth and the recombinant enzyme activity were improved when the carbon

flux was doubled and the specific growth rate reached the optimum value (section 5.2.3).

- However, as described in chapter 5, higher carbon feeding causes higher growth rates which lead to higher biomass levels but at the expense of the recombinant protein production.
- Reductions of the cost in both the *E.coli* and *P.pastoris* processes were achieved without compromising the yields. The replacement of expensive ingredients such as IPTG and YNB in the *E.coli* and *P.pastoris* process respectively and the reduction of the process time in *E.coli* culture through the increase of the feeding rate ultimately led to processes with increased yields, easy and cheap to reproduce and scale up.

6.2. Future work

The work presented in this thesis has provided much insight into the production of monoamine oxidase by various hosts, and significant optimisation in the bioprocesses has been achieved; however, in doing so it has generated various ideas and has widened the scope for future research.

Some suggestions regarding the work presented in chapter 3 are shown below:

- The use of a higher cell density *A.niger* culture for the homologous production of MAO. The use of a fed batch process would be an ideal situation to increase the volumetric productivity of the amine oxidase, however, such an attempt would necessitate an on- or at-line monitoring system for the determination of the concentration of the metabolisable inducer.
- As described earlier, the expression of amine oxidase in *A.niger* is limited by the ammonia produced from the inducers degradation. Therefore a way to alleviate this problem would be the search or construction of a slow or if possible non-metabolisable amine as an inducer which will induce the expression of amine oxidase and at the same time will not generate ammonia which represses the expression of the amine oxidase genes. Alternatively a successful strategy would be to place a strong and easy regulated promoter

upstream of the amine oxidase gene of interest so that the expression of the gene will be done under the regulatory control of a tight and easily induced promoter.

- It would be useful to repeat the experiment described in section 3.5. with the substitution of sodium nitrate with ammonia as primary nitrogen source. In this case the effect of butylamine formed would be easier to be observed as ammonia is assimilated through an $H^+/NH4^+$ antiport system which does not require the establishment of proton motive force and therefore cells can work in a wider range of pH's.

Regarding the work presented in chapter 4:

- It is of great interest to identify the exact nature of the proposed oxidation on the MAO-5N. The use of a mass spectrometric method enables the detection of the occurrence of particular amino acid modifications in proteins. The identification of the modification(s) can potentially give a solution based on the substitution of the oxidatively prone amino acid residues with other ones less prone to oxidations if these changes do not affect the catalytic properties of the enzyme. In addition, if the damage is attributable to reversible oxidative modifications, then strategies may be adopted to restore the activity of the enzyme after the end of the process.

As far as far as the work in chapter 5 is concerned:

- It would be interesting to determine if the negative effect of high oxygen availability on the activity of MAO-5N observed in the *E.coli* culture can occur in the MAO-5N in a *P.pastoris* culture. This can by achieved by comparing the enzyme activities between a culture run with dissolved oxygen set at very low dissolved oxygen tension with a culture run at relatively high dissolved oxygen levels.
- Regarding the biotransformations, the use of cell free extracts of MAO-5N produced by *P.pastoris* and *E.coli* cultures instead of whole cells would be an interesting comparison of the actual enzyme activity as the barriers of cell wall and membrane would be circumvented.

References

Ahamed A, Singh A, Ward O (2005) Culture-Based Strategies for Reduction of Protease Activity in Filtrates from *Aspergillus niger* NRRL-3. *World J Microb Biot* **21**(8)**:** 1577-1583

Ahamed A, Singh A, Ward O (2007) Chymostatin can combine with pepstatin to eliminate extracellular protease activity in cultures of *Aspergillus niger* NRRL-3. *J Ind Microbiol Biot* **34**(2)**:** 165-169

Ahmed S, Booth IR (1981) The effects of partial and selective reduction in the components of the proton-motive force on lactose uptake in *Escherichia coli*. *Biochem J* **200**(3)**:** 583-589

Åkesson M, Karlsson EN, Hagander P, Axelsson JP, Tocaj A (1999) On-line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. *Biotechnol Bioeng* **64**(5)**:** 590-598

Albert A (1985) *Selective Toxicity*, 7th edn. New York: John Wiley.

Alexeeva M, Enright A, Dawson MJ, Mahmoudian M, Turner NJ (2002) Deracemization of α - methylbenzylamine using an enzyme obtained by in vitro evolution. *Angew Chem Int Ed* **41**(17)**:** 3177-3180

Alonso J, Barredo JL, Armisen P, Dıez B, Salto F, Guisan JM, Garcia JL, Cortes E (1999) Engineering the D-amino acid oxidase from *Trigonopsis variabilis* to facilitate its overproduction in *Escherichia coli* and its downstream processing by tailor-made metal chelate supports. *Enzyme Microb Tech* **25:** 88–95

Altenbuchner J, Mattes R (2005) *Escherichia coli*. In *Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems*, Gellissen G (ed). Wiley-VCH

Andersen KB, von Meyenburg K (1980) Are growth rates of *Escherichia coli* in batch cultures limited by respiration? *J Bacteriol* **144**(1)**:** 114-123

Andersson L, Yang S, Neubauer P, Enfors S-O (1996) Impact of plasmid presence and induction on cellular responses in fed batch cultures of *Escherichia coli*. *J Biotechnol* **46:** 255-263

Andrews B, Adari H, Hannig G, Lahue E, Gosselin M, Martin S, Ahmed A, Ford PJ, Hayman EG, Makrides SC (1996) A tightly regulated high level expression vector that utilizes a thermosensitive lac repressor: production of the human T cell receptor V beta 5.3 in *Escherichia coli*. *Gene* **182**(1-2)**:** 101-109

Anthonsen T (2000) Reactions Catalyzed by Enzymes. In *Applied Biocatalysis*, Straathof AJJ, Adlercreutz P (eds), 2nd edn.: Harwood Academic Publishers

Archer DB, Jeenes DJ, Mackenzie DA (1994) Strategies for improving heterologous protein production from filamentous fungi. *Anton Leeuw* **65:** 245-250

Arís A, Corchero JL, Benito A, Carbonell X, Viaplana E, Villaverde A (1998) The expression of recombinant genes from bacteriophage lambda strong promoters triggers the SOS response in *Escherichia coli*. *Biotechnol Bioeng* **60**(5)**:** 551-559

Arnau C, Ramon R, Casas C, Valero F (2010) Optimization of the heterologous production of a Rhizopus oryzae lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. *Enzyme Microb Technol* **46**(6)**:** 494-500

Arst HN, MacDonald DW (1975) A gene cluster in Aspergillus nidulans with an internally located cis-acting regulatory region. *Nature* **254**(5495)**:** 26-31

Atkin KE, Reiss R, Koehler V, Bailey KR, Hart S, Turkenburg JP, Turner NJ, Brzozowski AM, Grogan G (2008) The structure of monoamine oxidase from *Aspergillus niger* provides a molecular context for improvements in activity obtained by directed evolution. *J Mol Biol* **384**(5)**:** 1218-1231

Axe DD, Bailey JE (1995) Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. *Biotechnol Bioeng* **47**(1)**:** 8-19

Ayadi DZ, Chouayekh H, Mhiri S, Zerria K, Fathallah DM, Bejar S (2007) Expression by *Streptomyces lividans* of the rat *a* Integrin CD11b A-Domain as a secreted and soluble recombinant protein. *J Biomed Biotechnol* **2007**(1)**:** Article ID 54327

Bailey KR, Ellis AJ, Reiss R, Snape TJ, Turner NJ (2007) A template-based mnemonic for monoamine oxidase (MAO-N) catalyzed reactions and its application to the chemo-enzymatic deracemisation of the alkaloid (+/-)-crispine A. *Chem Commun* (35)**:** 3640-3642

Baneyx F (1999) Recombinant protein expression in Escherichia coli. *Curr Opin Biotechnol* **10:** 411-421

Basker MJ, Comber KR, Sutherland R, Valler GH (1977) Carfecillin: Antibacterial activity *in vitro* and *in vivo*. *Chemotherapy* **23**(6)**:** 424-435

Belo I, Mota M (1998) Batch and fed-batch cultures of *E. coli* TB1 at different oxygen transfer rates. *Bioprocess Biosystems Eng* **18**(6)**:** 451-455

Bencina M, Bagar T, Lah L, Krasevec N (2009) A comparative genomic analysis of calcium and proton signaling/homeostasis in *Aspergillus* species. *Fungal Genet Biol* **46**(1, Supplement 1)**:** S93-S104

Berger M, Albrecht B, Berces A, Ettmayer P, Neruda W, Woisetschlager M (2001) S(+)-4-(1-Phenylethylamino)quinazolines as inhibitors of human immunoglobuline E synthesis: potency is Dictated by stereochemistry and atomic point charges at N-1. *J Med Chem* **44**(18)**:** 3031-3038

Berka RM, Dunn-Coleman NS, Ward M (1992) Industrial enzymes from *Aspergilli*. In *Aspergillus, biology and industrial applications*, Bennett JW, Klich MA (eds). Butterworth-Heinemann

Berlett BS, Levine RL, Stadtman ER (1996) Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* **271**(8)**:** 4177-4182

Bhattacharya SK, Dubey AK (1995) Metabolic burden as reflected by maintenance coefficient of recombinant *Escherichia coli* overexpressing target gene. *Biotechnol Lett* **17**(11)**:** 1155-1160

Bhattacharya SK, Dubey AK (1997) Effects of dissolved oxygen and oxygen mass transfer on overexpression of target gene in recombinant *E. coli*. *Enzyme Microb Technol* **20**(5)**:** 355-360

Blackwell JR, Horgan R (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Letters* **295**(1-3)**:** 10-12

Blaschko H (1963) *Amine oxidase*, Vol. VIII, London Academic Press.

Blaschko H, Richter D, Schlossmann H (1937) The oxidation of adrenaline and other amines. *Biochem J* **31**(12)**:** 2187–2196

Blatt MR, Maurousset L, Meharg AA (1997) High-Affinity NO^{-3}-H^{+} Cotransport in the Fungus *Neurospora*: Induction and control by pH and membrane voltage. *J Membr Biol* **160:** 59-76

Blommel PG, Becker KJ, Duvnjak P, Fox BG (2007) Enhanced bacterial protein expression during auto-induction obtained by alteration of Lac repressor dosage and medium composition. *Biotechnol Prog* **23:** 585-598

Boer L, Brouwer JW, Hassel CW, Levering PR, Dijkhuizen L (1989) Nitrogen metabolism in the facultative methylotroph *Arthrobacter* P1 grown with various amines or ammonia as nitrogen sources. *Anton Leeuw* **56**(3)**:** 221-232

Bommarius AS, Riebel BR (2004) *Biocatalysis, Fundamentals and Applications*: Wiley-VCH.

Borth N, Mitterbauer R, Mattanovich D, Kramer W, Bayer K, Katinger H (1998) Flow cytometric analysis of bacterial physiology during induction of foreign protein synthesis in recombinant *Escherichia coli* cells. *Cytometry* **31**(2)**:** 125-129

Boze H, Laborde C, Chemardin P, Richard F, Venturin C, Combarnous Y, Moulin G (2001) High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*. *Process Biochem* **36**(8-9)**:** 907-913

Brautaset T, Lale R, Valla S (2009) Positively regulated bacterial expression systems. *Microbial Biotech* **2**(1)**:** 15-30

Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS (1990) Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme. *Ann N Y Acad Sci* **589**(1)**:** 350-362

Buchholz KB, Poulsen PB (2000) Introduction. In *Applied Biocatalysis*, Straathof AJJ, Adlercreutz P (eds), 2nd edn.: Harwood Academic Publishers

Bukau B, Deuerling E, Pfund C, Craig EA (2000) getting newly synthesized proteins into shape. *Cell* **101**(2)**:** 119-122

Cabiscol E, Tamarit J, Ros J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* **3:** 3-8

Cabrita L, Bottomley S (2004) How do proteins avoid becoming too stable? Biophysical studies into metastable proteins. *Eur Biophys J* **33**(2)**:** 83-88

Carlile MJ, Gooday GW, Watkinson SC (2001) Fungal cells and vegetative growth. In *The Fungi*. 2nd edn.: Academic press

Carnicer M, Baumann K, Toplitz I, Sanchez-Ferrando F, Mattanovich D, Ferrer P, Albiol J (2009) Macromolecular and elemental composition analysis and extracellular metabolite balances of *Pichia pastoris* growing at different oxygen levels. *Microb Cell Fact* **8**(1)**:** 65

Carr R, Alexeeva M, Dawson MJ, Gotor-Fernαndez V, Humphrey CE, Turner NJ (2005) Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines. *ChemBioChem* **6**(4)**:** 637-639

Cashel M, Gentry DR, Hernandez VJ, Vinella D (1996) The stringent response. In *Escherichia coli and Salmonella – cellular and molecular biology*, Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Rfznikopp WS, Riley M, Schaechter M, Umbarger HE (eds). ASM Press

Castan A, Näsman A, Enfors S-O (2002a) Oxygen enriched air supply in *Escherichia coli* processes: production of biomass and recombinant human growth hormone. *Enzyme Microb Technol* **30**(7)**:** 847-854

Celik E, Calık P, Oliver SG (2009) Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. *Yeast* **26**(9)**:** 473-484

Cereghino GPL, Cereghino JL, Ilgen C, Cregg JM (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Curr Opin Biotechnol* **13**(4)**:** 329-332

Cereghino GPL, Cregg JM (1999) Applications of yeast in biotechnology: protein production and genetic analysis. *Curr Opin Biotechnol* **10**(5)**:** 422-427

Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris FEMS Microbiol Rev* **24**(1)**:** 45-66

Challener CA (2004) *Chiral Intermediates*: Wiley & Sons

Chang D-E, Smalley DJ, Conway T (2002) Gene expression profiling of growth transitions: an expanded stringent response model. *Mol Microbiol* **45**(2)**:** 289-306

Charoenrat T, Ketudat-Cairns M, Stendahl-Andersen H, Jahic M, Enfors S-O (2005) Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. *Bioprocess Biosystems Eng* **27**(6)**:** 399-406

Cheetham PSJ (1999) Enzymes, for flavor production. In *Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation*, Flickinger MC, Drew SW (eds). Wiley & Sons

Chen R (2007) Permeability issues in whole-cell bioprocesses and cellular membrane engineering. *Applied Microbiology and Biotechnology* **74**(4)**:** 730-738

Chen Y, Cino J, Hart G, Freedman D, White C, Komives EA (1997) High protein expression in fermentation of recombinant *Pichia pastoris* by a fed-batch process. *Process Biochem* **32**(2)**:** 107-111

Chew LC, Ramseier TM, Retallack DM, Schneider JC, Squires CH, Talbot HW (2005) *Pseudomonas fluorescens*. In *Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems*, Gellissen G (ed), pp 45-66. Wiley-VCH Verlag GmbH & Co

Chiruvolu V, Cregg JM, Meagher MM (1997) Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. *Enzyme Microb Technol* **21**(4)**:** 277-283

Chiruvolu V, Eskridge K, Cregg J, Meagher M (1998) Effects of glycerol concentration and pH on growth of recombinant *Pichia pastoris* yeast. *Appl Biochem Biotechnol* **75**(2)**:** 163-173

Chung JD (2000) Design of metabolic feed controllers: Application to high-density fermentations of *Pichia pastoris*. *Biotechnol Bioeng* **68**(3)**:** 298-307

Colby J, Zatman LJ (1975) Enzymological aspects of the pathways for trimethylamine oxidation and C1 assimilation of obligate methylotrophs and restricted facultative methylotrophs. *Biochem J* **148**(3)**:** 513-520

Contiero J, Beatty C, Kumari S, DeSanti CL, Strohl WR, Wolfe A (2000) Effects of mutations in acetate metabolism on high-cell-density growth of *Escherichia coli*. *J Ind Microbiol Biotechnol* **24**(6)**:** 421-430

Cos O, Ramon R, Montesinos J, Valero F (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. *Microbial Cell Factories* **5**(1)**:** 17

Cos O, Resina D, Ferrer P, Montesinos JL, Valero F (2005) Heterologous production of Rhizopus oryzae lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. *Biochem Eng J* **26**(2-3)**:** 86-94

Couderc R, Baratti J (1980) Oxidation of methanol by the yeast, *Pichia pastoris*. Purification and properties of the alcohol oxidase *Agric Biol Chem* **44**(10)**:** 2279-2289

Cregg J, Cereghino J, Shi J, Higgins D (2000) Recombinant protein expression in *Pichia pastoris Mol Biotechnol* **16**(1)**:** 23-52

Cregg JM, Madden KR (1988) Development of the methylotrophic yeast, *Pichia pastoris*, as a host system for the production of foreign proteins. *Dev Ind Microbiol* **29:** 33–41

Cregg JM, Vedvick TS, Raschke WC (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. *Nat Biotech* **11**(8)**:** 905-910

Crowley J, Arnold SA, Wood N, Harvey LM, McNeil B (2005) Monitoring a high cell density recombinant *Pichia pastoris* fed-batch bioprocess using transmission and reflectance near infrared spectroscopy. *Enzyme Microb Technol* **36**(5-6)**:** 621-628

Curran B, Bugeja V (2005) The biotechnological exploitation of heterologous protein production in fungi. In *Fungi biology and applications*, Kavanagh K (ed). John Wiley & Sons Ltd

Curvers S, Brixius P, Klauser T, Thömmes J, Weuster-Botz D, Takors R, Wandrey C (2001) Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Part 1. Fermentation. *Biotechnol Prog* **17**(3)**:** 495-502

D'Anjou MC, Daugulis AJ (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. *Biotechnol Bioeng* **72**(1)**:** 1-11

da Silva NA, Bailey JE (1986) Theoretical growth yield estimates for recombinant cells *Biotechnol Bioeng* **28**(5)**:** 741-746

Daly R, Hearn MTW (2005) Expression of heterologous proteins in *Pichia pastori*: a useful experimental tool in protein engineering and production. *J Mol Recognit* **18**(2)**:** 119-138

Davies J, Wright GD (1997) Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol* **5**(6)**:** 234-240

de la Mata I, Ramón F, Obregón V, Castillón MP, Acebal C (2000) Effect of hydrogen peroxide on D-amino acid oxidase from *Rhodotorula gracilis*. *Enzyme Microb Technol* **27**(3-5)**:** 234-239

De Leon A, Hernandez V, Galindo E, Ramvrez OT (2003) Effects of dissolved oxygen tension on the production of recombinant penicillin acylase in *Escherichia coli*. *Enzyme Microb Technol* **33**(5)**:** 689-697

De Mey M, De Maeseneire S, Soetaert W, Vandamme E (2007) Minimizing acetate formation in *E. coli* fermentations. *J Ind Microbiol Biotechnol* **34**(11)**:** 689-700

Dean RT, Fu S, Stocker R, Davies MJ (1997) Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* **324**(1)**:** 1-18

Debusk RM, Ogilvie S (1984) Participation of an extracellular deaminase in amino acid utilization by *Neurospora crassa*. *J bacteriology* **159:** 583-589

DeLisa MP, Li J, Rao G, Weigand WA, Bentley WE (1999) Monitoring GFP-operon fusion protein expression during high cell density cultivation of *Escherichia coli* using an on-line optical sensor. *Biotechnol Bioeng* **65**(1)**:** 54-64

Delroisse J-M, Dannau M, Gilsoul J-J, El Mejdoub T, Destain J, Portetelle D, Thonart P, Haubruge E, Vandenbol M (2005) Expression of a synthetic gene encoding a *Tribolium castaneum* carboxylesterase in *Pichia pastoris*. *Protein Expression Purif* **42**(2)**:** 286-294

Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. *Biotechnol Adv* **27**(3)**:** 297-306

Dijken JP, Bos P (1981) Utilization of amines by yeasts. *Arch Microbiol* **128**(3)**:** 320-324

Dijkhuizen L, Boer L, Boers RH, Harder W, Konings WN (1982) Uptake of methylamine via an inducible, energy-dependent transport system in the facultative methylotroph *Arthrobacter* P1. *Arch Microbiol* **133**(4)**:** 261-266

Dittrich CR, Vadali RV, Bennett GN, San K-Y (2005) Redistribution of metabolic fluxes in the central aerobic metabolic pathway of *E.coli*. Mutant strains with deletion of the *acka-pta* and *poxb* pathways for the synthesis of isoamyl acetate. *Biotechnol Prog* **21**(2)**:** 627-631

Dong H, Nilsson L, Kurland CG (1995) Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J Bacteriol* **177:** 1497–1504

Donovan RS, Robinson CW, Glick BR (1996) Optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. *J Ind Microbiol Biot* **16**(3)**:** 145-154

Döring F, Klapper M, Theis S, Daniel H (1998) Use of the glyceraldehyde-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast *Pichia pastoris*. *Biochem Biophys Res Commun* **250**(2)**:** 531-535

Downey RJ, AGedeon C (1994) Evidence for a H^+ nitrate symporter in *Aspergillus nidulans*. *Microbios* **78**(314)**:** 35-46

Dubendorff JW, Studier FW (1991) Creation of a T7 autogene : Cloning and expression of the gene for bacteriophage T7 RNA polymerase under control of its cognate promoter. *J Mol Biol* **219**(1)**:** 61-68

Dunsmore CJ, Carr R, Fleming T, Turner NJ (2006) A chemo-enzymatic route to enantiomerically pure cyclic tertiary amines. *J Am Chem Soc* **128**(7)**:** 2224-2225

Dunster CA, Cheeseman KH, Maddix SP (1997) The effect of oxidative stress on the production of the recombinant protein, interferon γ, produced by Chinese hamster ovary cells in stirred-batch culture. *Appl Microbiol Biotechnol* **48**(2)**:** 198-203

Dykhuizen D, Hartl D (1978) Transport by the lactose permease of *Escherichia coli* as the basis of lactose killing. *J Bacteriol* **135**(3)**:** 876-882

Eaton P (2006) Protein thiol oxidation in health and disease: Techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radical Biol Med* **40**(11)**:** 1889-1899

Eddy AA, Hopkins PG (1985) The putative electrogenic nitrate-proton symport of the yeast *Candida utilis*. Comparison with the systems absorbing glucose or lactate. *Biochem J* **231** (2)**:** 291–297

Eiteman MA, Altman E (2006) Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends Biotechnol* **24**(11)**:** 530-536

El-Mansi E (1997) Diversion of carbon flux from central metabolism to product formation. *Soc Gen Microbiol Symp* **145**(17)

El-Mansi EMT, Holms WH (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *J Gen Microbiol* **135**(11)**:** 2875-2883

El-Mansi M (2004) Flux to acetate and lactate excretions in industrial fermentations: physiological and biochemical implications. *J Ind Microbiol Biotechnol* **31**(7)**:** 295-300

Faber K (2000) *Biotransformations in organic chemistry* 4th edn.: Springer-Verlag.

Faber KN, Harder W, Ab G, Veenhuis M (1995) Methylotrophic yeasts as factories for the production of foreign proteins. *Yeast* **11**(14)**:** 1331-1344

Farr SB, Kogoma T (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Mol Biol Rev* **55**(4)**:** 561-585

Ferndahl C, Bonander N, Logez C, Wagner R, Gustafsson L, Larsson C, Hedfalk K, Darby R, Bill R (2010) Increasing cell biomass in *Saccharomyces cerevisiae* increases recombinant protein yield: the use of a respiratory strain as a microbial cell factory. *Microb Cell Fact* **9**(1)**:** 47

Files D, Ogawa M, Scaman CH, Baldwin SA (2001) A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-C. *Enzyme Microb Technol* **29**(6-7)**:** 335-340

Fleming GT, Patching JW (2008) The fermenter in research and development. In *Practical Fermentation Technology*, McNeil B, Harvey LM (eds). John Wiley & Sons

Fotheringham I, Archer I, Carr R, Speight R, Turner NJ (2006) Preparative deracemization of unnatural amino acids. *Biochem Soc Trans* **34**(Pt 2)**:** 287-290

Fraaije M, van Berkel W (2006) Flavin-Containing Oxidative Biocatalysts. In *Biocatalysis in the Pharmaceutical and Biotechnology Industries*. CRC Press

Frébort I, Adachi O (1995) Copper/quinone-containing amine oxidases, an exciting class of ubiquitous enzymes. *J Ferment Bioeng* **80**(6)**:** 625-632

Frébort I, Matsushita K, Adachi O (1997) Involvement of multiple copper/tops quinone-containing and flavin-containing amine oxidases and NAD(P)$^+$ aldehyde dehydrogenases in amine degradation by filamentous fungi. *J Ferment Bioeng* **84:** 200-212

Frébort I, Peg P, Luhovfi L, Toyama H, Matsushita K, Hirota S, Kitagawa T, Ueno T, Asano Y, Kato Y, Adachi O (1996) Two amine oxidases from *Aspergillus niger* AKU 3302 contain topa quinone as the cofactor: unusual cofactor link to the glutamyl residue occurs only at one of the enzymes. *Biochim Biophys Acta* **1295:** 59-72

Frébort I, Tanaka S, Matsushita K, Adachi O (2000) Cellular localization and metabolic function of n-butylamine-induced amine oxidases in the fungus *Aspergillus niger* AKU 3302. *Arch Microbiol* **173:** 358–365

Frère JM (1995) Beta-lactamases and bacterial resistance to antibiotics. *Mol Microbiol* **16**(3)**:** 385-395

Fucci L, Oliver CN, Coon MJ, Stadtman ER (1983) Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing. *Proc Natl Acad Sci U S A* **80**(6)**:** 1521-1525

Gamer AO, Heliwig J, van Ravenzwaay B (2002) Developmental toxicity of oral n-butylamine hydrochloride and inhaled n-butylamine in rats. *Food Chem Toxicol* **40**(12)**:** 1833-1842

Gasser B, Mattanovich D (2007) Antibody production with yeasts and filamentous fungi: on the road to large scale? *Biotechnol Lett* **29**(2)**:** 201-212

Gellissen G (2000) Heterologous protein production in methylotrophic yeasts. *Appl Microbiol Biotechnol* **54**(6)**:** 741-750

Ghezzi P, Bonetto V (2003) Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* **3**(7)**:** 1145-1153

Gill NK, Appleton M, Baganz F, Lye GJ (2008) Design and characterisation of a miniature stirred bioreactor system for parallel microbial fermentations. *Biochem Eng J* **39**(1)**:** 164-176

Glick BR (1995) Metabolic load and heterologous gene expression *Biotechnol Adv* **13:** 247-261

Goff SA, Goldberg AL (1987) An increased content of protease La, the *lon* gene product, increases protein degradation and blocks growth in *Escherichia coli*. *J Biol Chem* **262**(10)**:** 4508-4515

Gokarn RR, Evans JD, Walker JR, Martin SA, Eiteman MA, Altman E (2001) The physiological effects and metabolic alterations caused by the expression of *Rhizobium etli* pyruvate carboxylase in *Escherichia coli*. *Appl Microbiol Biotechnol* **56**(1)**:** 188-195

Gombert AK, Kilikian BV (1998) Recombinant gene expression in *Escherichia coli* cultivation using lactose as inducer. *J Biotechnol* **60:** 47–54

Gouka RJ, Punt PJ, Hondel CAMJJvd (1997) Efficient production of secreted proteins by *Aspergillus* : progress, limitations and prospects. *Appl Microbiol Biotechnol* **47:** 1-11

Goyal D, Sahni G, Sahoo DK (2009) Enhanced production of recombinant streptokinase in *Escherichia coli* using fed-batch culture. *Bioresour Technol* **100**(19)**:** 4468-4474

Grassin C, Fauguenbergue P. (1999) Enzymes, fruit juice processing. In Flickinger MC, Drew SW (eds.), *Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation*. Wiley & Sons.

Green J, Haywood GW, Large PJ (1983) Serological differences between the multiple amine oxidases of yeasts and comparison of the specificities of the purified enzymes from *Candida utilis* and *Pichia pastoris*. *Biochem J* **211:** 481-493

Green J, Large PJ (1983) Oxidation of dimethylamine and trimethylamine in methazotrophic yeasts by microsomal mono-oxygenases sensitive to carbon monoxide. *Biochem Biophys Res Commun* **113**(3)**:** 900-907

Gustafsson C, Govindarajan S, Minshull J (2004) Codon bias and heterologous protein expression. *Trends Biotechnol* **22**(7)**:** 346-353

Hacisalihoglu A, Jongejan JA, Duine JA (1997) Distribution of amine oxidases and amine dehydrogenases in bacteria grown on primary amines and characterization of the amine oxidase from *Klebsiella oxytoca*. *Microbiology* **143:** 505–512

Hammarström M, Hellgren N, van den Berg S, Berglund H, Härd T (2002) Rapid screening for improved solubility of small human proteins produced as fusion proteins in *Escherichia coli*. *Protein Sci* **11**(2)**:** 313-321

Han K, Lim HC, Hong J (1992) Acetic acid formation in *Escherichia coli* fermentation. *Biotechnol Bioeng* **39**(6)**:** 663-671

Hannig G, Makrides SC (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends Biotechnol* **16**(2)**:** 54-60

Harcum S, Bentley W (1999) Heat-shock and stringent responses have overlapping protease activity in *Escherichia coli*. *Appl Biochem Biotechnol* **80**(1)**:** 23-37

Harcum SW (2002) Structured model to predict intracellular amino acid shortages during recombinant protein overexpression in *E. coli*. *J Biotechnol* **93**(3)**:** 189-202

Hare ML (1928) Tyramine oxidase: A new enzyme system in liver. *Biochem J* **22**(4)**:** 968-979

Hartl F-U, Hlodan R, Langer T (1994) Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem Sci* **19**(1)**:** 20-25

Hartner F, Glieder A (2006) Regulation of methanol utilisation pathway genes in yeasts. *Microb Cell Fact* **5**(1)**:** 39

Hartsock CE, Lewis JK, Leslie I, Pope JA, Tsai LB, Sachdev R, Meng S-Y (1995) Assessing post-induction cellular response in a recombinant *E.coli* lactose-inducible system by monitoring β-galactosidase levels. *Biotechnol Lett* **17**(10)**:** 1025-1030

Henderson KW, Kerr WJ, Moir JH (2000) Enantioselective deprotonation reactions using a novel homochiral magnesium amide base. *Chem Commun* **31**(27)**:** 479 - 480

Hengge-Aronis R (1999) Interplay of global regulators and cell physiology in the general stress response of *Escherichia coli*. *Curr Opin Microbiol* **2**(2)**:** 148-152

Hoeks FW, Schmidhalter DR, Gloeckler R, Herwig C, Theriault K, S. VF, Broek Wd, Laukel F (2005) BPMSS Lonza's biopharmaceutical small scale plant started cGMP manufacturing in September 2004. *Chimia* **59:** 31-33

Hoffman BJ, Broadwater JA, Johnson P, Harper J, Fox BG, Kenealy WR (1995) Lactose fed-batch overexpression of recombinant metalloproteins in *Escherichia coli* BL21(DE3): Process control yielding high levels of metal-incorporated, soluble protein. *Protein Expression Purif* **6**(5)**:** 646-654

Hoffmann F, Arís A, Carbonell X, Rohde M, Corchero JL, Rinas U, Villaverde A (1999) Heat-inactivation of plasmid-encoded CI857 repressor induces gene expression from Ind− lambda prophage in recombinant Escherichia coli. *FEMS Microbiol Lett* **177**(2)**:** 327-334

Hoffmann F, Rinas U (2001) On-line estimation of the metabolic burden resulting from the synthesis of plasmid-encoded and heat-shock proteins by monitoring respiratory energy generation. *Biotechnol Bioeng* **76**(4)**:** 333-340

Hoffmann F, Rinas U (2004) Stress induced by recombinant protein production in *Escherichia coli*. *Adv Biochem Engin/Biotechnol* **89:** 73–92

Hoffmann F, Weber J, Rinas U (2002) Metabolic adaptation of *Escherichia coli* during temperature-induced recombinant protein production: 1. Readjustment of metabolic enzyme synthesis. *Biotechnol Bioeng* **80**(3)**:** 313-319

Höhne M, Bornscheuer UT (2009) Biocatalytic routes to optically active amines. *ChemCatChem* **1**(1)**:** 42-51

Holms H (1996) Flux analysis and control of the central metabolic pathways in *Escherichia coli*. *FEMS Microbiol Rev* **19**(2)**:** 85-116

Hopkins DJ, Betenbaugh MJ, Dhurjati P (1987) Effects of dissolved oxygen shock on the stability of recombinant *Escherichia coli* containing plasmid pKN401. *Biotechnol Bioeng* **29**(1)**:** 85-91

Horiguchi H, Yurimoto H, Goh T-K, Nakagawa T, Kato N, Sakai Y (2001) Peroxisomal catalase in the methylotrophic yeast *Candida boidinii*: transport efficiency and metabolic significance *J Bacteriol* **183**(21)**:** 6372-6383

Houard S, Heinderyckx M, Bollen A (2002) Engineering of non-conventional yeasts for efficient synthesis of macromolecules: the methylotrophic genera. *Biochimie* **84**(11)**:** 1089-1093

Ikura K, Kokubu T, Natsuka S, Ichikawa A, Adachi M, Nishihara K, Yanagi H, Utsumi S (2002) Co-overexpression of folding modulators improves the solubility of the recombinant guinea pig liver transglutaminase expressed in *Escherichia coli*. *Prep Biochem Biotechnol* **32**(2)**:** 189 - 205

Ilgen C, Lin-Cereghino J, Cregg JM (2005) *Pichia pastoris*. In *Production of recombinant proteins. Novel microbial and eucaryotic expression systems.*, Gellissen G (ed). Weinheim Wiley-VCH

Inan M, Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (*AOX1*) promoter of Pichia pastoris. *J Biosci Bioeng* **92**(6)**:** 585-589

Ishige T, Honda K, Shimizu S (2005) Whole organism biocatalysis. *Curr Opin Chem Biol* **9**(2)**:** 174–180

Iuchi S, Weiner L (1996) Cellular and molecular physiology of *Escherichia coli* in the adaptation to aerobic environments. *J Biochem* **120**(6)**:** 1055-1063

Jahic M (2003) Process techniques for production of recombinant proteins with *Pichia pastoris*. PhD Thesis, Department of Biotechnology, Royal Institute of Technology, Stockholm

Janes S, Mu D, Wemmer D, Smith A, Kaur S, Maltby D, Burlingame A, Klinman J (1990) A new redox cofactor in eukaryotic enzymes: 6-hydroxydopa at the active site of bovine serum amine oxidase. *Science* **248**(4958)**:** 981-987

Jensen EB, Carlsen S (1990) Production of recombinant human growth hormone in *Escherichia coli*: Expression of different precursors and physiological effects of glucose, acetate, and salts. *Biotechnol Bioeng* **36**(1)**:** 1-11

Johnston JP (1968) Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem Pharmacol* **17**(7)**:** 1285-1297

Jones MG (2007) The first filamentous fungal genome sequences: *Aspergillus* leads the way for essential everyday resources or dusty museum specimens? *Microbiology* **153**(1)**:** 1-6

Joosten V, van Berkel WJH (2007) Flavoenzymes. *Curr Opin Chem Biol* **11**(2)**:** 195-202

Ju S-S, Lin L-L, Chien HR, Hsu W-H (2000) Substitution of the critical methionine residues in *Trigonopsis variabilis* D-amino acid oxidase with leucine enhances its resistance to hydrogen peroxide. *FEMS Microbiol Lett* **186**(2)**:** 215-219

Jürgen B, Lin HY, Riemschneider S, Scharf C, Neubauer P, Schmid R, Hecker M, Schweder T (2000) Monitoring of genes that respond to overproduction of an insoluble recombinant protein in *Escherichia coli* glucose-limited fed-batch fermentations. *Biotechnol Bioeng* **70**(2)**:** 217-224

Jungo C, Marison I, von Stockar U (2007a) Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: A quantitative study based on concentration gradients in transient continuous cultures. *J Biotechnol* **128**(4)**:** 824-837

Jungo C, Schenk J, Pasquier M, Marison IW, von Stockar U (2007b) A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*. *J Biotechnol* **131**(1)**:** 57-66

Karthikeyan S, Zhou Q, Mseeh F, Grishin NV, Osterman AL, Zhang H (2003) Crystal structure of human riboflavin kinase reveals a barrel fold and a novel active site arch. *Structure* **11**(3)**:** 265-273

Keck RG (1996) The use of t-butyl hydroperoxide as a probe for methionine oxidation in proteins. *Anal Biochem* **236**(1)**:** 56-62

Kilikian BV, Suarez ID, Liria CW, Gombert AK (2000) Process strategies to improve heterologous protein production in *Escherichia coli* under lactose or IPTG induction. *Process Biochem* **35:** 1019–1025

Kim S-J, Kim N-J, Shin C-H, Kim C-W (2008) optimization of culture condition for the production of d-amino acid oxidase in a recombinant *Escherichia coli*. *Biotechnol. Bioprocess Eng.* **13**(2)**:** 144-149

Kim YH, Berry AH, Spencer DS, Stites WE (2001) Comparing the effect on protein stability of methionine oxidation versus mutagenesis: steps toward engineering oxidative resistance in proteins. *Protein Eng* **14**(5)**:** 343-347

Klinman JP (1996) New quinocofactors in eukaryotes. *J Biol Chem* **271**(44)**:** 27189–27192

Knoll J, Magyar K (1972) Some puzzling pharmacological effects of monoamine oxidase inhibitors. *Adv Biochem Psychopharmacol* **5:** 393-408

Koda A, Bogaki T, Minetoki T, Hirotsune M (2005) High expression of a synthetic gene encoding potato alpha-glucan phosphorylase in *Aspergillus niger*. *J Biosci Bioeng* **100**(5)**:** 531-537

Koh BT, Nakashimada U, Pfeiffer M, Yap MGS (1992) Comparison of acetate inhibition on growth of host and recombinant *E. coli* K12 strains. *Biotechnol Lett* **14**(12)**:** 1115-1118

Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ (2010) Efficient whole-cell biotransformation of 5-(hydroxymethyl)furfural into FDCA, 2,5-furandicarboxylic acid. *Bioresource Technol* **101**(16)**:** 6291-6296

Korant BD, Rizzo CJ (1991) An *E. coli* expression system which detoxifies the HIV protease. *Biomed Biochim Acta* **50:** 643-646

Korpimäki T, Kurittu J, Karp M (2003) Surprisingly fast disappearance of beta-lactam selection pressure in cultivation as detected with novel biosensing approaches. *J Microbiol Methods* **53**(1)**:** 37-42

Kuczynska-Wisnik D, Kedzierska S, Matuszewska E, Lund P, Taylor A, Lipinska B, Laskowska E (2002) The *Escherichia coli* small heat-shock proteins IbpA and IbpB prevent the aggregation of endogenous proteins denatured in vivo during extreme heat shock. *Microbiology* **148**(6)**:** 1757-1765

Kurland CG, Dong H (1996) Bacterial growth inhibition by overproduction of protein. *Mol Microbiol* **21**(1)**:** 1-4

Kuroki Y, Rao Juvvadi P, Arioka M, Nakajima H, Kitamoto K (2002) Cloning and characterization of vmaA, the gene encoding a 69-kDa catalytic subunit of the vacuolar H+-ATPase during alkaline pH mediated growth of *Aspergillus oryzae*. *FEMS Microbiol Lett* **209**(2)**:** 277-282

Lages F, Silva-Graca M, Lucas C (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. *Microbiology* **145**(9)**:** 2577-2585

Lederberg J, Lederberg EM (1952) Replica plating and indirect selection of bacterial mutants. *J Bacteriol* **63**(3)**:** 399-406

Lee B, Yurimoto H, Sakai Y, Kato N (2002a) Physiological role of the glutathione-dependent formaldehyde dehydrogenase in the methylotrophic yeast *Candida boidinii*. *Microbiology* **148**(9)**:** 2697-2704

Lee CY, Nakano A, Shiomi N, Lee EK, Katoh S (2003) Effects of substrate feed rates on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation. *Enzyme Microb Technol* **33**(4)**:** 358-365

Lee J, Kim H-C, Kim S-W, Kim SW, Hong SI, Park Y-H (2002b) Interplay of SOS induction, recombinant gene expression, and multimerization of plasmid vectors in *Escherichia coli*. *Biotechnol Bioeng* **80**(1)**:** 84-92

Levering PR, Dijken JPv, Veenhuis M, Harder W (1981) *Arthrobacter* P 1, a fast growing versatile methylotroph with amine oxidase as a key enzyme in the metabolism of methylated amines. *Arch Microbiol* **129:** 72-80

Li Q, Harvey LM, McNeil B (2008) The effects of elevated process temperature on the protein carbonyls in the filamentous fungus, *Aspergillus niger* B1-D. *Process Biochem* **43**(8)**:** 877-881

Li X, Robbins JW, Taylor KB (1992) Effect of the levels of dissolved oxygen on the expression of recombinant proteins in four recombinant *Escherichia coli* strains. *J Ind Microbiol Biotechnol* **9**(1)**:** 1-9

Lilie H, Schwarz E, Rudolph R (1998) Advances in refolding of proteins produced in *E. coli*. *Curr Opin Biotechnol* **9:** 497-501

Lindley ND (1987) Metabolism of Alkyl Amines by the Filamentous Fungus Aspergillus versicolor. *Appl Environ Microbiol* **53:** 246-248

Liu H, Tan X, Russell KA, Veenhuis M, Cregg JM (1995) PER3, a gene required for peroxisome biogenesis in *Pichia pastoris*, encodes a peroxisomal membrane protein involved in protein import. *J Biol Chem* **270**(18)**:** 10940-10951

Liu YC, Chang WM, Lee CY (1999) Effect of oxygen enrichment aeration on penicillin G acylase production in high cell density culture of recombinant *E. coli*. *Bioprocess Biosystems Eng* **21**(3)**:** 227-230

Loewen MC, Liu X, Davies PL, Daugulis AJ (1997) Biosynthetic production of type II fish antifreeze protein: fermentation by *Pichia pastoris*. *Appl Microbiol Biotechnol* **48**(4)**:** 480-486

Losen M, Frölich B, Pohl M, Büchs J (2004) Effect of oxygen limitation and medium composition on *Escherichia coli* fermentation in shake-flask cultures. *Biotechnol Prog* **20**(4)**:** 1062-1068

Lubertozzi D, Keasling JD (2009) Developing *Aspergillus* as a host for heterologous expression. *Biotechnol Adv* **27**(1)**:** 53-75

Luli GW, Strohl WR (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl Environ Microbiol* **56**(4)**:** 1004-1011

Macaloney G, Hall JW, Rollins MJ, Draper I, Anderson KB, Preston J, Thompson BG, McNeil B (1997) The utility and performance of near-infra red spectroscopy in simultaneous monitoring of multiple components in a high cell density recombinant *Escherichia coli* production process. *Bioprocess Biosystems Eng* **17**(3)**:** 157-167

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* **22**(4)**:** 249-270

Machida M (2002) Progress of *Aspergillus oryzae* genomics. In *Advances in Applied Microbiology* Vol. Volume 51, pp 81-98. Academic Press

Mack M, Wannemacher M, Hobl B, Pietschmann P, Hock B (2009) Comparison of two expression platforms in respect to protein yield and quality: *Pichia pastoris* versus *Pichia angusta*. *Protein Expression Purif* **66**(2)**:** 165-171

Makrides SC (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev* **60**(3)**:** 512–538

Manning M, Chou D, Murphy B, Payne R, Katayama D (2010) Stability of protein pharmaceuticals: an update. *Pharm Res* **27**(4)**:** 544-575

Manstein DJ, Pai EF (1986) Purification and characterization of FAD synthetase from *Brevibacterium ammoniagenes*. *J Biol Chem* **261**(34)**:** 16169-16173

March JC, Eiteman MA, Altman E (2002) Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in *Escherichia coli*. *Appl Environ Microbiol* **68**(11)**:** 5620-5624

Marx H, Mattanovich D, Sauer M (2008) Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris*. *Microbial Cell Factories* **7**(1)**:** 23

Marzluf G (1997) Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* **61**(1)**:** 17-32

Matsumoto, Takahashi, Kaieda, Ueda, Tanaka, Fukuda, Kondo (2001) Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae* lipase is applicable to biodiesel fuel production. *Appl Microbiol Biot* **57**(4)**:** 515-520

Mattanovich D, Gasser B, Hohenblum H, Sauer M (2004) Stress in recombinant protein producing yeasts. *J Biotechnol* **113**(1-3)**:** 121-135

Mattanovich D, Graf A, Stadlmann J, Dragosits M, Redl A, Maurer M, Kleinheinz M, Sauer M, Altmann F, Gasser B (2009) Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*. *Microbial Cell Factories* **8**(1)**:** 29

Mattevi A (2006) To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. *Trends Biochem Sci* **31**(5)**:** 276-283

Mattevi A, Vanoni MA, Todone F, Rizzi M, Teplyakov A, Coda A, Bolognesi M, Curti B (1996) Crystal structure of D-amino acid oxidase: a case of active site mirror-image convergent evolution with flavocytochrome b2. *Proc Natl Acad Sci U S A* **93**(15)**:** 7496-7501

Mayer M, Buchner J (2006) Refolding of inclusion body proteins *Methods Mol Med* **94**:239-254.

Menendez J, Valdes I, Cabrera N (2003) The ICL1 gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. *Yeast* **20**(13)**:** 1097-1108

Menzella HG, Ceccarelli EA, Gramajo HC (2003) Novel *Escherichia coli* strain allows efficient recombinant protein production using lactose as inducer. *Biotechnol Bioeng* **82**(7)**:** 809-817

Middelberg APJ (2002) Preparative protein refolding. *Trends Biotechnol* **20**(10)**:** 437-443

Middelhoven WJ, Niet MCH-t, Rij NJWK-v (1984) *Trichosporon adeninovorans* sp. nov., a yeast species utilizing adenine, xanthine, uric acid, putrescine and primary n-alkylamines as the sole source of carbon, nitrogen and energy. *Anton Leeuw* **50:** 369-378

Mierendorf RC, Morris BB, Hammer B, Novy RE (1998) Expression and purification of recombinant proteins using the pET system *Methods Mol Med* **13:** 257–292

Miller KD, Weaver-Feldhaus J, Gray SA, Siegel RW, Feldhaus MJ (2005) Production, purification, and characterization of human scFv antibodies expressed in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Escherichia coli*. *Protein Expression Purif* **42**(2)**:** 255-267

Mogk A, Mayer MP, Deuerling E (2002) Mechanisms of protein folding: molecular chaperones and their application in biotechnology. *ChemBioChem* **3**(9)**:** 807 - 814

Mohammadian-Mosaabadi J, Naderi-Manesh H, Maghsoudi N, Khalilzadeh R, Shojaosadati SA, Ebrahimi M (2005) Effect of oxidative stress on the production of recombinant human interferon-γ in *Escherichia coli*. *Biotechnol Appl Biochem* **41:** 37–42

Molla G, Motteran L, Piubelli L, Pilone MS, Pollegioni L (2003) Regulation of D-amino acid oxidase expression in the yeast *Rhodotorula gracilis*. *Yeast* **20**(12)**:** 1061-1069

Mousdale DM (2007) Metabolic analysis and optimization of microbial and animal cell bioprocesses In *Fermentation microbiology and biotechnology*, El-Mansi EMT, Bryce CFA, Demain AL, Allman AR (eds), 2nd edn.: Taylor & Francis

Müller S, Sandal T, Kamp-Hansen P, Dalbøge H (1998) Comparison of expression systems in the yeasts *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Klyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. *Yeast* **14**(14)**:** 1267-1283

Murooka Y, Doi N, Harada T (1979) Distribution of membrane-bound monoamine oxidase in bacteria. *Appl Environ Microbiol* **38:** 565-569

Murphy RA, Horgan KA (2005) Antibiotics, enzymes and chemical commodities from fungi. In *Fungi : Biology and applications*, Kavanagh K (ed). John Wiley & Sons

Nakano K, Rischke M, Sato S, Märkl H (1997) Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor. *Appl Microbiol Biotechnol* **48**(5)**:** 597-601

Narhi LO, Arakawa s, Aoki KH, Elmore R, Rohde MF, Boone T, Strickland TW (1991) The effect of carbohydrate on the structure and stability of erythropoietin. *J Biol Chem* **266**(34)**:** 23022-23026

Nelson DL, Cox MM (eds) (2004) *Lehninger Principles of Biochemistry*: W. H. Freeman

Nelson G, Kozlova-Zwinderman O, Collis AJ, Knight MR, Fincham JRS, Stanger CP, Renwick A, Hessing JGM, Punt PJ, Van Den Hondel CAMJJ, Read ND (2004) Calcium measurement in living filamentous fungi expressing codon-optimized aequorin. *Mol Microbiol* **52**(5)**:** 1437-1450

Nicholls DG, Ferguson SJ (2001) *Bioenergetics* 3rd edn. London: Academic Press.

Nieuwenhuijzen JW, Grimbergen RFP, Koopman C, Kellogg RM, Vries TR, Pouwer K, Echten Ev, Kaptein B, Hulshof LA, Broxterman QB (2002) The role of nucleation inhibition in optical resolutions with families of resolving agents13. *Angew Chem Int Edit* **41**(22)**:** 4281-4286

Nishihara K, Kanemori M, Yanagi H, Yura T (2000) Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Appl Environ Microbiol* **66**(3)**:** 884-889

O'Donnell D, Wang L, Xu J, Ridgway D, Gu T, Moo-Young M (2001) Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. *Biochem Eng J* **8**(3)**:** 187-193

Ochiai Y, Itoh K, Sakurai E, Adachi M, Tanaka Y (2006) Substrate selectivity of monoamine oxidase A, monoamine oxidase B, diamine oxidase, and semicarbazide-sensitive amine oxidase in COS-1 expression systems. *Biol Pharm Bull* **29**(12)**:** 2362-2366

Orman MA, Calik P, Özdamar TH (2009) The influence of carbon sources on recombinant-human- growth-hormone production by *Pichia pastoris* is dependent on phenotype: a comparison of Mut^s and Mut^+ strains. *Biotechnol Appl Biochem* **52**(3)**:** 245-255

Pakula TM, Salonen K, Uusitalo J, Penttila M (2005) The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. *Microbiology* **151**(1)**:** 135-143

Patel RN, Hou CT, Derelanko P (1983) Microbial oxidation of methanol: Purification and properties of formaldehyde dehydrogenase from a *Pichia* sp. NRRL-Y-11328. *Arch Biochem Biophys* **221**(1)**:** 135-142

Peng L, Zhong X, Ou J, Zheng S, Liao J, Wang L, Xu A (2004) High-level secretory production of recombinant bovine enterokinase light chain by *Pichia pastoris*. *J Biotechnol* **108**(2)**:** 185-192

Pestka S, Kivie Moldave LG (1974) [28] The use of inhibitors in studies of protein synthesis. In *Methods in Enzymology* Vol. Volume 30, pp 261-282. Academic Press

Petsch D, Anspach FB (2000) Endotoxin removal from protein solutions. *J Biotechnol* **76**(2-3)**:** 97-119

Pilone MS, Pollegioni L (2002) D-amino acid oxidase as an industrial biocatalyst. *Biocatal Biotransfor* **20:** 145–159

Pimienta E, Ayala J, Rodriguez C, Ramos A, Van Mellaert L, Vallin C, Anne J (2007) Recombinant production of *Streptococcus equisimilis* streptokinase by *Streptomyces lividans*. *Microb Cell Fact* **6**(1)**:** 20

Pollegioni L, Campaner S, Raibekas AA, Pilone MS (1997) Identification of a reactive cysteine in the flavin-binding domain of *Rhodotorula gracilis* D-amino acid oxidase. *Arch Biochem Biophys* **343**(1)**:** 1-5

Potvin G, Ahmad A, Zhang Z (2010) Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. *Biochem Eng J* **In Press, Corrected Proof**

Prior JE, Lynch MD, Gill RT (2010) Broad-host-range vectors for protein expression across gram negative hosts. *Biotechnol Bioeng* **106**(2)**:** 326-332

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol* **20**(5)**:** 200-206

Qoronfleh M (1999) Dissolved oxygen concentration affects the accumulation of HIV-1 recombinant proteins in *Escherichia coli*. *Appl Biochem Biotechnol* **80**(2)**:** 107-120

Ramon R, Feliu JX, Cos O, Montesinos JL, Berthet FX, Valero F (2004) Improving the monitoring of methanol concentration during high cell density fermentation of *Pichia pastoris*. *Biotechnol Lett* **26**(18)**:** 1447-1452

Ramon R, Ferrer P, Valero F (2007) Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*. *J Biotechnol* **130**(1)**:** 39-46

Ratledge C (2006) biochemistry and physiology of growth and metabolism. In *Basic Biotechnology* Ratledge C, Kristiansen B (eds), 3rd edn.

Ren H, Yuan J (2005) Model-based specific growth rate control for *Pichia pastoris* to improve recombinant protein production. *J Chem Tech Biot* **80**(11)**:** 1268-1272

Ren HT, Yuan JQ, Bellgardt KH (2003) Macrokinetic model for methylotrophic *Pichia pastoris* based on stoichiometric balance. *J Biotechnol* **106**(1)**:** 53-68

Repaske DR, Adler J (1981) Change in intracellular pH of *Escherichia coli* mediates the chemotactic response to certain attractants and repellents. *J Bacteriol* **145**(3)**:** 1196-1208

Resina D, Bollok M, Khatri N, Valero F, Neubauer P, Ferrer P (2007) Transcriptional response of P. pastoris in fed-batch cultivations to *Rhizopus oryzae* lipase production reveals UPR induction. *Microbial Cell Factories* **6**(1)**:** 21

Rinas U, Tsai LB, Lyons D, Fox GM, Stearns G, Fieschko J, Fenton D, Bailey JE (1992) Cysteine to serine substitutions in basic fibroblast growth factor: effect on inclusion body formation and proteolytic susceptibility during in vitro refolding. *Nat Biotech* **10**(4)**:** 435-440

Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* **8**(7)**:** 519-529

Sahin-Tóth M, Kaback HR (2001) Arg-302 facilitates deprotonation of Glu-325 in the transport mechanism of the lactose permease from *Escherichia coli*. *Proc Natl Acad Sci U S A* **98**(11)**:** 6068-6073

Sakai Y, Murdanoto A, Konishi T, Iwamatsu A, Kato N (1997) Regulation of the formate dehydrogenase gene, *FDH1*, in the methylotrophic yeast *Candida boidinii* and growth characteristics of an *FDH1*-disrupted strain on methanol, methylamine, and choline. *J Bacteriol* **179**(14)**:** 4480-4485

Sakai Y, Nakagawa T, Shimase M, Kato N (1998) Regulation and Physiological Role of the DAS1 gene, encoding dihydroxyacetone synthase, in the methylotrophic yeast *Candida boidinii*. *J Bacteriol* **180**(22)**:** 5885-5890

Schilling B, Lerch K (1995a) Amine oxidases from *Aspergillus niger*: identification of a novel flavin-dependent enzyme. *Biochim Biophys Acta* **1243:** 529-537

Schilling B, Lerch K (1995b) Cloning, sequencing and heterologous expression of the monoamine oxidase gene from *Aspergillus niger*. *Mol Gen Genet* **247:** 430-438

Schleif R (1996) Two Positively Regulated Systems, *ara* and *mal*. In *Escherichia coli and Salmonella – cellular and molecular biology*, Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Rfznikopp WS, Riley M, Schaechter M, Umbarger HE (eds). ASM Press

Schlieker C, Bukau B, Mogk A (2002) Prevention and reversion of protein aggregation by molecular chaperones in the *E. coli* cytosol: implications for their applicability in biotechnology. *J Biotechnol* **96:** 13–21

Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. *Nature* **409**(6817)**:** 258-268

Schmidt M, Viaplana E, Hoffmann F, Marten S, Villaverde A, Rinas U (1999) Secretion-dependent proteolysis of heterologous protein by recombinant *Escherichia coli* is connected to an increased activity of the energy-generating dissimilatory pathway. *Biotechnol Bioeng* **66**(1)**:** 61-67

Schuster, Dunn C, Frisvad, van D (2002) On the safety of *Aspergillus niger* – a review. *Appl Microbiol Biot* **59**(4)**:** 426-435

Sears IB, O'Connor J, Rossanese OW, Glick BS (1998) A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. *Yeast* **14**(8)**:** 783-790

Shacter E (2000) Quantification and significance of protein oxidation in biological samples. *Drug Metab Rev* **32**(3)**:** 307 - 326

Shen S, Sulter G, Jeffries TW, Cregg JM (1998) A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. *Gene* **216**(1)**:** 93-102

Shih JC, Thompson RF (1999) Monoamine oxidase in neuropsychiatry and behavior. *Am J Hum Genet* **65**(3)**:** 593-598

Shioya S (1992) Optimization and control in fed-batch bioreactors. In *Modern Biochemical Engineering* Vol. 46, pp 111-142. Springer Berlin / Heidelberg

Sies H (1993) Strategies of antioxidant defense. *Eur J Biochem* **215**(2)**:** 213-219

Sikora L, Marzluf GA (1982) Regulation of L-amino acid oxidase and of D-amino acid oxidase in *Neurospora crassa*. *Mol Gen Genet* **186:** 33-39

Slavica A, Dib I, Nidetzky B (2005) Single-site oxidation, cysteine 108 to cysteine sulfinic acid, in D-amino acid oxidase from *Trigonopsis variabilis* and its structural and functional consequences. *Appl Environ Microbiol* **71**(12)**:** 8061-8068

Sørensen HP, Mortensen KK (2005a) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J Biotechnol* **115:** 113–128

Sørensen HP, Mortensen KK (2005b) Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb Cell Fact* **4** doi: 10.1186/1475-2859-4-1.

Soriano E, Borth N, Katinger H, Mattanovich D (1999) Flow Cytometric Analysis of Metabolic Stress Effects Due to Recombinant Plasmids and Proteins in Escherichia coli Production Strains. *Metab Eng* **1**(3)**:** 270-274

Speers RA, Wan Y-Q, Jin Y-L, Stewart RJ (2006) Effects of fermentation parameters and cell wall properties on yeast flocculation. *J Inst Brew* **112:** 246–254

Spencer R, Fisher J, Walsh C (1976) Preparation, characterization, and chemical properties of the flavin coenzyme analogues 5-deazariboflavin, 5-deazariboflavin 5'-phosphate, and 5-deazariboflavin 5'-diphosphate, 5'leads to5'-adenosine ester. Biochemistry **15**(5)**:**1043-1053

Sreekrishna K, Flickinger MC (2009) *Pichia, Optimization of Protein Expression*: John Wiley & Sons, Inc.

Stadtman ER (1990) Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Radical Biol Med* **9**(4)**:** 315-325

Stadtman ER, Levine RL (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **25**(3)**:** 207-218

Straight JV, Ramkrishna D, Parulekar SJ, Norman B. Jansen (1989) Bacterial growth on lactose: An experimental investigation. *Biotechnol Bioeng* **34**(5)**:** 705-716

Stratford M (1993) Yeast flocculation: Flocculation onset and receptor availability. *Yeast* **9:** 85-94

Stratton J, Chiruvolu V, Meagher M (1998) High cell-density fermentation. In *Pichia Protocols* Vol. 103, pp 107-120, Springer-Verlag.

Straus DB, Walter WA, Gross CA (1988) *Escherichia coli* heat shock gene mutants are defective in proteolysis. *Genes Dev* **2**(12b)**:** 1851-1858

Studier FW (2005) Protein production by auto-induction in high-density shaking cultures. *Protein Expression Purif* **41:** 207–234

Studier WF, Rosenberg AH, Dunn JJ, Dubendorff JW, David VG (1990) [6] Use of T7 RNA polymerase to direct expression of cloned genes. In *Methods in Enzymology* Vol. Volume 185, pp 60-89. Academic Press

Summers DK (1996) *The Biology of Plasmids*: Blackwell Science.

Surribas A, Cos O, Montesinos JL, Valero F (2003) On-line monitoring of the methanol concentration in *Pichia pastoris* cultures producing an heterologous lipase by sequential injection analysis. *Biotechnol Lett* **25**(21)**:** 1795-1800

Szabo A, Langer T, Schröder H, Flanagan J, Bukau B, Hartl FU (1994) The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system DnaK, DnaJ, and GrpE. *Proc Natl Acad Sci U S A* **91**(22)**:** 10345-10349

Terpe K. (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biot* **72:** 211-222.

Thomas JA, Mallis RJ (2001) Aging and oxidation of reactive protein sulfhydryls. *Exp Gerontol* **36**(9)**:** 1519-1526

Thorpe ED, d'Anjou MC, Daugulis AJ (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnol Lett* **21**(8)**:** 669-672

Tobe S, Shimogaki H, Ohdera M, Asai Y, Oba K, Iwama M, Irie M (2006) Expression of *Bacillus* protease (Protease BYA) from *Bacillus* sp. Y in *Bacillus subtilis* and enhancement of its specific activity by site-directed mutagenesis-improvement in productivity of detergent enzyme-. *Biol Pharm Bull* **29**(1)**:** 26-33

Tokatlidis K, Dhurjati P, Millet J, Béguin P, Aubert J-P (1991) High activity of inclusion bodies formed in *Escherichia coli* overproducing *Clostridium thermocellum* endoglucanase D. *FEBS Letters* **282**(1)**:** 205-208

Tomazetto G, Mulinari F, Stanisçuaski F, Settembrini B, Carlini CR, Ayub MAZ (2007) Expression kinetics and plasmid stability of recombinant *E. coli* encoding urease-derived peptide with bioinsecticide activity. *Enzyme Microb Technol* **41**(6-7)**:** 821-827

Touati D (2000) Iron and oxidative stress in bacteria. *Arch Biochem Biophys* **373**(1)**:** 1-6

Turner N, Fotheringham I, Speight R (2005) Novel biocatalyst technology for the preparation of chiral amines. *ChemInform* **36**(35)

Turner NJ, Carr R (2007) Biocatalytic routes to nonracemic chiral amines. In *Biocatalysis in the pharmaceutical and biotechnology industries*, Patel RN (ed). Boca Raton, FL,: CRC Press LLC

Turner NJ, Truppo MD (2010) Biocatalytic Routes to Nonracemic Chiral Amines. In *Chiral Amine Synthesis Methods, Developments and Applications*, Nugent TC (ed). Weinheim: Wiley-VCH Verlag GmbH & Co

van den Hombergh JPTW, van de Vondervoort PJI, Fraissinet-Tachet L, Visser J (1997) *Aspergillus* as a host for heterologous protein production: the problem of proteases. *Trends in Biotechnology* **15**(7)**:** 256-263

van der Klei IJ, Yurimoto H, Sakai Y, Veenhuis M (2006) The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *BBA-Mol Cell Res* **1763**(12)**:** 1453-1462

van der Walt J, Johannsen E (1975) *Trichosporon terrestre* sp. nov. *Anton Leeuw* **41**(1)**:** 361-365

van Dijken J, Harder W, Beardsmore A, Quayle J (1987) Dihydroxyacetone: An intermediate in the assimilation of methanol by yeasts? *FEMS Microbiol Lett* **40**(2-3)**:** 97-102

Van Urk H, Postma E, Scheffers WA, Van Dijken JP (1989) Glucose transport in crabtree-positive and crabtree-negative yeasts. *J Gen Microbiol* **135**(9)**:** 2399-2406

Varga J, Kocsube S, Toth B, Frisvad JC, Perrone G, Susca A, Meijer M, Samson RA (2007) *Aspergillus brasiliensis* sp. nov., a biseriate black *Aspergillus* species with world-wide distribution. *Int J Syst Evol Microbiol* **57**(8)**:** 1925-1932

Varma A, Palsson BO (1994) Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type *Escherichia coli* W3110. *Appl Environ Microbiol* **60**(10)**:** 3724-3731

Vasic-Racki D (2000) History of Industrial Biotransformations – Dreams and Realities. In *Industrial Biotransformations*, Liese A, Seelbach K, Wandrey C (eds), 2nd edn.: Wiley

Vasina JA, Baneyx F (1997) Expression of aggregation-prone recombinant proteins at low temperatures: A Comparative study of the *Escherichia coli cspA* and *tac* promoter systems. *Protein Expression Purif* **9**(2)**:** 211-218

Ventura S, Villaverde A (2006) Protein quality in bacterial inclusion bodies. *Trends Biotechnol* **24:** 179-185

Verdoes JC, Punt PJ, Schrick JM, Verseveld HWv, Stouthamer AH, Hondel CAvd (1993) Glucoamylase overexpression in *Aspergillus niger*: molecular genetic analysis of strains containing multiple copies of the *glaA* gene. *Transgenic Res* **2:** 84-92

Verdoes JC, Punt PJ, Stouthamer AH, van den Hondel CA (1994) The effect of multiple copies of the upstream region on expression of the *Aspergillus niger* glucoamylase-encoding gene. *Gene* **145**(2)**:** 179-187

Villaverde A, Carrió MM (2003) Protein aggregation in recombinant bacteria: biological role of inclusion bodies. *Biotechnol Lett* **25:** 1385–1395

Vind J, Sørensen MA, Rasmussen MD, Pedersen S (1993) Synthesis of proteins in *Escherichia coli* is limited by the concentration of free ribosomes: Expression from reporter genes does not always reflect functional mRNA levels. *J Mol Biol* **231**(3)**:** 678-688

Vogel HJ (1956) Convenient growth medium for *Neurospora* (medium N). *Microbiol Genet Bull* **13:** 42-43

Vogt W (1995) Oxidation of methionyl residues in proteins: Tools, targets, and reversal. *Free Radical Biol Med* **18**(1)**:** 93-105

Walker GM (2004) Metals in yeast fermentation processes. In *Advances in Applied Microbiology*, Allen I. Laskin JWB, Geoffrey MG (eds), Vol. Volume 54, pp 197-229. Academic Press

Walt Jvd (1962) Utilization of ethylamine by yeasts. *Anton Leeuw* **28:** 91-96

Wan M, Takagi M, Loh BN, Imanaka T (1995) Comparison of HIV-1 protease expression in different fusion forms. *Biochem Mol Biol Int* **36:** 411-419

Wang L, Ridgway D, Gu T, Moo-Young M (2003) Effects of process parameters on heterologous protein production in *Aspergillus niger* fermentation. *J Chem Tech Biot* **78**(12)**:** 1259-1266

Wang Z, Wang Y, Zhang D, Li J, Hua Z, Du G, Chen J (2010) Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation. *Bioresour Technol* **101**(4)**:** 1318-1323

Ward OP, Qin WM, Dhanjoon J, Ye J, Singh A, Allen I. Laskin JWBGMG, Sima S (2005) Physiology and biotechnology of *Aspergillus*. In *Advances in Applied Microbiology* Vol. Volume 58, pp 1-75. Academic Press

Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* **186**(1)**:** 37-44

Weber J, Hoffmann F, Rinas U (2002) Metabolic adaptation of *Escherichia coli* during temperature-induced recombinant protein production: 2. Redirection of metabolic fluxes. *Biotechnol Bioeng* **80**(3)**:** 320-330

Welch RA (2006) The Genus *Escherichia*. In *The Prokaryotes*, pp 60-71. Springer New York

Werner RG, Kopp K, Schlueter M (2007) Glycosylation of therapeutic proteins in different production systems. *Acta Pædiatr* **96:** 17-22

Weyler W, Hsu Y-PP, Breakafield XO (1990) Biochemistry and genetics of monoamine oxidase. *Pharmacol Ther* **47**(3)**:** 391-417

Weyler W, Salach JI (1985) Purification and properties of mitochondrial monoamine oxidase type a from human placenta. *J Biol Chem* **260**(24)**:** 13199-13207

Wiame J-M, Grenson M, Ars Jr HN, Rose AH, Tempest DW (1985) Nitrogen catabolite repression in yeasts and filamentous fungi. In *Advances in Microbial Physiology* Vol. Volume 26, pp 1-88. Academic Press

Wilms B, Hauck A, Reuss M, Syldatk C, Mattes R, Siemann M, Altenbuchner J (2001) High-cell-density fermentation for production of L-N-carbamoylase using an expression system based on the *Escherichia coli rhaBAD* promoter. *Biotechnol Bioeng* **73**(2)**:** 95-103

Wülfing C, Plückthun A (1993) A versatile and highly repressible *Escherichia coli* expression system based on invertible promoters: expression of a gene encoding a toxic product. *Gene* **136**(1-2)**:** 199-203

Xu B, Jahic M, Enfors S-O (1999) Modeling of overflow metabolism in batch and fed-batch cultures of *Escherichia coli*. *Biotechnol Prog* **15**(1)**:** 81-90

Yagodina OV, Nikol'skaya EB, Khovanskikh AE, Kormilitsyn BN (2002) Amine oxidases of microorganisms. *J Evol Biochem Phys* **38**(3)**:** 251-258

Yazdani, Mukherjee (2002) Continuous-culture studies on the stability and expression of recombinant streptokinase in *Escherichia coli*. *Bioprocess Biosystems Eng* **24**(6)**:** 341-346

Yurimoto H (2009) Molecular basis of methanol-inducible gene expression and its application in the methylotrophic yeast *Candida boidinii*. *Biosci, Biotechnol, Biochem* **73**(4)**:** 793-800

Zabriskie DW, Arcuri EJ (1986) Factors influencing productivity of fermentations employing recombinant microorganisms. *Enzyme Microb Technol* **8**(12)**:** 706-717

Zeller A (1951) Oxidation of amines. In *The Enzymes* Vol. ii, pp 536-558. New York: Academic Press Inc.

Zhan X, Schwaller M, Gilbert HF, Georgiou G (1999) Facilitating the formation of disulfide bonds in the *Escherichia coli* periplasm via coexpression of yeast protein disulfide isomerase. *Biotechnol Progr* **15**(6)**:** 1033-1038

Zhang A-L, Luo J-X, Zhang T-Y, Pan Y-W, Tan Y-H, Fu C-Y, Tu F-z (2009) Recent advances on the *GAP* promoter derived expression system of *Pichia pastoris*. *Mol Biol Rep* **36:** 1611-1619

Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM (2000a) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. *Biotechnol Bioeng* **70**(1)**:** 1-8

Zhang W, Hywood Potter KJ, Plantz BA, Schlegel VL, Smith LA, Meagher MM (2003) *Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement. *J Ind Microbiol Biot* **30**(4)**:** 210-215

Zhang W, Inan M, Meagher M (2000b) Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. *Biotechnol Bioprocess Eng* **5**(4)**:** 275-287

Zhang W, Sinha J, Smith LA, Inan M, Meagher MM (2005) Maximization of production of secreted recombinant proteins in *Pichia pastoris* fed-batch fermentation. *Biotechnol Prog* **21**(2)**:** 386-393

Zhang Y, Olsen DR, Nguyen KB, Olson PS, Rhodes ET, Mascarenhas D (1998) Expression of eukaryotic proteins in soluble form in *Escherichia coli*. *Protein Expression Purif* **12**(2)**:** 159-165

Zhou X-S, Lu J, Fan W-M, Zhang Y-X (2002) Development of a responsive methanol sensor and its application in *Pichia pastoris* fermentation. *Biotechnol Lett* **24**(8)**:** 643-646

Zwart K, Veenhuis M, Dijken JPv, Harder W (1980) Development of amine oxidase-containing peroxisomes in yeasts during growth on glucose in the presence of methylamine as the sole source of nitrogen. *Arch Microbiol* **126:** 117-126

Zwart KB, Harder W (1983) Regulation of the metabolism of some alkylated amines in the yeasts *Candida utilis* and *Hansenula polymorpha*. *J Gen Microbiol* **129**(10)**:** 3157-3169

Zwart KB, Veenhuis M, Plat G, Harder W (1983) Characterization of glyoxysomes in yeasts and their transformation into peroxisomes in response to changes in environmental conditions. *Arch Microbiol* **136:** 28- 38

Chapter 7 Appendix

	Substrate	FAD	Cu
1	Amylamine	√√√	√√√
2	Putrescine	√√	√√
3	Cadaverine	-	-
4	Benzylamine	√√	√
5	R-a-Methylbenzylamine	-	-
6	S-a-Methylbenzylamine	√	-
7	R-N-a-Dimethylbenzylamine	-	-
8	S-N-a-Dimethylbenzylamine	-	-
9	R-a-Ethylbenzylamine	-	-
10	R-2-butylamine	-	-
11	2-aminopentane	-	-
12	R-2-aminohexane	-	-
13	2-Aminoheptane	-	-
14	R-2-Aminoheptane	-	-
15	D/L-2-Phenylglycinol	-	--
16	3-aminoproprionitrile	-	-

Reactivity of the *A.niger* 14900ii FAD and Cu containing amine oxidases against various amines.