PROCESS OPTIMISATION AND SCALE-UP OF INDUSTRIAL ENZYMES PRODUCTION

by

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of

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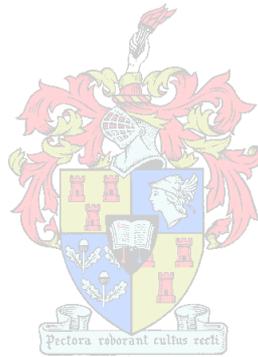
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Abstract

Industrial enzymes offer excellent prospects for the development of 'green' processes and high quality products with a diminished negative impact on the environment. This study endeavours to develop fed-batch process methods to improve the production of two industrially relevant enzymes in dedicated yeast systems, namely *Saccharomyces cerevisiae* and *Pichia pastoris,* at laboratory and pilot scale. This goal was achieved by specifically focussing on key bioprocessing parameters, namely the substrate feed rate during fed-batch fermentation, fermentation process conditions including the dissolved oxygen tension (DOT), growth medium improvement and scale-up effects during enzyme production. In the first system, the glucose feed rate was used to optimise the specific growth rate of recombinant *Saccharomyces cerevisiae* for the production of α-glucuronidase. In the second system, a semi-defined growth medium was developed, and both the substrate feed rate and DOT were optimised in the production of β-fructofuranosidase (FFase) by *Pichia pastoris*. These two systems serve to demonstrate the potential for optimisation of fed-batch cultures to maximise production of industrial enzymes by engineered yeasts.

α-Glucuronidase is a valuable enzyme for the production of insoluble xylan biopolymers, due to its unique ability to remove 4-O-methyl glucuronic acid side chains from polymeric xylan, without the requirement for endo-catalysts to first hydrolyse the polymer into xylo-oligosaccharides. The influence of specific growth rate on the production of *Scheffersomyces stipitis* α-glucuronidase by recombinant *S. cerevisiae* strain MH1000 pbk10D-glu was studied in glucose-limited fed-batch culture, including scale-up from 14 to 100 L. At and below the critical specific growth rate (μ_{crit}) of 0.12 h^{-1} at 14 L scale, the biomass yield coefficient ($Y_{x/s}$) remained constant at 0.4 g g^{-1} with no ethanol production, whereas ethanol yields ($k_{eth/x}$) of up to 0.54 g g^{-1} and a steady decrease in $Y_{x/s}$ were observed at $\mu > 0.12$ h^{-1}. Production of α- glucuronidase was growth associated at a product yield ($k_{\alpha\text{-}glu/x}$) of 0.45 mg g^{-1}, with the highest biomass (37.35 g/L) and α-glucuronidase (14.03 mg/L) concentrations recorded

during fed-batch culture at a growth rate equal to $\mu_{crit} = 0.12$ h^{-1}. Scale-up with constant k_La from 14 to 100 L resulted in ethanol concentrations of up to 2.5 g/L at $\mu = 0.12$ h^{-1}, apparently due to localised high glucose concentrations at the feed entry zone, which would induce oxido-reductive metabolism in the culture. At this larger scale, α-glucuronidase yield could be maximised at growth rates below μ_{crit} where ethanol production could be prevented.

The trans-fructosylating action of the β-fructofuranosidase (FFase) enzyme on sucrose produces short-chain fructooligosaccharides (1-nystose, 1-kestone and 1-fructofuranosyl-nystose) that can be used as sweeteners and pre-biotics in drug formulation and confectionary products. A semi-defined, industrial medium was developed for cultivation of *Pichia pastoris* for FFase production, as alternative to a commonly-used chemically defined laboratory medium, with a specific focus on the effect of the carbon source on the requirement for trace elements. Furthermore, statistical optimisation using a central composite design was applied to the glycerol fed-batch (GFB) phase of DO-stat fed-batch fermentations of *P. pastoris* to establish the optimal substrate (glycerol) feed rate and DOT for the production of FFase. Enzyme expression was under the control of two different promoters, namely the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) and alcohol oxidase (AOX), where the latter was induced by methanol after sufficient levels of biomass were produced in the GFB phase. Whereas the promoter that controls recombinant protein expression in *P. pastoris* determines which carbon source is used for fermentation, this study also established that the carbon source highlighted key nutritional requirements of the strain during heterologous enzyme production. Decreasing the concentration of basal salts in the semi-defined medium by a factor of two did not adversely affect enzyme production under either promoter. However, replacing the trace elements solution in the chemically defined medium with yeast extract (semi-defined medium) resulted in a decrease in the volumetric activity of FFase during expression under control of the AOX promoter by 54.3%, from 9238.27 U/ml to 4227.20 U/ml. Therefore, to maximise enzyme production, a distinct requirement for trace

elements was evident when methanol served as carbon source during induction. When glycerol served as carbon source, the change from the chemically defined medium to the semi-defined medium had no effect on enzyme expression under control of the GAP promoter, where similar volumetric FFase activities of 4648.68 U/ml and 4738.71 U/ml were recorded in the two respective media. Optimisation of DO-stat fed-batch fermentations of both strains using the semi-defined medium resulted in respective glycerol feed rates and DOT values of 40.3 g/h and 32.23% for the strain harbouring the GAP promoter, and 28.49 g/h and 48.54% for the AOX promoter strain. However, at these optimal conditions, the volumetric activity was 40% less than that from the AOX strain grown in the chemically defined medium. Hence, further optimisation, possibly at molecular level, may be required to match the expression level of the GAP promoter to that of the AOX promoter for FFase production.

A key observation from this research was that the mass of substrate available to yeast culture during fed-batch fermentation critically affects growth and production behaviour in terms of the metabolic state, biomass and product formation, irrespective of the yeast system used. Therefore, controlling the substrate feed rate proved to be a highly effective method to optimise recombinant enzyme production in fed-batch culture. Furthermore, to optimise product yield, the importance of careful control of key conditions during substrate-controlled fed-batch culture, including the dissolved oxygen concentration, the scale of the operation, the manner in which scale-up was carried out, and the nature of the growth medium was demonstrated through effective application to two different recombinant yeast expression systems. As such, this text would serve as a key reference for future fermentation development using fed-batch culture for the aerobic production of heterologous proteins by the Crabtree positive *S. cerevisiae* and the methylotrophic *P. pastoris*. Moreover, the data from the research provided valuable perspectives into the actual application for the

commercial production of α-glucuronidase and β-fructofuranosidase, both regarded as
biotechnological products of high commercial value.

Samevatting

Industriële ensieme bied uitstekende vooruitsigte vir die ontwikkeling van 'groen' prosesse en hoë-waarde produkte wat gelyktydig 'n verminderde negatiewe impak op die omgewing het. Die doel van hierdie studie was om semi-enkellading kultuur metodologieë te ontwikkel om die produksie van twee industrieel-relevante ensieme in twee toegewyde gisproduksiesisteme, naamlik *Saccharomyces cerevisiae* en *Pichia pastoris*, op laboratorium- en lootsaanlegskaal te verbeter. Hierdie doel is bereik deur spesifiek op sleutel bioprosesparameters, naamlik die substraat voersnelheid tydens semi-enkellading kultuur, fermentasie prosestoestande, ondermeer die opgeloste suurstofspanning (OSS), groeimediumverbetering en opskaleringseffekte tydens ensiemproduksie, te fokus. In die eerste gissisteem is die glukose voersnelheid gebruik om die spesifieke groeisnelheid van *S. cerevisiae* vir die produksie van α-glukuronidase te optimeer. 'n Semi-gedefinieerde groeimedium is vir die tweede gissisteem ontwikkel en beide die substraat voersnelheid en die OSS is vir die produksie van β-fruktofuranosidase (FFase) deur *Pichia pastoris* geoptimeer. Deur hierdie twee gissisteme te gebruik kon die potensiaal vir optimering van semi-enkellading kulture vir die maksimering van industriële ensiemproduksie deur geneties-gemanipuleerde giste op hoogs doeltreffende wyse gedemonstreer word.

α-Glukuronidase is 'n waardevolle ensiem wat vir die produksie van onoplosbare xilaan gebruik word. Hierdie ensiem het die unieke eienskap om 4-O-metiel glukuroonsuur sykettings van polimeriese xilaan te verwyder, sonder dat die polimeerketting 'n voorafgaande hidrolise stap moet ondergaan waar dit eers deur endo-katalisators na xilo-oligosakkariedeenhede afgebreek word. Die invloed van die spesifieke groeisnelheid is op die produksie van *Scheffersomyces stipitis* glukuronidase, wat deur rekombinante *S. cerevisiae* stam MH1000pbk10D-glu uitgedruk word, onder 'n glukosebeperking in semi-enkellading kulture ondersoek, wat ook 'n opskaleringstap van 14 na 100 L skaal ingesluit het. Op 14 L skaal, by en onder die kritiese spesifieke groeisnelheid (μ_{crit}) van 0.12 h^{-1}, kon 'n konstante

biomassa opbrengskoëffisiënt ($Y_{x/s}$) van 0.4 g g^{-1} gehandhaaf word sonder dat enige etanol produksie plaasgevind het. Daarteenoor is etanol opbrengskoëffisiënte ($k_{eth/x}$) van tot en met 0.54 g g^{-1} en 'n konstante toename in die $Y_{x/s}$ by $\mu > 0.12$ h^{-1} waargeneem. Die produksie van α-glukuronidase was groei-geassosieer, en 'n produkopbrengskoëffisiënt ($k_{\alpha\text{-}glu/x}$) van 0.45 mg g^{-1} en die hoogste biomassa (37.35 g/L) en α-glukuronidase (14.03 mg/L) konsentrasies is by 'n spesifieke groeisnelheid, gelykstaande aan $\mu_{crit} = 0.12$ h^{-1}, in semi-enkellading kulture waargeneem. 'n Toename in die werksvolume van 14 na 100 L by 'n konstante k_La het etanolkonsentrasies van tot en met 2.5 g/L by $\mu = 0.12$ h^{-1} tot gevolg gehad. Hierdie verskynsel kon waarskynlik aan gelokaliseerde hoë glukosekonsentrasies toegeskryf word op die plek waar die voermedium by die kultuur invloei. Die α-glukuronidase opbrengs kon dus gemaksimeer word deur die sisteem by spesifieke groeisnelhede laer as μ_{crit} op die groter skaal te bedryf waar etanolproduksie verhoed kon word.

Kort-ketting frukto-oligosaggariede (1-nystose, 1-kestose, en 1-fruktofuranosiel-nystose) kan deur die trans-fruktosileeringswerking van die β-fruktofuranosidase (FFase) ensiem vanaf sukrose geproduseer word, wat as kunsmatige versoeters, pre-biotiese middels in medisinale formulerings, of in fyngebak gebruik kan word. Met die oog op die effek van die koolstofbron op die spoorelementbehoefte van die mikro-organisme, is 'n semi-gedefinieerde medium vir die kweek van *Pichia pastoris* vir FFase produksie ontwikkel wat as alternatief tot die algemeen-gebruikte, chemies-gedefinieerde medium kon dien. 'n Statistiese optimering met behulp van 'n sentraal-saamgestelde ontwerp is verder op die gliserolvoerfase van die OS-staat, semi-enkellading kulture toegepas om die optimale substraat (gliserol) voersnelheid en OSS vir die produksie van FFase te bepaal. Die ensiem was onder beheer van twee verskillende promotors in twee verskillende gisstamme uitgedruk, naamlik die konstitutiewe gliseraldehied-3-fosfaat dehidrogenase (GAF) promotor en die alkohol oksidase (AO) promotor, waar laasgenoemde deur metanol geïnduseer word wanneer voldoende biomassa vlakke na die gliserolvoerfase bereik is. Terwyl die promotor wat die heteroloë proteïen-

uitdrukking beheer die aard van die koolstofbron tydens fermentasie bepaal, is daar ook bevind dat die aard van hierdie koolstofbron sleutel voedingsbehoeftes tydens rekombinante ensiemproduksie uitgelig het. Deur die spoorelementoplossing in die chemies-gedefinieerde medium met gisekstrak te vervang (semi-gedefinieerde medium) is daar 'n 54.3% afname, vanaf 9 238 na 4 227.20 U/ml, in die volumetriese aktiwiteit van FFase waargeneem, wanneer dit onder die beheer van die AO promotor uitgedruk was. 'n Behoefte aan spoorelemente was dus duidelik waarneembaar wanneer metanol as koolstofbron tydens die induksiefase gebruik is. Met slegs gliserol as koolstofbron het 'n verandering van 'n chemies-gedefinieerde na 'n semi-gedefinieerde medium geen effek op ensiem-uitdrukking onder beheer van die GAF promotor gehad nie, waar volumetriese FFase aktiwiteite van 4 648.68 en 4 738.71 U/ml in die onderskeie media waargeneem is. Optimering in OS-staat, semi-enkellading kulture van beide giste, in semi-gedefinieerde medium gekweek, het tot onderskeie gliserol voersnelhede en OSS waardes van 40.3 g/h en 32.23% vir die stam met die GAF promotor, en 28.49 g/h en 48.54% vir die stam met die AO promotor gelei. Die volumetriese aktiwiteit van die ensiem onder hierdie optimale toestande was egter steeds 40% minder vergeleke met die ensiemaktiwiteit wanneer die stam met die AO promotor in die chemies-gedefinieerde medium gekweek is. Verdere optimering, moontlik op molekulêre vlak, is dus aangedui om vergelykbare FFase uitdrukkingsvlakke onder beheer van die GAF en AO promotors te bewerkstellig.

'n Sleutelwaarneming is uit hierdie navorsing gemaak, naamlik dat die massa van die substraat, tydens semi-enkellading kultuur aan die mikro-organisme beskikbaar gestel en onafhangklik van gissisteem, 'n kritiese invloed op die gis se groei- en produksiegedrag gehad het, waar laasgenoemde na die metaboliese toestand, en biomassa- en produkopbrengs verwys. Deur die substraat se voersnelheid, nie op volume-basis nie, maar wel op massa-basis te baseer kon rekombinante ensiemproduksie op hoogs doeltreffende wyse geoptimeer word. Deur van massa-beheerde, semi-enkellading kulture te gebruik te maak kon die noukeurige

beheer van sleuteltoestande, insluitend die opgeloste suurstofkonsentrasie, die skaal van die operasie, die manier waarmee opskalering uitgevoer is, en die aard van die groeimedium ook gedemonstreer word, waar dit doeltreffend op twee verskillende rekombinante gissisteme toegepas is. Voorts sal hierdie studie in die toekoms as sleutelverwysingswerk vir fermentasie ontwikkeling kan dien waar semi-enkellading kulture met die Crabtree-positiewe *S. cerevisiae* en die metiel-trofiese *P. stipitis* vir die aërobiese produksie van heteroloë proteïen gebruik word. Ten slotte, die data wat in hierdie studie versamel is, het belangrike perspektiewe vir die kommersiële produksie van α-glukuronidase en β-fruktofuranosidase verskaf wat as biotegnologiese produkte van hoë waarde geag word.

Preface

This thesis discusses the development of fermentation processes for the production of two enzymes of industrial significance in yeast systems. It is presented in two major parts as Chapters 3 and 4, each chapter dealing with the production of a separate industrial enzyme using a suitable yeast-based expression system. A separate introduction and background information is given for each of chapters 3 and 4 (which may be quite repetitive of the literature review (Chapter 2) and the general introduction to the thesis (Chapter 1)) to reflect a stand-alone view where each of these chapters can be read independently of the rest of the thesis. Each chapter deals with the core concepts of bioprocess development for enzyme production by the particular yeast-based production host, namely optimisation—aimed at improving enzyme yield during fermentation and reducing production costs. Additionally, a scale-up section in Chapter 3 discusses typical methodology and engineering constraints during scale-up.

Chapter 3 covers the production of α-glucuronidase by *Saccharomyces cerevisiae* and is presented according to the style of *Biochemical Engineering Journal* where it was submitted for publication. The specific growth rate of *S. cerevisiae* was optimised to reduce the overflow of carbon to ethanol during fermentation, and thereby increase the yield of the enzyme. The chapter also includes an investigation of scale-up from laboratory scale bioreactors to pilot plant bioreactor.

Chapter 4 discusses the production of recombinant β-fructofuranosidase enzyme in *Pichia pastoris*. The focus is on modification of the growth medium to reduce production costs and to eliminate key problems associated with the widely used defined medium, and to optimise feeding rates and oxygen availability. The manuscript, as presented, is to be submitted to *Microbial Cell Factories Journal* for review and publication.

Dedication

To my beloved mother, Ms Obour Amma Gladys

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Nomenclature

AOX *1* : Alcohol oxidase promoter

D : Dilution rate, h^{-1}

D_i : Diameter of impeller, m

DO, DOT : Dissolved oxygen, Dissolved oxygen tension

F : Volumetric flow rate of substrate, Lh^{-1}

FFase : β-fructofuranosidase

fop-A : The gene that encodes the β-fructofuranosidase enzyme

GAP : Glyceraldehyde-3-phosphate dehydrogenase promoter

K_La : Volumetric mass transfer coefficient

N : impeller speed, rpm

OD_{600} : Optical density measured at 600 nm

PGK : Phosphoglycerate kinase promoter

P_g/V : Gassed power per unit volume

sc-FOS : Short chain fructooligosaccharides

μ_{max} : maximum specific growth rate, h^{-1}

μ_{crit} : Critical specific growth rate, h^{-1}

V : Volume of fermentation broth

V_s : Superficial gas velocity, m/s

vvm : Volume of air per volume of broth per minute

$Y_{x/s}$: Biomass yield on substrate

xx

CHAPTER ONE

1.0 Introduction

The increase in demand for sustainable technologies in industrialization calls for several changes in the procedures, processes and materials industries use to produce goods and services. Recent hike in carbon dioxide concentrations in the atmosphere, high energy costs, demand for high quality goods and services by the general public and excessive use of natural resources such as water in the industrialized world are major courses for concern, and various measures are being put in place to address these challenges. One of the solutions is the incorporation of microorganisms and microbial products such as enzymes into the products and services delivery line. Industries are constantly seeking to improve the economics of their processes in the most environmentally benign ways, either to meet regulatory demands or to improve the sustainability of their processes.

Enzymes are biological catalysts. They accelerate important biochemical reactions that occur in the cell and its environment to maintain life. Enzymes are proteins made up of several amino acids linked in a specific sequence with a specific structure. Several thousand enzymes are known, which act on a wide variety of substrates depending on their structure and cofactors involved. Enzymes catalyse chemical reactions under mild conditions of temperature and pressure at high reaction rates, and with great specificity which ensures that no unwanted side reactions occur. This reaction specificity increases product yield and ensures ease of downstream processing. Owing to the unique action of every enzyme, a variety of enzymes are present in cells to catalyse each of the numerous biochemical transformations that occur in the cell, (Damhus, et al., 2008).

The use of enzymes to augment industrial production of food and materials dates back to pre-historic times. According to ancient Greek epic poems, enzymes and microbes were used to

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make cheese around 800 BC (Schafe, et al., 2001). Traditional foods such as bread, yoghurt, *kefir*, vinegar, wine, beer and cheese as well as paper and textiles were produced using enzymes and microbes as early as 6000 BC in China, Sumer and Egypt (Ole, et al., 2002). The selection and improvement of microbial strains, coupled with technological advances in fermentation processes during the last century has led to the production of pure enzymes on large scale. Enzymes themselves are biodegradable, and therefore do not persist in the environment as compared to chemicals such as insecticides (Falch, 1991).

Enzymes are generally produced in bioreactors in fermentation processes during which microorganism synthesise the enzymes as metabolic products. Depending on the type of enzyme being produced, different microbes, different feeding materials, different types of bioreactors and different operating strategies are employed in the production of enzymes. The processes used in current production of enzymes are mostly aerobic, requiring large amount of natural air or pure oxygen.

Advances in recombinant DNA technology and protein engineering have led to the modification of microorganisms and enzymes to suit specific tasks. With genetically modified (GM) microorganisms and protein engineering, it is possible to produce enzymes and microbes that are more tolerant to harsh conditions such as high temperature oil wells and the highly alkaline environments in the textile and clothing industry (Maure *et al*., 1999). These enzymes are tailored to give higher specificity and higher product purity under specific conditions. The result of this scientific advancement is a highly diversified industry that is still growing in terms of size and complexity (Estell, 1993).

This research focuses on using two yeast systems to produce two industrially significant enzymes: α-glucuronidase and β-fructofuranosidase. α-Glucuronidase is an enzyme with the unique ability to cleave 4-O-methyl glucuronic acid side chains of polymeric xylan, making it insoluble in the process and therefore more useful as a biopolymer (Xue, et al., 2008; Gomes,

2012). β-Fructofuranosidase produces short chain fructooligosaccharides (sc-FOS) from sucrose. These sugars are used as components of functional foods (prebiotics) and as sweeteners in drug formulation (Fernandez, et al., 2007). Previous production of different classes of α-glucuronidsase and β-fructofuranosidase has been done with the native organisms from which these enzymes were obtained, i.e. *Schizophyllum commune* and *Aspergillus niger*, respectively, which expressed the enzymes in relatively low concentrations (Xue, et al., 2008; Yanai, et al., 2001). In the present work, these enzymes are therefore produced using different recombinant yeasts capable of producing enzymes in higher titres in fed-batch fermentation. *Saccharomyces cerevisiae* was selected for production of α-glucuronidase and *Pichia pastoris* for production of β-fructofuranosidase. The selection of host strains and development of production strains were performed outside of the scope of the present study, and was based primarily on success in genetic engineering and production levels observed in shakeflaks (Gomes, 2012; Coetzee, et al., 2013). In the present study, the fed-batch production process for α-glucuronidase, performed with recombinant *S. cerevisiae* in a bioreactor, was optimised in terms of the specific growth rate of the culture to maximize enzyme yield. Furthermore, the effect of scale on the yield of α-glucuronidase by the *S. cerevisiae* production system in fed-batch is studied by performing scale-up experiments based on data from small scale bioreactors. The present work also developed a complex growth medium and optimized a fed-batch process to maximize the yield of β-fructofuranosidase using two strains of *P. pastoris*.

CHAPTER TWO

Review of Literature

2.1 Introduction

This chapter considers various industries and the class of enzymes they use, current trends in the world enzyme market, the enzyme expression systems to be used in this research work and how the production of other enzymes with these yeasts has been optimised by other researchers. It also considers fed-batch fermentation processes for enzyme production with the selected yeasts, and the different methods available for scale-up of a bioprocess such as enzyme production.

2.2 Industrial Enzymes and their Applications

The apparent benefits of enzymes make them universally acceptable for applications in various industrial and domestic processes. Due to their effectiveness in catalysis, enzymes are ingredients that are required in small quantities in the formulation of the overall product. Currently, the common use of industrial enzymes involves the breakdown of large molecules into smaller units, usually in aqueous media (van Beilen & Li, 2002). A wide variety of enzymes are used for several purposes in various industries as summarised in Table 2.1.

2.2.1 Food Industry and Beverages

One of the oldest industries utilising enzymes is the food industry. In the early 1960s the first major use of microbial enzymes in the food processing was reported. This involved the conversion of starch to glucose using glucoamylase instead of the conventional acid hydrolysis process. The enzyme-catalysed process reduced the cost of steam by 30% and ash and by-products formation by 50% and 90%,, respectively (Falch, 1991). Subsequently, nearly all glucose production from starch changed from the old acid hydrolysis process to

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enzymatic hydrolysis. In the baking industry, supplementary enzymes such as glucose oxidases, lipoxygenase and phospholipase are added to the dough to ensure high crumb uniformity, better volume and a longer shelf life. In cheesemaking (dairy industry), lipases are used to accelerate the coagulation of milk (van Beilen & Li, 2002). Many large breweries use several enzymes to control the fermentation process in order to produce consistent, high quality beer (Ogawa & Shimizu, 1999). In food processing, smaller protein molecules with improved nutritional value and functional properties are obtained by enzyme-catalysed hydrolysis of proteins (Falch, 1991). In the processing of fruit juice, enzymes are used to break down the cell walls of plant material before the juice is extracted. This results in improvement in the colour and aroma of juice with correspondingly high volumetric yields (Schafe, et al., 2001).

The present work includes process optimisation for the production of β-fructofuranosidase enzyme used for the production of confectionary products and prebiotics in the food industry.

2.2.2 Textiles and Leather Industries

Most of the operations in the textiles and leather industries also use enzymes. A typical example in the textile industry is enzymatic scouring. Scouring is the process of cleaning fabrics by removing impurities such as pectins, waxes, mineral salts and hemicelluloses from cellulosic materials. Conventionally, scouring is done by soaking the cellulosic fibre in sodium hydroxide and then rinsing it with a large volume of water. Apart from removing the impurities, the highly alkaline sodium hydroxide also attacks the fibre and reduces its weight and strength significantly. An alternative enzymatic scouring based on pectate lyase is now being widely accepted as it has no impact on the cellulose and also uses less amount of water, at a lower temperature (van Beilen & Li, 2002). Other applications of enzymes in textiles include bioposlishing (cellulase), denim finishing, bleaching and desizing of cotton fabric. In

the leather industry, enzymes are used for bating, degreasing, leather expansion and in making waterproof leather (Damhus, et al., 2008)

2.2.3 Detergent and Cleaning Industry

The detergent industry heavily relies on enzymes for improved cleaning. The first use of enzymes for cleaning was reported in 1913 by the German scientist Otto Röhm who used pancreatic juice to make pre-soak solutions (Damhus, et al., 2008). The pancreatic juice obtained from animals contains trypsin, chymotrypsin, carboxypeptidases, alpha-amylases, lactases, sucrases, maltases and lipases. Today, several enzymes are incorporated into detergents to facilitate cleaning processes and improve material quality. Proteases, amylases and mannanase in strong detergents break down and dissolve dirt that is attached to fabrics to make it easily removable. Glycosidic bonds that link carbohydrates to surfaces are easily broken by cellulases in detergents used for cleaning food processing equipment. When cellulases are used in detergents for cleaning, they give greater smoothness and enhance the colour of damaged cotton surfaces (Schmid, et al., 2002). However, enzymes used in the detergent industry must be capable of working in alkaline media because most processes in cleaning occur at high pH values.

2.2.4 Paper and Pulp Industry

In the pulp and paper industry, traditional processing of paper from wood did not use enzymes and accordingly produced low grade paper with high costs of chemicals and energy. Modern paper processing involves the use of enzymes at several stages. For instance, xylanases are used for boosting bleaching, amylases for modification of starch for paper coating, lipases for pitch (resin) control and a combination of lipases, cellulases and amylases for deinking of paper during paper recycling (Schmid, et al., 2002). As in the detergent industry, enzymes used in the paper and pulp industry must also work in alkaline conditions because most of the processes that occur prior to the enzymatic treatment are in alkaline solutions.

The present study includes fed-batch fermentation process optimisation for the production of α-glucuronidase used to convert xylan, a by-product of paper processing, into a useful biopolymer.

2.2.5 Biofuels

The increase in the emission of greenhouse gases from fossil fuels and the ever increasing crude oil prices have increased the interests of global bodies and governments in biofuels during the past decade. Lignocellulosic and starch biomasses that are not readily fermentable are hydrolysed into fermentable sugars by enzymes. The enzymes used for hydrolysis of lignocellulosic materials are mainly cellulases and hemicellulases, (xylanases) but there is a growing interest in incorporating accessory enzymes such as pectinases, acetyl esterase and laccases in the enzyme cocktails (Aro, et al., 2005). Starch materials can be hydrolysed simultaneously or sequentially by the action of α-amylases and gluco-amylases. This conversion enables non-sugar raw materials to be used for the production of ethanol (Sánchez & Cardona, 2008).

2.2.6 Organic Synthesis and Biopharmaceutical Industries (BPI)

The high reaction rates, together with the highly selective nature of enzymes and the mild reaction conditions associated with enzymatic reactions make them suitable for use in organic synthesis reactions. Manufacturing organic molecules requires strict adherence to reaction conditions that minimize side reactions and ensure pure products with accurate regioselective and stereoselective orientations. Lipases work well in organic solvents and are among the most useful class of enzymes for organic synthesis. (Ole, et al., 2002). For example, single enantiomer intermediates used in making drugs and agrochemicals and enantiopure alcohols and amides are synthesized with lipases, enantiopure carboxylic acids are synthesized with nitrilases and artificial penicillin is synthesised with acylases (Damhus, et al., 2008).

2.2.7 Agriculture

In agriculture, enzymes have also been used to increase the quality of poultry and pig feed. Premix feeds that are added to grains for poultry may contain enzymes, vitamins and minerals salts (Schafe, et al., 2001). Plant-based feeds are rich in hemicelluloses and celluloses and monogastrics that lack certain enzymes are not able to fully digest these feeds as opposed to ruminants.When enzymes such as xylanases and β-glucanases are added to the feed, they increase the digestibility of the feed and therefore prevent feed wastage (Ole, et al., 2002; van Beilen & Li, 2002).

2.2.8 Oil Drilling and Recovery

In the oil and gas industry, highly pressurized guar-based derivatives (gel) are pumped into underground rock formation to create fractures through which oil flows freely into wells. After the fractures are created, a mannanase-based enzyme preparation is used to liquefy the gel to open up the channels for oil flow (Novozymes AS, 2008; van Beilen & Li, 2002). Additionally, drilling muds that are used to cool the drill bit during drilling contain starch and cellulose derivatives, which eventually form a solid coating on the wall of the well. After drilling, a clean-up process to remove this coating uses enzyme (cellulase, amylase) based processes (Novozymes AS, 2008).

Table 2.1 Classes of enzymes used in various industries

Industry	Class of Enzymes	Application(s)
Leather Industries	Protease	Bating
	Lipase	De-pickling, making waterproof leather
	Elastase	Increasing leather softness
Paper and Pulp	Xylanase	Bleach boosting, reduction in refining energy
	Cellulase	Deinking, drainage improvement, fibre
	Amylase	Starch coating, cleaning
	Protease	Bio-film removal
	Esterases	Pitch control, Reduction of anionic trash.
Agriculture	Phytase	Improve digestibility, phosphorous release
	Xylanase	Improve digestibility in non-ruminants
	β-glucanase	Improve digestibility in farm animals
	ICPs	Bioinsecticides
Organic synthesis (Pharmaceuticals)	Oxyreductases	Enantioselective reduction of ketones
	Nitrilases, nitrile	Synthesis of enantiopure carboxylic acids,
	Esterases	Trans-esterification, aminolysis, hydrolysis of
	Acylase	Synthesis of semi-synthetic penicillin, antibiotics
	Lipase	Resolution of chiral alcohols and amides
	D-amino oxidase	Semi-synthetic pharmaceutical intermediates
	Dehalogenase	Intermediate for herbicides
	Ammonia lyase	Intermediates for aspartame (artificial sweetener)

Sources : Falch (1999), Damhus, *et al.*(2008), Ole, *et al.*(2002), Beilen & Li (2002) , Schafe, *et al*. (2001), Estell (1993), Flores, *et al*., (1997), Li *et al* (2012)

Table 2.1 (Continued)

Industry	Class of Enzymes	Application(s)
Oil drilling and recovery	Mannanase (gel breaker)	Fracturing
	Alpha-amylases	Drill bit cleaning
	Lipases	Used as biosurfactant for emulsification and bioremediation of oily waste
Biofuels	Xylanase, cellulase and phospholiapse	Converting hemicellulose materials (biomass) into fermentable sugars
	β-glucosidase	Degumming
Detergent and cleaning	Proteases	Stain removal
	Amylases	Stain removal
	Lipases	Colour clarification
	Cellulases	Anti-redeposition
	Mannanases	Removal of recurring stains
Textiles	Cellulase	Denim finishing, cotton softening, bio-polishing
	Amylase	De-sizing of cotton
	Pectate Lyase	Scouring
	Laccase	Bleaching
	Catalase	Bleach termination
	Peroxidase	Removal of excess dye

Sources : Falch (1999), Damhus, *et al.*(2008), Ole, *et al.*(2002), Beilen & Li (2002) , Schafe, *et al*. (2001), Estell (1993), Flores, *et al*., (1997), Li *et al* (2012)

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Table 2.1 (Continued)

Industry	Class of Enzymes	Application(s)
Food and Beverages	Xylanases	Dough conditioning
	Lipases	Cheese flavouring, dough stability
	Glucose oxidases	Dough strengthening
	β-glucanases	Digestibility
	Amylases	Low calorie beer, juice treatment
	Pectinases	Firming fruit based products
	Acetolactate decarboxylase	Beer maturation
	Phospholipase	*in situ* dough emulsification
	Lipoxygenase	Bread whitening, dough strengthening
	Laccase	Juice clarification
	Fructosyl transferase	Production of functional foods
	Glucose oxidase	Cross-links gluten to make weak dough stronger, drier and more elastic
	Asparaginase	Reduces acrylamide formation during baking
	Protease	Milk cuddling, infant formulas

Sources : Falch (1999), Damhus, *et al*.(2008), Ole, *et al*.(2002), Beilen & Li (2002) , Schafe, *et al*. (2001), Estell (1993), Flores, *et al*., (1997), Li *et al* (2012)

2.2.9 Global Enzyme Market

The global industrial enzyme market is one of the fastest growing markets. Key factors driving market growth in the area include new enzyme technologies endeavouring to enhance cost efficiencies and productivity, and growing interest among consumers in substituting chemical based products with organic products such as enzymes. Other factors propelling market growth include surging demand from textile manufacturers, animal feed producers, detergent manufacturers, pharmaceutical companies, and cosmetics vendors (Li, et al., 2012).

In 1998, world-wide enzyme sales was valued at US$1.5 billion, with a projected annual growth rate of 2% in the leather industry, 15% in paper and pulp industry and 25% in animal feed enzymes (van Beilen & Li, 2002). With increase in global demand for enzymes, the market value rose steadily between 6.5% and 7.6% annually to US$ 3.3 billion in 2010 and is estimated to reach US$ 8 billion by 2015 (Li, et al., 2012). Figure 2.1, reproduced from Li *et al* (2012) shows the growth of the enzymes market from 2008 to 2010 with a projected value of US$ 8 billion for 2015. Figure 2.2 and 2.3, respectively show the distribution of enzyme sales by industrial sector and geographical region in the first quarter of 2011 by *Novozymes AS*, a global enzyme production company.
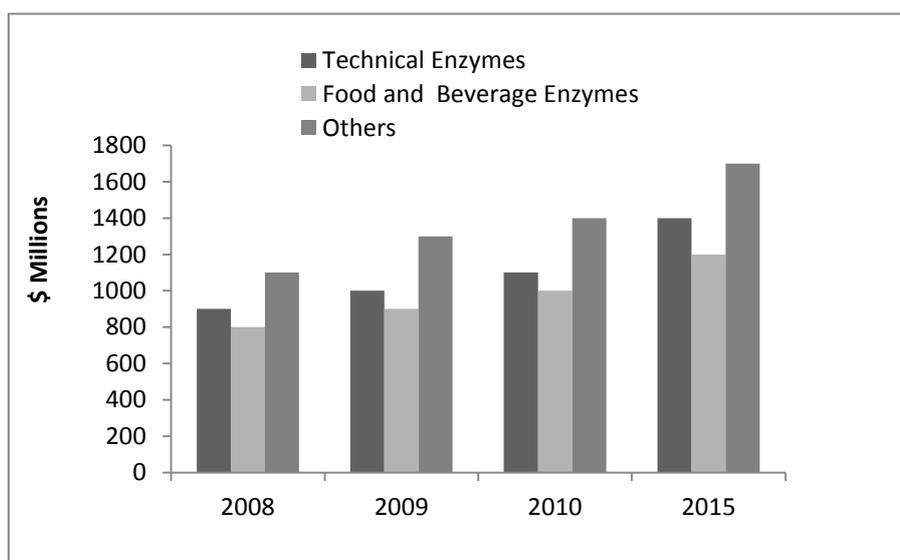
Figure 2.1 Global industrial enzymes market, 2008-2015 (Li *et al*, 2012)
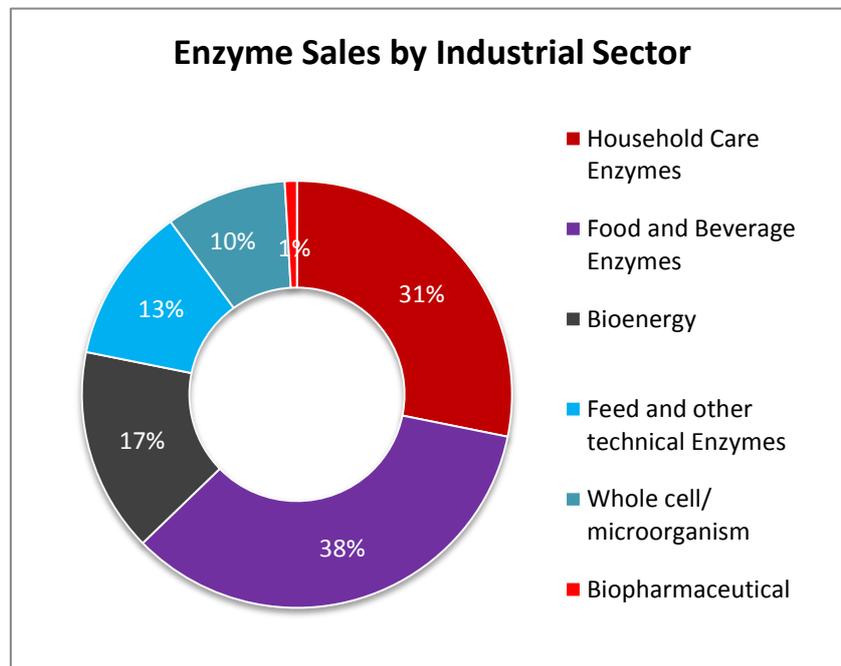
Figure 2.2 Distribution of enzyme usage by type of industry (Novozymes AS, 2011)
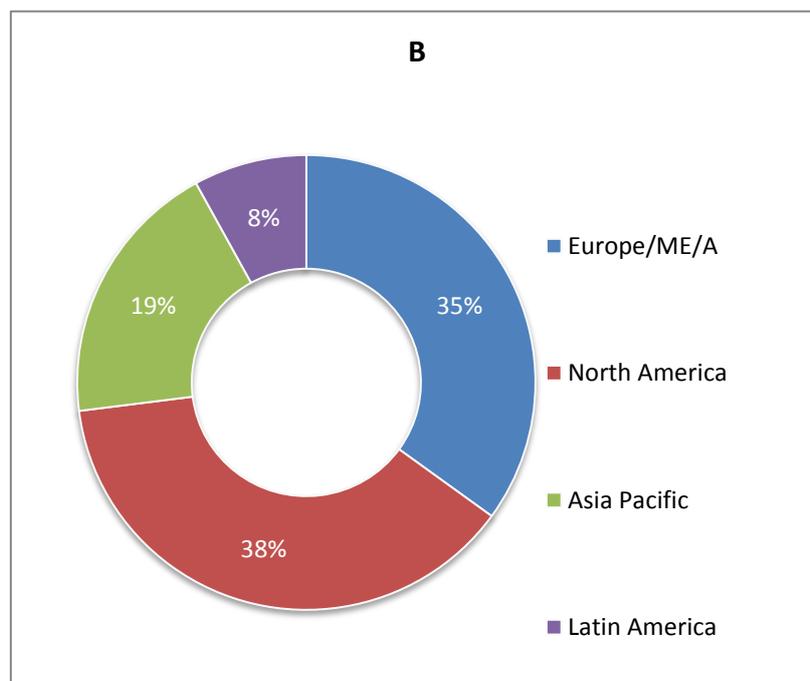
Figure 2.3 Enzyme sales by geographical area (Novozymes AS, 2011)

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2.2.10 Fructooligosaccharides and β-Fructofuranosidase

During the last decade, there has been a rapid development in a group of food additive products called nutraceuticals or functional foods that have the ability to prevent and treat diseases in addition to their fundamental nutritional value. Short chain fructooligosaccharides (sc-FOS) obtained from sucrose are an example of these foods. sc-FOS form part of Foods of Specified Health Use (FOSHU), which consist of dietary fibre, sugar alcohols, peptides and proteins, prebiotics, phytochemicals and antioxidants and polyunsaturated fatty acids (Yanai, et al., 2001). These products were estimated to have a market value of US$2 billion in the year 2000. The major producers and consumer countries were the United States, the United Kingdom, Germany France and Japan (Sangeetha, et al., 2005).

sc-FOS are non-cariogenic sugars with low calorie contents, because they are not broken down in the gastro-intestinal tract. They enhance the selective proliferation of bifidobacteria in the colon (prebiotic effect) at the expense of harmful microbial species that cause colon diseases (Fernandez, et al., 2007). sc-FOS also reduce cholesterol, triglyceride and glucose levels in blood and are therefore used as sweeteners for foods for *type 2* diabetic patients (Sangeetha, et al., 2005). Apart from their pre-biotic, anti-cancerous and anti-diabetic functions, sc-FOS are also used in the formulation of light jams, ice cream and confectionary products all in the category of food processing, as dietary fibre to aid fermentation in the large intestines in humans and as an aid in mineral absorption and lipid metabolism in both humans and farm animals (Chen, et al., 2011)

Naturally, sc-FOS are present in small quantities in vegetables (onion, tomato and garlic), cereals (rye, barley), brown sugar and honey. Industrially, sc-FOS are obtained from sucrose by the action of β-fructofuranosidase on sucrose. This enzyme has high transfructosylating

activity and is natively expressed in several bacteria and fungi such as *Aureobasidium pullulans* and *Aspergillus niger* (Chen, et al., 2011; Maiorano, et al., 2008). Optimum temperature and pH for the enzymatic reaction for conversion of sucrose to sc-FOS have been reported with maximum conversion efficiency of 60% w/w (Fernandez, et al., 2007; Chen, et al., 2011). sc-FOS can also be produced by the action of inulinase on inulin (Rocha, et al., 2006). In the current research, the production the β-fructofuranosidase enzyme, produced by expression of a synthetic gene in recombinant *Pichia pastoris* is optimised. The composition of the medium used in fermentation is altered to reduce the production cost of the enzyme, and therefore the final cost of the sc-FOS product, while the feeding rates and oxygen availability for the process were also optimised.

2.2.11 Xylan and α-Glucuronidase

The major hemicellulose in hardwood is xylan. Due to the presence of polar side chains in the molecule, xylan is highly soluble in water and therefore not applicable as a biopolymer compared to the insoluble cellulosic and starch components of wood (Polizeli, et al., 2005). To increase the industrial value of xylans extracted from woody biomass, it is modified by removing the polar side chains from the backbone to enable formation of insoluble precipitates (Gomes, 2012). Conventionally, physical methods such as ultrasound and chemical treatment methods are used for structural modification of xylan (Xue, et al., 2008). Enzymatic modification has also been successfully applied, and holds significant advantages such as having greater substrate specificity and control over the side-chain removal process (Gomes, 2012; Chimphango, et al., 2012). Xylan biopolymers modified with an α-glucuronidase, for side chain removal resulting in precipitation in water, can be used for the production of hydrogels for encapsulation of drugs, as dietary additives in animal feed to improve digestibility and as bio-films for coating paper (Ebringerová, 2005; Chimphango, et al., 2012).

α-Glucuronidase (EC 3.2.1.139) is an accessory enzyme used for enzymatic modification of xylan from lignocellulosic biomass into insoluble biopolymers (Polizeli, et al., 2005). The unique property of this enzyme resides in its ability to remove *4-O*-methyl glucuronic acid side groups from xylan without hydrolysing the xylan backbone, thus retaining the xylan biopolymer structure and potential end-applications (Tenkanen & Siika-aho, 2000; Gomes, 2012). Generally, there are two classes of α-glucuronidases—the first with activity towards xylan oligomers (only cleaves side-chains from short chain xylan) and the second with activity towards polymeric xylan, naturally expressed only in *Schizophyllum commune* and *Scheffersomyces stipitis* (Gomes, 2012; Tenkanen & Siika-aho, 2000). Natively expressed α-glucuronidases from fungi (Heneghan, et al., 2007; Siika-aho, et al., 1994) and bacteria (Nurizzo, et al., 2002) with activity towards oligmeric xylan have been isolated and characterised by several researchers. However, a distinct paucity exists in the literature on the production of the α-glucuronidases from *S. commune* and *S. stipitis* with polymeric activity, for commercial application by recombinant expression systems. The use of α-glucuronidases with activity towards polymeric xylan prevents the requirement for endocatalysts (xylanases) to first degrade the polymer before enzymatic modification is applied. Additionally, hydrogels produced from polymeric xylan offer superior properties in their applications compared to oligomeric xylan hydrogels (Chimpango, et al., 2012; Gomes, 2012). Therefore the production of *S. stipitis* α-glucuronidase by recombinant *S. cerevisiae* is of interest, and is studied in this work, together with scale-up effects on the enzyme production.

2.3 Enzyme Production Systems

Enzymes of industrial significance that are expressed naturally by organisms may not be suitable for industrial scale production, due to low production levels and low purity (Delroisse, et al., 2005). Therefore the production of enzymes for both research and industrial applications is typically performed with an engineered expression system—an organism that

has been genetically engineered to incorporate a gene encoding the enzyme (protein) of interest into its genomic DNA (Falch, 1991). As part of the engineering strategy, the expression of the gene is placed under control of a particular promoter, which determines the levels and frequency of expression of the foreign gene, and therefore the recombinant enzyme production. Promoters that are commonly used to control gene expression in yeast strains include *PGK, AOX, ENO1, GAP, ADH1* and *GAL10*. Each promoter has specific cultivation conditions that maximise the expression of genes under its control and fermentation technology seeks to meet these demands. Recombinant organisms obtained by genetic engineering produce relatively pure enzymes in large quantities, especially when secreted into the culture supernatant, as compared to the natural strains that may produce a variety of enzymes at the same time (Rai & Padh, 2001; Estell, 1993; van Beilen & Li, 2002). During genetic engineering, a secretory pathway may be triggered for the release of recombinant enzymes into the supernatant to facilitate protein separation and purification (Shuler & Kargi, 2002; Rai & Padh, 2001; Prescott, 2002).

Escherichia coli, Aspergillus niger, Bacillus spp., Pichia pastoris, *Saccharomyces cerevisiae, Hansenula polymorpha* and *Yarrowia lipolytica* are significant expression systems that have been genetically modified for the successful production of recombinant proteins such as lipases, cellulases, amylase, β-fructofuranosidas and other enzymes for industrial applications (van Beilen & Li, 2002). The last four of these expression systems are eukaryotic fungi and therefore produce recombinant proteins with post-translational modifications similar to proteins produced by higher plants and animals, making these systems more preferable for protein expression as compared to bacterial systems.

2.3.1 *Pichia pastoris* expression system

2.3.1.1 Overview

Pichia pastoris is a single-cell fungus (yeast) that is easy to grow and handle. It has been successfully used for production of several recombinant proteins owing to its high proliferation rate, ability to grow in simple, inexpensive media and the presence of a promoter originating from the alcohol oxidase I (AOX*1*) gene that is exclusively suitable for the regulated production of proteins (Romanos, 1995). Like most eukaryotes, proteins produced by *P. pastoris* undergo post-translational modifications such as disulphide bond formation, folding and glycosylation as in animal and plant cells. Therefore recombinant proteins produced by *P. pastoris* are fully functional upon secretion, compared to the lethargic cell inclusions or inactive peptides produced in bacterial systems (Creg, 1985). Moreover, it does not produce toxins during cultivation and it is generally safe to cultivate. Heterologous proteins produced by *P. pastoris* are free from contamination by bacteria or viruses (Cino, 2009).

2.3.1.2 A Brief History of *Pichia pastoris*

Successful transformation of *P. pastoris* for the production of recombinant proteins was first reported in 1985 by Salk Institute of Biotechnology (SIB). In the late 1970s, Phillips Petroleum Company established fermentation procedures and developed media required for cultivating *P. pastoris* on methanol to reach cell densities above 130 g/L dry cell weight for the production of single cell proteins (SCP). The SCP production by *P. pastoris*, however, was marred by high costs of methanol (Creg, 1985).

Researchers at SIB were contracted by Philips Petroleum in the early 80s to do more research on the organism. They isolated the AOX*1* gene together with its promoter and further developed strains, vectors and genetic engineering methods for *P. pastoris.* In 1993, Invitrogen Inc. acquired patents and licences, respectively, from Phillips Petroleum on *P.*

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pastoris expression system, allowing them to sell the *P. pastoris* system to researchers and commercial facilities all over the world. (Creg, 1985)

2.3.1.3 Gene Expression and Secretion of Heterologous Proteins in *P. pastoris*

P. pastoris is methylotrophic yeast capable of metabolising methanol as carbon and energy source. The metabolism of methanol in *P. pastoris* starts with conversion of methanol to formaldehyde in an oxidation process that finally yields hydrogen peroxide. The reaction is catalysed by alcohol oxidase *(*AOX*)* enzyme. There are two genes coding for AOX in *P. pastoris* —AOX*1* and AOX*2*, which are induced by methanol (Daly & Hearn, 2005). In recombinant DNA technology, the gene coding for the recombinant protein is incorporated into the *P. pastoris* genome such that it is regulated by the *AOX1* promoter. Thus the expression of the recombinant protein is activated by the presence and metabolism of methanol in the growth medium. The AOX*1* gene uses oxygen poorly (Higgins, 2001) therefore it is expressed to high levels in *P. pastoris* to compensate for its deficiency in oxygen uptake. This effect leads to elevated expression of the recombinant gene that is controlled by this promoter and up to 30% of total cell protein (TCP) can be AOX*1* enzyme if methanol is used solely as the carbon source to cultivate *P. pastoris* (Creg, 1985). Glucose, however, represses the expression of the AOX*1* gene (Daly & Hearn, 2005).

Another promoter that has been used for the successful expression of proteins in *P. pastoris* is the glyceraldehyde-3-phosphatedehydrogenase (GAP*DH*) constitutive promoter *(*GAP*),* which was first isolated in 1997 (Potvin, et al., 2010). Unlike the inducible AOX*1* promoter that requires methanol for protein production, the GAP promoter is constitutive; hence biomass production and recombinant protein expression occur simultaneously and are directly linked to the GAP-controlled gene copy number. Whereas process conditions in AOX cultivation need precise control, especially during the methanol induction phase, GAP systems have minimal control requirements but the organism must be maintained in growth phase for longer periods. Steady and high-throughput cultivation using the GAP promoter can

be achieved in continuous culture with longer production periods. GAP-controlled genes are expressed in high quantities when *P. pastoris* is cultivated on glucose, glycerol or methanol (Potvin, et al., 2010).
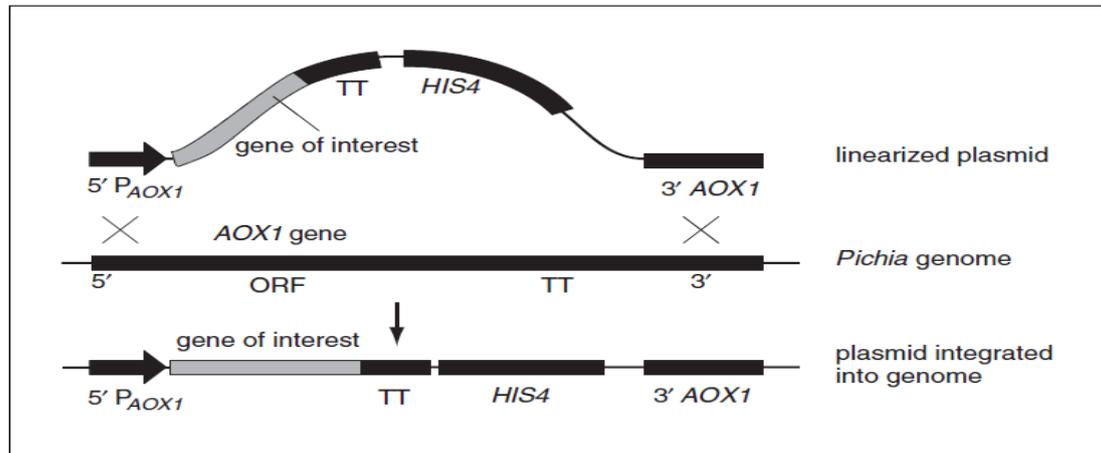
Figure 2.4 A gene replacement event in *P. pastoris* (Adapted from Higgins, 2001)

The recombinant gene is integrated into the *P. pastoris* system through homologous recombination. When the gene of interest contains regions that are homologous to the host gene sequence, the two genes can be matched which eventually leads to insertion of the foreign gene into the host genome. Figure 2.4 shows a gene replacement event at the AOX*1* locus by a plasmid fragment carrying an expression cassette containing AOX*1* promoter (Higgins, 2001).

Secretion of proteins into the extracellular medium requires a signal sequence on the produced proteins to direct them to the secretory pathway. In genetic engineering of *P. pastoris*, a gene sequence called the alpha-mating factor (α-MF) of *S. cerevisiae* is incorporated into popular vectors as a secretion signal. This ensures that the expressed proteins are secreted into the growth medium, which greatly enhances subsequent downstream processing and purification of proteins (Creg, 1985). Extracellular secretion of proteins also ensures that the protein has undergone essential post-translational modifications

such as folding, disulphide bridge formation and glycosylation, and is therefore fully functional upon purification. Secretion also holds several advantages in downstream processing, with secreted enzymes often being of sufficient purity to allow direct application after concentration, with no further purification requirements, as was also done with the two enzymes produced in the present study (van Beilen & Li, 2002).

2.3.1.4. Medium and Culture Conditions for *P. pastoris* Cultivation

The medium for laboratory cultivation of *P. pastoris* as proposed by Invitrogen (USA) is composed of Basal Salts medium (BSM) and Pichia Trace Metals (PTM1) solution with varying carbon sources depending on the promoter driving the recombinant protein production. Table 2.2 shows the composition of the BSM. It contains Mg^{2+}, K^+, SO_4^{2-} and other macronutrients that are required for proper cell growth (Vogel & Todaro, 1997).

Table 2.2 Composition of BSM according to the Invitrogen Protocol

Component	Quantity (per litre)
Phosphoric acid, 85%	26.7 ml
Calcium sulphate	0.93 g
Potassium sulphate	18.2 g
Magnesium sulphate-7H_2O	14.9 g
Potassium hydroxide	4.13 g
Ammonium hydroxide	Variable (pH control)

Source: Invitrogen Protocol, 2002.

The composition of PTM1 solution used in laboratory fermentation of *P. pastoris,* according to the Invitrogen Protocol, is given in Table 2.3. Essentially, it contains relatively low concentrations of trace elements used in transport processes in cells, maintenance of osmotic balance, and serve as growth factors and enzyme cofactors (Ghosalkar, et al., 2008; Zhao, et

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al., 2008). Trace elements like cobalt, zinc and iron that serve as micronutrients and have been shown to decrease the duration of the lag phase (Zhang, et al., 2009) are contained in the trace salts solution.

Researchers have shown that by adding 1% v/v YNB (yeast nitrogen base), casamino acids and EDTA to the growth medium, the level of proteolysis in human interferon-α2b antigen produced in *P. pastoris* decreased significantly and relatively pure protein was obtained (Ayed, et al., 2008). It has been suggested that the casamino acids are preferentially attacked by proteases, EDTA chelates the secreted proteases to render them inactive whilst YNB serves as a source of nitrogen to augment cell growth.

Table 2.3 Composition of PTM1 Solution for *P. pastoris* cultivation

Component	Quantity (per litre)
Copper sulphate-5H_2O	6.0 g
Sodium iodide	0.08 g
Manganese sulphate-H_2O	3.0 g
Sodium molybdate-2H_2O	0.2 g
Boric acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Iron sulphate-7H_2O	65 g
Biotin	0.2 g
Sulphuric acid ,98%	5 ml

According to Cino (2009) and other researchers, protein production in *P. pastoris* is efficient at 30°C, and almost all protein expression stops at 32°C (Cino, 2009; Inan, et al., 1999). By

lowering the induction temperature from 30°C to 23°C, researchers recorded a tenfold increase in the production of herring anti-freeze protein with a corresponding reduction in protein degradation (Daly & Hearn, 2005). It has been proposed that these observations may be due to higher stability of the cell membrane and a reduction in the rate at which enzyme-degrading proteases are released into the extracellular medium at lower temperatures (Prescott, 2002; Wang, et al., 2009).

Daly and Hearn (2005) also investigated the effect of pH on the expression levels and quality of recombinant protein production in *P. pastoris* and reported a wide range of pH, from 3 to 6. Being an aerobic microbe, *P. pastoris* requires high amounts of oxygen for growth and product formation. Scientists at New Brunswick Scientific (Edison, NJ) observed that by changing from shake flask fermentations to bioreactors, production of thrombomodulin in *P. pastoris* increased by over 140% (Chen & Krol, 1996). In the fermenter, high oxygen concentrations can be attained to meet oxygen demands of highly proliferating culture by increasing agitation, the rate of air flow and by adding pure oxygen to the broth.

2.3.1.5 Fed-Batch Fermentation of *P. pastoris*

Production of recombinant proteins using *P. pastoris* with the inducible AOX*1* promoter requires methanol for the induction and subsequent expression of the foreign gene. However, residual methanol concentrations above 4 g/L in the broth may suppress cell growth (Celik & Calik, 2011). Therefore part of the optimization process in *P. pastoris* involves modifications in the actual fermentation process as well as using different carbon sources such as glycerol and sorbitol to achieve high cell densities in batch and fed-batch cultures before initiating the induction phase.

The first stage of *P. pastoris* cultivation is the glycerol batch phase (GBP) aimed at increasing cell concentration by growing the culture on selective media with glycerol as the only carbon

and energy source. Sorbitol has also been shown to be an effective carbon source and can be used to supplement glycerol in the batch phase (Zhang, et al., 2008). Secondly, a glycerol fed-batch (GFB) phase is started with glycerol to promote additional cell growth and de-repress the AOX1 gene. The methanol induction phase (MIP) is then begun by gradually switching the feed from glycerol to methanol which triggers the expression of the AOX 1 gene, and hence the recombinant protein. The addition of methanol as a carbon source during the production phase is regulated to levels that would only induce the AOX1 promoter without inhibiting cell growth. Cino (2009) reported that an increase in concentration of methanol from 0.15 to 1.0% v/v resulted in an increase in the levels of human β-2-glycoprotein I (domain V) production for Mut+ transformants by a factor of 10. The accumulation of methanol in the growth medium can inhibit cell growth (Higgins, 2001) and result in low levels of recombinant protein production. Therefore the feed rate is regulated so as not to accumulate methanol in the bioreactor which may inhibit further cell growth (Plantz, et al., 2006). Different feeding mechanisms and inclusion of a transition phase between the GFB and MIP can be included in the fed-batch process to increase the pre-induction cell concentrations and protein production levels. DO-stat, μ-stat and transition-phase methods of improving fed-batch culture productivity with *P. pastoris* are described below as examples of these.

In constant dissolved oxygen (DO-stat) fed-batch fermentation the substrate feed rate is coupled with the concentration of dissolved oxygen in the medium during GFB phase in both GAP and AOX strain cultivation. A rise of DOT above the set-point causes substrate (glycerol, sorbitol and methanol) to be fed into the bioreactor. When the DOT level falls below the set-point, the feed is stopped to reduce the metabolic rate of the culture and hence maintain the DOT at the set point. This so called on-demand feeding ensures that only the amount of substrate required is fed and there is no accumulation of substrate, which is especially important with methanol feeding, to avoid inhibition of growth and enzyme

production. The culture growth under these conditions is maintained near the maximum specific growth rate for the particular carbon source, leading to maximum biomass and recombinant enzyme production (Jimenez, et al., 1997; Lee, et al., 2003). Ferreira *et al* (2012) used DO-stat technique to overcome acute oxygen limitation in a 50 L bioreactor during cultivation of *P. pastoris* to produce single chain Antibody fragment (scFv).

Another technique employed in the GFB phase of both GAP and AOX strains is exponential feeding mechanism. This pre-programmed feeding profile maintains a constant specific growth rate throughout the fed-batch phase; it is thus called the μ-stat fed-batch. The maximum specific growth rate, or near-maximum specific growth rate achieved in DO-stat fermentation might not be the optimum for enzyme production, therefore μ-stat fed-batch may be more desirable to regulate the specific growth rate at values below the maximum specific growth rate. Bahrami and coworkers used μ-stat optimized at specific growth rate of 0.2 h^{-1} to achieve high biomass concentrations of $120g_{DW}/L$ and 1.4 g/L of recombinant human granulocyte colony stimulating factor (rhG-CSF) in *P. pastoris* culture (Bahrami, et al., 2008).

Another method used to increase the pre-induction biomass in AOX cultivations is the addition of a transition phase (TP) after the GBP, before the MIP. A dual substrate feed where the inducer (methanol) is fed together with glycerol is implemented. It serves to de-repress the AOX1 gene and simultaneously provide energy source (glycerol) for synthesis of enzymes in methanol metabolism. The TP typically lasts for 4-5hours (Cos, et al., 2006).

The last technique used by researchers to increase the biomass concentration, and consequently recombinant enzyme productivity during MIP of the AOX strain is to feed mixed carbon sources during the induction phase. In AOX fermentation, substrates such as sorbitol (Arnau, et al., 2010), glucose (Paulová *et al*., 2012), trehalose and mannitol (Xie, et al., 2005) that do not repress the *AOX1* gene are fed concurrently with the inducer (methanol).

They serve as sources of carbon and energy for further biomass production during the induction phase because *P. pastoris* grows poorly on methanol alone (Cino, 2009). Xie *et al* (2005) used mixed feed of methanol, sorbitol and lactic acid to double angiostatin production levels from 108 mg/l on methanol alone to 191mg/l on the mixed feed.

A relatively recent and emerging technique for expressing recombinant proteins in *P. pastoris* is to place the recombinant gene under the control of glyceraldehyde-3-phosphate (GAP) promoter during the genetic engineering. Due to the constitutive nature of this promoter, GAP strains offer flexibility in fermentation by eliminating the enzyme induction phase during production. The GAP strain is mainly grown on glycerol as carbon source and the enzyme is produced concomitantly with cell growth. This strain is more desirable for industrial scale production of enzyme and metabolites using *P. pastoris* in continuous fermentation (Cos, et al., 2006).

The fermentation techniques of the GAP strain are similar to those of the AOX strain, with the exception being the MIP. The GAP strain is cultivated in glycerol batch phase followed by the glycerol fed-batch phase. Being constitutive, most of the enzyme production occurs during the glycerol fed-batch phase. Delroisse *et al* (2005) reported of a two-fold increase in protein production in the GAP strain over AOX strain in shake flask cultures. Other researchers have reported high throughput steady human chitinase production rate of 300 mg/L in continuous fermentation of GAP strain lasting for 1 month with cell densities reaching 100 g/L dry cell weight (Goodrick, et al., 2001).

In the present study, FFase production under control of both the AOX and GAP promoters were studied, both in terms of a change of medium from defined medium (Invitrogen Protocol) to a semi-defined industrial medium. Additionally, statistical optimisation was applied to the glycerol feed rate (during GFB phase of both GAP and AOX strains) and DOT in DO-stat fed-batch fermentation with the semi-defined medium to maximize the yield of Ffase.

2.3.2 *Saccharomyces cerevisiae* Expression System

2.3.2.1 Overview

Saccharomyces cerevisiae, commonly known as baker's yeast or brewer's yeast, is the most successful fungus for production of recombinant proteins for the past three decades. Several reasons account for the success of *S. cerevisiae* in the production of foreign proteins. It has a GRAS (generally recognised as safe) status, with extensive knowledge of its genetic make-up and relevant metabolic pathways in the literature. It has a DNA that is easily manipulated with a variety of vectors. Being eukaryotic, it is able to secrete proteins that have gone through the necessary post-translational processing and are fully functional as compared to bacterial proteins (Strausberg & Strausberg, 1995).

Cavalieri *et al* reported of *S. cerevisiae* DNA in the residue of an Egyptian wine jar from 3150 BC (Cavalieri, et al., 2003). In 1680, Antonie van Leeuwenhoek observed yeast cells under a microscope, but considered them to be globular structures rather than living organisms. In 1857, French microbiologist Louis Pasteur proved that living yeast cells cause alcoholic fermentation and not chemical catalysts (Huxley, 1871). Pasteur demonstrated that sparging oxygen into the broth increased the yeast cell growth but inhibited alcohol production via fermentation − a phenomenon known as the Pasteur Effect. Natural strains of *S. cerevisiae* were used over the years in both the baking and wine industries for fermentation purposes, until 1978, when it was first used to produce recombinant proteins following its successful genetic transformation (Romano, et al., 2007). In April 1996, the *S. cerevisiae* genome project was completed, with a complete characterization and mapping of the genetic make-up of the organism. Apart from allowing further research into higher eukaryotic genomes, the *S. cerevisiae* genome program opened new doors for genetic manipulation of the organism to suit several needs in its applications (Dujon, 1996). Several commercial products such as the Hepatitis B surface antigen (HBsAg), insulin, granulocyte macrophage colony stimulating

factor (GM-CSF) and human growth factors are produced by *S. cerevisiae* expression system under industrial conditions (Celik & Calik, 2011).

2.3.2.2. Gene expression and foreign protein secretion in *S. cerevisiae*

Several promoters are available for gene regulation and control of recombinant protein expression in *S. cerevisiae*. These include MET3 expressed in the presence of the amino acid methionine, PHO5 controlled by inorganic phosphate, CUP1 triggered by Cu^{2+} ions and GAL1 and GAL10 which are both controlled by galactose. Table 2.4 shows the various promoters used in *S. cerevisiae* expression vectors and the corresponding conditions for expressing the gene.

Production of heterologous proteins in *S. cerevisiae* can be achieved through simple manipulation of the growth medium by adding nutrients or chemicals to trigger expression under the respective promoter; thus galactose for *GAL1*, phosphate for *PHO5*, and copper for *CUP1* (Strausberg & Strausberg, 1995). By using these promoters, it is fairly easy to control the time of product formation in fermentation involving high cell densities. Like *P. pastoris*, the *α-MF* signal sequence is also used in some of the vectors in *S. cerevisiae* to direct synthesised proteins to a secretory pathway (Hardjito, et al., 1993).

S. cerevisiae is capable of post translational maturation of synthesised proteins such as disulphide isomerization and both N- and O-linked glycosylation, although the glycosylation patterns may be quite different from mammalian glycosylation (Strausberg & Strausberg, 1995). Therefore recombinant proteins secreted by conventional *S. cerevisiae* have extensive applications in fields such as biofuels, food processing, detergent industry and leather making where the presence or absence of extra glycol-chains on the protein does not significantly affect its functionality. However, *S. cerevisiae* can be genetically modified to produce proteins that are accurately glycosylated; hence it has been used for the production of several

therapeutic proteins such as human growth factors, human hormones and human blood proteins (Erlend, et al., 2006).

Table 2.4 *Saccharomyces cerevisiae* promoters

Promoter	Expression Conditions	Mode of expression
Acid phosphatase (*PHO5*)	Phosphate-deficient medium	Inducible
Alcohol dehydrogenase I (*ADH I*)	2-5% glucose	Constitutive
Alcohol dehydrogenase II (*ADH II*)	0.1-0.2% glucose	Inducible
Cytochrome c_1 (*CYC1*)	Glucose	Repressive
Gal-1-P Glc-1-P uridyltransferase	Galactose	Inducible
Galactokinase (*GAL1*)	Galactose	Inducible
Glyceraldehyde-3-phosphate Dehydrogenase (GAP*D,* GAP*DH*)	2-5% glucose	Constitutive
Metallothionein (*CUP1)*	0.03-0.1mM copper	Inducible
Phosphoglycerate kinase (*PGK*)	2-5% glucose	Constitutive
Triose phosphate isomerase (*TPI*)	2-5% glucose	Constitutive
UDP galactose epimerase (*GAL10*)	Galactose	Inducible

(Source: Strausberg & Strausberg, 1995)

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2.3.2.3 Media and Culture Conditions in *S. cerevisiae* cultivation

S. cerevisiae basically grows on non-selective media which may be composed of yeast nitrogen base (YNB) as nitrogen source, a mineral salts solution to provide both major and minor nutrients and glucose as carbon source. For high level recombinant protein expression, casamino acids or single amino acids such as uracil, histidine and tryptophan that may serve as alternative sources of nitrogen are essential components of the growth medium. Echegaray *et al* reported of achieving biomass concentration of 75g l^{-1} and specific invertase activity of 175 U/mg by growing *S. cerevisiae* on sugar-cane blackstrap molasses supplemented with urea and penicillin (Echegaray, et al., 2000).

Several researchers and mass production facilities have cultivated *S. cerevisiae* at a temperature of 30°C and pH of between 5.0 and 6.5 for successful production of recombinant proteins (van Rooyen, *et al*., 2005; Krogha, *et al*., 2008). However, Miguel *et al* reported that maximum production of glucose-6-phosphate dehydrogenase (G6PD) enzyme in *S. cerevisiae* was achieved at a temperature of 35°C and a pH of 4 (Miguel, et al., 2007).

Dissolved oxygen concentration is one of the most critical factors in *S. cerevisiae* fermentation, considering that even at low specific growth rates, oxygen-limiting conditions can induce ethanol production in this yeast system (Lei, et al., 2001). Oxygen demand can be high in high cell density cultivations of *S. cerevisiae*. In large scale fermenters, vigorous agitation and aeration with intermittent supply of pure oxygen are used to meet oxygen demands in *S. cerevisiae* cultures. To prevent the culture from undergoing fermentative metabolism under anaerobic conditions, there should be enough oxygen to drive glucose through oxidative metabolism and this is achieved by keeping the dissolved oxygen concentration at levels above 20% saturation (Kim, et al., 2005). Once this dissolved oxygen level is maintained in *S. cerevisiae* culture, Kim et al (2005) explain that it is only the

Crabtree effect that induces ethanol production in the culture. Like *P. pastoris*, *S. cerevisiae* is also resistant to shear forces generated by rotating Rushton impellers up to speeds of 1200rpm (Boswell, et al., 2003) hence intensive agitation can be used to improve mixing and enhance oxygen transfer in the culture broth.

After a total cultivation period of 72 hours, Park *et al* (2009) recorded the highest activity of hepatitis B surface antigen produced in *S. cerevisiae* under the GAL1 constitutive promoter at 40 hours, during the exponential growth phase. Kumagai *et al* (1990) also showed that the maximum activity of α-amylase expressed in *S. cerevisiae* with the constitutive ENO promoter coincided with the maximum biomass concentration achieved in exponential growth phase, and both declined afterwards. However, proteins expressed under inducible promoters may be secreted in *S. cerevisiae* during the stationary and death phases. La Grange *et al* (1996) observed maximum activity during the stationary phase in *S. cerevisiae* culture expressing β-xylanase under ADH2 inducible promoter. However, two problems may be associated with prolonged fermentations: first, secreted enzymes may be degraded in the broth as the fermentations stays for longer periods. Secondly, the dying cells may lyse and release other proteins into the broth. This can significantly increase the impurity levels in the supernatant and make separation and purification of the desired product difficult and costly (Rai & Padh, 2001). Therefore the overall cultivation time is dependent on the recombinant protein; whether it is growth associated or not, as well as the nature of the promoter driving the gene expression.

2.3.2.4 Process optimization in *S. cerevisiae* expression system

S. cerevisiae has a complex metabolic mechanism, which oxidises sugars (glucose, sucrose and xylose) through the EMP pathway to carbon (IV) oxide or ethanol. *S. cerevisiae* is Crabtree positive, thus in the presence of excess carbon, the cells take up more of the carbon source than the amount that can go through the tricarboxylic acid cycle (Kreb's cycle) at a time. This results in a metabolic bottleneck that channels the excess carbon into an oxido-

reductive pathway, producing acetaldehyde which is eventually reduced to ethanol (Lei, Rotbøll and Jørgensen, 2001). Under aerobic conditions, high residual glucose concentrations in the growth medium coupled with high specific growth rates may promote oxido-reductive metabolism leading to loss of carbon to ethanol at the expense of biomass. This phenomenon is known as catabolite repression or the Crabtree effect (Paciello, 2009). However, ethanol in the growth medium suppresses cell growth and minimizes recombinant protein production (Mendoza-Vega, et al., 1994). Optimization of *S. cerevisiae* systems therefore looks at the possibility of producing high cell densities without triggering the Crabtree effect by regulating the amount of carbon available to the culture using fed-batch or continuous fermentation processes.

Fed-batch cultivation of *S. cerevisiae* for recombinant protein production is started with an initial batch phase at low glucose concentration, typically in the range of 5 g/L to 20 g/L (Guillou, et al., 2004). A fed-batch phase is initiated after the batch phase with constant glucose supply at a rate that allows the culture to grow below the *critical specific growth rate* (μ_{crit}) —the growth rate above which oxido-reductive metabolism starts; which is usually a fraction of the maximum specific growth rate (μ_{max}) (Paalme *et al*., 1997). Under fully aerobic conditions, the flow rate and concentration of feed are therefore regulated to maintain the substrate concentration at levels below the threshold that would initiate the Crabtree effect.

Mendoza-Vega *et al* investigated the effect of dilution rate on recombinant hirudin production in fed-batch fermentation and recorded maximum biomass and product yields at $\mu = 0.2$ h^{-1} (Mendoza-Vega, et al., 1994). Paalme *et al*. (1997), using continuous culture to cultivate *S. cerevisiae* on glucose recorded μ_{max} of 0.44 h^{-1} and μ_{crit} of 0.25 h^{-1}. Mendoza-Vega *et al* (1995) observed a μ_{crit} of 0.15 h^{-1} and μ_{max} of 0.32 h^{-1}. Cortassa and Miguel (1998) also using continuous cultures recorded μ_{max} of 0.35 and 0.325h^{-1} and μ_{crit} of 0.15 h^{-1} and 0.17h^{-1},, respectively for two different recombinant *S. cerevisiae* strains. From the literature, it is

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apparent that both the maximum and critical specific growth rates are intrinsic properties of the particular strain based on the set of genetic modifications it has undergone. Different strains may be engineered for different purposes, such as for ethanol production or enzyme production; and therefore may have different metabolic capabilities and constraints. The foreign genes incorporated into the strains may further impose different levels of burden on the metabolic activities of the host strain (Görgens *et al*., 2001), giving rise to different rates of growth and ethanol production thresholds.

In the current work, fed-batch culture is used to determine the maximum and critical specific growth rates of *S. cerevisiae* MH1000 pbk10D-glu under fully aerobic conditions. The overflow of carbon to ethanol, as a function of the specific growth rate and its impact on cell growth and expression of the α-glucuronidase gene under the control of the constitutive phosphoglycerate kinase (*PGK*) promoter is also investigated.

2.4 Fermentation Processes for Recombinant Enzyme Production

The term fermentation technically refers to the anaerobic activity of microbes on sugars to produce ethanol and carbon dioxide. Fermentation is a type of respiration in which the final electron acceptor in glycolysis is an inorganic species such as NO_3^-, Fe^{3+}, Cu^{2+} or SO_4^{3-} but not oxygen, resulting in the conversion of pyruvate to lactic acid, ethanol, acetone and butanol (Shuler & Kargi, 2002). Today the meaning of the word has been extended to include a wide range of enzymatic and microbial conversions, even in the presence of oxygen.

Most recombinant enzymes used for industrial applications are produced in submerged fermentation (SmF) in bioreactors with volumes of up to 1,000 cubic meters. In submerged fermentation, carefully selected microorganisms are grown in liquid or semi-liquid media enriched with nutrients and energy sources (Panke & Wubbolts, 2002). Depending on the microbe and recombinant protein being produced, it may be carried out under aerobic conditions with high concentrations of oxygen or under anaerobic conditions. As the

microorganisms metabolize the energy source and grow, the desired enzymes are produced in the process (Ogawa & Shimizu, 1999). The enzyme may either be secreted into the fermentation medium or it may be retained intracellularly. Depending on the type of enzyme being produced and the expression system used, different types of bioreactors with different feeds, different feeding strategies and different control methods may be employed (Doran, 1995; van Beilen & Li, 2002). In the current research, fully aerobic cultivations are used for the production of two industrially relevant enzymes with *S. cerevisiae* and *P. pastoris*. The enzyme in each case is secreted into the extracellular medium.

2.4.1 The Bioreactor

The bioreactor is the heart of every bioprocess. Conversion of substrates into desired products by microorganisms is achieved in the bioreactor. It provides a conducive and well monitored environment where the particular expression system employed can achieve optimal growth and product formation. Several types of bioreactors ranging from internal mechanical agitation to bubble columns and loop bioreactors (Najafpour, 2007) are used in microbial fermentation processes. The most common type of bioreactor used in the production of recombinant proteins is the continuously stirred tank reactor (CSTR) which employs internal mechanical agitation for mixing purposes. Bubble columns and loop bioreactors are used for cultivations where the cells are sensitive to mechanical agitation (shear sensitive), such as in cultivation of mammalian and insect cells. Although these reactors have high energy efficiencies in low-viscosity Newtonian broths, their major drawback is inadequate mixing in highly viscous broths (Shuler & Kargi, 2002).

A stirred-tank reactor is shown in Figure 2.5. Typically, it consists of a cylindrical vessel made of either glass; for small reactors or stainless steel for larger reactors. It is fitted with a mechanically rotated shaft, which may have one or more impellers mounted on it depending on the size of the reactor and is driven by an external motor. The rotational motion of the

shaft creates a vortex characterised by laminar flow and inefficient mixing. Thus, baffles are installed on the wall of the vessel to break fluid packets and ensure turbulent conditions for efficient mixing. Bioreactors for cultivating shear sensitive cells do not usually have baffles.
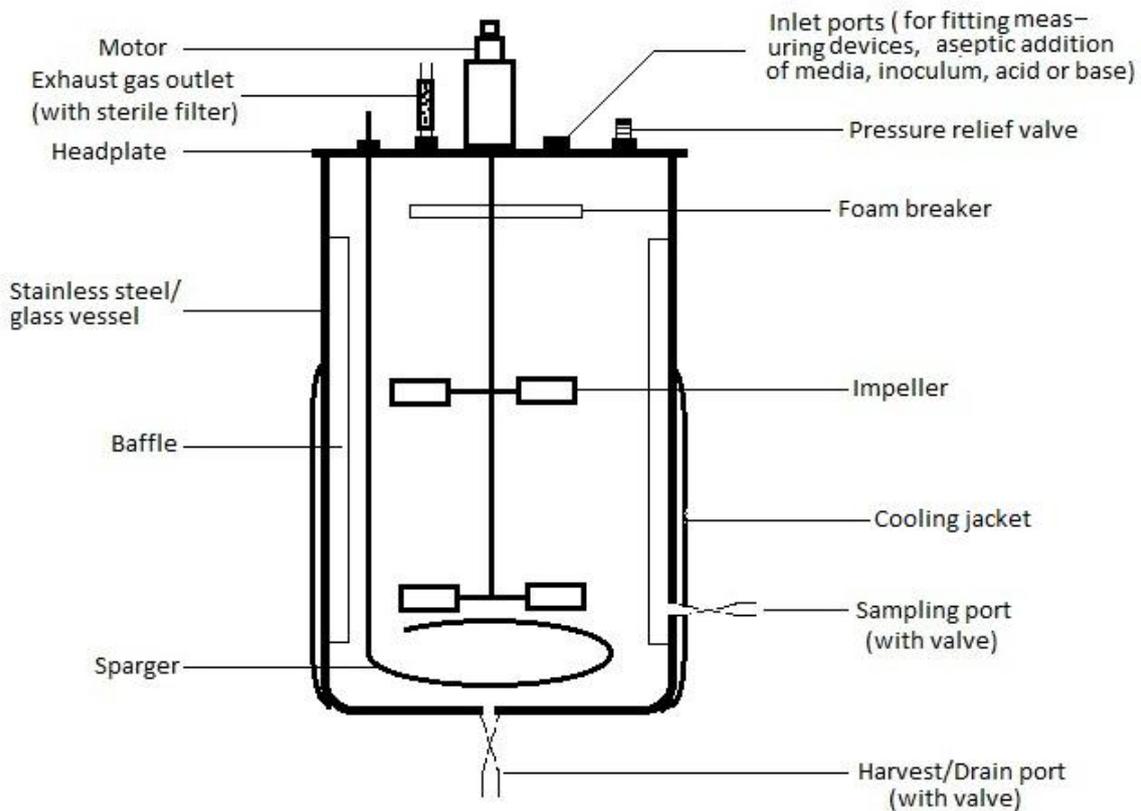
Figure 2.5 Stirred tank bioreactor

Foam formation is a common occurrence at high agitation and aeration rates, especially if the concentration of proteins is quite high. Foaming is undesirable as it may trap cells, impede product formation and fill up the bioreactor headspace. A flat blade mounted on the shaft at the surface of the broth may be used to break foam. Chemical anti-foaming agents may also be added to the media in small amounts as large amounts may interfere with microbial growth. The bioreactors used in this research work are the CSTR type of reactors with air sparging.

2.4.2 Fed-Batch Fermentation Techniques

Fed-batch culture is operated by feeding fresh media into the bioreactor after an initial batch period without removal of media. In this way, microbial populations can be maintained in exponential growth phase for longer periods of time as the growth limiting substrate is in constant supply (Ferreira, 2001). The growth rate of many recombinant protein production systems is slowed down by the presence of large amounts of residual substrates in the bioreactor due to substrate inhibition or catabolite repression. For instance, *P. pastoris* growth is inhibited by excess methanol in the broth whilst *S. cerevisiae* may produces ethanol in the presence of excess glucose (Creg, 1985; Strausberg & Strausberg, 1995). These phenomena are avoided in enzyme fermentations by using fed-batch culture. The substrate feed rate is regulated such that residual substrate concentration is maintained below the threshold for catabolite repression or substrate inhibition and both biomass and enzyme production rates are regulated by the substrate feed rate. For instance, Rojas *et al* (2011) recorded biomass concentration of 48 g/L in fed-batch culture of *S. cerevisiae* compared to 2.2g/l in batch culture due to elimination of catabolite repression in the former. Fed-batch culture is also important during protein expression under inducible promoters like the AOX*1* promoter in *P. pastoris* because the culture can be cultivated to high cell densities before initiating protein induction (Ferreira, 2001; Zhang, et al., 2009). The continuous supply of fresh medium dilutes the fermentation broth so as not to accumulate metabolites to concentrations that may have deleterious effects on cell growth and recombinant product formation (Chen, et al., 2004; Chen & Krol, 1996).

An important parameter used to control the performance of fed-batch fermentation is the dilution rate, D expressed as

$$D = \frac{F}{V} \tag{2.1}$$

where F is the substrate flow rate (Lh^{-1}) and V is the volume of the fermentation broth in litres. The dilution rate and feed concentration determine the amount of substrate available to the culture at any point in time. Thus, in principle, *D* controls the growth rate of the culture at quasi steady state. Therefore the dilution rate can be used to keep the growth rate at a particular level if quasi steady state conditions are achieved in the fermentation (Najafpour, 2007; Ramirez, et al., 1994). However, this steady state condition is difficult to attain in practice (Maurer, et al., 2006). A relatively new fed-batch fermentation technique based on the method used by James *et al* (2012) is used in this research to study the metabolic responses (in terms of biomass and product yields) of *S. cerevisiae* to different specific growth rates and substrate concentrations. The method allows the direct control of the specific growth rate with the amount of substrate fed. It is based on mathematical formulations derived from fundamental growth relations and mass balances. It enables prediction of biomass concentration and volume of broth in the bioreactor at any point in time, so that the required amount of glucose that will drive the culture to grow at a pre-determined growth rate is fed into the bioreactor at an exponential feed rate.

Due to the industrial orientation of the present study, the methods developed are those that can easily be translated into full production plants. Hence fed-batch culture, as opposed to chemostat culture is used in the research. Typically, chemostats are good for academic investigation of physiological phenomena in yeasts (Shuler & Kargi, 2002) but they are not frequently used in industrial settings, because:

1. chemostats lack the level of traceability of products offered by batch and fed-batch culture. Traceability is a core requirement of good manufacturing practices (GMP) in both food and pharmaceutical industries.

2. plasmid shedding—genetically modified strains tend to lose the integrated foreign gene after replicating over a large number of generations (Calik & Calik, 2012).

Therefore in a chemostat, plasmid shedding and minor mutations are a common occurrence since the culture growth can run for a long time.

3. chemostats are prone to contamination problems on the industrial scale.

2.4.3 Bioreactor Monitoring and Control

Most operating parameters in a bioreactor must be kept constant or nearly constant throughout a fermentation run. The success of fermentation depends on the constancy of the environment surrounding the cells, at least within some experimental error. Significant fluctuations in process variables can lead to low biomass and recombinant protein yields due to creation of unfavourable conditions for cell proliferation under such variations. Therefore as part of bioprocess optimization, process variables such as temperature, pH, dissolved oxygen (DO) and foam must be maintained at their set-point values throughout the cultivation (Harms, et al., 2002; Alford, 2006).

Using a feedback control system, a PID control scheme maintains the temperature at the set point by either turning electric heating elements in the wall of the vessel on/off or by regulating the inflow of steam or cooling water in the bioreactor jacket. The cultivation temperature is usually monitored with an accuracy of not less than ± 0.5°C from the set point in both *P. pastoris* and *S. cerevisiae* cultivations (Romanos, 1995; Rojas, et al., 2011).

In certain cultures, the products of microbial metabolism released into the broth may be acidic or basic and may alter the pH of the growth medium. The pH of the broth is measured with a pH probe connected to the control console. Peristaltic pumps connected to the control console regulate the pH at the set point by pumping either acid or base solution into the broth depending on the measured pH value.

In aerobic fermentations, it is necessary to maintain the dissolved oxygen concentration above a certain critical level. The concentration of dissolved oxygen in a fermenter is measured with

a dissolved oxygen probe. There are two types in common use: galvanic electrodes and polarographic electrodes. Both types of probes have membranes that are permeable to oxygen and are used to measure the partial pressure of oxygen in the broth. The levels of dissolved oxygen in high cell density cultures of both *P. pastoris* and *S. cerevisiae* fermentation at large scale are controlled by increasing the air flow rate, mixing the air stream with pure oxygen, increasing the degree of agitation or a combination of the three. In modern bioreactors control of the impeller speed and gas quality are usually cascaded with the measured oxygen levels. Increase in aeration rate above certain levels in vvm (volumes of air/volume of broth) should be accompanied by corresponding increase in agitation to prevent gas channelling (Garcia-Ochoaa, et al., 2010).

Intense agitation and aeration coupled with the presence of surfactants in the culture broth, may lead to formation of foam. Foam results from the dispersion of gas bubbles in the liquid medium. Foam formation may be enhanced when proteins adsorb to the gas-liquid interface (Kordialik-Bogacka & Ambroziak, 2007). At concentrations as low as 1mg/l, proteins can adsorb at interfaces and cause excessive foaming (Prins & van't Riet, 1987). Hence fermentations for the production of heterologous proteins are almost invariably susceptible to the problem of foaming. Even though mild foaming conditions improve mass transfer in the reactor (Prins & van't Riet, 1987), excessive foaming should be avoided because it contributes significantly to fouling and contamination, especially in *S. cerevisiae* systems (Čepononytė, et al., 2008). Entrapment of cells and substrate in the foam and filling up of reactor headspace also reduce productivity. Excessive foaming can be stopped by adding chemical anti-foam agents or by mechanical foam breakers. Chemical anti-foams are made of silicon-based solvents and act by decreasing the interfacial tension of the broth. Anti-foaming agents may be added prior to foam formation (Varley, et al., 2004) or during fermentation with an appropriate dosing pump.

2.5 Scale-up of Fermentation Processes

Large scale production of recombinant proteins for industrial applications requires operation of bioreactors larger than those used in laboratory and pilot scales. According to the *Six-tenths* rule, production costs in a typical fermentation process for recombinant proteins increase by only $2^{0.6}$ if production capacity is doubled (Votruba & Sobotka, 1992). Transfer of microbial processes from laboratory or pilot scale to industrial scale however, presents some challenges such as poor mixing, inadequate oxygen supply in intensive cell cultures and high risk of contamination. The physical environment of the cells, such as the hydrostatic pressure and temperature distribution, as well as biological factors like the number of generations the inoculum goes through are affected by the scale of operation (Junker, 2004). The main objective of bioreactor scale-up for heterologous protein production is therefore to replicate in the larger bioreactor the conditions of a fermentation process performed at a laboratory scale (Diaz & Acevedo, 1999).

Several approaches to bioprocess scale-up based on the theory of models and principles of geometric similarity are reported in the literature (Vogel & Todaro, 1997). Scale-up parameters are usually combinations of process variables and coefficients that affect oxygen transfer, heat removal and the homogeneity of the broth and therefore have some impact on the physiology of the microorganism. (Diaz & Acevedo, 1999).

2.5.1 Constant $k_L a$ Scale-up

The volumetric mass transfer coefficient ($k_L a$) governs oxygen transfer from the gas phase to the liquid phase for use by microorganisms. The basis of a constant $k_L a$ scale-up is to ensure the maintenance of a certain mass transfer capability to meet the oxygen demand of the culture at the larger scale (Ozbek & Gayik, 2001). The overall mass transfer potential of the broth is the product of $k_L a$ and the oxygen gradient. Sparging with pure oxygen increases the solubility of oxygen in the broth, and therefore increases the oxygen gradient but $k_L a$ remains

constant. Adding oxygen-vectors however, increases $k_L a$ by improving the diffusion of oxygen across the hydrodynamic boundary layer surrounding the cells, (Galaction, et al., 2004; Fyferling, et al., 2008).

The agitation and aeration rates in a bioreactor are related to $k_L a$ by a semi-empirical correlation given in Equation 2.2 (Stanbury, et al., 1995; Fyferling, et al., 2008)

$$k_L a = \alpha \left(\frac{P_g}{V_L} \right)^a v_s^{\ b} \tag{2.2}$$

where P_g represents the power of the impeller motor (W) under gassing conditions, V_L the broth volume (m^3), v_s the gas superficial velocity (volumetric gas flow rate/cross-sectional area of vessel, ms^{-1}) and α, a and b are empirical constants specific for the broth and impeller type and determine the dependency of $k_L a$ on mixing and aeration rates. At aeration rates less than 1 vvm, the gas holdup in the bioreactor and the concomitant reduction in broth density are small, therefore the ratio of gassed power to ungassed power approaches 1 (Hari-Prajtino, et al., 1998). Under these conditions, the degree of mixing (P_g/V) can be approximated with the correlation for ungassed systems (Diaz & Acevedo, 1999; Junker, 2004)

$$\left(\frac{P_g}{V} \right) = \frac{\rho N^3 D_i^5}{D_i^3} \tag{2.3}$$

and the gas flow rate (v_s) calculated with the equation

$$v_s = \frac{\dot{V}}{A} \tag{2.4}$$

where ρ represents the broth density (kg/m^3), N the impeller speed (Hz), D_i the impeller diameter, $\dot{V}$ the volumetric air flow rate and A the cross-sectional area of the bioreactor.

In constant $k_L a$ scale-up, the value of $k_L a$ is experimentally determined in the small bioreactor and Equation 2.2 is used to determine the agitation and aeration rates to give equal $k_L a$ in the

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larger bioreactor after the constants α, a and b are experimentally determined in the small scale. In the present study, these constants are experimentally determined in *S. cerevisiae* broth and used to formulate an equation of the form of Equation 2.2 for calculation of agitation rate and gas flow rate at the larger scale.

The methods for measuring $k_L a$ have been discussed by Garcia-Ochoa and Emilio (2009) and include the sulphite oxidation (SO) method, dynamic gassing out method, direct measurement with online gas analysers and using the oxygen yield coefficient. In the SO method, all the dissolved oxygen in the bioreactor at an instant is used to oxidise a known mass of sodium sulphite (Na_2SO_3) to sodium sulphate (Na_2SO_4). The rate determining step in this reaction is the transfer of oxygen from the gaseous phase into the liquid medium, therefore the rate of the reaction can be used to determine the rate of oxygen transfer, hence $k_L a$ (Garcia-Ochoaa, et al., 2010).

The most common method of determining $k_L a$ is the dynamic gassing out method because it is applied *in situ* with the actual fermentation broth. In this method, the air supply to the bioreactor is stopped, and the fall in DO level is plotted against time. The slope of the curve obtained is exclusively the oxygen uptake rate (OUR) of the culture. Upon resumption of air supply, the rise in DO levels (dC/dt) with time depends on both the oxygen uptake rate and oxygen transfer rate (OTR) according to Equations 2.5 and 2.6:

$$\frac{dC}{dt} = OTR - OUR \qquad (2.5)$$

$$\frac{dC}{dt} = K_L a\left(C^* - C\right) - q_{O_2} X \qquad (2.6)$$

where q_{O2} is the specific oxygen consumption rate (g O_2 $g_x^{-1}s^{-1}$), X the biomass concentration (g/L) C* and C the dissolved oxygen concentration at maximum solubility and at the current conditions, respectively. After some time of sparging, the rate of oxygen transfer equals the

rate of oxygen uptake and dC/dt remains constant. If the DOT measured at this equilibrium is C_e, integration of Equation 2.6 yields (Appendix II, Doran, 1995)

$$\ln\left(\frac{C_e - C_1}{C_e - C_2}\right) = k_L a(t_2 - t_1)$$

$$(2.7)$$

where C_1 and C_2 represent the dissolved oxygen concentrations measured at times t_1 and t_2, respectively. Hence $k_L a$ can be determined from Fig. 2.6.
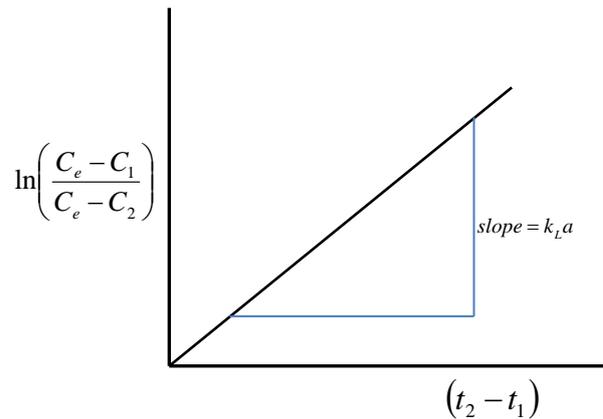
Figure 2.6 $k_L a$ determination using the dynamic gassing out technique

In the method of direct measurement, oxygen analysers are used to measure the molar flow rate of oxygen both in the inlet (F_{O2}^{in}) and exhaust (F_{O2}^{out}) gas streams with the assumption that the oxygen uptake rate is equal to the oxygen transfer rate (Fyferling, et al., 2008). Knowing the broth volume (V), the flow rates are used to calculate the oxygen uptake rate, which is related to $k_L a$ as shown in Equation 2.7 (Fyferling, et al., 2008).

$$OTR = K_L a\left(C^* - C\right) = \left(F_{O_2}^{in} - F_{O_2}^{out}\right)\frac{1}{V}$$

$$(2.8)$$

Flores *et al* (1997) used constant $k_L a$ to scale-up *Bacillus thuringiensis* fermentation from 14 L bioreactor to 1100 L with similar biomass and product yields as in small scale. In the

present study, the dynamic gassing out method is used to determine k_La for scale-up of *S. cerevisiae* system from 14 L laboratory scale bioreactor to 100 L pilot plant bioreactor.

2.5.2 Constant $\frac{P_g}{V}$ Scale-up

The volumetric dissipated power in aerated systems (P_g/V) or the gassed power per unit volume can also be kept constant upon scale-up. The mixing time and impeller tip speed may increase upon scale-up with constant Pg/V but the degree of turbulence does not change due to constant size of eddies upon scale-up (Junker, 2004). The degree of turbulence has profound effects on both mass transfer and homogeneity of the broth. At low aeration rates, the gassed power per unit volume (W/m^3) can be approximated with Equation 2.3 (Diaz & Acevedo, 1999). However, at aeration rates above 1 vvm, the gassed power may be 50-65% less than the ungassed power due to the loss in broth density upon aeration (Hari-Prajtino, et al., 1998).

2.5.3 Scale-up with constant impeller tip speed (ITS)

The impeller tip speed used for scale up purposes is expressed as

$$\frac{N_2}{N_1} = \frac{D_{t,1}}{D_{t,2}} = \left(\frac{V_1}{V_2}\right)^{\frac{1}{3}} \tag{2.9}$$

which assumes that $ITS = \pi N D_i$, where N$_1$ and N$_2$ are the impeller speeds (rpm) of the small scale and large scale bioreactors,, respectively, D$_t$ and D$_i$ are,, respectively the vessel and impeller diameters, and *V* the vessel volume (Junker, 2004). The *ITS* gives a measure of the degree of shear forces that exist at the tip of the impeller blade and it is particularly useful for scale-up of shear sensitive culture such as filamentous fungi and mammalian cell culture.

2.5.4 Scale-Down approach

Operating bioreactors at the pilot and industrial scales for the purpose of scale-up studies can be expensive and time consuming. An alternative approach to study scale-up behaviour is to operate a smaller bioreactor at the operating conditions of the bigger reactor in order to simulate the level of heterogeneity in environment existing in the larger bioreactor, a method called scale-down approach (Shuler & Kargi, 2002). The scale-down approach can be used to identify and evaluate problems at the large scale.

To do a scaled-down study, the appropriate method of scale-up is used to calculate the operating variables of the bigger reactor based on experimental data from a laboratory scale reactor. Instead of applying these calculated variables to a larger reactor, they are used to operate the laboratory bioreactor, with the assumption that the small reactor is a control volume extracted from the bigger reactor and that it adequately represents the environmental conditions in the larger bioreactor at the prevailing operating conditions (Junker, 2004). The approach has been used to investigate the type of variations in dissolved oxygen concentrations, CO_2 evolutions rate and substrate distribution in microbial processes at large scale (Sweere, et al., 1987). One operating variable that is not adequately modelled in the scale-down approach is heat distribution, so modifications to control temperature separately are necessary (Neubauer & Junne, 2010).

2.6 Project Aims and Objectives

The aim of this study is to improve fed-batch fermentation processes for the production of industrial enzymes with recombinant yeast-based systems, specifically production of α-glucuronidase in *S. cerevisiae* and β-fructofuranosidase in *P. pastoris*. The research seeks to elucidate the relationship between enzyme yields and key fed-batch fermentation parameters such as the substrate feed rate, dissolved oxygen concentration, the type of medium used as

well as change of production scale in fermentation, though not all these parameters are studied in one system. The effect of substrate feed rate on enzyme yield in fed-batch culture is evaluated in the two yeast systems: in *Saccharomyces cerevisiae* careful control of the substrate feed rate is used to keep the growth rate low to avoid ethanol production whereas in *Pichia pastoris*, on-demand control of substrate feed rate is used to maintain the culture at near maximum growth rates on both growth and protein-inducing substrates. To further investigate the sensitivity of the substrate feed control strategy to change of production scale, data from laboratory scale experiments is used to design and run fed-batch fermentation in a pilot scale bioreactor for the production of α-glucuronidase in *S. cerevisiae*. The impact of external process variables on enzyme production, specifically the dissolved oxygen concentration maintained in the fermentation broth is also studied in the *Pichia pastoris* system.

The medium used in fermentation has profound effects on microbial growth and consequently, recombinant enzyme yield. To evaluate the impact of medium on industrial enzyme production in fed-batch processes with yeasts, the effect of a change of medium from a chemically defined medium to semi-defined medium is investigated in the production of β-fructofuranosidase by *Pichia pastoris*. The specific objectives of the two sections of the research are summarized next.

2.6.1 α-Glucuronidase Production in *S. cerevisiae*

Saccharomyces cerevisiae is a Crabtree positive yeast and therefore produces ethanol under certain conditions in fed-batch culture. To maximise enzyme yield in *S. cerevisiae* culture, ethanol production must be minimised, therefore the objectives are:

1. To use the substrate feed rate to regulate the growth rate of the culture, and to find the critical specific growth rate above which ethanol production starts in glucose limited fed-batch fermentation of *S. cerevisiae* MH1000 pbk10D-Glu;

2.   To investigate the effect of specific growth rate on the expression levels of the α-glucuronidase gene under the constitutive *PGK* promoter in *S. cerevisiae* MH1000 pbk10D-glu;

3.   To study the effect of scale-up on the process from 14 L laboratory fermenter to 100 L pilot scale.

2.6.2 β-Fructofuranosidase Production in *Pichia pastoris*

Fed-batch fermentation of *Pichia pastoris* for production of recombinant enzymes is done predominantly using a chemically defined medium. Apart from high production costs, this medium also shows salt precipitation during autoclaving.

In the production of recombinant enzyme with *P. pastoris* under the constitutive GAP promoter, the enzyme expression occurs during the glycerol fed batch phase (GFB). Under the AOX promoter, GFB phase is used to maximise the biomass concentration prior to protein induction with methanol. The MIP is the most critical phase in enzyme production under AOX promoter and much research was conducted on this phase (Cereghino, et al., 2002; Lee, et al., 2003) , and standard operating protocols exist in the literature for managing MIP. The optimisation work in this research was therefore limited to the GFB to improve the pre-induction biomass production. The specific objectives were:

1.   To develop a semi-defined medium as an alternative to the chemically defined medium for *Pichia pastoris* fermentation.

2.   To use response surface methodology (RSM) to establish optimal dissolved oxygen concentration and glycerol feed rate during GFB phase of *Pichia pastoris* fermentation, using the semi-defined medium.

CHAPTER THREE

Production of α-Glucuronidase by Recombinant *Saccharomyces cerevisiae*

Chapter accepted for publication:

Abstract

During recombinant enzyme production in yeast systems using fed-batch culture, there can be significant changes in enzyme production levels with a change of production scale. This chapter discusses the production of α-glucuronidase enzyme in recombinant *S. cerevisiae* using fed-batch culture at laboratory and pilot scales. The process optimisation at laboratory scale in a 14 L bioreactor was aimed at using the substrate feed rate to control the specific growth rate of the culture and thereby minimise ethanol production in the fed-batch culture. A critical specific growth rate (μ_{crit}) of 0.12 h^{-1} was recorded in the fed-batch culture. Whereas a constant biomass yield coefficient ($Y_{x/s}$) of 0.4 g g^{-1} was recorded below this μ_{crit}, a steady decrease in $Y_{x/s}$ was observed at growth rates above μ_{crit} with increase in ethanol production. Thus, optimal biomass and α-glucuronidase concentrations of 37.35 g/L and 14.03 mg/L, respectively were recorded during fed-batch culture at a growth rate equal to $\mu_{crit} = 0.12$ h^{-1}.

To investigate process reproducibility between laboratory and pilot scales during enzyme production in this yeast system, scale-up from 14 to 100 L bioreactor was performed on the basis of constant $k_L a$ at $\mu_{crit} = 0.12$ h^{-1}. At the larger scale, respective biomass and

α-glucuronidase concentrations of 31.99 g/L and 13.92 mg/L were recorded, which were similar to values obtained at 14 L scale. However, the scale-up resulted in a shift of μ from the set $\mu_{crit} = 0.12$ h^{-1} to 0.13 h^{-1} during the fed-batch phase and accumulation of ethanol up to 2.5 g/L was observed. This shift in μ and the associated ethanol production were attributed to inefficient mixing at the larger scale, which would induce oxido-reductive metabolism in substrate-rich zones in the larger bioreactor. The work presented in this chapter provides valuable information on the effect of substrate feed rate on recombinant enzyme production in fed-batch culture of the Crabtree positive *Saccharomyces cerevisiae*, as well as the effect of change of scale on the overall performance of the fed-batch culture.

The current chapter was submitted to Biochemical Engineering Journal for publication as an original research article on 13th May 2013, under the title "Optimisation and Scale-up of α-Glucuronidase Production by Recombinant *Saccharomyces cerevisiae* in Aerobic Fed-Batch Culture with Constant Growth Rate". After an initial review process, the manuscript was re-submitted with major revisions on 18th July 2013. After final review, it was accepted for publication on 15th September 2013. Dr Eugéne van Rensburg and Prof Johann Görgens, all of the Department of Process Engineering, Stellenbosch University co-authored the article.

Declaration by Candidate

With regards to Chapter 3 (pages 47-69), the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Physical experimental work, generation of results, analyses and data processing	100 %
Chapter/article write-up	70 %

The following co-authors have contributed to Chapter 3 as follows

Name	email	Nature of contribution	Extent of contribution (%)
Eugene van Rensburg	eugenevrb@sun.ac.za	Critical reading/ supervision	15 %
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Signature of candidate…………Emmanuel Anane………..

Date …………………………………………22nd November 2013……………..

Declaration by co-authors

The undersigned hereby confirm that:

1. The declaration above accurately reflects the nature and extent of the contribution of the candidate and co-authors to Chapter 3,

2. No other authors contributed to chapter 3 beside those specified above, and

3. Potential conflicts of interests have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 3.

Signature	Institution of affiliation	Date
Eugene van Rensburg	Dept of Process Engineering, Stellenbosch University	22-11-2013
Johann F Gorgens	Dept of Process Engineering, Stellenbosch University	22-11-2013

3.1   Introduction

α-Glucuronidase (EC 3.2.1.139) is an enzyme used for enzymatic modification of xylan from lignocellulosic biomass into insoluble biopolymers (Polizeli, et al., 2005). The distinctive property of this enzyme belonging to family GH 115 resides in its ability to remove *4-O*-methyl glucuronic acid side groups from polymeric xylan, without the requirement for degradation of the xylan backbone to produce xylooligomers, thus allowing the production of water-insoluble biopolymers (Tenkanen & Siika-aho, 2000; Gomes, 2012). Modified xylan can be used to make hydrogels for drug encapsulation, together with applications in pharmaceutical and cosmetic industries (Gomes, 2012; Ebringerová, 2005; Chimphango, et al., 2012). Several reports are available on the native expression of α-glucuronidase by fungi (Heneghan, et al., 2007; Siika-aho, et al., 1994) and bacteria (Nurizzo, et al., 2002). In all of these studies, the α-glucuronidases that were investigated remove *4-O*-methyl glucuronic acid side chains only from xylooligomers (Siika-aho, et al., 1994; de Wet, et al., 2006). Only the α-glucuronidases produced by *Schizophyllum commune* (Tenkanen & Siika-aho, 2000) and *Scheffersomyces stipitis* (Gomes, 2012) (EC 3.2.1.139; present study) possess the unique ability to remove side chains from polymeric xylan. Production of the α-glucuronidase from *Scheffersomyces stipitis* (EC 3.2.1.139) with fed-batch culture at pilot scale is therefore of interest, to allow development of enzyme applications.

Saccharomyces cerevisiae is a versatile host that has seen the successful expression of a variety of heterologous proteins. Although recombinant proteins produced by *S. cerevisiae* may be hyperglycosylated, this expression host has found extensive application in the production of other technical enzymes used in the textile, paper and biofuel industries (Strausberg & Strausberg, 1995). *S. cerevisiae* is also generally regarded as safe (GRAS), further adding to the appeal of this host as heterologous expression system (Calik & Calik,

2012). Being a strong ethanologen (Calik & Calik, 2012), controlling ethanol production by *S. cerevisiae* during the production of recombinant protein in aerobic culture is critical to maximising the biomass concentration and product yield.

Being Crabtree-positive (Lei, et al., 2001), ethanol production occurs during the so-called overflow metabolism when the culture is grown above a critical specific growth rate and in the presence of excess glucose (Lei, et al., 2001; Rodrigues, et al., 2006). The Crabtree effect is based on the pyruvate dehydrogenase (*Pdh*) enzyme complex, responsible for converting pyruvate to acetyl Co-A, which has a ten-fold lower affinity for pyruvate than pyruvate decarboxylase (*Pdc*) (Lei, et al., 2001; van Dijken, et al., 1993; Rodrigues, et al., 2006). Hence, at high carbon flux, *Pdh* becomes easily saturated with excess pyruvate converted to acetaldehyde by *Pdc*, and eventually to ethanol through alcohol dehydrogenase (ADH1) (Lin & Tanaka, 2006).

To circumvent carbon overflow to ethanol, the rate of glycolysis (glucose flux through the Embden-Meyerhof-Parmas (EMP) pathway) must be regulated to maintain the culture in fully-oxidative respiration state. During fermentation, this is usually achieved by controlling the glucose available to the culture using either fed-batch or continuous culture where the rate of glucose supply, and associated growth rate, is limited by the rate at which respiration can occur (Paalme, et al., 1997).

The purpose of this study was to maximise the production of recombinant *S. stipitis* α-glucuronidase under control of the constitutive phosphoglycerate kinase promoter (*pPGK*) (Schena, et al., 1991) in *S. cerevisiae* using fed-batch culture. This was achieved by controlling the specific growth rate through control of the substrate feed rate to maximise heterologous protein production using fed-batch culture at 14 L scale, specifically by determining the growth rate at which carbon overflow to ethanol production occurred (μ_{crit}). Because quasi-steady state is difficult to achieve in fed-batch culture (Lee, et al., 1999), the

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fed-batch method used in this study was based on a dynamic control strategy using the glucose concentration and flow rate to directly regulate the carbon flux through the EMP pathway, thereby controlling the specific growth rate of the culture (James, et al., 2012). To bring the commercial production of α-glucuronidase using the *S. cerevisiae* MH1000pbk10D-glu production system closer to fruition, the system response to a change from laboratory to 100 L pilot scale was investigated on the basis of a constant k_La. Accurate control of the feed rate proved critical to ensure maximum enzyme production at 100 L pilot scale, to counter apparent mixing inefficiencies.

3.2   Materials and Methods

3.2.1   Yeast Strain and Inoculum Preparation

Saccharomyces Saccharomyces cerevisiae MH1000 pbk10D-glu used in all experiments was deposited in the yeast culture collection of the Dept. of Microbiology, Stellenbosch University, South Africa and was engineered to chromosomally express *Scheffersomyces stipitis* α-glucuronidase under control of a *PGK* promoter and terminator (data not shown). Stock cultures of this strain were stored in 1 ml aliquots with 20% (w/v) glycerol as cryoprotectant at -80°C. Cultures were routinely grown for 48 h at 30 °C on YPD agar plates, consisting out of (per litre) 10 g yeast extract, 20 g peptone, 20 g glucose and 13 g agar (Sigma-Aldrich, Kempton Park, South Africa). Starter cultures were grown in capped test tubes by incubating 5 ml YPD medium inoculated with several colonies from agar-grown culture on an orbital shaker (New Brunswick Scientific, Enfield, CT, USA) at 30 °C for 24 hours. Of these cultures, 30 ml was used to inoculate four 500 ml Erlenmeyer flasks, each containing 150 ml medium that consisted out of (per litre): 6.7 g yeast nitrogen base (YNB) without amino acids, 10g $(NH_4)_2SO_4$, 2.4g KH_2PO_4 and 10 g casamino acids (all Sigma-Aldrich, Kempton Park, South Africa) and sterilised in an autoclave at 121 °C for 15 minutes. Glucose was sterilised separately at a concentration of 500 g/L and added aseptically to the

culture medium to a final concentration of 20 g/L. Shake flask cultures were incubated at 30 °C on an orbital shaker (Yihder Technology Co. Ltd, Taipei, Taiwan) adjusted to 150 rpm for 24 h. The contents of the four flasks were used to inoculate the bioreactor containing 6 L medium with the same composition as the medium used in the previous cultivation step, with the only difference being a lower glucose concentration of 15 g/L.

3.2.2   Experimental Design

Single parameter optimization experiments were designed to determine the critical specific growth rate and to study the effect of specific growth rate on both enzyme yield and biomass production in *S. cerevisiae*. Based on literature (Strausberg & Strausberg, 1995), specific growth rates were chosen to be 0.08 h^{-1}, 0.12 h^{-1}, 0.15 h^{-1} and 0.25 h^{-1} in the range of ca. 25-85% of the maximum specific growth rate (μ_{max}). Triplicate fed-batch fermentation experiments were performed at each specific growth rate.

3.2.3   Fermentation

All fermentations at laboratory scale were conducted in a 20 L NBS Bioflo IV (New Brunswick Scientific, Enfield, CT, USA) with 14 L working volume. Throughout the cultivation, dissolved oxygen tension (DOT) was measured with a polarographic probe (Endress Hauser, Reinach, Switzerland) and the oxygen level was maintained above 30% of saturation using a control loop linking the agitation rate to the DOT. A constant aeration rate of 0.8 vvm corresponding to air flow rate ranging between 5 and 10 L/min was maintained throughout the cultivation by manually adjusting the air flow rate as the culture volume increased. The temperature was maintained at 30 °C, whereas the pH was monitored using combination glass pH electrode (Endress Hauser, Reinach, Switzerland) and controlled at pH 5.5 with 3N KOH. Foam formation was controlled by supplementing the medium feed with 300 μl/L antifoam (Antifoam 204, Sigma-Aldrich, Kempton Park, South Africa).

3.2.4   Exponential Feeding Profile

The fed-batch phase of all cultivations was conducted using an exponential feeding regime and was implemented upon exhaustion of glucose at the end of the initial batch phase, that was signalled by a spike in the DOT. The mass of cells could be predicted at any time and the specific growth rate controlled during the fed-batch phase using the exponential growth equation (Daramola & Zampraka, 2008)

$$X_t = X_o e^{\mu t} \tag{3.1}$$

where X_t is the mass of cells in grams at any time t, X_o the mass of cells at the beginning of the fed-batch phase in grams and μ is the specific growth rate (h^{-1}). As opposed to the control of μ with the dilution rate (D) under quasi-steady state conditions, a transient control regime was implemented where the rate at which glucose (the primary growth-limiting nutrient) was fed to the culture was used to maintain a desired specific growth rate. The mass of glucose added to the culture to achieve cell growth from an initial X_o to a final X_t can be described using the equation (James, et al., 2012; Chen, et al., 2008) (Appendix I).

$$S_m(t) = \frac{x_0 V_0 \left(e^{\mu t} - 1\right)}{Y_{x/s}} + S_0 \tag{3.2}$$

where, $S_m(t)$ is the mass of glucose (g$_{glu}$) added to the bioreactor at time t, S_0 the mass of glucose fed at time t_{i-1}, x_0 and V_0 the biomass concentration (g/L) and broth volume (L),, respectively and $Y_{x/s}$ the biomass yield coefficient. The input values for the biomass yield coefficient into this equation were determined in preliminary fermentations. For a typical fermentation, the fed-batch culture commenced at $x_o = 6.7$ g/L and $V_o = 6$ L after exhaustion of the initial glucose in the batch phase.

3.2.5   Specific growth rate calculation

The specific growth rate achieved in the fed-batch phase during transient control with glucose flux was calculated using the total mass of cells at each sampling point, according to the equation (James, et al., 2012; Miguel, et al., 2007) (Appendix I)

$$\mu = \left(\frac{1}{X_m}\right)\left(\frac{dX_m}{dt}\right) = \frac{d(\ln X_m)}{dt} \qquad (3.3)$$

where $X_m = xV$ and where X_m represents the total mass of cells (g), x the concentration of cells (g/L) and V the volume of the broth (L). The specific growth rate was therefore obtained from the natural logarithm of the mass of cells plotted as a function of time, with μ calculated from the slope using Equation 3.3. By using graduated containers, the volumes of KOH, glucose feed and samples were monitored and the overall volume (V) of the broth was corrected for the volume of KOH added for pH control, the volume of glucose feed added and all sample volumes to ensure accurate estimation of the true volume of the broth at all sampling times.

3.2.6   Sampling

During the batch phase, 15 ml samples were drawn every hour to determine the biomass concentration used to determine the exponential feeding profile. During the fed-batch phase, samples were taken in two-hour intervals to monitor cell growth and for subsequent analysis for enzyme, ethanol and glucose profiles. After the fed-batch phase, sampling time was increased to 4 hour intervals.

3.2.7   Analytical Methods

The dry biomass concentration of samples was determined gravimetrically by filtering triplicate 5 ml culture broth samples through marked and weighed 0.22 μm glass microfiber

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filter paper disks (Whatman Plc, Kent, UK), followed by washing and drying in a microwave oven. Residual glucose and ethanol in the fermentation supernatant were analysed using high performance liquid chromatograph (HPLC) equipped with a Cation-H Micro-Guard Cartridge, an Aminex HPX-87H Column (all Bio-Rad, Johannesburg, South Africa) maintained at 65 °C and an RI detector (Shodex, RI-101). Analytes were eluted using 5 mmol/L sulphuric acid at a flow rate of 0.6 ml/min and the area under peaks of eluted glucose and ethanol were related to concentration based on commercially available standards (Sigma-Aldrich, Kempton Park, South Africa).

3.2.8   α-Glucuronidase Quantification

The α-glucuronidase enzyme protein secreted into the supernatant was partially purified by centrifugation for 10 min at 8 000 rpm followed by ultrafiltration through a 30 kDA membrane (Amicon Ultra®, Merck Millipore). Due to the novelty of this particular α-glucuronidase enzyme having activity specifically directed at polymeric xylan, there was no established enzymatic assay to quantify the enzyme product, since the colorimetric assay described in the literature (Siika-aho, et al., 1994) for α-glucuronidase with activity towards oligomeric xylan lacked the required selectivity for the enzyme produced in the present study. Additionally, 4-O-methyl glucuronic acid was not commercially available as standard for quantification using HPLC techniques. Therefore the enzyme protein was quantified using standard scanning densitometry of an SDS-PAGE gel by serial dilution of samples to fall within the linear range of measurement (Bromage & Kaattari, 2007). Samples from fermentation runs together with samples from the supernatant of control experiments of wild-type *S. cerevisiae* MH1000 without the α-glucuronidase gene were separated on a 10% SDS-PAGE gel stained with silver staining according to the method described by Laemmli (Laemmli, 1970), using bovine serum albumin (BSA, Thermo Scientific, Waltham, MA, USA) as standard (Fig. 3.1). The α-glucuronidase protein with a molecular weight of 125

kDA (Tenkanen & Siika-aho, 2000) was identified relative to the molecular weight protein markers also included in the gel (Thermo Scientific, Waltham, MA, USA). Importantly, the lane containing the control sample did not show any protein band at 125 kDa (Fig. 3.1), thus confirming that this band was indeed α-glucuronidase produced by the recombinant strain. Stained protein bands were quantified according to their densities, relative to the BSA standard using Image J® software (v 1.45s, National Institute of Health, USA).
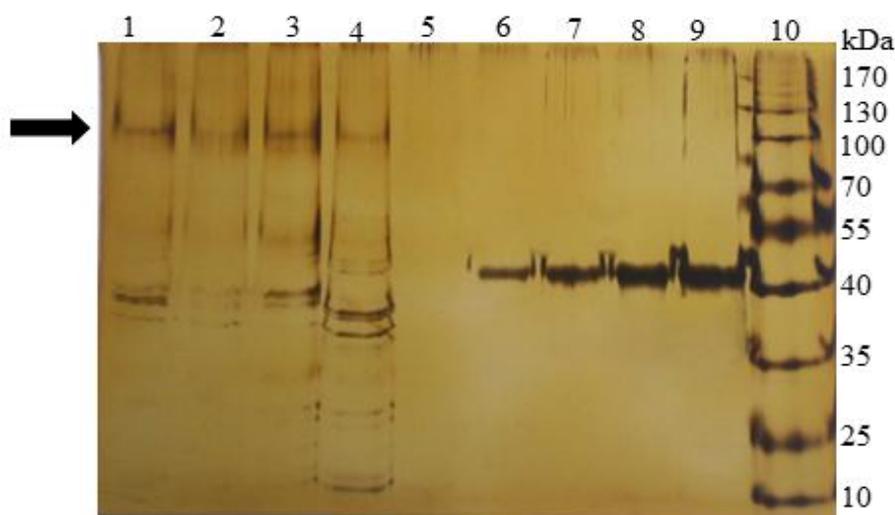
Figure 3.1 SDS-PAGE of α-glucuronidase during fermentation at $\mu = 0.08$ h^{-1}. Samples shown were taken at 12 hours (lane1), 18 hours (lane 2), 22 hours (lane 3), and 28 hours (lane 4) (10x diluted). Lane 5 was the control sample taken at 35 hours of cultivating wild type *S. cerevisiae* MH1000 in the same medium. Lanes 6, 7, 8 and 9 are BSA standards (10, 20, 30 and 40 mg/L, respectively), Lane 10 is molecular weight marker.

3.2.9   Scale-up Fermentation

Scale up experiments were done in a 150 L NBS Fermentation System bioreactor (New Brunswick Scientific, Enfield, CT, USA) with 100 L working volume, equipped with two Rushton impellers, a polarographic dissolved oxygen probe, a combination glass pH electrode (all Endress Hauser, Reinach, Switzerland), an EL-FLOW® air flow meter (Bronkhorst High-Tech B.V., Ruurlo, The Netherlands) and an Indusoft® Supervisory Control and Data Acquisition (SCADA) system (Indusoft Inc., Austin, TX, USA). During the fed-batch phase,

the mass of the vessel containing the glucose feed was monitored gravimetrically using an electronic balance and the feed was administered using a peristaltic pump (Watson Marlow, Cornwall, UK), controlled through the SCADA system to maintain the feed rate according to Equation 3.2. All process variables were maintained as at the laboratory scale.

The scale-up was based on a constant volumetric mass transfer coefficient ($k_L a$) to ensure that the mass transfer capability could be maintained at a sufficient level in the larger bioreactor vessel to satisfy the oxygen demand of the culture (Garcia-Ochoa & Emilio, 2009). The $k_L a$ in the 14 L bioreactor was determined using the dynamic gassing out technique in the presence of an actively growing culture as described by Doran (1995) and based on the equation (derivation given in Appendix II):

$$\ln\left(\frac{C_e - C_1}{C_e - C_2}\right) = k_L a(t_2 - t_1)$$
(3.4)

where C_e represents the equilibrium DOT in the fermentation broth at the prevailing aeration and agitation rates, and C_1 and C_2 the DOT recorded at times t_1 and t_2,, respectively, during re-oxygenation. To maintain a specific $k_L a$ during scale-up, calculation of agitation and aeration rates at the larger scale was conducted according to the empirical correlation (Junker, 2004)

$$k_L a = \alpha\left(\frac{P_g}{V}\right)^a v_s^b$$
(3.5)

where α, a and b are empirical coefficients determined by measuring $k_L a$ at different agitation and aeration rates and used to determine the dependency of $k_L a$ on the mixing intensity of the impeller (P_g/V) and the aeration rate (v_s) . In Equation 3.5, P_g represents the gassed power of impeller motor (W), V the volume of broth (m^3) and v_s the superficial gas velocity (m/s). The gassed power was calculated indirectly by determining the power ratio between the gassed and un-gassed broths. During the fed-batch phase, a power ratio of 0.83 was calculated using the equation (Doran, 1995)

$$\frac{P_g}{P_0} = 0.10 \left(\frac{F_g}{NV} \right)^{-0.25} \left(\frac{N^2 D_i^4}{g W_i V^{2/3}} \right)^{-0.20}$$

(3.6)

where P_0 represents the power consumption of the un-gassed broth, F_g the gas flow rate (m^3/s), N the impeller speed (hertz), g the acceleration due to gravity (9.82 m/s^2), W_i and D_i the width and diameter of the impeller,, respectively. Hence the power consumption per unit volume under gassing conditions was calculated using the equation (Diaz and Acevedo, 1999)

$$\left(\frac{P_g}{V} \right) = \frac{0.83 \rho N^3 D_i^5}{D_i^3}$$

(3.7)

where ρ is the broth density (kg/m^3).

3.3   Results

To determine the effect of specific growth rate on α-glucuronidase yield and ethanol production by recombinant *S. cerevisiae* MH1000 pbk10D-glu, four growth rates were selected for investigation in glucose-limited fed-batch fermentation. Initial batch fermentation gave a maximum specific growth rate of 0.3 ± 0.015 h^{-1}. Triplicate fed-batch fermentations were run for each pre-determined specific growth rate using exponential feeding mechanism to maintain a constant growth rate throughout the fermentation.

3.3.1   Fed-batch fermentations at 14 L scale

Growth curves depicting plots of the natural logarithm of the mass of cells during the fed-batch phase of cultivation at the four specific growth rates of 0.08, 0.12, 0.15 and 0.25 h^{-1} are shown in Fig. 3.2. The values for μ, calculated from the slopes of the curves, deviated by less than 5% from the values at which the specific growth rates were set. However, in Fig. 3.2, curves B and C showed a little flattening out getting to the end of the fermentation, which was

a deviation from exponential growth. Since these fermentations recorded the highest biomass concentrations, it may be explained that depletion of a medium component other than the carbon-source in the later stages of the culture resulted in this non-exponential growth. That notwithstanding, the linearity of the plotted data ($R^2 > 0.93$) proved that exponential growth at constant growth rate was maintained at most times during the fed-batch phase, and thus that the fed-batch control strategy was effective in controlling the culture growth rate.
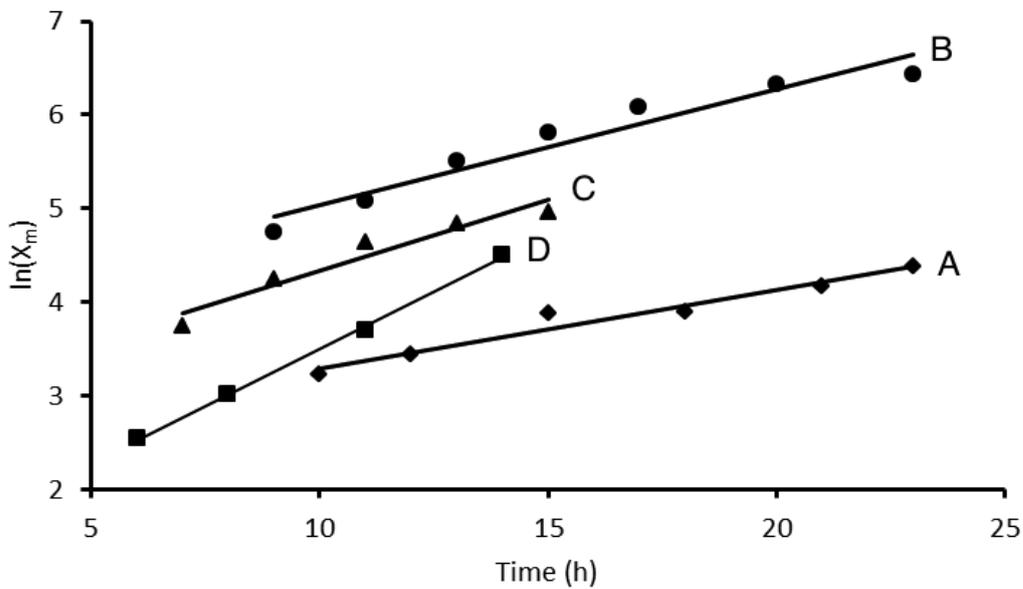
Figure 3.2 Plots of the natural logarithm of biomass accumulation during fed-batch phase of carbon-limited fed-batch cultures of *S. cerevisiae* MH1000pbk10D-glu at set points $\mu = 0.08$ h^{-1} (A); $\mu = 0.12$ h^{-1} (B); $\mu = 0.15$ h^{-1} (C) and $\mu = 0.25$ h^{-1} (D). Actual values for the specific growth rate, calculated from the slopes of the curves were $\mu = 0.084$ h^{-1} (A); $\mu = 0.124$ h^{-1} (B); $\mu = 0.152$ h^{-1} (C) and $\mu = 0.244$ h^{-1} (D), with the $R^2 > 0.93$ for all curve

Profiles depicting the masses of biomass, residual glucose, ethanol and α-glucuronidase at 14 L scale in cultures grown at the four specific growth rates during the fed-batch phase are shown in Fig. 3.3, with product concentrations and yields shown in Table 3.1. The mean maximum specific growth rate (μ_{max}) during the initial batch growth phase, prior to start of the fed-batch feeding, was 0.3 ± 0.015 h$^{-1.}$ The highest biomass concentration of 37.34 ± 0.65

g/L, corresponding to a mass of 528.26 g in fed-batch culture was recorded at a specific growth rate of 0.12 h^{-1}, and was significantly greater than the highest biomass concentrations recorded at specific growth rates of 0.15 and 0.25 h^{-1} (Table 3.1; Fig. 3.4). During the fed-batch phase, levels of residual glucose (Fig. 3.3) were close to zero in all fermentations, except at a specific growth rate of 0.25 h^{-1} where accumulation of low levels of glucose was evident, but was consumed immediately after cessation of feeding (Fig. 3.3D).

Table 3.1 Growth and product formation parameters at different specific growth rates in fed-batch culture of *S. cerevisiae* MH1000pbk10D-glu at 30 °C and pH 5.5 at 14 and 100 L scale. All experiments were conducted in triplicate with the standard deviation shown in brackets.

Parameter[a]	Set specific growth rate (h^{-1})				
	0.08	0.12	0.15	0.25	0.12[b]
Biomass (g/L)[c]	27.68 (0.97)	37.34 (0.65)	19.48 (1.6)	15.14 (1.2)	31.99 (0.31)
Ethanol (g/L)[d]	0.00	0.00	7.65 (0.62)	12.13 (1.02)	2.5 (0.014)
α-Glu (mg/L)	10.31(0.46)	14.74 (0.72)	9.58 (1.42)	5.49 (1.08)	13.92 (0.42)
$Y_{x/s}$ (g g^{-1})	0.40 (0.01)	0.40 (0.02)	0.24 (0.02)	0.15 (0.05)	0.41 (0.01)
$Y_{eth/s}$ (g g^{-1})	0.00	0.00	0.08 (0.0001)	0.08 (0.0003)	0.08 (0.008)
$k_{eth/x}$ (g g^{-1})	0.00	0.00	0.31(0.08)	0.54 (0.1)	0.20 (0.06)
$Y_{\alpha\text{-glu/s}}$ (mg g^{-1})	0.17 (0.01)	0.18 (0.03)	0.10 (0.001)	0.05 (0.004)	0.18 (0.09)
$k_{\alpha\text{-glu/x}}$ (mg g^{-1})	0.45 (0.001)	0.44 (0.03)	0.45 (0.08)	0.45 (0.006)	0.45 (0.05)

[a] Values shown for yields were determined by linear regression of data for biomass plotted as a function of substrate or product plotted as a function of substrate or biomass ($R^2 > 0.89$). All experiments were carried out in triplicate with the standard deviation shown in brackets. α-Glu: α-glucuronidase, eth: ethanol, x : biomass, s: substrate.
[b] Specific growth rate at 100 L scale, whereas all other experiments were conducted at 14 L scale
[c] Dry weight basis
[d] These values are ethanol concentrations produced during the fed-batch phase and do not include ethanol produced prior to the fed-batch phase

Similar ethanol concentrations were recorded in all cases during batch growth, reaching a mean concentration of 5.12 ± 0.87 g/L (corresponding to 30.72 g) before the feeding phase commenced (Fig. 3.3). Whereas the mass of ethanol remained constant or declined during the subsequent fed-batch phase at $\mu = 0.08$ h^{-1} and 0.12 h^{-1}, the level of ethanol increased at $\mu =$ 0.15 h^{-1} and 0.25 h^{-1} to respective average masses of 115.52 ± 4.33 and 199.28 ± 3.27 g/L (Figs. 3.3 C and D) at the end of fed-batch phase. The decrease in ethanol subsequent to the feeding phase could not be correlated to an increase in the mass of cells, and could possibly be attributed to evaporation, in addition to assimilation by the yeast as carbon source under glucose-limited conditions. Generally, the concentration of α-glucuronidase increased gradually, with the most prominent increase during the fed-batch phase of cultivation, irrespective of specific growth rate (Fig. 3.3). Furthermore, cessation of enzyme protein production clearly coincided with the end of the fed-batch phase, confirming that enzyme production was strongly growth-associated (Fig. 3.3).
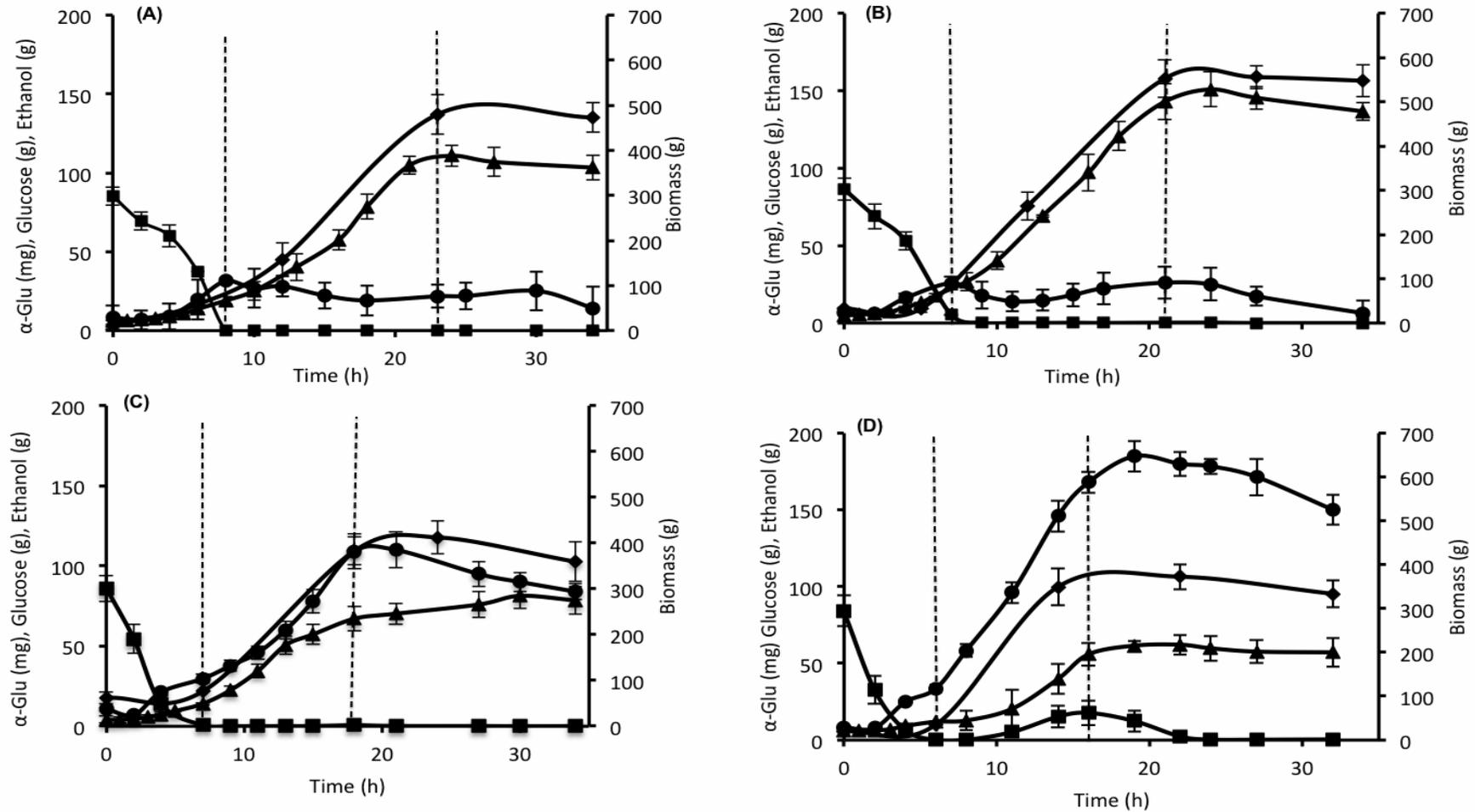
Figure 3.3 Growth and production profiles of *S. cerevisiae* MH1000pbk10D-glu showing the mass of biomass (▲), residual glucose (■), ethanol (●) and α-glucuronidase (♦) in carbon-limited fed-batch cultures using an exponential feeding profile at specific growth rates of $\mu = 0.08$ h^{-1} (A); $\mu = 0.12$ h^{-1} (B); $\mu = 0.15$ h^{-1} (C) and $\mu = 0.25$ h^{-1} (D) at 30 °C and pH 5.5. The vertical dotted lines indicate the start and end of fed-batch phase

3.3.2   The Influence of Specific Growth Rate on Product Formation

To illustrate the effect of specific growth rate on product formation, the maximum mass of biomass, ethanol and α-glucuronidase recorded during the fed-batch phase at different exponential feed rates were plotted as a function of specific growth rate (Fig. 3.4).
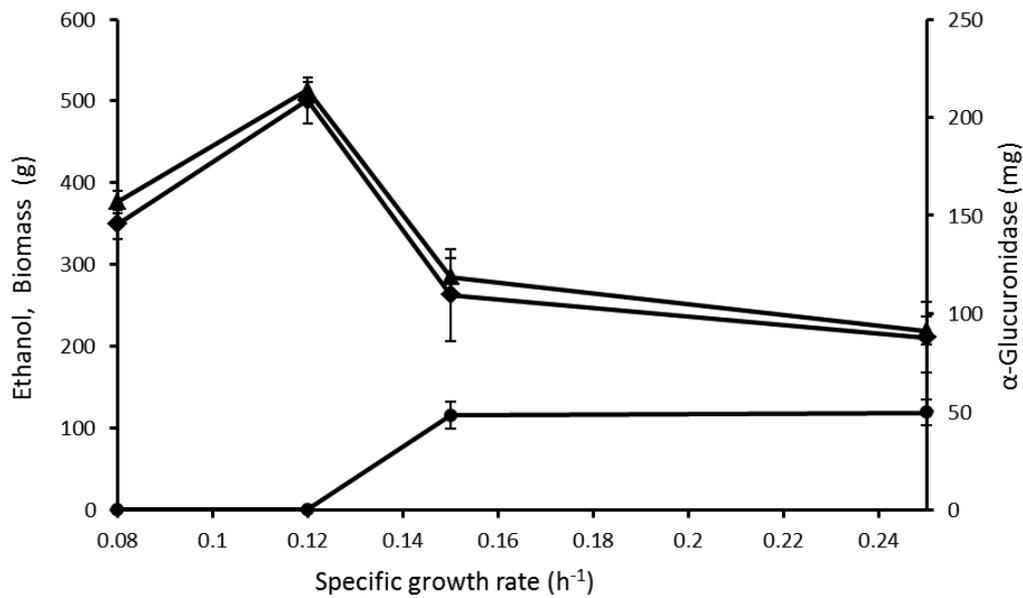
Figure 3. 4 Mass of biomass (▲), ethanol (●) and α-glucuronidase (♦) plotted as a function of the specific growth rate. Values are average of triplicate experiments where the error bars represent the standard deviation of each data point.

Generally, there was a close relationship between concentrations of biomass and α-glucuronidase with the highest masses recorded at $\mu = 0.12$ h^{-1}. Furthermore, ethanol accumulation at $\mu > 0.12$ h^{-1} suggested that this specific growth rate was close to the critical specific growth rate where carbon overflow occurred. Whereas the biomass yield coefficient of 0.4 g g^{-1} remained constant below $\mu = 0.12$ h^{-1}, further increases in the specific growth rate resulted in a marked decrease in the biomass yield ($Y_{x/s}$) with the lowest value of 0.15 g g^{-1} calculated at $\mu = 0.25$ h^{-1} (Table 3.1). Linear regression of data where $Y_{x/s}$ was plotted as a

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function of μ (Appendix IV) revealed that with an increase in the specific growth rate to μ_{max}, the biomass yield coefficient decreased linearly ($R^2 = 0.89$) and could be described by the relationship

$$Y_{x/s} = -1.9388\mu + 0.5927 \tag{3.7a}$$

$$0.12h^{-1} \leq \mu \leq 0.3h^{-1} \tag{3.7b}$$

Therefore, a strong negative correlation between $Y_{x/s}$ and μ during the fed-batch phase in the range specified in Equation 3.7b was identified. The close relationship between the biomass concentration and enzyme produced (Fig. 3.4) resulted in a constant α-glucuronidase yield relative to biomass ($k_{\alpha\text{-}glu/x}$) of 0.45 mg g^{-1} irrespective of the specific growth rate. Since the enzyme product was growth-associated, and the total biomass was influenced by the growth rate, the total enzyme was, consequently, indirectly related to the growth rate.

3.3.3   Fed-batch Fermentation at 100 L Scale

A volumetric mass transfer coefficient (k_La) of 108 h^{-1} was recorded in the fed-batch phase in the 14 L bioreactor. A series of k_La measurements at different agitation and aeration rates were used to establish according to Equation 3.5 the relationship between the gassed power of impeller motor per unit volume (P_g/V), the superficial gas velocity (v_s) and k_La, which can be expressed as

$$k_La = 1.62\left(\frac{P_g}{V}\right)^{0.66} v_s^{0.15} \tag{3.8}$$

The greater index of mixing (P_g/V) compared to that of the gas flow rate (v_s) suggested that the rate of oxygen transfer from the gas phase into the broth was more dependent on mixing than on the gas flow rate. Using Equation 3.8 and an aeration rate of 0.8 vvm, corresponding to 32 L/min, the minimum agitation rate to maintain a k_La value of 108 h^{-1} at the larger scale with 40 L of starting culture was calculated to be 75.2 rpm. At these operating conditions, a

k_La value of 102.34 h^{-1} was recorded at the larger scale, deviating by 5.2% from the set value. Although the mixing efficiency in the larger reactor would undoubtedly be less than that at 14 L scale, Equation 3.8 still proved an effective method to correlate k_La with the operating parameters, since the DOT could be maintained at saturation levels greater than 30% throughout the larger scale cultivations.

Profiles for biomass, ethanol and α-glucuronidase production at 100 L scale are shown in Fig. 3.5, with growth and production parameters summarised in Table 3.1. Given that the highest masses of biomass and α-glucuronidase were recorded at a specific growth rate of 0.12 h^{-1}at the 14 L scale, this specific growth rate was selected for the fed-batch regimen during scale-up. Generally, the trends for growth and product formation at 100 L scale closely resembled the profiles presented for fed-batch cultivations at the smaller scale (Figs. 3.3 B and 3.5).
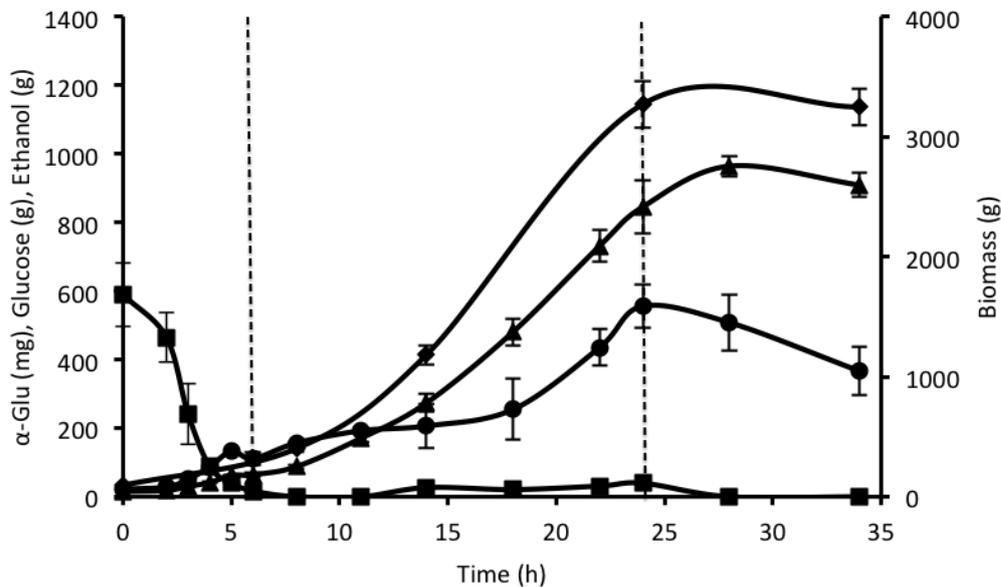
Figure 3.5 Mass of biomass (▲), residual glucose (■), ethanol (●) and α-glucuronidase (♦) in carbon-limited fed-batch culture of *S. cerevisiae* MH1000pbk10D-glu at 100 L scale maintained at $\mu = 0.12$ h^{-1} using an exponential feeding profile. The vertical dotted lines indicate the start and end of fed-batch phase.

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However, in contrast to smaller scale, the specific growth rate attained by the culture in the fed-batch phase at larger scale was $\mu = 0.131$ h^{-1}, which was 8.3% greater than the set $\mu = 0.12$ h^{-1}. Additionally, cultivations at larger scale were characterised by the accumulation of ethanol, reaching a maximum mean concentration of 2.5 ± 0.92 g/L, corresponding to a mass of 243.61 g (excluding ethanol produced in batch phase) at the end of the feeding phase. Although the biomass concentration of 31.99 ± 0.31 g/L (2753. 91 g, Fig. 3.5) at 100 L scale was almost 15% lower than that recorded at 14 L scale (Table 3.1), this decrease did not appear to influence α-glucuronidase production, with similar concentrations of 14.74 ± 0.72 and 13.92 ± 0.42 mg/L recorded at 14 and 100 L scale,, respectively (Table 3.1).

3.4   Discussion

Fed-batch culture has seen many applications for the production of yeast biomass and for the production of high value products by recombinant organisms (Minihane & Brown, 1986), including *S. cerevisiae.* To the knowledge of the authors, this is the first instance where α-glucuronidase (EC 3.2.1.139), with de-branching activity towards polymeric xylan, was produced using recombinant *S. cerevisiae* in fed-batch culture. When producing *S. cerevisiae* biomass for bakers' yeast (Daramola & Zampraka, 2008) or when the yeast is used as a host for heterologous protein production (Calik & Calik, 2012), careful control of the fermentation production system is critical to prevent the loss of carbon to ethanol. Ethanol production by *S. cerevisiae* during aerobic growth in a glucose carbon-limited culture depends on the metabolic route by which the glucose is catabolised. In turn, these routes are influenced by the concentration at which the carbon substrate is supplied to the culture and in the case of fed-batch or continuous culture, the rate at which the substrate is supplied, which determines the specific growth rate of the cells (Rodrigues, et al., 2006; James, et al., 2012).

In this study, a transient control of fed-batch culture based on glucose flux was used to great effect to control the specific growth rate of the culture and hence maximise the biomass and

growth-associated recombinant α-glucuronidase production. Compared to conventional quasi-steady state control with dilution rate, the method implemented in this work allowed the regulation of the specific growth rate under transient conditions when quasi-steady state has not been established. The rate at which substrate should be fed per unit time could be inferred from a pre-determined rate at which biomass would accumulate, given the biomass yield and the increase in culture volume per unit time. The data shown in Fig. 3.2 clearly attested to the efficacy of this method to maintain exponential growth at pre-determined specific growth rates. More specifically, the specific growth rate could be maintained at values below μ_{crit} where the biomass yield was at its maximum and ethanol production was minimised by not allowing the culture to enter respiro-fermentative growth. Massimo *et al.* (1991) used a similar inferential control strategy based on the oxygen uptake rate (OUR) to optimise the specific growth rate for penicillin production by *Penicillium spp.*

Controlling the growth rate of the culture at a range of exponential feed rates in fed-batch culture, the μ_{crit} for the strain used in this study was established to be close to 0.12 h^{-1}. At growth rates below this value, the data suggested that the glucose consumed by the yeast was completely oxidised, as evident from the absence of ethanol in the culture and the constant biomass yield coefficient of 0.4 g g^{-1}, thus approaching the theoretical maximum yield of 0.51 g g^{-1} (Table 3.1).

At $\mu > \mu_{crit}$, the conversion of a greater proportion of glucose to ethanol at the expense of biomass was clearly evident from the increase in the ethanol yield on biomass ($Y_{eth/x}$), whereas the $Y_{x/s}$ decreased substantially (Table 3.1, Fig. 3.3), suggesting the overflow of carbon via the *Pdc* pathway. These results were similar to that of Dantigny (1995) where a steady decrease in the biomass yield was observed at dilution rates greater than μ_{crit} in chemostat cultures of *S. cerevisiae* H1022. Moreover, the μ_{crit} observed in this work was 40% of μ_{max}, in agreement

with values reported in literature where μ_{crit} values range from 40 – 55 % of μ_{max} (for example, Mendoza-Vega *et al* (1994) and Paalme (1997)).

At specific growth rates greater than the critical growth rate the carbon overflow metabolism, and associated decrease in the biomass concentration, significantly reduced production of the constitutively-expressed α-glucoronidase (Fig. 3.4). Controlling μ at values lower than but close to μ_{crit} resulted in maximising the biomass yield and hence, enzyme protein. Similarly, Biener *et al.* also observed the highest biomass concentration at μ_{crit} (Biener, et al., 2012) using calorimetric control methods to maintain a constant μ in fed-batch cultures of *S. cerevisiae*. Therefore, the use of the *PGK* promoter, originating from the EMP pathway, for the constitutive expression of α-glucuronidase proved an effective strategy to maximise α-glucuronidase production in fed-batch culture. Due to constitutive control, the $k_{\alpha\text{-}glu/x}$ remained relatively constant, irrespective of growth rate (Table 3.1), which resulted in the greatest enzyme titre at μ close to μ_{crit} where $Y_{x/s}$ was at its maximum. Moreover, due to little to none carbon flow towards ethanol at $\mu < \mu_{crit}$, more carbon would be available for biomass production, which benefitted recombinant protein production, as evident from the enzyme yield on substrate ($Y_{\alpha\text{-}gluc/s}$) that were almost two-fold greater than when $\mu > \mu_{crit}$.

Scale-up on the basis of constant k_La was successfully applied, as revealed by the similarity in the trends of biomass and α-glucuronidase production at 14 and 100 L scale at $\mu = 0.12$ h^{-1} (Fig. 3.5). Two reasons can be proposed for the accumulation of ethanol at the larger 100 L scale at this specific growth rate. The accumulation of residual glucose and the associated accumulation of ethanol during the fed-batch phase could possibly be attributed to accumulation of glucose at the feed entry zone due to longer mixing times at the larger scale, where mixing might be less efficient than in the smaller bioreactor. Therefore, localised exposure of *S. cerevisiae* cells to high concentrations of glucose could trigger ethanol production, referred to as the short-term Crabtree effect (Rodrigues, et al., 2006). On the other

hand, calculating μ from the natural logarithm of mass of cells plotted as a function of time revealed a specific growth rate of 0.131 h^{-1} that was either very close to or perhaps slightly in excess of μ_{crit}, which would result in carbon overflow to ethanol. Nevertheless, the production of ethanol at these low levels did not appear to negatively impact α-glucuronidase production, possibly due to the ability of the culture to still undergo oxidative metabolism in the presence of low levels of ethanol (< 5 g/L), as observed by Paalme *et al* (Paalme, et al., 1997).

3.5   Conclusions

To maximise growth-associated α-glucuronidase production under control of the constitutive *PGK* promoter, the *S. cerevisiae* MH1000pbk10D-glu should be grown in exponential fed-batch culture at specific growth rates close to the μ_{crit} value of 0.12 h^{-1} where the biomass yield is at its maximum due to minimal ethanol production. The highest final concentration of α-glucuronidase obtained from such a culture was 14.03 mg/L.

Using constant $k_L a$ as basis for process scale-up proved to be an effective strategy, given the close to theoretical biomass yield attained in aerobic cultures of *S. cerevisiae* at both laboratory and pilot scale. At larger scale, the specific growth rate should be carefully controlled well below μ_{crit} to avoid the short-term Crabtree effect, possibly resulting from localised pockets of high glucose concentration at the feed entry point due to less efficient mixing.

Acknowledgements

CHAPTER FOUR

Process Optimization for the production of β-fructofuranosidase by *P. pastoris* grown in Semi-Defined Medium using Response Surface Methodology

Abstract

Apart from the substrate feed rate, other fermentation parameters such as the level of dissolved oxygen available to the culture and the type of medium used for fermentation affect the overall recombinant enzyme production levels in fed-batch culture of yeast systems. In this chapter, the influence of dissolved oxygen tension, substrate feed rate as well as a change of medium from a chemically defined medium to a semi-defined medium on the expression of β-fructofuranosidase (FFase) in the methylotrophic *Pichia pastoris* yeast is presented.

Short-chain fructooligosaccharides (sc-FOS), comprising 1-nystose, 1-kestone and 1-fructofuranosyl-nystose produced from sucrose by the action of β-fructofuranosidase enzyme can be used as sweeteners and pre-biotics in drug formulation and in confectionary products. As an alternative to a commonly-used chemically defined medium, a semi-defined industrial medium was developed for cultivation of *Pichia pastoris* for the production of FFase. Using the semi-defined medium, statistical optimisation was further applied to the glycerol fed-batch (GFB) phase of DO-stat fed-batch fermentations to establish optimal glycerol feed rate and DOT for the production of FFase under the control of two different promoters, namely glyceraldehyde-3-phosphate dehydrogenase (GAP) and alcohol oxidase (AOX). Decreasing the concentration of basal salts in the semi-defined medium by a factor of two did not adversely affect enzyme production under either promoter. However, replacing the trace elements solution in the chemically defined medium with yeast extract (semi-defined medium) resulted in a decrease in the volumetric activity of FFase expressed under control of the AOX promoter by 54.3%, from 9238.27 U/ml to 4227.20 U/ml signifying a distinct

requirement for trace elements when methanol served as carbon source during protein induction. When glycerol served as carbon source, the change from the chemically defined medium to the semi-defined medium had no effect on enzyme expression under control of the GAP promoter, where similar volumetric FFase activities of 4648.68 U/ml and 4738.71 U/ml were recorded in the two respective media. Optimisation of DO-stat fed-batch fermentations of both strains using the semi-defined medium resulted in respective glycerol feed rates and DOT values of 40.3 g/h and 32.23% for the strain harbouring the GAP promoter, and 28.49 g/h and 48.54% for the AOX promoter strain. However, at these optimal conditions, the volumetric activity was 40% less than that from the AOX strain grown in the chemically defined medium. Hence, further optimisation, possibly at molecular level, may be required to match the expression level of the GAP promoter to that of the AOX promoter for FFase production.

In this chapter, the influence of carbon source on the nutritional requirements of *P. pastoris* during heterologous enzyme production under the two commonly-used promoters was highlighted. Additionally, a fundamental dependency of enzyme production levels on both substrate feed rate and DOT, as well as minor limitations in bioprocess optimisation during enzyme production in fed-batch culture were identified.

Declaration by Candidate

With regards to Chapter 4 (pages 71-93), the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Physical experimental work, generation of results, analyses and data processing	100 %
Chapter/article write-up	70 %

The following co-authors have contributed to Chapter 4 as follows

Name	email	Nature of contribution	Extent of contribution (%)
Eugene van Rensburg	eugenevrb@sun.ac.za	Critical reading/ supervision	15 %
Johann Gorgens	jgorgens@sun.ac.za	Critical reading/ supervision	15 %

Signature of candidate……………Emmanuel Anane…………

Date …………22-11-2013…………………..

Declaration by co-authors

The undersigned hereby confirm that:

1. The declaration above accurately reflects the nature and extent of the contribution of the candidate and co-authors to Chapter 3,

2. No other authors contributed to chapter 3 beside those specified above, and

3. Potential conflicts of interests have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 3.

Signature	Institution of affiliation	Date
Eugene van Rensburg	Dept of Process Engineering, Stellenbosch University	22-11-2013
Johann F Gorgens	Dept of Process Engineering, Stellenbosch University	22-11-2013

4.1   Introduction

Short chain fructooligosaccharides (scFOS) are sugars with low calorie content used as sweeteners in diabetic drug formulation, light jams, ice cream and confectionary products (Sangeetha, et al., 2005). sc-FOS are important nutraceuticals comprising 1-kestose, 1-nystose and 1-fructofuranosyl-nystose obtained from the hydrolysis-transferase action of the enzyme β-fructofuranosidase on sucrose (Chen, et al., 2011; Maiorano, et al., 2008). β-Fructofuranosidase (EC 3.2.1.26) has been isolated and expressed natively in several bacteria and fungi, including *Aspergillus spp* and *Bacillus macerans* (Sangeetha, et al., 2005; Fernandez, et al., 2007), while production in the methylotrophic yeast *Pichia pastoris* has not been reported.

P. pastoris is one of the most versatile yeasts capable of producing a wide range of recombinant protein products such as vaccine sub-units, single cell proteins and fully functional enzymes (Calik & Calik, 2012; Cos, et al., 2006). The success of *P. pastoris* as an expression host resides in high cell densities achieved in fed-batch culture, the presence of easily manipulated promoters and a wealth of information regarding its genome (Creg, 1985). Additionally, it is non-fermentative and easily secretes most recombinant proteins into the fermentation broth through the *α-MF pre-pro* peptide sequence derived from *S. cerevisiae* (Higgins, 2001), making it a suitable host for recombinant expression of β-fructofuranosidase enzyme.

The basic medium for growing *P. pastoris* for laboratory research is a defined medium developed by Invitrogen Inc. in the 1970s and includes basal salts, the so-called *Pichia* trace metals solution and a carbon source (Invitrogen, 2002). The carbon source would depend on the promoter driving the expression of the recombinant protein. During protein expression under the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter, the fermentation is started with the glycerol batch phase (GB) which usually lasts 24 hours. This

is followed by the glycerol fed-batch phase (GFB) during which enzyme production is achieved as the culture grows on a continuous glycerol feed. A strain with recombinant gene expression under the inducible AOX promoter, on the other hand, is initially grown in GB and GFB phases to produce a high concentration of biomass before switching to methanol for induction of protein expression, during the so-called methanol induction phase (MIP). High levels of glycerol in the growth medium can repress the AOX gene whereas methanol accumulation can cause cell death (Cos, et al., 2006; Cereghino, et al., 2002), therefore substrate levels should be carefully controlled during fed-batch fermentation. Moreover, the precipitation of salts, high ionic strength, high cost and unbalanced composition have been cited as major problems associated with this defined medium (Cos, et al., 2006; Cereghino, et al., 2002), therefore a complex medium alternative is preferred for industrial cultivation of *P. pastoris* for recombinant protein production. Although much research was conducted on the effect of carbon substrate concentration during fed-batch fermentation, there exists a distinct paucity in the literature on the influence of the other components of the defined medium on recombinant protein production (Cos, et al., 2006), specifically the effect of the *Pichia* trace salts solution on protein expression under the GAP promoter.

Fed-batch fermentation where the substrate feed is regulated by a constant dissolved oxygen concentration (DO-stat) has been shown to significantly improve recombinant protein production in both AOX and GAP strains of *P. pastoris* (Ferreira, et al., 2012; Lee, et al., 2003). In this method, the substrate feeding is controlled by the dissolved oxygen set-point, such that the pump switches on when the dissolved oxygen tension (DOT) rises above the set-point, based on the premise that a rise in DOT results from accumulation of oxygen due to depletion of substrate in the medium (Hu, et al., 2010). Therefore, the substrate feed is controlled by the internal metabolic rate of the culture and in using this system of *on-demand feeding*, accumulation of residual substrate is minimal.

The purpose of this study was to modify the commonly used defined medium of *P. pastoris* to include complex medium components suitable for industrial application. This was achieved by replacing *Pichia* trace metals solution in the defined medium with yeast extract to obtain a semi-defined medium, and by reducing the concentration of basal salts specified in the defined medium by a factor of 2. Additionally, response surface methodology (RSM) was used to establish optimal glycerol feed rate and DOT during the glycerol fed-batch (GFB) phase of both the AOX and GAP strains cultivated in the semi-defined medium. In the GAP strain, the GFB phase determines the overall productivity of the fermentation, whereas in the AOX strain, GFB determines the pre-induction biomass concentration, which directly affects the final enzyme production levels (Gao et al, 2012). Therefore, in order to maximise recombinant Ffase production in both strains using the newly developed medium, it was desirable to determine optimal glycerol feed rate and DOT in the GFB phase during cultivation of both AOX and GAP strains.

4.2   Materials and Methods

4.2.1 Strains and Inoculum preparation

P. pastoris DSMZ 70382 was used in this research. Detailed construction of the strains is out of the scope of this work and is described elsewhere (Coetzee *et al*, unpublished); to summarise the method briefly: Two sub-strains were transformed with synthetic expression plasmids containing the *FopA* gene encoding the β-fructofuranosidase enzyme (DNA 2.0, CA, USA) using the electroporation method described by Becker and Guarenthe (1991). The *FopA* gene was placed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (pGAP*)* promoter and the methanol-inducible alcohol oxidase (pAOX) promoter to give rise to two strains, which will be referred to as the GAP and AOX strains,, respectively. Stock cultures of the strains were stored at -80 $^{\circ}$C in 1 ml aliquots containing

30% (w/v) glycerol as cryoprotectant. Both strains were routinely grown for 72 h at 30 °C on YPD agar plates, consisting out of (per litre) 10 g yeast extract, 20 g peptone, 20 g glucose and 13 g agar (Sigma-Aldrich, Kempton Park, South Africa). Fresh colonies from these plates were used to inoculate four 500 ml Erlenmeyer flasks, each containing 100 ml medium that consisted out of (per litre): 40 ml of a 1 mol/L solution of KH_2PO_4/K_2HPO_4 buffer, 13.4 g YNB, 10 g $(NH_4)_2SO_4$ and 10 g glycerol and sterilised in an autoclave at 121 °C for 15 minutes. Shake flask cultures were grown at 30 °C on an orbital shaker (Yihder Technology Co. Ltd, Taipei, Taiwan) adjusted to 200 rpm for 24 h. The contents of the four flasks were used to inoculate 4 L medium in bioreactors.

4.2.2 Fermentations

Fermentations were carried out in two 10.5 L NBS BioFlo 110 bioreactors with 8 L working volume, each equipped with a polarographic DO probe, a combination glass pH electrode (all Mettler Toledo, Sandton, South Africa) and controlled using BioCommand® bioprocess supervisory software (New Brunswick Scientific Co. Inc., CT, USA) . Since this research was limited to optimisation of dissolved oxygen tension and glycerol feed rate during the fed-batch phase, the temperature, pH and aeration rate were maintained at specific values of 30°C, pH 5.5 and 1.2 vvm,, respectively, through the entire cultivation. The pH was controlled by automatic titration with 25% (v/v) NH_4OH and foaming was controlled by adding 300 µL of Antifoam 204 (Sigma, Kempton Park, South Africa) per litre into the initial fermentation broth.

The duration of the glycerol batch phase varied between 24 h and 27 h. Glycerol depletion was determined from a spike in DOT with a corresponding decrease in the agitation rate. This signal was used to initiate the glycerol fed-batch (GFB) phase with glycerol feed rate and DOT adjusted to values specified in the central composite design. The GFB phase lasted 72 h and 24 h for the GAP and AOX strains,, respectively. In the culture of the AOX strain, the

GFB phase was followed by MIP at a feed flow rate of 0.525 ml methanol min^{-1} L^{-1} for the first 4 h, followed by a feed flow rate of 1.1 ml min^{-1} L^{-1} until the end of the fermentation. Substrate feeding during GFB and MIP was controlled by DO-stat technique implemented through the BioCommand® supervisory software.

4.2.3 Experimental Design

4.2.3.1 Growth Medium

Both GAP and AOX strains were grown in the complete defined medium (medium 1) as described in the Invitrogen protocol (Invitrogen, 2002) using basal salts (BS) comprised out of (per litre): 26.7 ml of 85% w/v H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH and 12 ml/L of *Pichia* trace metals (PTM_1) solution comprising out of per litre: 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5 ml H_2SO_4. Medium 2 consisted of the BS as in medium 1, supplemented with 10 g/L yeast extract (YE) without PTM_1 solution whereas in medium 3, the concentration of the BS was decreased by a factor of 2, with 10 g/L YE without PTM_1 solution. In the culture of the GAP strain, glycerol was used as the carbon source in each medium whereas glycerol and methanol were used sequentially in the culture of the AOX strain. Volumetric activities of β-fructofuranosidase from each medium were compared to select the best medium for further process optimisation.

4.2.3.2 Optimisation Experiments

The volumetric activity of β-fructofuranosidase was optimised as a function of DOT and glycerol feed rate during GFB phase using response surface methodology (RSM) with a two factor central composite experimental design (CCD) (Table 1). The CCD is a suitable experimental design technique that gives a lower number of experiments, yet can be used to

navigate a spherical design space and construct a three-dimensional surface of the response variable(s) taking into account interaction between the input variables. The design was comprised of 4 replicated centre points, 4 axial points and 4 star points distributed evenly in a sphere. Based on the results of Lee *et al* (2003) who investigated the effects of substrate feed rate and DOT independently in DO-stat fed-batch culture of recombinant *P. pastoris* expressing α-amylase enzyme, the input factors in the design were selected in the ranges of 8 g/h $\leq$ A $\leq$ 50 g/h and 5% $\leq$ B $\leq$ 60%, where A represents the glycerol feed rate and B the dissolved oxygen tension. Based on this design, a total of 12 experiments were carried out with each strain, with volumetric activity as the response variable. Design and analysis of the experiments was done using Design Expert$^{®}$ software (Stat-Ease Inc., Minneapolis, USA). During fermentation, the MIP was carefully controlled based on a standard protocol (Invitrogen, 2002) such that the impact of changes in the GFB phase were clearly manifested in the volumetric activity of the enzyme at the different operating conditions specified in the CCD.

4.2.4 β-Fructofuranosidase Assay

One unit of enzyme activity (U) was defined as the amount of enzyme required to produce one micromole of glucose per minute from a 100 g/L sucrose solution incubated at 40°C and pH of 5.5 (Hidaka, 1988). To determine the enzymatic activity, 13.3 g of sucrose (Sigma Aldrich, Kempton Park, South Africa) was dissolved in 100 ml of citrate-phosphate buffer at pH 5.5 (424 ml of 0.1 M citric acid and 576 ml of 0.2 M Na_2HPO_4). Aliquots of 0.75 ml of this solution were added to 0.25 ml of fermentation supernatant and incubated in a water bath at 40°C. After 60 minutes, 61 µl of 35% perchloric acid (PCA) was added to stop the reaction. The solution was centrifuged and the clear liquid analysed with high performance liquid chromatography (HPLC) to determine the concentrations of glucose, 1-kestose and 1-nystose (*FOS*) produced during the reaction. Although only glucose concentration was used to

calculate the enzyme activity, the analysis for 1-kestose and 1-nystose was done to confirm that the other components of scFOS were produced by the enzyme.

4.2.5 Analyses

Glucose, 1-kestose and 1-nystose contents were determined by HPLC equipped with an Xbridge^{TM} Amide column (4.6 x 250 mm, 3.5 μm particle size) (Waters Corporation, Milford, MA, USA) with column temperature adjusted to 30 °C. The mobile phases used for elution were 0.0125% ammonium hydroxide in water and 0.0125% ammonium hydroxide in 90% acetonitrile at a flow rate of 0.7 ml/min. Peaks were detected by a Varian 380-LC evaporative light-scattering detector. External standard calibration curves were used to quantify the compounds in each sample.

Biomass concentration was determined gravimetrically by centrifuging 5 ml sample of fermentation broth in an oven-dried, pre-weighed tube at 8000 rpm for 5 minutes. The pellet was washed twice with deionized water and dried at 105 ºC in an oven to a constant mass. Each concentration measurement was done in duplicate and the average was calculated.

4.3 Results and Discussion

4.3.1 Medium Development

To develop a cost-effective growth medium for cultivating *P. pastoris* for the production of β-fructofuranosidase on industrial scale, the conventional defined medium for *P. pastoris* cultivation (according to the Invitrogen Protocol) was modified into a semi-defined medium by substituting the Pichia trace salts solution with yeast extract, whilst reducing the specified concentrations of basal salts by half.

4.3.1.1 The Influence of *Pichia* Trace Metals Solution

Volumetric activity and biomass concentrations obtained from cultivating *P. pastoris* on the different media are shown in Fig. 4.1. In replacing PTM_1 solution in the defined medium (medium 1) with yeast extract (medium 2), no significant differences in the biomass

concentration and volumetric activity were recorded in the culture of the GAP strain (Fig. 4.1(I)). However, the volumetric activity and biomass concentration in the culture of the AOX strain decreased by 54% and 20%,, respectively (Fig. 4.1(II)) relative to AOX strain cultivated in the defined medium (medium 1). The impact of PTM_1 solution on recombinant protein production by *P. pastoris* has not been reported extensively in the literature (Cos, et al., 2006), although Wanderley *et al* (2013) recorded a 2.5-fold decrease in the concentration of frutalin expressed in *P. pastoris* under control of the AOX promoter by eliminating PTM_1 solution from the growth medium.
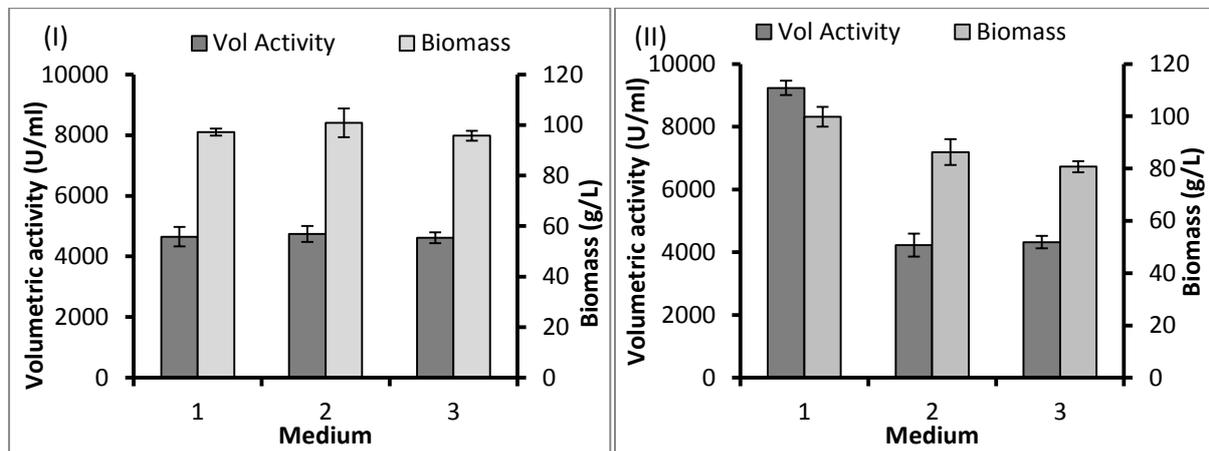
Figure 4 1 Volumetric FFase activity and biomass produced in cultivating GAP (I) and AOX (II) strains of *P. pastoris* on different media with glycerol or methanol as carbon source. Medium 1 (control experiment) comprises basal salts and Pichia trace metals; Medium 2, basal salts and yeast extract; Medium 3, half the concentrations of basal salts in 1 and yeast extract.

The results (Fig. 4.1), together with the literature suggest that the essential micronutrients supplied by PTM_1 solution may be more vital for recombinant protein production under the AOX promoter than under the GAP promoter due to the difference in carbon sources used in the fermentation. Thus, the nutritional requirement of the host strain may change with

different carbon sources, possibly due to different routes through which metabolism and biosynthesis occur under each carbon source and this was clearly accentuated when methanol was used as carbon source. Although the exact nature of the interaction between methanol (as carbon source) and the trace elements during recombinant protein expression in *P. pastoris* is not yet clear, Hartner and Glieder (2006) propose a complex methanol metabolism route with several metal ions acting as enzyme co-factors and precursors, therefore the need to supply defined amounts of trace elements for efficient utilisation of methanol. On the other hand, glycerol, with a higher carbon-content is metabolised through the relatively simple EMP pathway (Sola, et al., 2004), and hence yeast extract, containing minimal amounts of the micronutrients (Vogel & Todaro, 1997) can substitute PTM_1 solution in fermentation of the GAP strain. At the molecular level, the effect of these two carbon sources on the nutritional requirements and metabolic behaviour of *P. pastoris* during recombinant protein production largely remains unexplored.

3.3.1.2   The Influence of Concentration of Basal Salts

Compared to medium 2, there was no marked difference in volumetric activity of β-fructofuranosidase and biomass concentrations for both AOX and GAP strains cultivated in medium 3 (Fig. 4.1) in which the concentrations of BS components in medium 2 were reduced by a factor of 2, with 10 g/L yeast extract. Additionally, the salt precipitation observed during autoclaving and upon storage of supernatant from media 1 and 2 [filtered through 0.22 µm membrane and stored at 4 ℃ (Fig. 4.2(I))] was not observed in medium 3 (Fig. 4.2 (II)).

From the results, it is apparent that the concentrations of BS components in the defined medium may be overestimated such that excess salts precipitate in the supernatant during autoclaving and upon storage. The precipitation of salts, on one hand, may alter the composition of the medium, and make certain key nutrients unavailable to the culture. On the

other hand, even if the extra salts did not precipitate, they would not be used up because the culture has only a specific nutrient requirement.
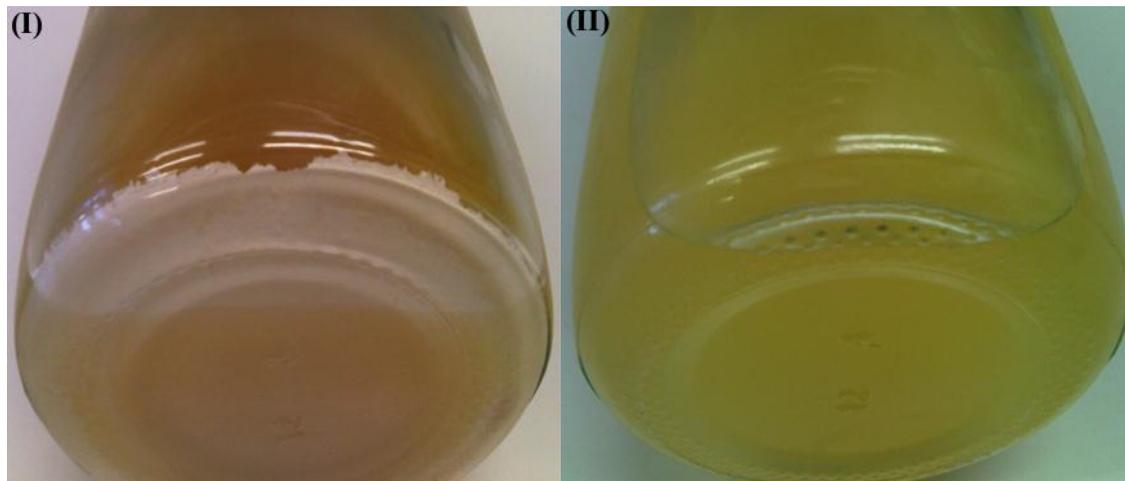
Figure 4.2 Supernatant from fermentation showing salt precipitation in medium 1 (I) and no precipitation in medium 3 (II). Medium 1 contains basal salts at concentrations specified in the Invitrogen protocol whereas the concentration of basal salts in medium 3 is reduced by a factor of 2.

In similar results, Brady *et al* (2001) observed no difference in cell growth and concentration of recombinant *Plasmodium falciparum* merozoite protein I expressed in *P. pastoris* by reducing the concentration of chemicals in the Invitrogen protocol to 25%. Therefore in large scale production of β-fructofuranosidase and other recombinant proteins by *P. pastoris*, half of the concentrations specified in basal salts medium may be used to achieve the same production levels as in the full defined medium.

4.3.2 DO-Stat Fed-Batch Optimisation

Substitution of PTM$_1$ solution with yeast extract (to get a semi-defined medium) did not have any adverse effect on recombinant FFase production under the GAP promoter, although volumetric activity under this promoter (both in defined and semi-defined media) was 54% of that obtained under the AOX promoter cultivated in defined medium (Fig. 4.1). Moreover, the β-fructofuranosidase activity under the AOX promoter decreased by a factor of 2 in the semi-defined medium compared to the defined medium. Optimisation of the GFB phase of fed-

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batch cultivation processes with the AOX and GAP strains in semi-defined medium (medium 3) was therefore aimed at achieving the volumetric FFase production levels observed with the AOX strain cultivated on the defined medium. Improvement of recombinant enzyme expression levels in the GAP strain is particularly desirable due to the use of glycerol throughout the fermentation, compared to the use of methanol in the MIP phase of AOX culture as methanol poses the problems of high flammability, high volatility (requiring the use of special explosion-proof bioreactors on large scale) and the requirement of ultra-low levels of methanol in food products (Waterham, et al., 1997).

The fed-batch cultivation of *P. pastoris* in medium 3 was optimised in terms of glycerol feed rate and DOT during GFB phase of both GAP and AOX strains using a central composite design (CCD). In the culture of the AOX strain, the DOT used in the GFB phase (according to the CCD) was maintained throughout the MIP phase. Table 4.1 shows the CCD with 12 different combinations of factors and the responses in biomass concentration (X) and volumetric activity (U_f) from the experiments for both the GAP and AOX strains. The data was fitted using quadratic regression of U_f as a function of glycerol feed rate (A) and DOT (B) at 95% confidence level. The models obtained are given in Equations 4.1 and 4.2 for the GAP and AOX strains, respectively and the analysis of variance (ANOVA) for the regressions are given in Tables 4.1 and 4.2. Response surface plots of U_f based on Equations 4.1 and 4.2 are shown in Fig. 4.3 (I) and (II), respectively.

$$U_f = 2838.8 + 80.9A + 22.8B - 0.1AB - 0.965A^2 - 0.292B^2 \qquad (4.1)$$

$$U_f = 1436.41 + 143.73A - 26.84B - 1.39AB - 1.29A^2 + 1.54B^2 \qquad (4.2)$$

The volumetric activity (response) for the GAP strain (Fig. 4.3 (I)) showed a smooth parabolic curvature with a weak dependency on the DOT and strong dependency on the glycerol feed rate (ANOVA, Table 4.2). This was as expected, due to the growth associated

and constitutive nature of this promoter (Zhang, et al., 2009). Enzyme expression levels under the GAP promoter was shown to depend on the type and concentration of carbon source available to the culture (Waterham, et al., 1997; Zhang, et al., 2009). Low DOT with a corresponding low glycerol feed rate would produce less biomass, hence less recombinant enzyme under the GAP promoter whereas glycerol feed rates that are too high may result in substrate accumulation, which can induce cell death (Romanos, 1995).

Table 4.1 Central composite design for glycerol feed rate (A) and dissolved oxygen tension (B) for *P. pastoris* growth on semi-defined medium with results of biomass concentration and volumetric activity achieved in each experimental run for both GAP and AOX strains.

Run No.	Coded values		Experimental values		GAP[a]	AOX[b]
	X_A	x_B	A (g/h)	B (%)	U_f (U/ml)[b]	U_f (U/ml)
1	0	0	35	30	4738.67	4337.46
2	0	1.5	35	60	4392.14	5703.55
3	-1	1	15	50	4141.55	5064.53
4	0	0	35	30	4535.33	4097.65
5	1	1	55	50	4888.72	4819.36
6	1	-1	55	10	4569.25	4355.22
7	0	-1.25	35	5	4854.04	3534.47
8	0	0	35	30	4896.28	4227.61
9	-1.35	0	8	30	4121.01	2685.93
10	1.4	0	63	30	4047.17	3475.85
11	0	0	35	30	4956.65	4000.44
12	-1	-1	15	10	3661.71	3228.06

[a,b] These are the responses from the experimental runs in the CCD. [c] On dry cell weight basis

The curvature of volumetric Ffase production levels for the AOX strain (Fig 4.3 (II)) showed greater dependency on DOT than on the glycerol feed rate during GFB phase with p-values of 0.0064 and 0.0264, respectively (ANOVA, Table 4.3). The response also showed a distinct saddle point with increasing volumetric activity at both low and high DOT values relative to the mid-point of the experimental range. The improved enzyme expression levels under low oxygen concentration was also observed in other AOX strains of *P. pastoris* where productivity of Fab antibody fragment increased 2.5-fold under hypoxic (low oxygen) conditions (Baumann, et al., 2008). The authors proposed that oxygen limitation in the AOX strain may up-regulate the transcription rate of intracellular enzymes, leading to higher metabolic rates. At high DOT levels, on the other hand, it was suggested that the oxidation of primary alcohols in *P. pastoris* by the alcohol oxidase enzyme requires substantial moles of oxygen; hence higher DOT would favour efficient utilisation of methanol (Daly & Hearn, 2005).
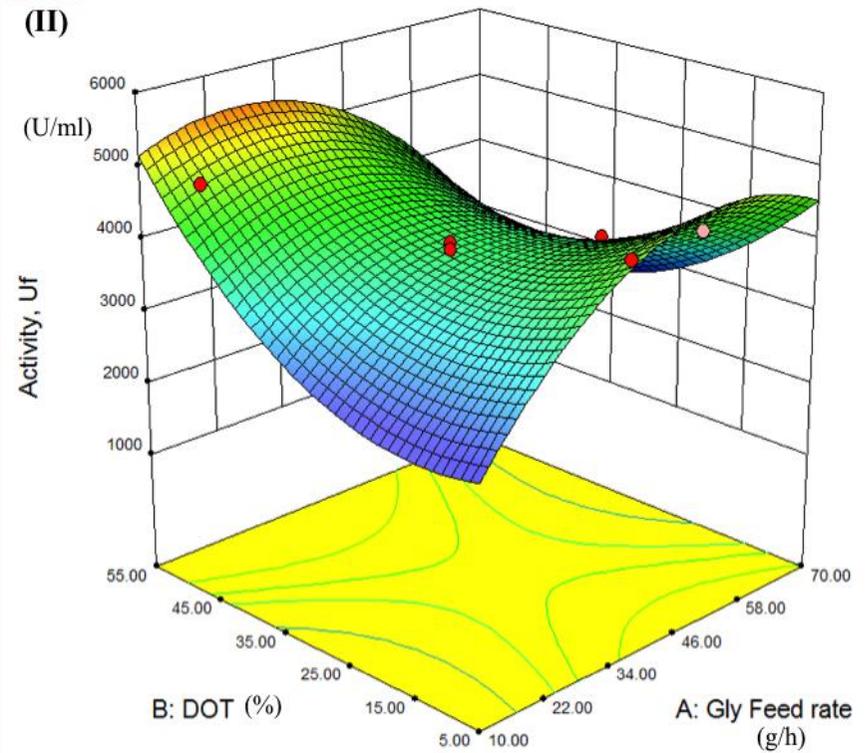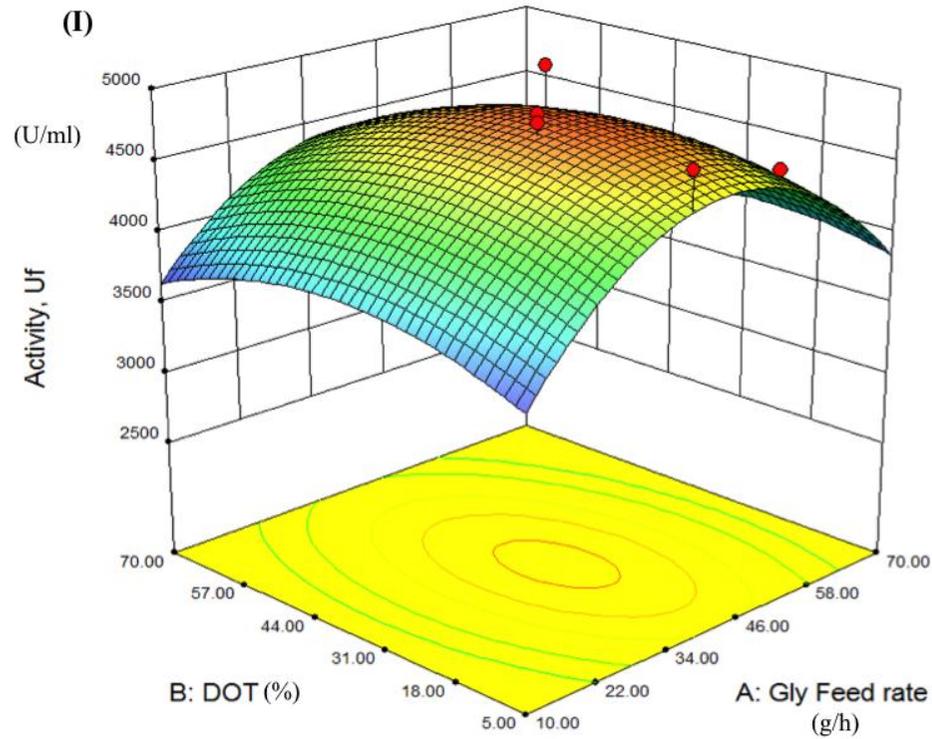
Figure 4.3 Response surface plots of volumetric activity (U_f) of β-fructofuranosidase as a function of glycerol feed rate (A) and dissolved oxygen tension (DOT, B) for GAP (I) and AOX (II) strains of *P. pastoris* grown in medium 3
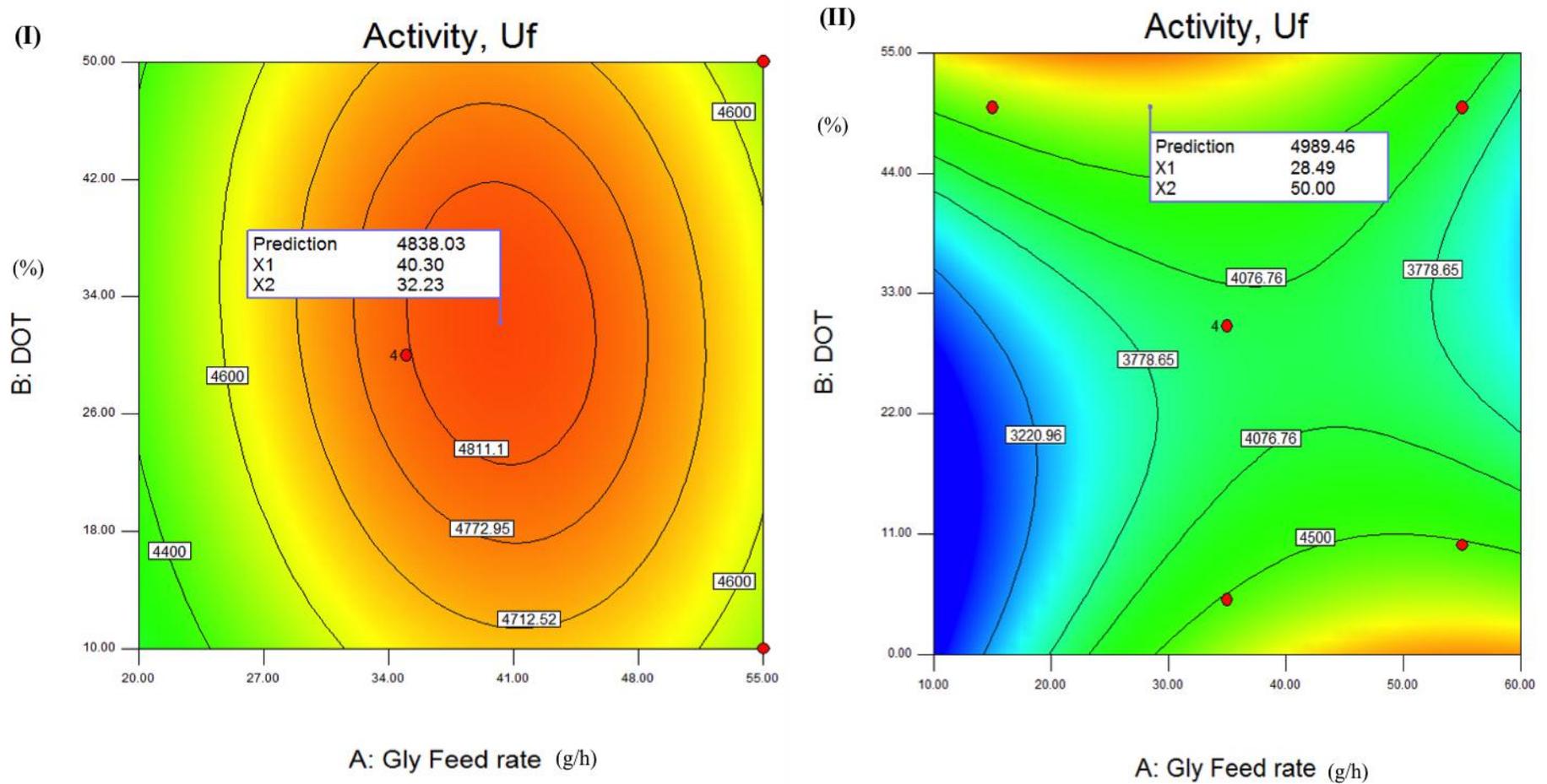
Figure 4.4 Contour plots of volumetric activity of β-fructofuranosidase at varying glycerol feed rate (A) and dissolved oxygen tension (DOT, B) for GAP (I) and AOX (II) strains of *P. pastoris* cultivated in a semi-defined medium

Table 4. 2 ANOVA for regression analysis of volumetric activity (U_f) based on Equation 4.1 for the GAP strain

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Model	4218.08	5	843.62	35.38	0.0002
A	700.60	1	700.60	29.38	0.0016
B	130.05	1	130.05	5.45	0.0582
AB	0.20	1	0.20	0.00849	0.9296
A^2	3387.07	1	3387.07	142.04	<0.0001
B^2	1.53	1	1.53	0.064	0.8086
Lack of fit	126.80	3	42.27	7.79	0.0628

Table 4.3 ANOVA for the regression of U_f with Equation 4.2 for the AOX strain

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Model	6.448 E006	5	1.290E006	46.88	0.0003
A	2.672E005	1	2.672E005	9.71	0.0264
B	5.567E005	1	5.567E005	20.24	0.0064
AB	7.082E005	1	7.082E005	25.74	0.0039
A^2	1.449E006	1	1.449E006	52.66	0.0008
B^2	1.559E006	1	1.559E006	71.22	0.0004
Lack of fit	1.094E005	2	54707.03	5.83	0.0925

The optimal glycerol feed rate and DOT during GFB phase based on the surface plots were, respectively 40.3 g/h and 32.23% with a predicted U_f of 4838.03 U/ml for the GAP strain and 28.49 g/h and 48.54% with predicted U_f of 4989.46 U/ml for the AOX strain (Fig 4.4). These glycerol feed rates and DOT values are in close agreement with other reported values for other strains of *P. pastoris* in both GFB and MIP (Arnau, et al., 2010; Cos, et al., 2006; Potvin, et al., 2010; Lee, et al., 2003). The volumetric activity values at the optimum conditions for the GAP and AOX strains in the semi-defined medium improved by 4.8% and 15.4%, respectively, relative to the base case (without optimisation) although these values were about 40% less than the volumetric activity recorded in the AOX strain in the defined medium. The percentage increases in the production levels of both AOX and GAP strains with optimised fed-batch, compared to the base case were not sufficient to achieve the desired production levels observed under the *AOX* promoter in defined medium, therefore confirmatory experiments were not done to check the predicted volumetric activities at the optimal conditions. The difference in expression levels between the two promoters has been reported (Waterham, et al., 1997; Zhang, et al., 2009; Potvin, et al., 2010), where the tight regulation of the alcohol oxidase gene (hence the *AOX* promoter) by methanol sometimes results in better expression levels than in the constitutive *GAP* promoter (Hartner & Glieder, 2006; Kim, et al., 2013). Other optimisation methods at the molecular level, such as increasing the gene copy number and gene sequence optimisation may improve the expression of β-fructofuranosidase under the GAP promoter.

4.4 Conclusions

Similar β-fructofuranosidase production levels were achieved in the GAP strain cultivated in defined and semi-defined media. PTM_1 solution in the defined medium may therefore be substituted with yeast extract (semi-defined medium) for industrial scale FFase production under the *GAP* promoter without compromising the enzyme yield. Moreover, reducing the concentration of basal salt medium components in the semi-defined medium with

optimisation of DOT and glycerol feed rate during GFB phase resulted in 4.8% and 15.4% increase in FFase production levels in GAP and AOX, respectively, signifying that the concentration of these salts in the defined medium may be overestimated.

The alternative medium manipulations, however, did not result in better performance for enzyme expression under the AOX promoter and the data confirms that the (Invitrogen) defined medium remains the best medium to use for recombinant enzyme production under this promoter. Elimination or substitution of any medium components may lead to less enzyme production.

CHAPTER FIVE

Conclusions and Recommendations

The use of enzymes offers great potential in the development of sustainable processes in various industries. However, owing to limitations in enzyme production levels by microbial systems, the implementation of enzyme-catalysed processes remains challenging. This research focused on optimisation of key bioprocessing aspects of fed-batch culture including the substrate feed rate, dissolved oxygen tension and growth medium with the aim to improve recombinant enzyme expression in two yeast systems, namely *S. cerevisiae* and *P. pastoris*.

From the two yeast systems studied, it was apparent that the substrate feed rate in fed-batch culture is a key determinant of enzyme production levels in yeasts as it directly affects the metabolic state of the culture, biomass and product formation rates. In the ethanol-producing *Saccharomyces cerevisiae*, the optimum feed rate was used to maintain the culture in an oxidative metabolic state to maximise biomass and hence α-glucuronidase yields, whereas in the fast-growing *Pichia pastoris*, optimal feed rates were used to maintain the culture at near maximum growth rate to maximise biomass and FFase production levels. In the two systems, recombinant enzyme production was closely correlated with the biomass concentration. It was thus established that optimisation of the feed rate is necessary to maximise enzyme yield in fed-batch culture, but the control strategy would depend on intrinsic properties of the particular yeast system employed. Other fed-batch fermentation parameters, such as the dissolved oxygen tension and the growth medium were also shown to influence both culture growth and recombinant enzyme production in yeasts (*Pichia pastoris*); hence careful optimisation of these factors may be required to balance efficient enzyme production levels with low production costs. Additionally, typical scale-up challenges such as inefficient mixing and non-predictive culture growth at larger scale were identified in *Saccharomyces*

cerevisiae yeast, therefore careful choice of scale-up criteria, such as the use of constant mixing time and constant impeller tip speed to minimise the impact of inefficient mixing would be advantageous in large scale cultivation of this yeast for recombinant enzyme production. However, in the use of the above-mentioned scale-up criteria, dissolved oxygen monitoring is compromised and the culture may run into anaerobic fermentation. Therefore the overall scale-up of *S. cerevisiae* system is a trade-off between mixing and oxygen availability.

Although optimisation of the fed-batch parameters discussed above may significantly improve recombinant enzyme yields in yeasts, the enzyme expression may be limited by inherent molecular phenomena such as the strength of promoters used to drive expression of a foreign gene, the metabolic burden of heterologous protein production on the strain and insufficient gene copy number. Such a limitation was clearly accentuated in this research by the inability of the GAP strain of *P. pastoris* to match FFase expression levels of the AOX strain after fed-batch process optimisation. In such cases, more fundamental optimisation methods such as gene sequence optimisation, protein engineering and selection of better transformants may be necessary to improve the enzyme production.

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APPENDIX I : Fed-batch culture for α-glucuronidase production by *S. cerevisiae*

Introduction

Fed-batch culture is operated by feeding medium into the bioreactor after an initial batch period without removal of medium, to continuously supply a growth limiting substrate that maintains the culture in exponential growth phase over long periods of time. In variable volume fed-batch fermentation, a concentrated feed of the limiting substrate is introduced at a flow rate that increases the volume of the broth, and there may be constant changes in concentration of cells, metabolites and other products over time.

Mass balances of a fed-batch bioreactor

The overall material balance equation can be applied to the conventional fed-batch bioreactor shown:

F = flow rate (L/h); X = mass of cells (g)

S = Substrate conc. (g/L); V = volume of broth (L)
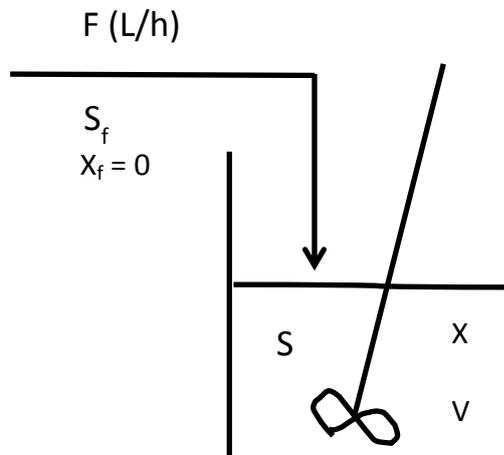
Figure AI-1 Fed-batch bioreactor

i. Overall balance: the change in broth volume with time depends on the feed flow rate.

$$\frac{dV}{dt} = F \tag{AI-1}$$

ii. Biomass balance

Biomass accumulation = biomass in + biomass produced – death

But Biomass In = 0 {sterile feed}

$$\frac{dX}{dt} = 0 + r_x V - r_d V \tag{AI-2}$$

$$\left\{ \begin{array}{c} r_x = rate\ of\ cell\ generation\ = \mu x \\ r_d = rate\ of\ cell\ death = k_d x \\ X = mass\ of\ cells = xV \\ x = concentration\ of\ cells \end{array} \right\}$$

if $r_x \gg r_d$, (cell death is negligible) then

$$\frac{d(xV)}{dt} = \mu xV \tag{AI-3}$$

At the beginning of the fed-batch phase, both biomass concentration and broth volume are changing. Hence the Product Rule (Calculus, Differentiation) is applied to the LHS of Equation AI-3 to yield

$$\frac{xdV}{dt} + \frac{Vdx}{dt} = \mu xV$$

$$xF + \frac{Vdx}{dt} = \mu xV$$

$$\frac{dx}{dt} = x(\mu - \frac{F}{V}) \tag{AI-4}$$

111

After a period of operation, the total mass of biomass increases with time, so does the volume; therefore if the *increments are equivalent*, the biomass concentration does not change with time, a condition called **quasi-steady state** (*qss*). At qss

$$\frac{dx}{dt} = 0$$

$$0 = x(u - \frac{F}{V})$$

$$\mu = \frac{F}{V} = D \qquad\qquad (AI-5)$$

Therefore in a conventional fed-batch culture, the dilution rate (D)—a mechanical parameter can be used to control the specific growth rate (μ--a biological parameter) (Dane-Wittrup, 2007) at *qss*. However, the qss approach has certain limitations:

i.   **Achieving qss**-According to Maurer *et al* (2006), it takes a period of operation equivalent to 4 - 6 residence times in a bioreactor before steady state condition is achieved. The residence time (τ) of a reactor is the inverse of the rate constant of the reaction; in a bioreactor the rate constant is the specific growth rate of the culture. Therefore, to operate a fed-batch culture at μ =D = 0.1 h^{-1} in a 14 L bioreactor with 4 L of batch culture (This is an arbitrary example),

$$\tau = \frac{1}{\mu} = \frac{1}{D} \qquad\qquad (AI-6)$$

$$\tau = \frac{1}{0.1} = 10 \ h$$

$$t_{qss} = 4\tau = 40 \ h$$

where t$_{qss}$ is the time required to achieve qss. A minimum fed-batch operation period of 40 h is required to achieve qss. To operate the bioreactor at D = 0.1 h^{-1},

$$D = \frac{F}{V},$$

$$0.1 \ h^{-1} = \frac{F}{4L}$$

$$F = 0.4 \ Lh^{-1}$$

With a remaining volume of 10 L for the fed-batch phase, the bioreactor is filled up in 25 hours if the flow rate of 0.4 L h^{-1} is maintained (40 h is required for qss). The time is

much shorter if an exponential fed-batch mode is implemented. Therefore in the qss method, the bioreactor may be filled up before qss is actually achieved.

ii. **Loss of production time**- under certain circumstances (e.g. if larger volumes are available), the bioreactor may be operated until qss is achieved to allow control of μ with D. In such a procedure, a growth rate curve similar to Fig AI-2 may be observed (see results of Ramirez,*et al*., 1994 who claimed to have achieved qss in fed-batch culture. The first 70% of total fed-batch time was characterised by wide fluctuations in biomass concentration).
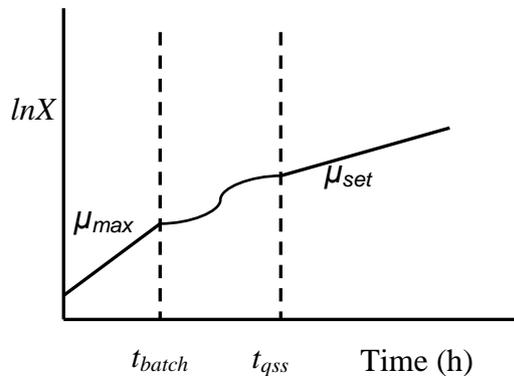

Fig. AI-2 Variation of specific growth rate during quasi-steady state fed-batch operation

The time interval between t_{batch} (end of batch phase) and t_{qss} may be characterised by unpredictable and uncontrolled growth patterns. For instance, in *S. cerevisiae* cultures, the cells may be plunged into permanent oxido-reductive metabolism leading to production of ethanol; in *Escherichia coli* there may be excessive accumulation of acetic acid leading to cell death whereas in penicillin production by *Penicillium spp*, lack of control of *μ* may induce stresses leading to pre-mature and therefore inefficient production of penicillin (Minihane & Brown, 1986).

iii. Product purity—to obtain pure enzyme and other metabolic products, the culture broth should not be contaminated by other proteins. In the case of *Saccharomyces cerevisiae*-Glucuronidase project, SDS-PAGE showed the secretion of other extracellular

proteins into the broth at 62 hours of cultivation. In such cases, fermentation may be stopped before the attainment of qss.

Therefore alternative methods, generally described as *dynamic methods* of fed-batch control have been adopted to control μ at specific values under both *transient and quasi-steady state* conditions (in the dynamic methods, qss may eventually be achieved if operation goes beyond t_{qss}.But that condition is not required to control μ)

Dynamic methods of controlling μ in fed-batch culture

i. **Inferential control methods**—these methods control μ based on a measured state variable (a parameter that describes the current state of a system) such as oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), culture volume, inlet air flow rate, respiratory quotient (RQ), pH and metabolic heat generation rate (calorimetric control). Inferential control strategies do not require qss to be established before μ can be controlled. Typically, they consist of two parts: system prediction and control (feeding) (Lee, et al., 1999). The predictive part is based on mathematical models of the system whereas the control part is based on an inference from a measured state variable. Accurate estimation of the system and robust models linking the measured state variable to μ are required to implement such methods. They are usually implemented through fuzzy logic control and neural networks (artificial intelligence). The major limitation is the availability and cost of sensors used to measure the required state variables and the non-linearity of mathematical models developed for bioprocesses.

ii. **Predictive control method**: The molecular mechanism underlying the control of μ with D is that the dilution rate controls the amount of glucose available to the culture (Lee, et al., 1999; Minihane & Brown, 1986). Therefore a direct method of control involving the glucose flux is also applicable in controlling μ at pre-determined values (James, et al., 2012). This method also has two parts: system prediction (mass of cells) and calculation of the glucose demand (GD) that would drive growth of the culture at a specified μ given the current biomass concentration and broth volume. To determine the substrate requirements in this

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method, the following derivation based on *substrate balance* in a fed batch bioreactor (refer to Figure AI- 1) is necessary:

mass accumulation = mass fed - mass consumed   (on unit time basis)

$$\frac{d(SV)}{dt} = S_f F - q_s x V \qquad \text{(AI-7)}$$

$$
\left\{
\begin{array}{c}
S = residual\ substrate\ concentration\ (\frac{g}{L}) \\
V = culture\ volume(L) \\
q_s = \frac{\mu}{Y_{x/s}} = specific\ substrate\ consumption\ rate \\
x = biomass\ concentration\ \left(\frac{g}{L}\right) \\
F = volumetric\ flow\ rate\ of\ feed\ (\frac{L}{h})
\end{array}
\right\}
$$

$$\frac{d(SV)}{dt} = S_f F - \frac{\mu x V}{Y_{x/s}} \qquad \text{(AI-8)}$$

Applying Product Rule to the LHS of Equation AI-8, (because both the volume and residual glucose (substrate) are constantly changing)

$$\frac{SdV}{dt} + \frac{VdS}{dt} = S_f F - \frac{\mu x V}{Y_{x/s}} \qquad \text{(AI-9)}$$

If we assume the *rate of change* in substrate accumulation is zero (or close to zero compared to S_f), then

$\frac{dS}{dt} = 0$, and given that $\frac{dV}{dt} = F$,

$$SF = S_f F - \frac{\mu x V}{Y_{x/s}} \qquad \text{(AI-10)}$$

$$\frac{\mu x V}{Y_{x/s}} = F(S_f - S)$$

$$Y_{x/s} = \frac{\mu x V}{F(S_f - S)} \tag{AI-11}$$

From biomass balance, $\frac{V}{F} = \frac{1}{D}$, and if D = μ, then

$$Y_{x/s} = \frac{x}{S_f - S} = \frac{dx}{ds} \tag{AI-12}$$

In other words, the substrate balance on the fed-batch bioreactor can be used to derive the biomass yield coefficient, $Y_{x/s}$, where x is concentration of biomass produced, S_f -S the biomass consumed. Therefore Equation AI-12 can be written as

$$Y_{x/s} = \frac{x_1 - x_0}{S_f - S}$$

$$S_f = \frac{x_1 - x_0}{Y_{x/s}} + S \tag{AI-13}$$

But $X_1 = X_0 e^{\mu t}$ and $X = xV$, [a mass-concentration conversion is assumed here, see section on specific growth rate calculation]

$$S_f = \frac{x_0 V_0 (e^{\mu t} - 1)}{Y_{x/s}} + S \tag{AI-14}$$

Hence there is a functional relationship between the substrate feed rate (S_f) and the specific growth rate (μ) based on the biomass yield coefficient, $Y_{x/s}$ (James, et al., 2012). Equation AI-14 can be used to control μ at a specific value based on S_f. The application of this method is subject to two conditions:

a. The cells must utilise the substrate according to the estimated or pre-determined biomass yield coefficient. In most organisms, $Y_{x/s}$ is constant for a specific substrate at specific pH and temperature. Therefore the $Y_{x/s}$ determined in previous experiments can be used to program the feed in subsequent experiments. However, in others (like *S. cerevisiae*) the biomass yield coefficient changes with the metabolic regime (Paalme, et al., 1997). In such cases, preliminary runs to determine $Y_{x/s}$ under each set of new circumstances are necessary to accurately determine the required feed rate at

various time points in the culture. In the current work, an equaiton relating the biomass yield coefficient to the specific growth rate was established and used to predict $Y_{x/s}$ at the various specific growth rates.

b. Substrate accumulation in the bioreactor must remain at minimal or very low levels – thus, substrate must be consumed as soon as it enters the bioreactor.

Equation AI-14 represents the dynamic control of µ using the glucose flux (glucose feed rate, S_f). It is more of a predictive model than an inferential model because no state variable is measured during feeding. The feed control is based on a lumped model comprising a predictive part, [$(e^{\mu t} - 1)$]—that predicts exponential cell growth; and a substrate utilisation part, [$\dfrac{x_0 V_0}{Y_{x/s}} + S$] that describes the mass of substrate required to achieve the predicted biomass. The predictive part depends on μ whereas the substrate supply (utilisation) part depends on $Y_{x/s}$. The substrate feed equation also incorporates an exponential function, and therefore works best in a culture undergoing exponential growth, otherwise substrate accumulation may be observed.

Specific growth rate calculation

Calculation of μ concentration basis—from mass balance on biomass:

$$\frac{d(xV)}{dt} = \mu x V \qquad\qquad \text{[Same as eqn AI-3]}$$

Applying Product rule,

$$\frac{x dV}{dt} + \frac{V dx}{dt} = \mu x V$$

Dividing through by xV

$$\frac{1}{V}\frac{dV}{dt} + \frac{1}{x}\frac{dx}{dt} = \mu \qquad\qquad\qquad (\text{AI-15})$$

Applying the property that: $\dfrac{d(\ln a)}{dt} = \dfrac{1}{a}\dfrac{d(a)}{dt}$

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$$\mu = \frac{d(\ln V)}{dt} + \frac{d(\ln x)}{dt} \tag{AI-16}$$

Applying the logarithmic property that: $\ln a + \ln b = \ln(ab)$,

$$\mu = \frac{d(\ln xV)}{dt}$$

$$\mu = \frac{d(\ln X_m)}{dt} \tag{AI-17}$$

where X_m is the mass of cells obtained from the product of culture volume and biomass concentration. (The same formula is derived below on mass basis)

Calculation of μ on mass basis: in the dynamic method, there is no qss; therefore both the biomass concentration and volume are constantly changing. Since the mass of cells is a product of concentration and volume, a differential equation can be written for the mass of cells (X_m)—see *concentration to mass conversion* (Johnson, 1987; James, et al., 2012). The mass equivalence of the biomass (concentration) balance equation is

$$\frac{d(X_m)}{dt} = \mu X_m \tag{AI-18}$$

$$\mu = \frac{1}{X_m}\frac{dX_m}{dt} \tag{AI-19}$$

$$\mu = \frac{d(\ln X_m)}{dt} \tag{AI-20}$$
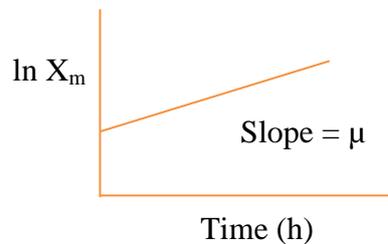
Fig. AI-3 Calculation of μ in dynamic fed-batch culture

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Therefore a plot of the natural logarithm of *total mass of cells in the bioreactor at various time point* (X_m) against time (Equation 17 or 20, Figure AI-3) gives a straight line with slope as the specific growth rate (μ). On the contrary, in qss method, μ is calculated from the slope of a plot of the natural logarithm of the *concentration of cells* against time.

Variation in substrate concentration:

During the glucose flux control method, the most important parameter that controls μ is the substrate feed rate (g/h) and not the substrate concentration (g/L). Therefore adjustments in substrate concentration that are accompanied by equivalent adjustments in flow rate (L/h) to maintain the feed rate at a specified value will not affect the specific growth rate. This offers flexibility in operation because volume limitation can easily be eliminated. [Use a highly concentrated feed (g/L) at a low flow rate (L/h) to achieve the same feed rate (g/h) as using a dilute feed at a high flow rate. Since the volume-filling time depends on flow rate (L/h), the operator can decide when the bioreactor should be filled, by adjusting the feed concentration].

Contrary to the qss method where an increase in substrate concentration would increase biomass concentration (*x*) (Shuler & Kargi, 2002) without any effect on *μ*, changes in substrate concentration in the glucose flux method [can] change both *μ* and *x*.

Table AI-1 Variation in feed rate, substrate concentration and flow rate in the dynamic glucose flux method.

Feed rate (g/h) (calculated from Equation AI-14)	Substrate conc (g/L)	Flow rate (L/h)	Effect on *μ*
1.5	20	0.08	Constant
1.5	30	0.05	Constant
1.5	40	0.04	Constant
1.6	20	0.08	Increases
2.4	30	0.08	Increases
1.2	15	0.08	Decreases

For instance, in the first 3 rows of Table AI-1, there is an increase in substrate concentration but with an equivalent decrease in the flow rate hence the feed rate remains constant, therefore μ and *x* will remain constant. [It is actually the g/h that determines μ]. On the other hand, the last 3 rows show an increase in substrate concentration and a constant flow rate, resulting in an increase in the feed rate. This will increase both μ and *x.* Therefore the final biomass concentration in this method is determined by the feed rate (hence μ) and the duration of operation. Perhaps a challenge in the method is [as opposed to qss] the inability to control *x* without altering μ. The predictive feed (feeding based on biomass) creates a direct link between μ and *x*, and the two parameters are not independent. To maximise biomass production with the method, the fed-batch culture should be operated

i.   at the highest possible μ
ii.   for long periods of time (means with a highly concentrated feed at low flow rates)

Implication of the dynamic (glucose flux) method on growth kinetics

Equation AI-14 suggests an unbound model relating the substrate feed, S_f to the specific growth rate, μ - in other words, μ increases indefinitely with increasing S_f. This is, however, not true because at a later phase in the fermentation when balanced growth is achieved, substrate utilisation will be governed by saturation kinetics according to the Monod model.

$$\mu = \frac{\mu_{max} S_f}{K_s + S_f}$$
(AI-21)

Therefore in the application of the dynamic method, it is important to use μ_{max} determined in batch culture to calculate the maximum allowable S_f (S_{fmax}) and then operate the fed-batch below this S_{fmax}. This will result in operating at μ lower than μ_{max}. Any feed rate higher than S_{fmax} will result in substrate accumulation and growth at μ_{max} (like batch culture).

Implementation of the method

The following is the account of the exponential fed-batch mode based on the glucose flux method implemented in the fed-batch cultivations of *S. cerevisiae* for production of α-glucuronidase. The method (as derived above) is based on the fundamental growth equations

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and mass balances used to predict the mass of biomass in the bioreactor to feed the required amount of glucose that would drive the culture growth at a pre-determined specific growth rate. Table AI-2 gives the growth data and other relevant parameters that were used to implement this feeding mechanism. Some of these parameters were obtained from preliminary batch fermentation whilst others were set at values specific for the example in this appendix.

Table AI-2 Growth and bioreactor parameters for *S. cerevisiae* cultivation

Parameter	Value	Unit	Source/Reference
Maximum specific growth rate (μ_{max})	0.3	h^{-1}	Batch experiment
Pre-determined specific growth rate (μ)	0.12	h^{-1}	Specified
Glucose concentration in feed solution (S)	70	g/L	Specified
Volume of broth at start of fed-batch phase	6	L	Measured
Biomass concentration at start of fed-batch phase (x_0)	6.2	g/L	Measured at the end of batch phase
Biomass yield coefficient ($Y_{x/s}$)	0.4	$g\ g^{-1}$	Batch experiment

The batch phase preceding the fed-batch lasted approximately 5 hours, during which a total of 15g/L of glucose was depleted. The depletion of glucose in the broth was signalled by a sudden drop in the carbon dioxide concentration in the exhaust gas (Appendix IV) and a sudden spike in DOT. It was also confirmed qualitatively with AccuCheck® glucose strips. This signal was used to initiate the fed-batch phase. The feeding profile was determined as follows:

Prediction of Biomass

The mass of cells in the reactor was predicted with the general growth equation

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$$X_t = X_0 e^{\mu t}$$
(AI-22)

Where X_0 is the mass of cells at the start of the fed-batch phase and X_t the mass of cells at any time t after feeding is initiated. The growth rate to be achieved is μ. At the beginning of the fed-batch phase, the biomass concentration was $6.2 g_{DW}/L$ with a total broth volume of 6L, therefore,

$X_0 = 6.2 g_{DW}/L \ * \ 6L = 37.2 g_{DW}$

Hence for the specific growth rate of $0.12 \ h^{-1}$ ($0.12/60 = 0.002 \ min^{-1}$)

$X_t = 37.2 e^{0.002t}$
(AI-23)

where t is time in minutes and X_t the mass of cells in the bioreactor. The biomass profile for the entire duration of the fermentation as predicted by Equation AI-23 and plotted in Microsoft Maths® is shown in Figure A1 below.
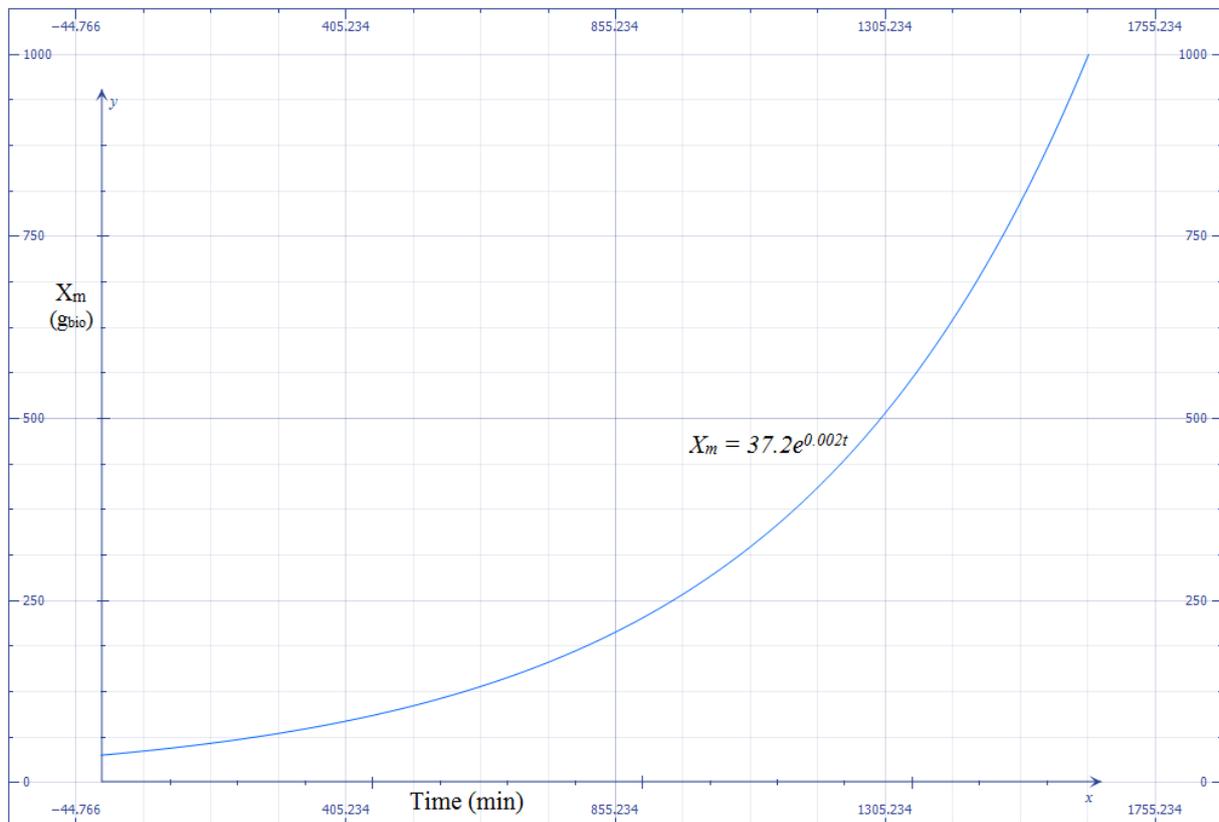

Figure AI-4 Exponential increase in mass of cells (X_m) as predicted by Equation AI-22

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Calculation of the Amount of Substrate

The equation for the amount of substrate calculation used in developing the exponential feed profile is given as (Kerste, et al., 2008; Ramirez, et al., 1994; James, 2012; Dane-Wittrup, 2007).

$$S_f = \frac{x_0 V_0 \left(e^{ut} - 1\right)}{Y_{x/s}} + S_0 \qquad \text{(AI-14)}$$

where S_f is the mass of glucose (g_{glu}) that must be pumped into the bioreactor within the time t_i to enable the cells to grow at the set growth rate of μ, S_0 the amount of glucose fed at time t_{i-1}, x_0 and V_0 the biomass concentration (g_{DW}/L) and broth volume (L), respectively and $Y_{x/s}$ the biomass yield coefficient.

For a typical calculation using the data in Table AI-2 and considering the first minute after fed-batch was started, with a pre-determined specific growth rate of 0.12 h^{-1},

$$\mu = \frac{0.12 h^{-1}}{60 \min} \times 1h = 0.002 \min^{-1} \quad ; \quad \Delta t = (1-0) \min ;$$

$$S_{f1} = \frac{6.2 \frac{g}{L} \times 6L \left(e^{0.002 \times 1} - 1\right)}{0.4} + 0 = 0.1862 g_{glu} \qquad \text{(AI-24)}$$

S_o is zero because there was no feed at time t_o. Thus the mass of glucose to be pumped into the vessel was determined. Similarly, in the second minute after feeding was started;

$$X_2 = 37.2 e^{0.002 \times 2} = 37.274 g_{DW}$$

$$S_{f2} = \frac{37.274 \left(e^{0.002 \times (2-1)} - 1\right)}{0.4} + 0.1862 = 0.3728 g_{glu} \qquad \text{(AI-25)}$$

Therefore the amount of glucose required by the cells from the first to the second minute was

$$\Delta S_f = S_{f2} - S_{f1} = 0.3728 - 0.1862 \qquad\qquad\qquad (AI\text{-}26)$$

$$\Delta S_f = 0.1866 g_{glu}$$

These series of calculations for the amount of glucose required per minute during the fed-batch phase were calculated in a spread sheet. An excerpt of the first 15 minutes from the spread sheet for the fed-batch phase is given in Table AI-3.

Table AI-3 Amount of Substrate and Flow rate Calculations

| Time (min)	X (g_{DW})	ΔS_f (g_{glu}/min)	F (ml/min)
1	37.2	0.186186124	2.659802
2	37.27447445	0.186558869	2.665127
3	37.3490988	0.18693236	2.670462
4	37.42387094	0.187306599	2.675809
5	37.49879358	0.187681587	2.681166
6	37.57386622	0.188057326	2.686533
7	37.64908915	0.188433817	2.691912
8	37.72446267	0.188811061	2.697301
9	37.7999871	0.189189061	2.702701
10	37.87566272	0.189567818	2.708112
11	37.95148985	0.189947333	2.713533
12	38.02746878	0.190327608	2.718966
13	38.10359983	0.190708644	2.724409
14	38.17988328	0.191090443	2.729863
15	38.25631946	0.191473006	2.735329

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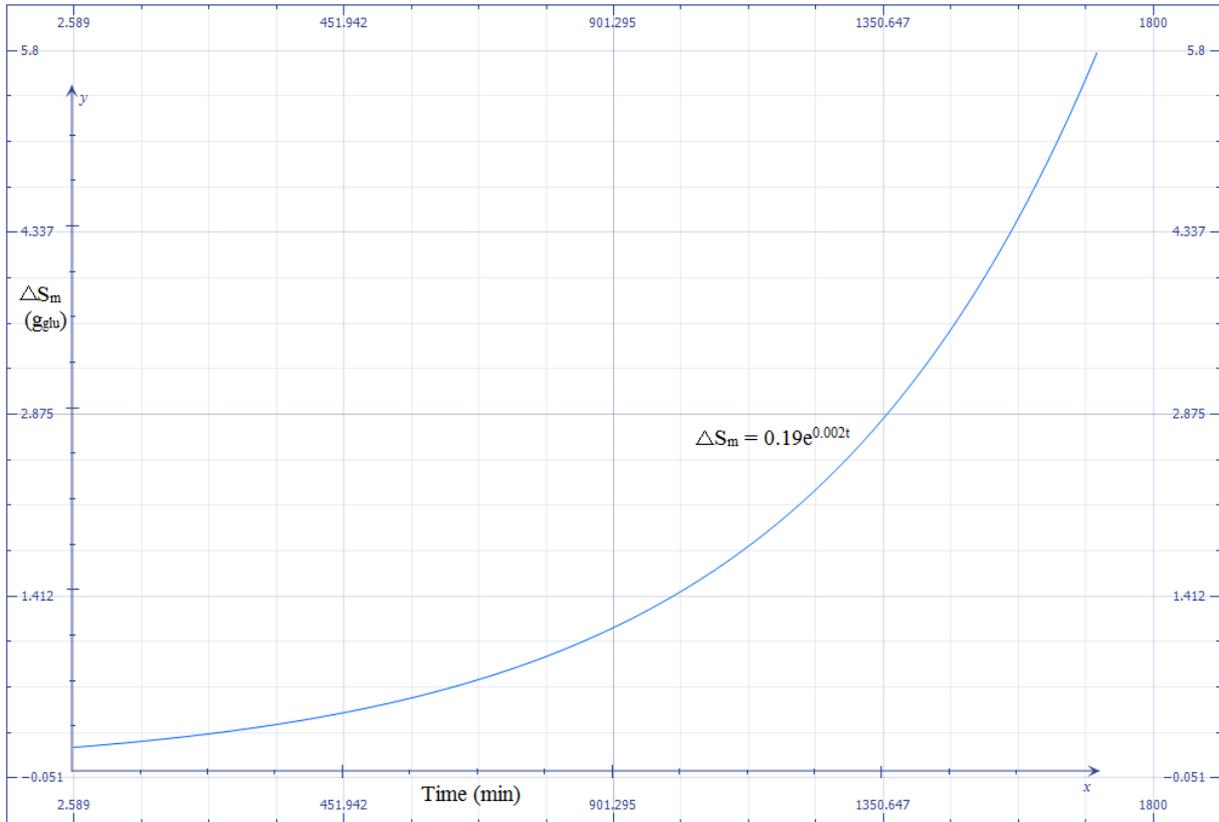
Figure AI-5 Exponential profile for glucose requirements at $\mu = 0.12 \text{ h}^{-1}$

In Table AI-3, for example in the 3rd minute, the mass of cells was predicted to be 37.349098 g_{DW}. From the third minute to the fourth minute, the culture would grow at 0.12h^{-1} to a new mass of 37.42387094 g_{DW} and the amount of glucose to effect this growth was 0.18693236 g_{glu}.

With a 70g/L reservoir of glucose solution, the volumetric flow rate was calculated as:

$$F = \frac{0.1862g \text{ min}^{-1}}{70\text{g L}^{-1}} = 0.00266L\text{ min}^{-1} = 2.66ml\text{ min}^{-1} \tag{AI-27}$$

Thus, to meet the substrate requirement of the culture, the feed pump was programmed to administer 2.66 mL of the 70g/L glucose solution within that minute. Figures AI-5 and AI-6

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show the exponential profiles of the glucose requirements of the culture in the course of the fermentation as predicted by the cell growth and the corresponding feed flow rates to meet the substrate requirements.
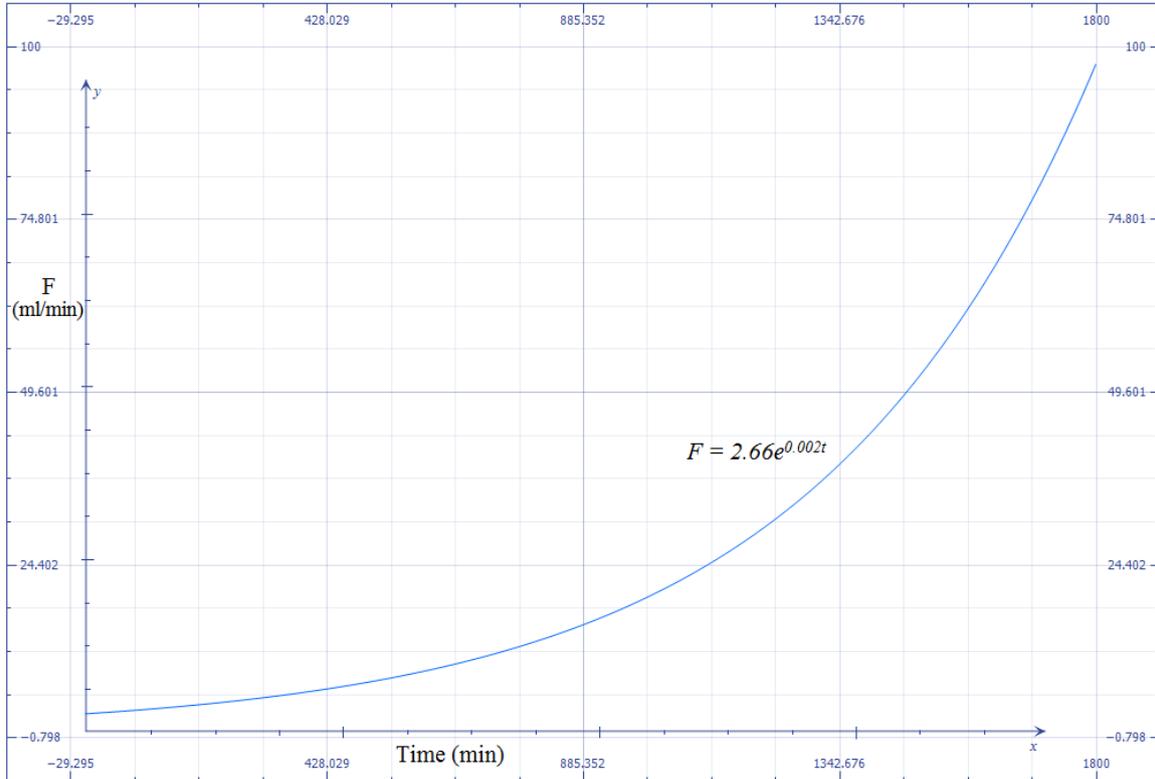

Figure AI-6 Exponential profile for feed flow rate

The flow rate data derived from the calculations and shown in Table AI-3 was plotted in Microsoft Maths[®] to obtain the curve and equation in Figure AI-6. This equation was used to program the programmable logic controller (PLC) according to an exponential feeding profile that operated on an algorithm of the format

$$y = Ae^{xt}$$

(AI-28)

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where y represents the pump set point, A is a pre-exponential factor representing the starting volumetric flow rate and x the specific growth rate of the culture in the fed-batch phase. The programme calculated the new flow rate every 10 seconds, and the Watson-Marlow® U505A pump set point was automatically adjusted accordingly to achieve this flow rate with a particular size of silicone tubing.

Figure AI-7 compares the predicted biomass with the actual biomass obtained at the end of the exponential feeding phase of the fermentation.
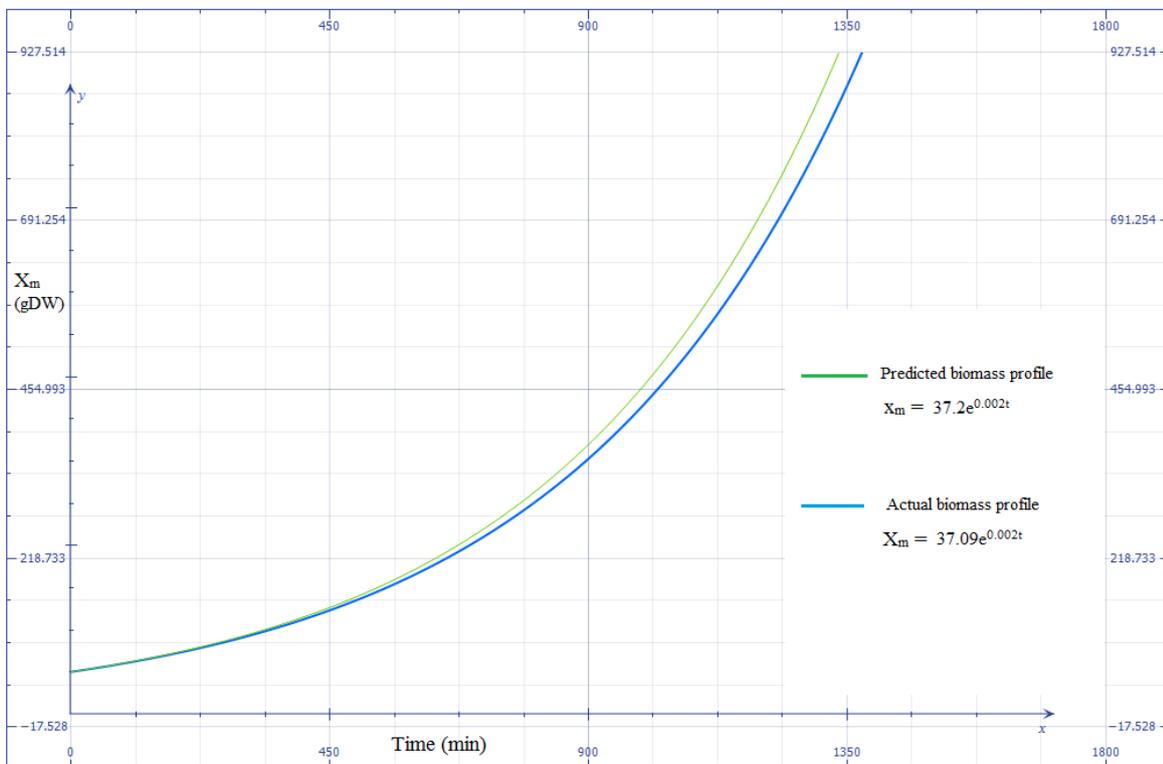

Figure AI-7 Predicted and Actual biomass profiles for growth rate $\mu = 0.12$ h^{-1}

Control of Dissolved Oxygen Concentration

The concentration of dissolved oxygen in the fermentation broth was regulated through a cascade control system between the DOT set-point and the agitation rate. The DOT set-point was set to 30% saturation, whereas the agitation limits were set at 150 rpm (lower limit) and 1000 rpm (upper limit). Thus, the agitation rate was increased automatically to compensate for a drop in the DOT due to metabolic activity, in order to maintain the DOT at 30 %. During operation, the maximum agitation rate reached was ca. 650 rpm, in the *S. cerevisiae* culture growing at $\mu = 0.12 \text{ h}^{-1}$.

Air flow rate was also monitored manually, and was increased intermittently as the broth volume increased, to maintain aeration rate of about 0.8vvm. The cascade control, together with the manual control of airflow rate was able to maintain the DOT above 30 % in all fermentations.

APPENDIX II : Scale-up Procedure

k_La Determination

The dynamic gassing out technique as described by Doran (1995) was used to calculate the volumetric mass transfer coefficient, k_La. A typical dissolved oxygen profile for gassing out-gassing in experiment is shown in Figure AII-1.
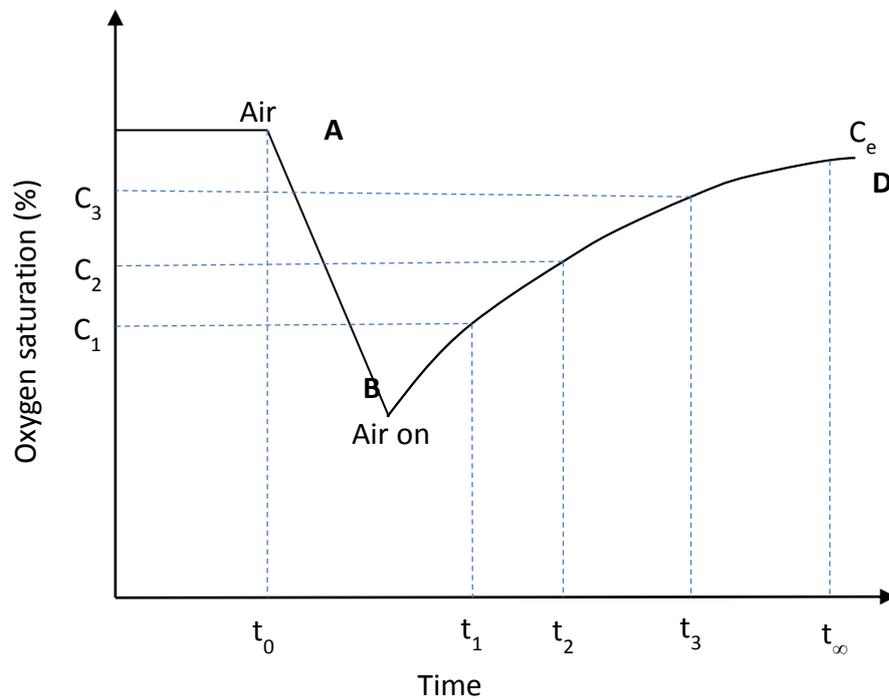

Figure AII-1 Dissolved oxygen profile during dynamic gassing out-gassing in experiment

The overall equation governing the transfer and usage of oxygen in an active fermentation broth is given as (Garcia-Ochoa & Emilio, 2009)

$$\frac{dC}{dt} = k_L a\left(C^* - C\right) - q_{o2}X \tag{AII-1}$$

During gassing-out (when air supply is turned off), there is no external supply of oxygen hence the term $k_La(C^* - C)$ is equal to zero and $q_{O2}X$ is equal to the slope of the curve AB in

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Figure AII-1. During gassing-in (when air supply is turned on again), the differential $\frac{dC}{dt}$ decreases along the curve BD until it drops to zero at some infinite time (t_∞), at which point the rate of oxygen transfer from the gas phase into the broth is equal to the rate of oxygen consumption by the microorganisms. The oxygen concentration measured at this steady state condition is the equilibrium concentration (C_e) at the prevailing conditions of aeration and agitation rates.

When this equilibrium is achieved, $C = C_e$,

$$\frac{dC}{dt} = k_L a \left(C^* - C_e \right) - q_{o2} X \qquad\qquad\qquad\text{(AII-2)}$$

But $\frac{dC}{dt} = 0$. Applying this condition to Equation AII-2 yields

$$q_{O2} X = k_L a \left(C^* - C_e \right) \qquad\qquad\qquad\text{(AII-3)}$$

Replacing $q_{O2}X$ in Equation AII-1 with the result of Equation AII-3 gives

$$\frac{dC}{dt} = k_L a \left(C^* - C \right) - k_L a \left(C^* - C_e \right)$$

Simplifying this expression yields

$$\frac{dC}{dt} = k_L a \left(C_e - C \right) \qquad\qquad\qquad\text{(AII-4)}$$

Separation and integration of Equation AII-4 along the path BD at points $t_1, t_2, t_3 \ldots$ gives points that can be plotted as shown in Figure AII-2.

$$\int_{C_1}^{C_2} \frac{1}{C_e - C} dC = \int_{t_1}^{t_2} k_L a \, dt$$

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$$\ln\left(\frac{C_e - C_1}{C_e - C_2}\right) = k_L a(t_2 - t_1) \tag{AII-5}$$
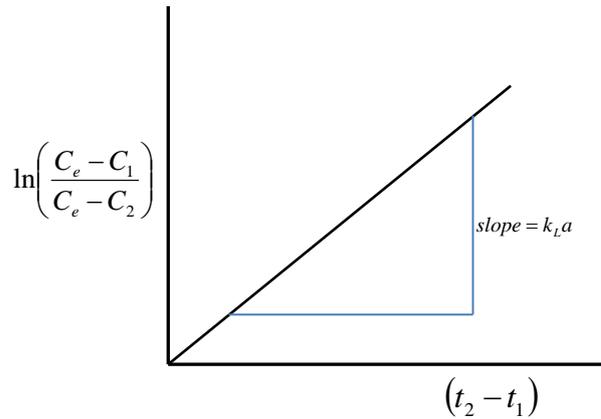
Figure AII-2 $k_L a$ from integration methods

Table AII-1 shows four different gassing out-gassing in measurements from which $k_L a$ values were calculated. These were at different aeration and agitation rates, and the combinations of these parameters were used for the calculation of scale-up parameters as given in the following section.

Plots of the first and second measurements are given in Figure AII-3. The term $ln\left(\frac{C_e - C_1}{C_e - C_2}\right)$ was calculated from the rising part (gassing-in period) of each of the curves and plotted against the respective time intervals to determine $k_L a$ as shown in Figure AII-3 (B). The equilibrium concentrations (C_e) for the dissolved oxygen were the values at which the DO became stable during gassing in, at the given air flow and agitation rates

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Table AII-1 Dissolved oxygen tension during gassing in-gassing out experiment

| Time (s)	DO (%)-1st	DO (%)-2nd	DO (%)-3rd	DO (%)-4th
0	30	31.9	30	76.3
10	29.5	32.0	29.7	76.4
20	20.2	31.8	28.5	76.4
30	19.1	29.8	24.9	75.1
40	17	26.4	17.2	72.5
50	14.9	20.8	9.5	69.6
60	11.1	14.3	4.4	65.1
70	7.9	10.6	2.3	62.1
80	5.9	6.4	2.2	59.1
90	4.6	4.8	3.3	56.5
100	3.7	3.8	5.7	54.1
110	3.7	5.4	10.1	52.1
120	4.4	10.7	14.8	49.8
130	8.0	17.8	18.8	49.1
140	13.3	24.8	21.8	51.4
150	18.7	30.9	24.1	55.6
160	23.2	36.2	25.9	60.2
170	26.3	39.2	27.5	64.5
180	28.2	40.8	28.6	67.6
190	29.1	41.5	29.5	70.6
200	29.5	41.4	29.9	73.4
210	29.7	40.7	29.9	74.7
220	29.9	40	29.9	75.9
230	29.9	39	29.9	77
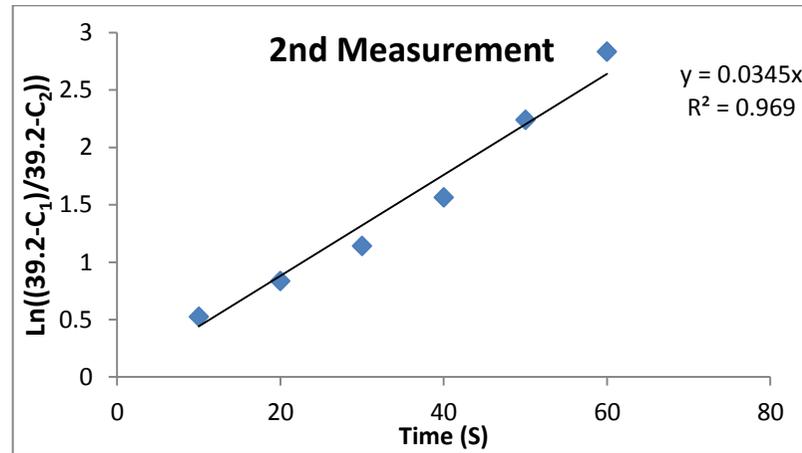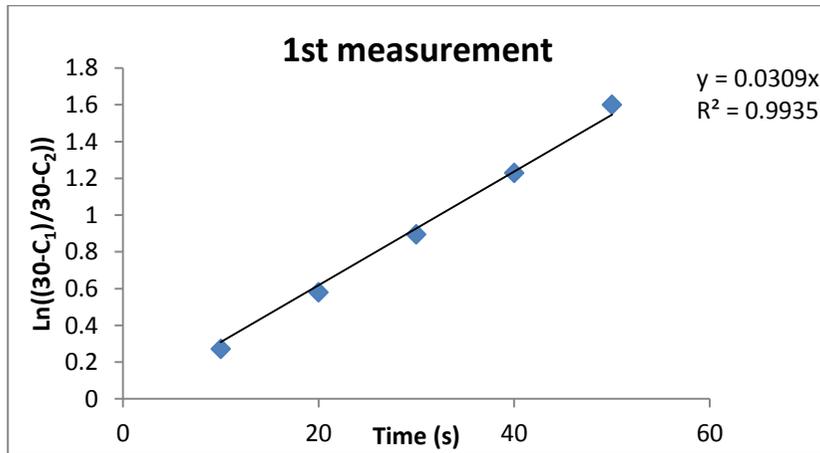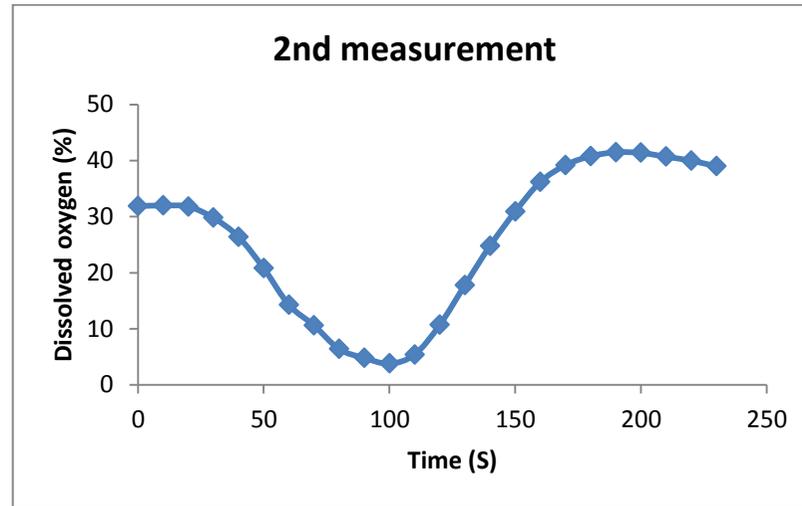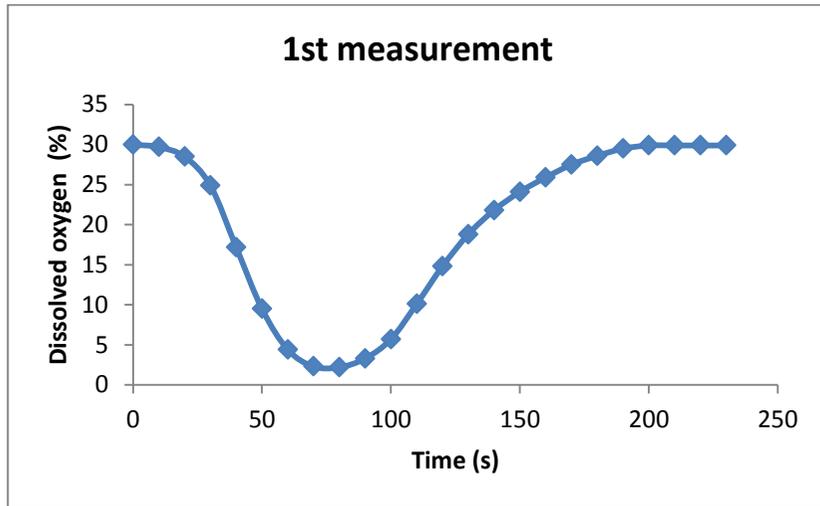
Figure AII-3 (A) Dissolved oxygen profiles (B) $k_L a$ values from semi-log graphs

Constant k$_L$a Scale-up

The volumetric mass transfer coefficient (k$_L$a) is related to the power consumption per unit volume and superficial gas velocity according to the following relation (Doran, 1995; Garcia-Ochoa & Emilio, 2009; Diaz & Acevedo, 1999)

$$k_L a \ = \ \alpha \left(\frac{P_g}{V_L} \right)^a v_s^{\ b} \qquad \text{(AII-6)}$$

The following are the conditions under which the different measurements of k$_L$a (Table AII-2) were done. The bioreactor has an impeller diameter of 93 mm and a tank diameter of 240 mm

Table AII-2 Conditions of k$_L$a measurement

#	Agitation (rpm)	Air flow rate (L/min)	Broth density (g/ml)
1	420	4.0	1.0437
2	498	4.0	1.0292
3	543	4.0	1.0292
4	420	5.3	1.0437

From Table AII-2 the following parameters can be calculated after the necessary conversions are made. For instance, in the 1st measurement,

i. $\quad \dfrac{P_g}{V} = \dfrac{0.83 \rho N^3 D_i^5}{D_i^3} = \dfrac{0.83 \cdot 1043.7 kg \cdot 7^3 \cdot 0.093^2 \, m^2}{m^3 \cdot s^3} = 2569.88 \dfrac{W}{m^3} \qquad$ (AII-7)

ii. $\quad A = \dfrac{\pi D_t^{\ 2}}{4} = \dfrac{\pi (0.24)^2}{4} = 0.0452 \, m^2 \qquad$ (AII-8)

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iii. $\quad v_s = \dfrac{\dot{V}}{A} = \dfrac{6.67 \times 10^{-5} \, m^3}{0.0452 \cdot m^2 \cdot s} = 0.00148 m s^{-1}$ $\hspace{2cm}$ (AII-9)

From these calculations, Table AII-3 was constructed.

Table AII-3   Parameters for mass transfer correlation

#	$k_L a \; (s^{-1})$	$\dfrac{P_g}{V}\left(\dfrac{W}{m^3}\right)$	v_s (m/s)
1	0.0309	2569.88	0.00148
2	0.0345	4224.53	0.00148
3	0.0409	5476.32	0.00148
4	0.0322	2569.88	0.00196

From Table AII-3, the variables α, *a* and *b* in Equation AII-6 can be calculated as follows:

$$\dfrac{\#3}{\#2} = \dfrac{0.0409}{0.0345} = \left(\dfrac{5476.32}{4224.53}\right)^a ; \quad a = 0.66 \hspace{2cm} (AII\text{-}10)$$

$$\dfrac{\#4}{\#1} = \dfrac{0.0322}{0.0309} = \left(\dfrac{0.00196}{0.00148}\right)^b ; \quad b = 0.15 \hspace{2cm} (AII\text{-}11)$$

Substituting the values of *a* and *b* into #1, converting $k_L a$ to units of h^{-1}

$$108.3 = \alpha (2569.88)^{0.66}(0.00148)^{0.15}; \alpha = 1.62 \hspace{2cm} (AII\text{-}12)$$

Therefore the relationship between the volumetric mass transfer coefficient, the gassed power per unit volume and the superficial gas velocity was found to be

$$k_L a = 1.62 \left(\frac{P_g}{V} \right)^{0.66} v_s^{0.15} \qquad \qquad \text{(AII-13)}$$

Due to physical constraints in operating the 150L bioreactor, such as maximum allowable gas flow rate, maximum allowable gas hold-up, gas bubble channelling and foam formation, a suitable superficial gas velocity (v_s) was chosen. Furthermore, the exponent of v_s in Equation AII-13 (i.e 0.15) is less than the exponent of P_g/V hence changes in the former do not affect the $k_L a$ value as much as changes in the latter (Table AII-3). Therefore a value of 0.00314m/s (equivalent to 32 L/min) was chosen for v_s.

Table AII-4 Geometric specifications of the 14 L and 100 L bioreactors

Parameter	14 L Bioreactor	100 L Bioreactor
Working Volume (L)	14	100
Vessel Diameter, D_t (mm)	240	450
Vessel height, H_t (mm)	485	950
H_t/D_t ratio	2	2.1
Impeller Diameter, D_i (mm)	93	180
D_i/D_t as a %	38.75	40
Number of impellers	2	2
Impeller separation, h_i (mm)	170	300
Impeller Spacing criterion	$D_i < h_i < 2D_i$	$D_i < h_i < 2D_i$
Type of impeller	Rushton Impeller	Rushton Impeller
Baffle width, W_b (mm)	25	37.5
W_b/D_t ratio	0.1	0.08

To maintain a constant $k_L a$ of 0.0309 s^{-1} (108 h^{-1}) (determined from the fed-batch phase in 14 L bioreactor) in the 100L bioreactor, the required agitation rate was calculated as follows:

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$$108.3h^{-1} = 1.62 \left(\frac{P_g}{V} \right)^{0.66} (0.00314)^{0.15} \tag{AII-14}$$

On per second basis, Equation AII-14 can be simplified to

$$\left(\frac{P_g}{V} \right) = 82.0808 \frac{W}{m^3} = \frac{0.83 \rho N^3 D_i^5}{D_i^3} \tag{AII-15}$$

$$N = \left(\frac{68.12 \cdot D_i^3}{\rho D_i^5} \right)^{\frac{1}{3}} \tag{AII-16}$$

With average broth density of 1.0365 g/ml and impeller diameter of 180 mm

$$N = 1.253 s^{-1} = 75.2 rpm \tag{AII-17}$$

Thus operating the 100 L bioreactor at agitation rate starting from 75.2 rpm with air flow rate of 32 L/min will achieve a minimum volumetric mass transfer coefficient of 108 h^{-1}.

APPENDIX III: DO-stat Fed-batch Methodology

In the fermentation of *P. pastoris* for Ffase production, both the GFB and MIP of the fed-batch culture were done based on DO-stat fed-batch technique. The methodology is based on the premise that lack of carbon substrate in the medium leads to an increase in the dissolved oxygen concentration. An increase in feeding of the growth-limiting substrate (usually carbon) would then result in a decrease in DO concentration, thereby allowing control of substrate feeding based on the actual consumption rate thereof. In circumstances where the fermentation equipment do not allow on-line monitoring of both/either the glycerol and methanol concentrations (such as in this research), the DO-stat technique is an efficient way of preventing substrate accumulation.

In the method implemented in this work, two dissolved oxygen set points were used. An upper DOT set-point, typically 30%, on which the substrate feed was based (value specified in the CCD) and a lower DOT set-point, typically 25%, which regulated the cascade control of the agitation rate to prevent the culture from running into oxygen-limiting conditions (Figure AIII-1).
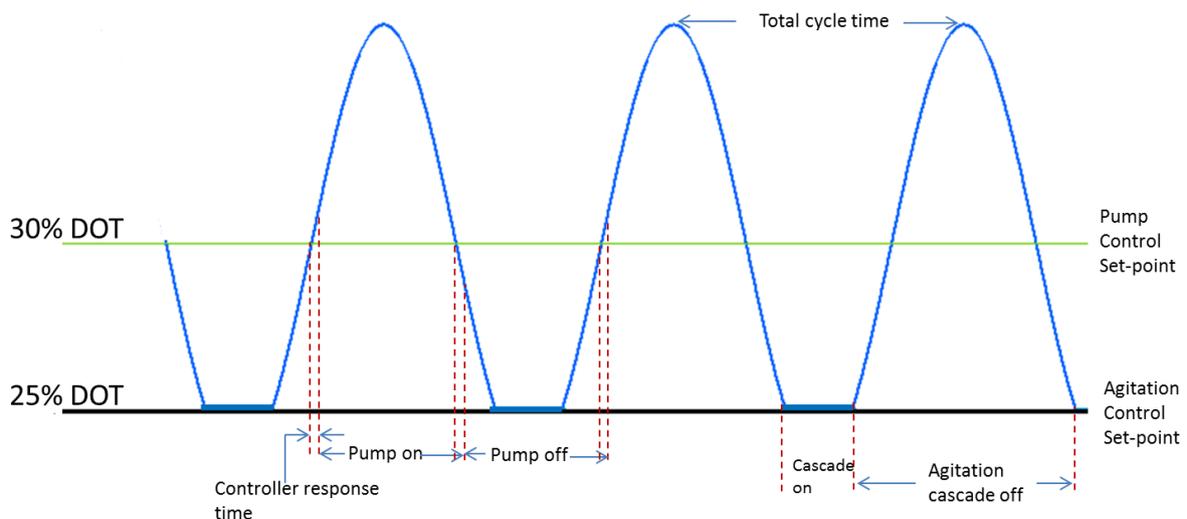

Figure AIII-1 DO-stat fed-batch scheme during the glycerol fed-batch (GFB) and methanol induction phases (MIP) of *P. pastoris* cultivation.

The substrate feed pump was switched on only when the measured DOT rose above the pump control set-point. During the 'on' time, substrate was fed at a rate specified in the CCD using a particular silicone tube size and a pre-calibrated pump. The typical cycle time for the feed ranged from 100 s at the beginning of fed-batch to 55 s by the end of the fermentation. The controller had a constant response or lag time of 28 s. Thus, both the 'on' and 'off' times of the pump (Figure AIII-1) were shifted forward by equal time periods (25 s) relative to the times the DOT wave crossed the pump control set-point. The agitation control set-point was selected to be sufficiently low so that the sustained DOT due to agitation cascade would not cause the pump to continue feeding outside of the controller lag time. Thus, overfeeding of substrate was prevented. The full feeding profile charts are given in Figure AIII-2.
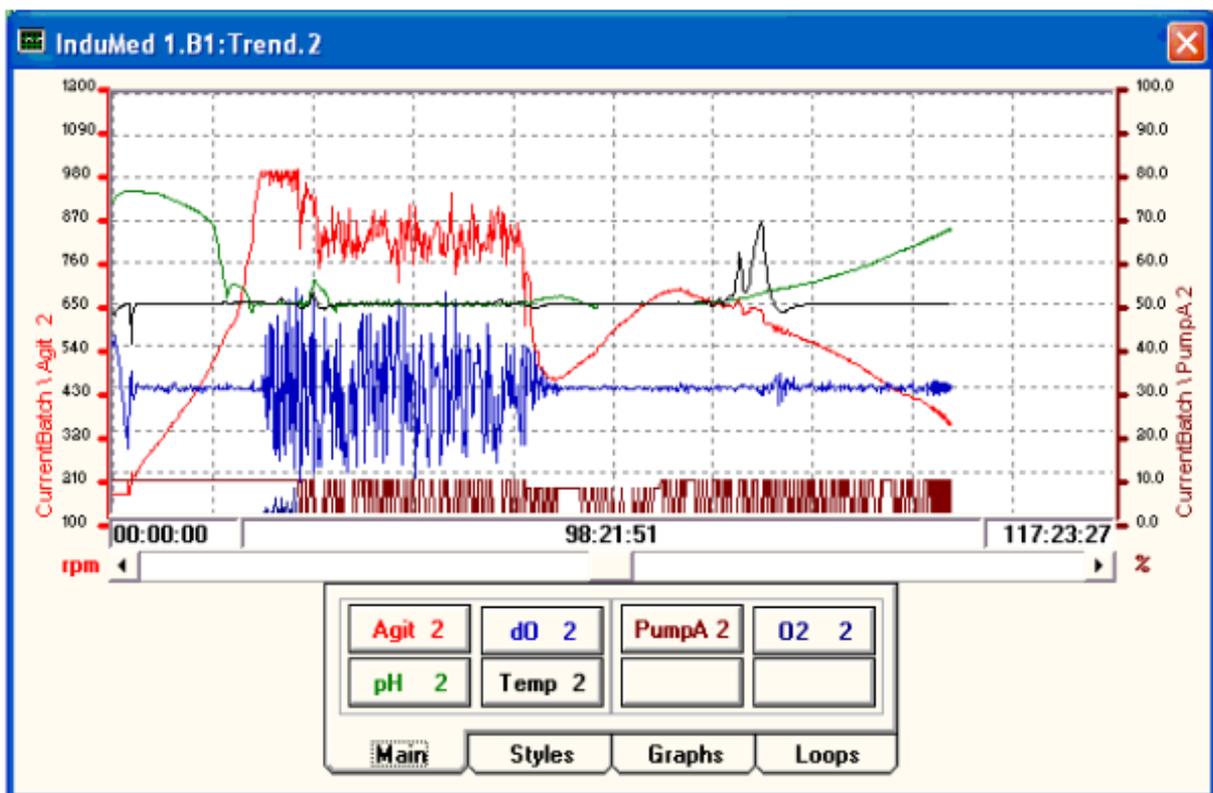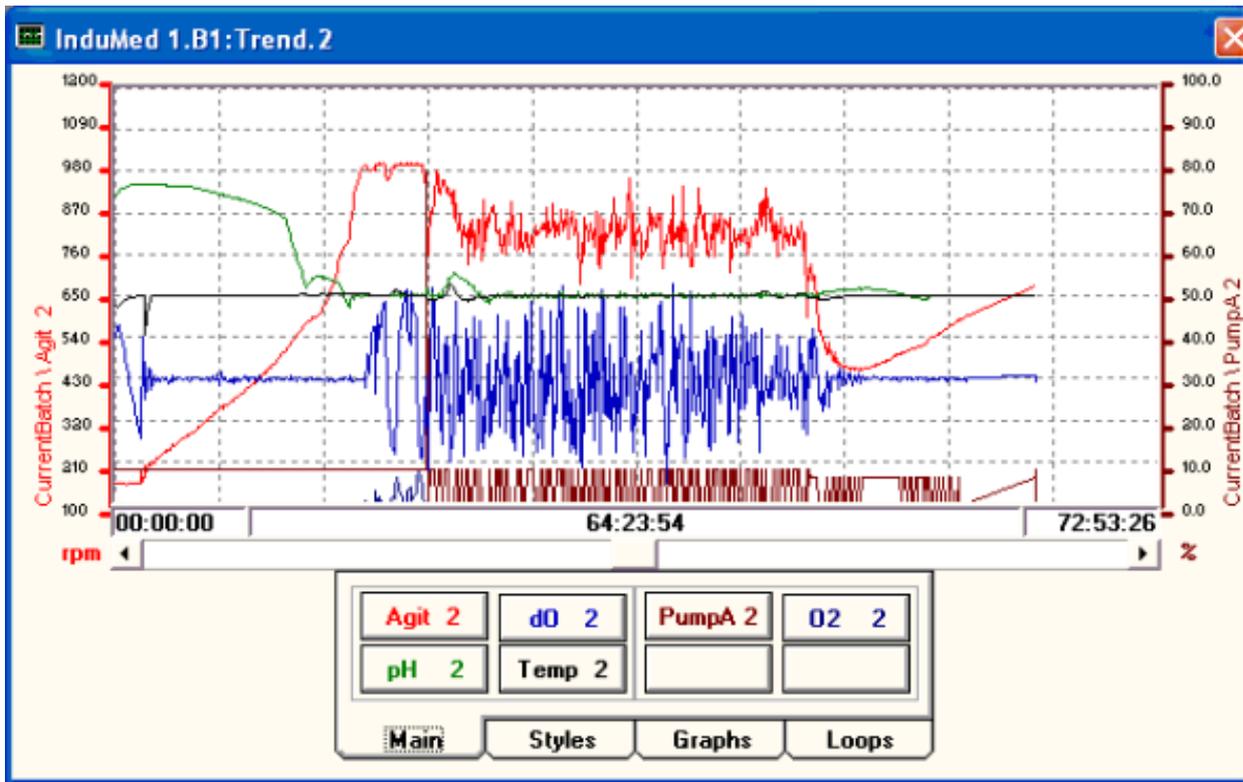
Figure AIII-2 DO-stat fed-batch trends during cultivation of *P. pastoris* for production of Ffase.

APPENDIX IV: Miscellaneous Data
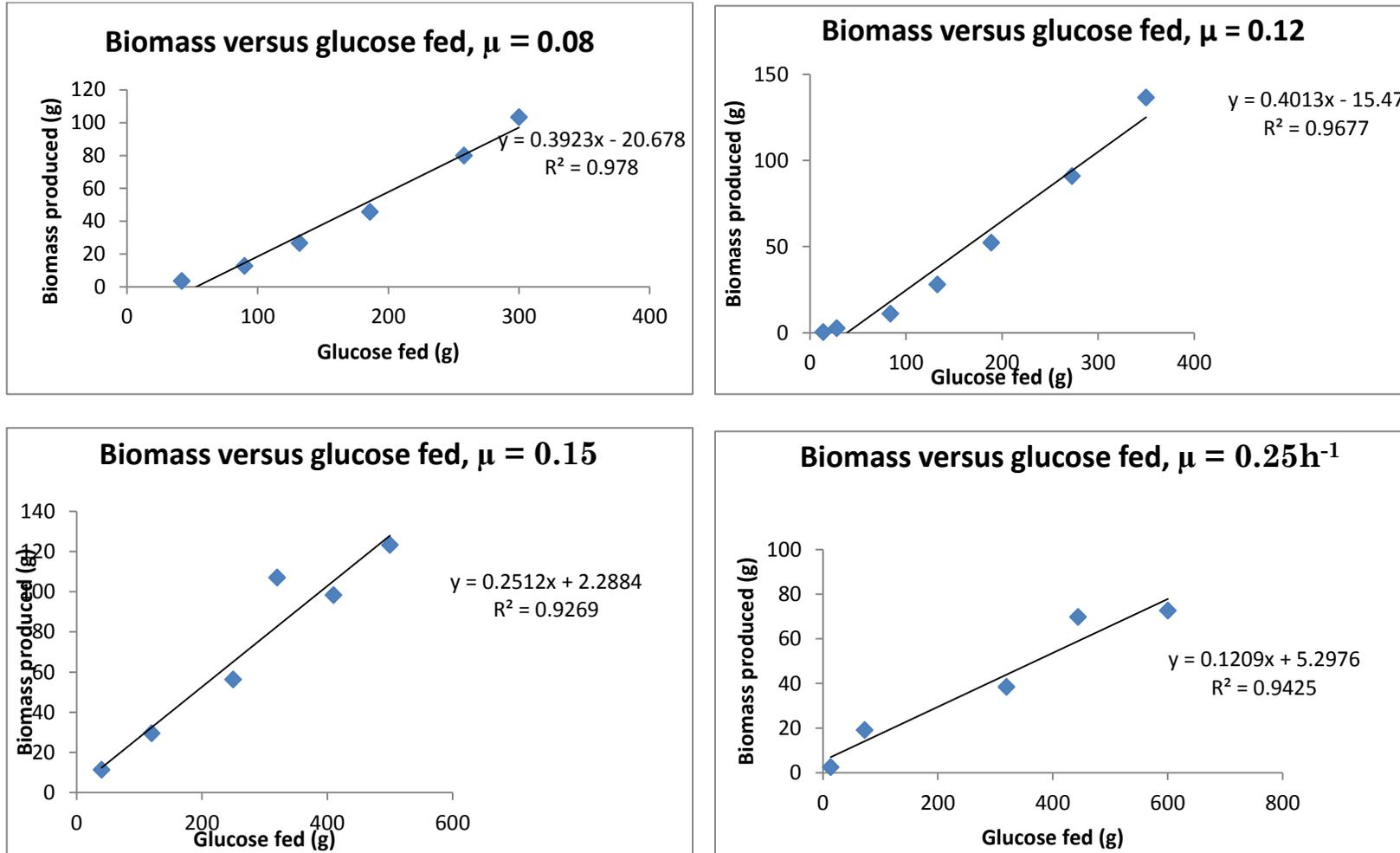
Figure AIV-1 Determination of biomass yield coefficients at different specific growth rates in *S. cerevisiae* cultivation.
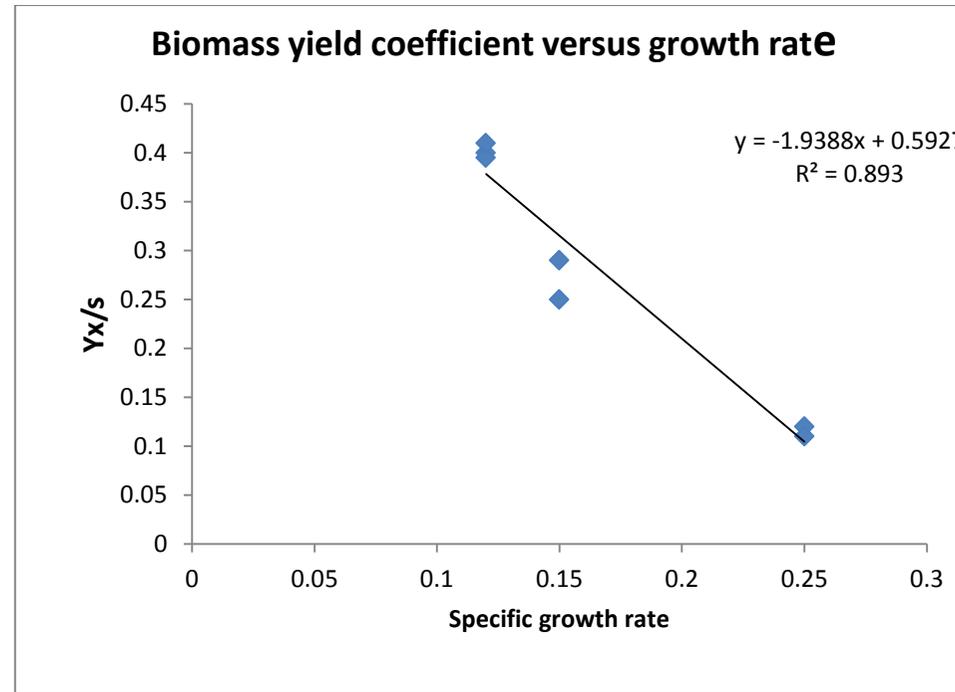
Figure AIV-2 Correlation between the biomass yield coefficient and specific growth rate in *S. cerevisiae* cultivation.

	Label	Current	Cursor	Max	Min	Eng. U...	Description
	Gas Flow, in	19.20	19.70	300.00	0.00	SLPM	Gas Flow Rate In
	% Oxygen,in	20.40	21.20	110.00	0.00	%	% Oxygen in Gas in
	% CO2 Out	2.74	2.66	50.00	0.00	%	% CO2 in gas out
	% O2 Out	16.20	15.70	25.00	0.00	%	% O2 in gas out
	Gas Flow, o...	27.10	26.80	300.00	0.00	SLPM	Gas Flowrate out
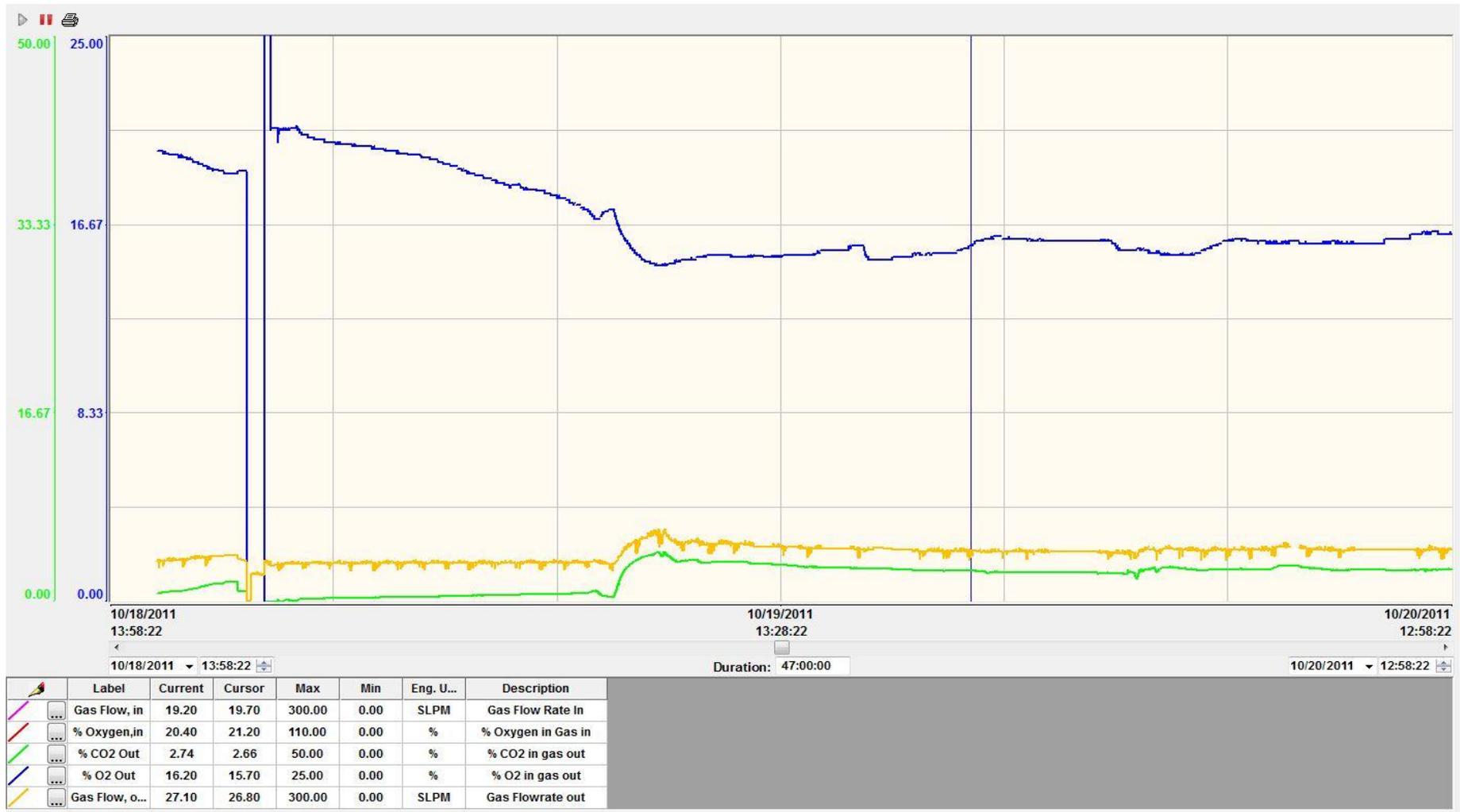
Figure AIV-3 Typical carbon dioxide profile (green curve) during fermentation of S. cerevisiae. Notable in the curve are the sudden fall indicating the end of batch phase and the sharp increase in CO_2 at the beginning of fed-batch phase.

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