Process integration in the optimisation of amidase production from recombinant *Escherichia coli*

By

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Abstract

The use of enzymes as biocatalysts in the process industries have been prominent in recent times. Biocatalysis is favoured for use due to operation at moderate energy conditions such as low temperature, pressures and moderate pH values. Besides, enzymes accept a wide range of substrates and are specific in nature, thus exhibiting regio- or stereo- selectivity. Biocatalysis typically generate less waste, thereby making it an environmentally friendly process.

For large scale production, enzymes are preferably expressed by recombinant organisms due to the properties conferred by their host cell. Such properties include enzyme stability, wide substrate specificity and high productivity. Processes for production of recombinant enzymes require both cultivation and downstream phases with combined optimisation critical to the economical production of the biocatalyst. Since the product yield is a direct function of biomass concentration, productivity is maximised by high density cell culture as well as optimised induction conditions to achieve maximum protein expression and hence optimal translation of the recombinant gene. Extracellular enzymes can then be recovered from the extracellular fluid, while intracellular enzymes are recovered from the cell lysate. Typically, the downstream operation may include up to 8 process units operations to enable solid-liquid separation, the isolation of the product, purification and polishing or formulation. All these steps impact the total achievable yield as well as the equipment, maintenance and production cost. Hence, it is necessary to purify the protein in a minimum number of steps to improve yield without compromising the degree of purity.

This thesis presents the investigation of the production of a novel thermostable amidase (EC 3.5.1.4) from *Geobacillus pallidus* RAPc8 using recombinant *E.coli* BL21 (DE3). The choice of growth medium and induction strategy were optimised under bioreactor conditions to enhance amidase productivity. Further, expanded bed adsorption (EBA) was assessed as a tool for minimising the unit operations in the amidase purification train. The EBA process can integrate up to four steps by merging processes involved in centrifugation, microfiltration and initial adsorption into one unit operation.

At the bioreactor stage, the volumetric enzyme activity and specific activity achieved are functions of the biomass concentration, protein expression, transcription and translation of the amidase gene. These were investigated in turn, initially using a batch method and subsequently a fed batch process. Bacterial cell density was studied as a function of the growth medium used. A defined medium with glucose as the carbon source and a complex Luria broth (LB) with or without glucose supplementation were used. The dry biomass on reaching stationary phase in the batch process using defined media reached 9.40 g l^{-1} compared to 1.80 g l^{-1} in LB. Further studies were therefore continued using the defined medium. Induction studies were conducted using Isopropyl thiogalactoside (IPTG). Biomass production was not suppressed by the IPTG concentration across the range 0 to 1000 µM. Induction of recombinant protein expression by 400 µM IPTG at early exponential phase of growth (5^{th} hour) gave a four-fold increase in specific amidase activity compared to induction at mid exponential phase (8^{th} hour). Protein production increased by a factor of two with IPTG addition. This was independent of IPTG concentration in the range of 40 to 1000 µM. Amidase activity increased with increasing IPTG concentration up to 400 µM. Hence, there was an increase in specific amidase activity and amidase activity per unit biomass increased with increasing IPTG concentration up to 400 µM. This optimal inducer concentration translates to 80 µmol IPTG g^{-1} cell on biomass basis. This represented a 6 fold increase over the highest value reported to date for this system. Further, results from this study showed that biomass concentration should be taken into consideration prior to choosing inducer concentration. Hence, induction was considered as biomass based. Amidase was shown to be dominantly cytoplasmic with less than 3 % amidase retained within the cell debris.

To further increase volumetric amidase concentration, the role of cell culture was investigated through fed batch studies. An exponential feeding method was developed to support increase in biomass for the fed batch process. Induction and maintenance of selection pressure for the plasmid was carried out using three different approaches: en-bloc induction at the start of feeding with a final IPTG concentration of 400 µM (100 µg ml^{-1} ampicillin added in batch and continuously), en-bloc induction at the early exponential phase in batch phase with further continuous induction at the start of feeding (final IPTG concentration of 400 µM and 100 µg ml^{-1} ampicillin added in batch and continuously) and intermittent addition of inducer over the fed batch duration culminating in a total IPTG concentration of 1.6 mM (400 µg ml^{-1} ampicillin added continuously and

intermittently). Biomass concentration increased from 9.40 g l^{-1} in the batch phase to approximately 40 g l^{-1} across all fed-batch experiments, representing a four fold increase in cell density. The initial specific growth rate at the start of feeding was maintained at 0.2 h^{-1} to prevent acetate formation and also support plasmid stability. There was a loss in population of recombinant cells as indicated by reduced plasmid stability. Less than 2% of cells contained plasmid at the end of the fed batch process. This in turn led to a large reduction in specific amidase activity across all fed batch experiments conducted. The lack of maintenance of selective pressure by the ampicillin addition was postulated to be responsible for loss of the amidase-carrying plasmid of the recombinant strain. It is likely that the ampicillin was degraded by β-lactamase, a by-product of recombinant *E. coli* cells displaying ampicillin resistance. This was confirmed using a simple agar-plate well experiment. The control sample prevented growth of the wild type *E. coli* around its well, across a radius of 10 mm, while no inhibition was observed around the wells into which supernatant of the culture medium (sample taken from 0 to 24 hrs of cultivation) was pipetted. This shows that agents within the culture medium facilitated ampicillin degradation and hence selective pressure was not maintained for the mutant strain. Thus, the fed batch process did not improve amidase expression.

In the downstream processing of amidase, expanded bed adsorption (EBA) was investigated for enzyme recovery directly from crude cell homogenate. Critical factors for efficient EBA operation investigated included choice of adsorbent, operating conditions such as pH and ionic strength and the physical characteristics of the feedstock. Recovery and purification of amidase was mediated through surface charge effects. This allowed for the use of ion exchange matrices offering advantages such as high capacities and considerable selectivity with reasonable cost. Initial studies were carried out in batch shake flask to determine the best adsorbent for amidase retention and conditions for selective adsorption of amidase over other proteins. The 80% amidase adsorption exhibited by the STREAMLINE anionic diethylaminoethyl, DEAE, was higher than the enzyme retention observed for STREAMLINE sulfopropyl (SP) adsorbent (25%). Optimal binding to the DEAE adsorbent was observed at pH 7.7 using 50 mM phosphate buffer (corresponding to an ionic strength of 15.60 mS).

Further experiments were conducted to determine the influence of particulates and biomass in the crude homogenate on protein adsorption to the DEAE matrices. Clarified

samples used in packed bed (the unit used in conventional purification train) were compared to the typical unclarified feedstock applied in EBA processes to determine the extent of interaction between contaminating biomolecules (especially cell debris) and the DEAE adsorbent. Feedstocks consisting of whole *E. coli* cells in bovine serum albumin (BSA) solution as well as partially and wholly disrupted cell suspensions in crude protein lysates were used as unclarified samples for this study. For the column, the extent of cell debris influence was characterised by assessing the bed stability, the breakthrough curve and the dynamic binding capacity. The interaction between biomass and DEAE adsorbent was minimal in the shake flask experiment as less than 2% adsorption difference was found using clarified feed over crude homogenised samples. Studies in the expanded bed column showed an increase in bed height when unclarified feedstock was used but it did not affect bed stability throughout all the experiments. However, the adsorption performance of the EBA operation was influenced by electrostatic interaction between cell debris and the anionic Streamline DEAE adsorbent under process conditions. The influence of whole cells was generally minimal on BSA adsorption. Although the sigmoid shaped biomass breakthrough profile was similar across BSA concentrations of 0.24 to 5.12 mg ml^{-1} feed, the biomass from the highest BSA concentration (5.12 mg ml^{-1} feed) broke through earlier, suggesting competition between biomass and protein. However, microscope studies showed that the influence of whole *E. coli* whole cells was minimal, as the cells barely bonded to the DEAE adsorbent. Biomass influence was pronounced when partially and completely homogenised samples were used as feedstock. The application of these feeds resulted in early protein and amidase breakthrough and a lower dynamic binding capacity compared with clarified lysates, thus indicating reduction in adsorption performance for the EBA process when unclarified feedstock were used. This was more pronounced with the completely disrupted cell homogenate. The gradual biomass breakthrough and high retention time suggests that finer cell fragments generated during homogenisation interacted more with the DEAE adsorbent. Despite the interference of biomass with protein adsorption, the EBA process has shown that amidase can be selectively captured from crude homogenate, although further studies are needed to optimise the process.

Generally, this study has provided fundamental understanding of recombinant protein expression and its subsequent recovery through process integration. This can further assist in process development and optimisation.

Declaration

I know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledge, is my own.

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Oluwafemi Ayokunle OLAOFE

Dedication

To my parents, Olorunfemi and Kikelomo Olaofe, and my dear siblings Oluwaseun, Temitope, Aderonke and Adekunle for your love, care and support throughout my studies

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Nomenclature and Abbreviations

A/Ao Ratio of biomass absorbance at the outlet as a fraction of the inlet concentration

ATP Adenosine triphosphate

BSA Bovine serum albumin

C/Co Ratio of protein concentration at the outlet as a fraction of the inlet concentration

CAP Catabolite activator proteins

CIP Cleaning in place

DBC Dynamic binding capacity

DCW Dry cell weight

DEAE Diethylaminoethyl

DNA Deoxyribonucleic acid

DNS Dinitrosalicylic acid

EBA Expanded bed adsorption

HCDC High cell density cultivation

HPLC High pressure liquid chromatography

IPTG Iso propyl thio galactoside

LB Luria broth

n Number of observations

RNA Ribonucleic acid

RTD Residence time distribution

SAB Sample application buffer

SP Sulfopropyl

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

vvm Volume of air per volume of medium per minute

w/v Weight per volume

ROMAN SYMBOLS		UNIT
C_x	Concentration of biomass	$g\ l^{-1}$
C_s	Concentration of substrate	$g\ l^{-1}$
K_d	Specific death rate constant	
K_s	Saturation constant	$mg\ l^{-1}$
q_p	Rate of product formation	
$Y_{x/s}$	Yield coefficient of biomass based on substrate	$g\ g^{-1}$

GREEK SYMBOLS

μ	Specific microbial growth rate	h^{-1}
μ_{max}	Maximum specific microbial growth rate	h^{-1}

Chapter 1

Introduction

1.1 BACKGROUND

Biocatalysis harnesses the catalytic potential of enzymes to produce building blocks and end products for the pharmaceutical and chemical industry. The use of enzymes, rather than the conventional chemical methods, has advantages such as relatively mild conditions of temperature, pH and pressure, chiral specificity of the biocatalytic activity, wide substrate acceptability and the potential for immobilisation and re-use of the biocatalyst. Moreover, these properties afford efficient reactions with few by-products, making biocatalysts environmentally friendly compared to the conventional chemical processes (Dordick *et al.*, 1998; Zhao *et al.*, 2002; Bommarus and Polizzi, 2006). The classes of enzymes and different reactions they catalyse are presented in Table 1.1. The use of enzymes as biocatalysts has shown vast potential for industrial syntheses and are used in numerous new applications such as food, agriculture, paper, leather and textile industries (Table 1.1), resulting in significant reductions in costs relative to conventional chemical processes (Beilen and Li, 2002). Biocatalysts have also been used in the production of chemicals such as acrylamide as well as in chiral drug production in pharmaceuticals (Held *et al.*, 2000). Recent advancement in recombinant DNA technologies, genomics and proteomics, through directed evolution and mutagenesis, will help enhance the potential usefulness of biocatalysts.

Recombinant micro-organisms are becoming the main source of enzymes, due to the ease of genetic manipulation and the wide variety of enzymes available from micro-organisms found in diverse and extreme environments (Lowe, 2001).

Table 1.1: Classes of enzymes, reaction catalysed and their potentials as biocatalyst

Class of Enzyme	Reaction catalysed	Potentials	Reference
Oxido-reductases	The exchange of electrons between donor and acceptor molecules e.g. hydroxylation of alkanes	Glucose oxidase is used in the food industry; Laccase to modify phenolic saccharides in fruit juice/fermented alcohol; Laccase and peroxidase used to degrade plastic waste having olefin units	Xu, 2005
Transferases	Transfer of chemical groups	Glutathione transferase catalyses the detoxification of chemical compounds such as herbicides	Sheesan *et al.*, 2001
Hydrolases	Bond cleavage and synthesis of ester and amide bonds	Used in the production of laundry detergents (lipases, proteases); Food industry (amylases, proteases, lipases, esterases)	Hasan *et al.*, 2006
Lyases	Addition and removal of groups to form double bonds	Applications range from the manufacture of chiral intermediates to recycling of chlorinated byproducts from chemical manufacturing and selective treatment of process waste streams.	Swanson, 1999
Isomerases	Intramolecular re-arrangement	Glucose isomerase is used for the manufacture of high-fructose syrups from corn; Syrups are used in soft drinks, baking etc; Isomerases are also used to racemise unwanted stereoisomers in amino acid resolutions.	Held *et al.*, 2000
Ligases	Join two substrates at the expense of ATP forming a covalent bond	Used as a tool for DNA amplification	Glick and Pasternak, 2003

The expression of protein by a recombinant host, such as *Escherichia coli*, requires that the foreign gene be inserted into a multi-copy plasmid vector under the control of a promoter. The ideal promoter is tightly regulated to minimise metabolic burdens and toxic effects while directing efficient transcription to allow high level protein production. The level of transcription of the recombinant gene can be regulated by using an appropriate level of an inducing agent to maximise yield. One of the most commonly used promoters for expression of recombinant genes in bacteria is the *lac* promoter from the *E. coli* lactose operon. It is well characterised and understood among the bacterial promoters. Moreover, induction can be performed at low temperature and a wide range of induction level is possible (Donovan *et al.*, 1996; Weickert *et al.*, 1996).

The microbial production of the enzymes requires that the recombinant cells be grown up in a bioreactor and the enzyme must then be recovered in subsequent steps. The economic optimisation of heterologous protein production, through bioprocess-based approaches for maximal volumetric productivities of recombinant proteins, include growing the host strain to high concentration using a high cell density cultivation method (HCDC) and manipulation of induction conditions for effective transcription of the recombinant gene (Kilikian *et al.*, 2000; Kim *et al.*, 2007). The choice of medium, its composition and the type of feeding strategy adopted in a fed batch process play a major role in attaining high biomass concentration and high product yield, the latter being dependent on the former (Choi *et al.*, 2006; Shiloach and Fass, 2005). For recombinant protein expression, induction conditions such as inducer concentration and time of induction (before or after accumulation of biomass) have to be considered (Lowe, 2001; Yildirim *et al.*, 2007). This will inform the best way to optimise protein production at the bioreactor stage prior to downstream processing.

The process for protein recovery varies depending on whether the enzyme is produced extracellularly or intracellularly. As shown in Figure 1.1, proteins secreted into the growth medium are easily recovered from the supernatant in a minimal number of steps, while for the recovery of intracellular proteins, the microbial cells produced at the bioreactor stage are concentrated in a solid-liquid separation process and subjected to cell disruption or permeabilisation for release of products. The soluble protein is separated from the cell debris in another solid-liquid operation, using centrifugation or filtration or a combination of both.

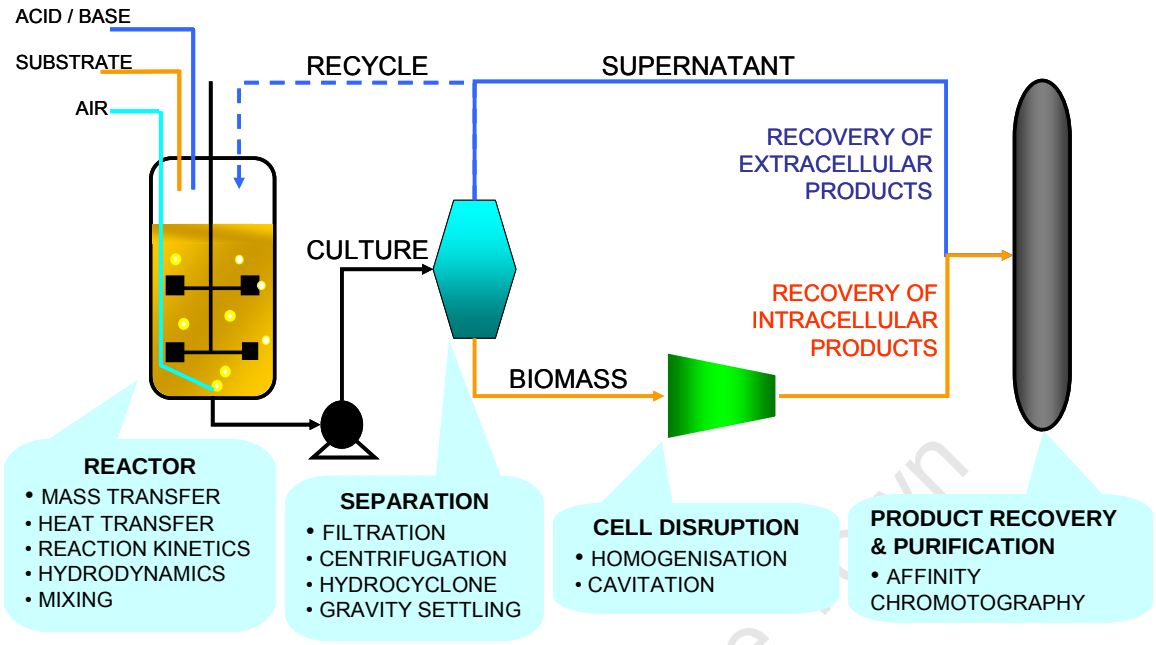


Figure 1.1: A typical bioprocess for the production of enzymes

The desired protein is separated from the crude protein mixture through a series of purification steps utilising a range of specific interactions to effect separation. The complexity of the downstream sequence is determined by both the complexity of the crude protein preparation and the required purity of the product, which is in turn dependent on the intended use of the product. Each of these steps in the recovery scheme will impact on the overall process economy by increasing operational cost and process time, and also by reducing the overall achievable yield. The influence of increasing number of steps on overall yield is illustrated in Figure 1.2. For instance, in a process train consisting of 8 steps with a yield of 90% from each step (i.e. 90% of the product entering the unit operation is found in the desired output stream) then an overall yield of less than 45% is achieved. This means that over 50% of the product produced has been lost in the process. A reduction of the number of steps to four while maintaining yield per step, would increase the output by a further 35% which would considerably impact on the product cost price. As such, the merging of unit operations is desirable to reduce the number of processing steps and hence offer an efficient way of reaching high process economy in the general production process. This helps to avoid capital expenditure and recurrent maintenance cost and time attached to a longer

processing train (Krijgsman, 1992). Further, it contributes to reduction in raw material requirements and waste materials for treatment.

This thesis investigates the bioprocess optimisation of amidase enzyme production from recombinant *E. coli* through optimising the upstream and downstream design with the aim of maximising enzyme yield. Amidases belong to the nitrile-degrading enzymes. They hydrolyse amides to free carboxylic acids and ammonia. The amidase enzyme from *Geobacillus pallidus* used in this study has been previously characterised by Cameron *et al.* (2005), Agarkar *et al.* (2006) and Makhongela *et al.* (2007). It has been shown that this enzyme is thermostable, thereby enabling operation at high temperature where high reaction rates can be achieved. The enhanced stability shown by the immobilised enzyme preparation coupled with its D-selectivity towards amide hydrolysis for production of enantiomerically pure carboxylic acids further enhances its potential for application as an industrial biocatalyst. Hence, it is desirable to optimise the production of this enzyme.

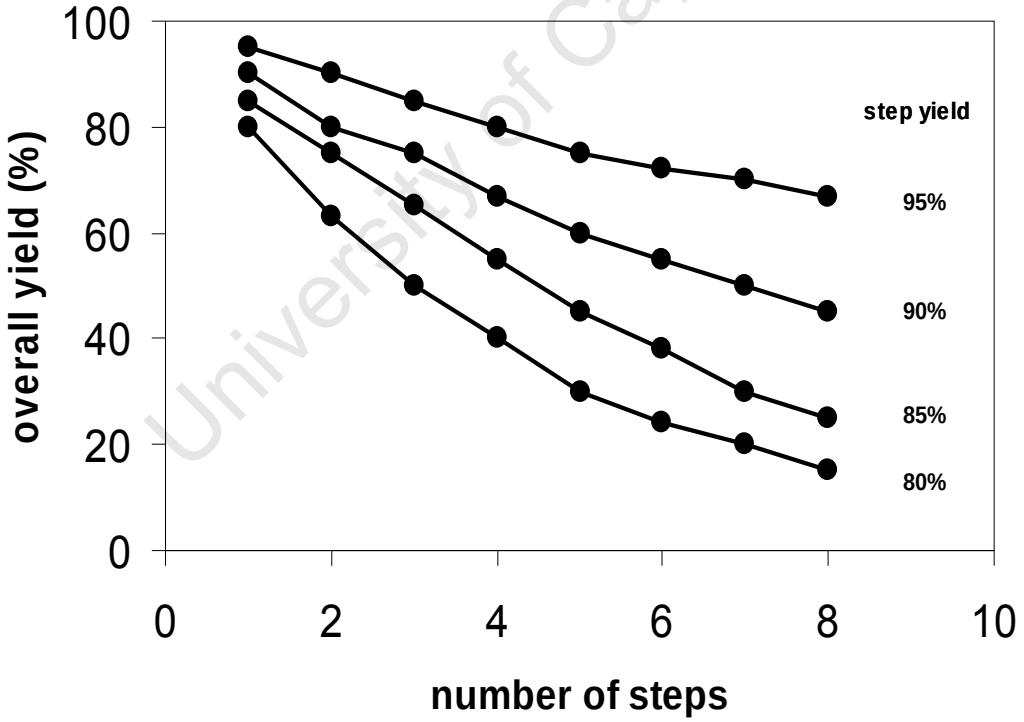


Figure 1.2: Overall yield as a function of number of process steps and yield of individual operation, (Krijgsman, 1992)

This study was conducted to enable development of an efficient route for biomass production and effective transcription and translation of the amidase gene. The use of expanded bed adsorption was proposed as a means to process cell suspensions directly from the crude lysate with a view to reducing the process steps for recovery of desired protein, such that the need for three to four processing steps (centrifugation, microfiltration, concentration and adsorption) would be incorporated into a single process operation.

In general, it was expected that bioreactor optimisation for amidase enzyme production and the subsequent recovery by integration of downstream processing steps, while maintaining or enhancing product quality, could contribute favourably toward an economical process design.

1.2 THESIS STRUCTURE

Extensive literature reviews of recombinant protein induction, high cell density cultivation and protein recovery using expanded bed adsorption as an integrated unit operation are presented in **Chapter 2**. Sections 2.1 and 2.2 provide information about the amidase enzyme, its biochemical characteristics and its current applications in various bioprocesses. Section 2.3 describes *E. coli* as the production system, and provides further information about its growth kinetics. Sections 2.4 to 2.6 describe recombinant protein expression, the motivation for use of IPTG as an inducer for the current study, and the influence of inducer concentration and time of induction in maximising protein expression. The impact of high density cell culture in increasing product yield, as well as the feeding strategies in fed batch processes, are reviewed in the subsequent Section 2.7. A typical description of downstream processing for recovery of intracellular proteins is presented in Sections 2.8 to 2.10. A general review on the recovery of proteins directly from cell lysate using expanded bed adsorption was also presented, and various factors involved in the process discussed. The concluding part of this Chapter (Sections 2.11 to 2.14) presents the motivation and objectives of this study.

A detailed description of experimental apparatus and methods used in this thesis are presented in **Chapter 3**.

In **Chapter 4,** the results from the bioreactor optimisation studies are presented and discussed. The effect of medium composition on biomass concentration is discussed. Further, results of induction studies for optimal transcription and translation of the cloned amidase gene conducted in batch and fed-batch processes are discussed. The effect of the inducer concentration was analysed on both volumetric and biomass basis with a view to concluding whether protein induction is based on volumetric or specific biomass concentration.

Results obtained from experiments involving protein purified directly from the cell lysate using expanded bed adsorption are presented and discussed in **Chapter 5**. The conditions for optimal interaction between the desired protein and adsorbent were determined and are discussed, as well as the influence of cell-adsorbent interaction on column performance and the general process.

A summary of the general conclusions of the study is presented in **Chapter 6**. These are discussed in terms of their potential application. Further, recommendations are presented for future work in this research field to enhance understanding and process efficiency.

Chapter 2

Literature Review

This literature review presents the background and previous research on optimisation of recombinant protein production and the premise on which this study is based. The biochemical properties of the amidase enzyme are stated, with particular reference to its potential as "an ideal" biocatalyst for use on an industrial scale. The maximum expression and production of this enzyme from recombinant *E. coli* depend on the efficient transcription and translation of the recombinant gene and design of an effective downstream process for its recovery. The bioprocess approaches by different researchers for optimal production of recombinant protein through induction studies and high cell density cultivation (HCDC) is presented in this review. The subsequent steps for protein recovery in the downstream processing are highlighted with a view to minimising the number of unit operations to increase product yield. The use of expanded bed adsorption (EBA) for processing protein directly from the cell lysate has been studied and proposed as a way of minimising the number of steps through integration of up to four processes. The underlying principles of this method and the requirements for a successful operation are discussed. From this literature review, the motivation and research objectives of this study is presented.

2.1 AMIDASES

2.1.1 SOURCES OF AMIDASE

Amidase (amidohydrolase; EC 3.5.1.4) catalyses the hydrolysis of carboxylic acid amides to free carboxylic acids and ammonia. Amidases exist in 3 of 21 plant families (*Gramineae, Cruciferae*, and *Musaceae*) (Banerjee *et al.*, 2002) and also in a limited number of fungal genera (*Fusarium, Aspergillus* and *Penicillium*) (Harper, 1977). Mostly, amidases have been isolated, purified and described in bacterial genera such as *Rhodococcus* (Hirrlinger *et al.*, 1996), *Geobacillus* (Cameron *et al.*, 2005),

Brevibacterium (Baek *et al.*, 2003), *Microbacterium* (Doran *et al.*, 2005) and *Micrococcus* (Fournand and Arnand, 2001).

2.1.2 AMIDASE METABOLISM

Amidases are involved in nitrogen metabolism in both prokaryotic and eukaryotic cells through amide hydrolysis (Banerjee *et al.*, 2002). Amidase classification, as reviewed by Fournand and Arnand (2001), showed that bacterial aliphatic amidases fall into two groups. The first type includes aliphatic amidases that hydrolyse only short chain aliphatic amides. The second type are aliphatic amidases that hydrolyse mid-chain length amides. These are usually coupled with nitrile hydratase in microbial nitrile metabolism. Both classes of amidases exhibit an acyl transfer activity leading to the formation of hydroxamic acids (Fournand and Arnand, 2001). Further, amidases exhibit wide substrate specificities (Table 2.1). Some amidases catalyse the hydrolysis of aliphatic amides (Asano *et al.*, 1982; Kotlova *et al.*, 1999), while some hydrolyse amides of aromatic acids (Hirrlinger *et al.*, 1996; Trott *et al.*, 2002) and yet others act on amides of alpha-or-omega-amino acids. Cheong and Oriel (2000) regarded some as wide spectrum amidases.

Hydrolysis catalysed by nitrile degrading enzymes is the most common pathway for microbial metabolism of nitriles (Banerjee *et al.*, 2002; Cowan *et al.*, 1998). Various enzymes are responsible for nitrile metabolism in micro-organisms. In Figure 2.1, nitrile hydrolysis is shown to occur via two routes: In the first route, nitrilase catalyses conversion of organic nitriles to corresponding acids and ammonia. In the second two-step route, nitrile hydratase hydrolyses nitriles to form amides, which are subsequently converted to acids and ammonia by amidases. The reaction mechanism for the amidase conversion reaction, which also involves nitrile hydrolysis as proposed by Banerjee *et al.* (2002), shows that the carbonyl group of the amide undergoes nucleophilic attack, which results in the formation of a tetrahedral intermediate (Figure 2.2). This, in turn, is converted to an acyl-enzyme with the removal of ammonia and subsequent hydrolysis to the carboxylic acid.

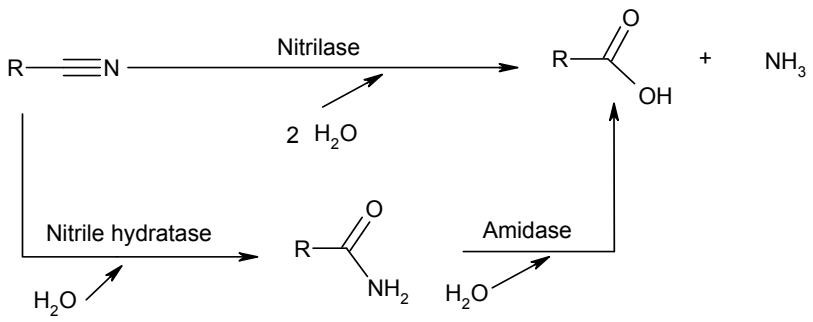


Figure 2.1: Biochemical pathway for enzymatic nitrile hydrolysis (Cowan *et al.*, 1998)

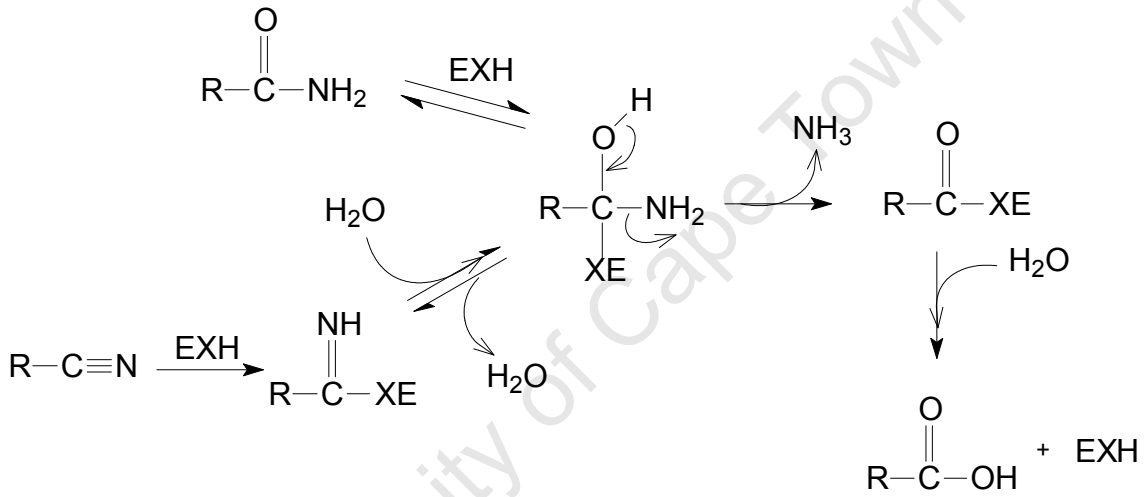


Figure 2.2: Mechanism of catalysis by amidases, redrawn from Banerjee *et al.* (2002).

2.1.3 PROPERTIES OF AMIDASES

Enzyme properties such as charge, molecular weight, isoelectric point, optimal reaction temperature and pH at which the enzymes are stable, are important for design of protein purification in the downstream processes (Hatti-Kaul and Mattiasson, 2001). Table 2.1 is a summarised list of the characteristics of amidases isolated from different micro-organisms. These enzymes exhibit varied substrate specificities, ranging from aliphatic and aromatic amides, to wide spectrum specificity. Further, the biochemical properties of amidase such as its molecular weight, charge and hydrophobicity have been exploited for its purification (Table 2.2). Often, the purification train (following liberation and

preceding formulation) consists of three steps, though the purity and the intended use of the product are major determining factors.

The display of D-selectivity by some amidases is an added advantage due to the potential to exploit this property for production of optically pure chiral compounds. Moreover, selective hydrolysis of D-enantiomers is preferred as this results in metabolically stable and enhanced products (Baek *et al.*, 2003). Examples include the cleavage of D-peptides and enantioselective synthesis of D-amino acids from the DL amino acid racemic mixtures.

Some amidases are reported to have thermostable properties. This is a major benefit as thermostable enzymes exhibit high stability with respect to heat, organic solvents, pH and chemical denaturants (Baek *et al.*, 2003). Specifically, Egorova *et al.* (2004), Baek *et al.* (2003) and Makhongela *et al.* (2007) have reported thermostable amidase active between 40 to 80ºC and pH 7 to 10 from the thermophilic actinomycetes *Pseudonocardia thermophila* and the bacteria *Brevibacillus borstelensis* BCS-1 and *Geobacillus pallidus* respectively. Biocatalyst stability is a major factor influencing productivity in enzyme biotransformation both in small and industrial scale operations (Cabral, 2001). Benefits of operating at high temperature include high reaction rates, inhibition of other potentially contaminating micro-organisms and enhanced solubility of the substrate so that higher product yield (Baek *et al.*, 2003; Egorova *et al.*, 2004). The thermostable properties have also been utilised for amidase purification using heat treatment (Baek *et al.*, 2003; Egorova *et al.*, 2004; Makhongela *et al.*, 2007) (Table 2.2). Findings reported by Makhongela *et al*. (2007) showed that a selection of other proteins in the crude extract precipitated out at 55ºC, resulting in a four-fold purification of amidase.

Further to selection of a heat-stable amidase for use as an industrial biocatalyst, enhanced enzyme stability can be conferred using immobilisation. Makhongela *et al.* (2007) reported improved *G. pallidus* amidase stability with an immobilised preparation showing optimal activity in a broadened pH range, with stability enhanced by 67% at 80ºC. Also, immobilisation can reduce process cost because it allows enzyme re-use.

Table 2.1: Characteristics of amidase from different micro-organisms

Micro-organism and recombinant system	Molecular weight (kDa), No of subunits	Optimum		Stability		Comments	Reference
		Temp. (°C)	pH	Temp. (°C)	pH		
Rhodococcus sp.	44.5, 8	40	8.5		5.5-9	pI=4.0, aliphatic amide substrate	Nawaz *et al.*, 1994
Rhodococcus erythropolis MP50	61 , 8	55	7.5	30-60	6 - 9	Aromatic and aliphatic amide substrate	Hirrlinger *et al.,* 1996
Microbacterium sp. *AJ115, E. coli*	54.7,	50				Displayed enantioselectivity for S enantiomer	Doran *et al.*, 2005
Brevibacillus borstelensis BCS-1, *E. coli*	30, 6	85	9	70	7 -10	Thermostable amidase, D selectivity towards aromatic, aliphatic& branched amino acids	Baek *et al.*, 2003
Klebsiella pneumoniae	62, 1	65	7.0	30-65	5 - 8	Thermostable amidase, aliphatic amide substrate,	Nawaz *et al.*, 1996
Geobacillus pallidus, E. coli	38, 6	50	7.0	50-70	6 - 8	Thermostability enhanced by immobilisation, hydrolyses low molecular weight aliphatic amides, D-selectivity towards lactamide	Makhongela *et al.*, 2007
Rhodococcus rhodochrous M8	42, 4	55-60	7.6		5 - 8	Aliphatic amide substrate	Kotlova *et al.*, 1999
Pseudonocardia thermophila	52, 2	60	7.0	40-80	5 - 9	pI = 4.2, aromatic, aliphatic and amino acid amides	Egorova *et al.*, 2004
Bacillus stearothermophilus BR388, *E. coli*	(-), 2	55	7.0			Wide spectrum amidase, 23 fold increase in mutant strain compared with wild type	Cheong and Oriel, 2000
Rhodococcus erythropolis MP50, *E. coli*	63,(-)					Aromatic amide substrate	Trott *et al.*, 2002
Rhodococcus sp. R312, *E. coli*			7-8			Linear aliphatic amides substrate, production of wide range of hydroxamic acids	Fournand *et al.*, 1998
Brevibacterium sp., *E. coli*	46, 2					Aryloxypropionamides as substrates	Mayaux *et al.*, 1990

Table 2.2: Amidase purification methods and percentage recovery

Method of Purification	Comment	References
A single step from the crude lysate using the calmodulin-binding peptide affinity tag	Recovery: 28.5% Purification: 3.2 fold	Doran *et al*., 2005
Ion exchange and hydrophobic chromatography	Recovery: 20% Purification: 6 fold	Hirrlinger *et al.*, 1996
Acetone precipitation, ammonium sulphate fractionation, ion exchange and hydrophobic chromatography	Recovery: 20% Purification: 138 fold	Nawaz *et al.*, 1994
Hydrophobic chromatography, anionic exchange and gel filtration	Recovery: 26% Purification: 48 fold	Egorova *et al.*, 2004
Heat treatment, ion exchange and hydrophobic interaction	Recovery: 25.4% Purification: 5.5 fold	Baek *et al.*, 2003
Heat treatment, hydrophobic interaction chromatography and anion exchange chromatography	98% homogenous enzyme	Agarkar *et al.*, 2006
Heat treatment and gel permeation chromatography	Purification: 6 fold	Makhongela *et al.*, 2007

Bioprocess operation is dependent on the water solubility of the reactants and products which, in turn is a function of medium pH and temperature (Burton *et al.*, 2002). Hence, the properties exhibited by the free and immobilised preparation of the amidase enzyme (stability at broad pH and temperature range and wide substrate acceptability) show its potentials as an “ideal” biocatalyst in large scale bioprocesses.

2.1.4 APPLICATIONS OF AMIDASE

The use of chemical methods for the conversion of nitriles to amides and acids has several disadvantages including extreme pH conditions, high reaction temperature and the formation of toxic by-products and a large quantity of salts (Cowan *et al.*, 1998; Banerjee *et al.*, 2002). The bio-conversion of nitrile (Figure 2.1) has advantages such as its potential for carrying out chemo-, regio-, and enantio-selective transformation (Fournand and Arnand, 2001). Several important organic acids, such as *p*-aminobenzoic acid, acrylic acid, nicotinic acid, pyrazinoic acid and 3-indole acetic acid, have been produced commercially through biotransformations of corresponding nitriles, using amidase activity in microbial cells (Banerjee *et al.*, 2002; Fournand and Arnand, 2001). Superficial nitrile groups on acrylic fibres were converted to the corresponding amides by *Rhodococcus rhodochrous* NCIMB 11216 containing amidase activity (Tauber *et al.*, 2000).

Amidases can catalyse stereoselective transformations to produce important optically active compounds. Thus, the stereoselectivity of amidases are used in the production of antibiotics (Robas *et al.*, 1992) and other optically active compounds that are useful in the chemical and pharmaceutical industries (Mylerova and Martinkova, 2003). Penicillin G amidase has been widely used on an industrial scale for the production of penicillin G derivatives used in therapeutics (Robas *et al.*, 1992). This amidase catalyses the hydrolysis of penicillin G to phenylacetic acid and 6–aminopenicillanic acid (6–APA), the precursor for the synthesis of many β-lactam antibiotics.

Bacteria containing amidase activity have been used in bioremediation, waste treatment and the production of herbicide–resistant plants. Bacteria containing nitrile-hydrolysing enzymes degrade the effluents containing nitrile compounds, removing toxic waste. For example, bromoxynil-degrading soil bacterium, *Agrobacterium radiobacter*, was used for the degradation of herbicide, bromoxynil (Muller and Gabriel, 1999), and immobilised cells of *Rhodococcus erythropolis* were used to treat waste effluents containing propionitrile from synthetic fibre production. The propionitrile was converted to ammonium propionate, which can serve as a feed supplement (Mylerova and Martinkova, 2003).

Considering the potential of amidase enzymes highlighted above, a thorough knowledge on means of producing this enzyme optimally on a process scale will be useful.

2.2 MICROBIAL PRODUCTION OF AMIDASES

The use of wild type organisms for production of enzymes may increase difficulty in scaling up to improve yield, and in addition, some wild strains are not generally regarded as safe. In these cases, a recombinant system for production of enzymes is favoured. Further, a greater range of enzymes with varying properties may be produced (Lowe, 2001). Other advantages include the use of safe host organisms, reduced content of foreign proteins, economically feasible replacement of chemical procedures, waste streams with less toxic components, and environmentally cleaner enzymatic solutions.

Amidases have mostly been produced by recombinant *E. coli*, cloned with a vector carrying antibiotic resistant marker genes (Table 2.1) (Baek *et al.*, 2003; Doran *et al.*, 2005; Fournand *et al.*, 1998; Makhongela *et al.*, 2007; Mayaux *et al.*, 1990; Trott *et al.*, 2002). This is due to difficulty in culturing the wild strains from which amidases originate (Makhongela, 2006), the narrow substrate acceptability displayed by the wild type enzyme (Martins *et al.*, 2006) and increased activity expressed by the mutant strains (Cheong and Oriel, 2000).

E. coli has been described as the most common bacterial host used in recombinant DNA technology due to its well characterised molecular genetics, physiology and expression systems. It has a rapid metabolic rate, can grow to high density using inexpensive media and has the ability to over-express enzymes (Shiloach and Fass, 2005; Choi *et al.*, 2006). The specific growth rate of *E. coli* has been described generally by the Monod model of kinetics. According to Malthus, the rate of biomass production is proportional to biomass where the specific growth rate provides the proportionality constant.

As such, it follows first order kinetics. Mathematically, this can be expressed as

$$\frac{dC_x}{dt} = \mu C_x \tag{2.1}$$

where C_x is the concentration of biomass, t is the time and μ is the specific growth rate for the biomass. This has been shown to be dependent on the rate limiting substrate concentration as expressed by the Monod equation (Equation 2.2) (Bailey and Ollis, 1986).

$$\mu = \frac{\mu_{\max} C_s}{K_s + C_s} \tag{2.2}$$

where $\mu_{\max}$ is maximum specific growth rate (h^{-1}) of the microorganism; C_s and K are the concentration (mg l^{-1}) and half-saturation constant (mg l^{-1}) of the rate-limiting substrate respectively. The rate limiting substrate is often a carbon or nitrogen source, although in some cases, it is oxygen (Doran, 1995). At limiting substrate concentration, μ is directly proportional to C_s, resulting in first-order reaction kinetics. At high C_s, μ becomes independent of the substrate concentration, resulting in zero-order kinetics.

Where death of *E. coli* is significant, the net growth rate can be described by Equation 2.3

$$\frac{dCx}{dt} = \mu Cx - k_d Cx \tag{2.3}$$

where k_d is the specific death rate constant.

The production of intracellular protein from recombinant organisms is an example of a growth-associated product, that is, the enzyme is produced simultaneously with microbial growth under all growth conditions. The specific rate of product formation is proportional to the specific rate of growth (Equation 2.4)

$$q_p = \frac{1}{C_x}\frac{dP}{dt} = Y_{P/C_x}\,\mu \tag{2.4}$$

where q_p is the rate of product formation and Y_{P/C_x} is yield of coefficient of product based on biomass.

Various growth rates have been reported for *E. coli* based on the type of system (mutant or recombinant) and the substrate providing the carbon source. Typically, as shown in Table 2.3, the growth rate for a wild-type *E. coli* strain ranges between 0.80 and 1.1 h^{-1}, while values in the range 0.30 to 0.80 h^{-1} have been reported for recombinant systems. Although the recombinant strain can exhibit a similar growth rate to the plasmid-free wild type, the reduced specific growth rates mostly observed for recombinant systems have been postulated to be due to the extra load, or metabolic burden that arises from plasmid maintenance (Durany *et al.*, 2004; Nayak and Vyas, 1999). Thus, the plasmid-host relationship is a major factor in determining the cell growth rate.

Table 2.3: Kinetic parameters reported for *E. coli*

Carbon source (rate limiting substrate)	μ (h^{-1})	K_s (mg l^{-1})	Comments and Reference
Glucose	1.10	3	Wild type *E. coli*
Glycerol	0.87	2	Blanch and Clarke,
Lactose	0.80	20	1996
Glucose	0.99 – 1.10	4.0	Wild type *E. coli*
Lactose		20	Doran, 1995
Glucose	0.60		Recombinant strain Durany *et al.*, 2004
Glucose	0.54		Recombinant strain Kilikian *et al.*, 2000
Glycerol	0.30		Recombinant strain Lutomski *et al.*, 2004
Glucose	0.65		Recombinant strain Hu *et al.*, 2004
Glucose	0.80		Recombinant strain Vidal *et al.*, 2005

2.3 RECOMBINANT PROTEIN INDUCTION

2.3.1 CHOICE OF PROMOTER

Heterologous production of protein may be used to augment expression levels of the desired product (Walsh, 2002). Protein expression in a recombinant system involves the insertion of the foreign gene encoding the protein of choice into a multi-copy plasmid vector under the transcription control of either a constitutive promoter or a strongly regulated promoter. Recombinant *E. coli* strains have been used for the expression of an extensive range of commercially important proteins such as penicillin acylase, human insulin, human growth hormone, human Interleukin and Interferon-α (Spiridonova *et al.*, 2007; Xu *et al.*, 2006; Walsh, 2002; Lim *et al.*, 2000).

In a constitutive promoter system, the target protein is continually expressed thereby leading to the over-production of the foreign protein. Continuous high level expression often leads to a metabolic burden on the cell's energy resources, resulting in a reduced growth rate or inhibition in cell growth (Donovan *et al.*, 1996). A reduction in cell growth rate will lead to a reduced yield of the target protein, since the overall protein yield is dependent on the cell yield and yield of product per cell (Equation 2.4). Therefore, a promoter system which can be regulated is preferred, since this offers the facility to trigger recombinant gene expression by varying environmental factors such as temperature (Xu *et al.*, 2006; Schmidt *et al.*, 1999; Seeger *et al.*, 1995), dissolved oxygen tension (Donovan *et al.*, 1996), pH (Chou *et al.*, 1996) or the concentration of a constituent in the growth medium (Lee *et al.*, 1997). The choice of regulatable promoter in a recombinant system is based on a variety of criteria such as strength and control, a low basal expression level (i.e., it is tightly regulated), ease of utilisation, cost effectiveness and the conditions under which the promoter is to be used (e.g., temperature and choice of host strains or medium) (Terpe, 2006). The *lac* promoter from the *E. coli* lactose operon is favoured for recombinant gene expression in bacteria as it is well understood and characterised at the molecular level (Donovan *et al.*, 1996; Terpe, 2006).

2.3.2 REGULATION OF THE *LAC* PROMOTER IN *E. COLI*

Lactose is a disaccharide that consists of a single unit of glucose and galactose each. Prior to utilisation by the cells, lactose is transported from the extracellular surroundings

into the cell and then broken down to glucose and galactose by hydrolysis. These reactions are catalysed by three enzymes. Lactose permease transports lactose across the cytoplasmic membrane into the cell, β-galactosidase is involved in breaking down lactose to glucose and galactose, and β-galactoside transacetylase plays a secondary role in the hydrolysis reaction and may also function to detoxify lactose analogs which are harmful to the cells (Brown, 1989). Each of these enzymes is encoded by a gene, and together these genes form an operon. The lactose (*lac*) operon in *E. coli* consists of three structural genes; *lac*Z (β-galactosidase), *lac*Y (permease) and *lac*A (transacetylase) (Figure 2.3).

The expression of the structural genes is controlled by a regulatory gene called *lac*I. Transcription from the *lac* promoter is regulated by the *lac* repressor, the product of the *lac*I gene. In the absence of an inducer, lactose or IPTG, transcription from the *lac* promoter is inhibited by the *lac* repressor which binds to the operator region of the *lac* operon. The presence of the bound repressor protein to the operator prevents the proper binding of RNA polymerase to the promoter region, and transcription of the *lac* genes cannot occur. However, the operator site is not always occupied by the *lac* repressor since equilibrium exists between bound and unbound repressor molecules. Thus, there is a low basal level of transcription of the *lac* genes.

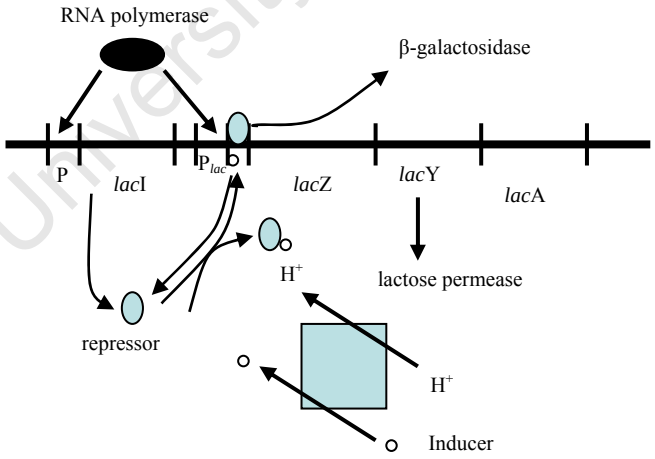
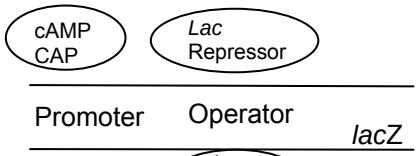
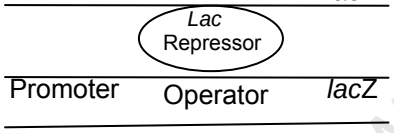
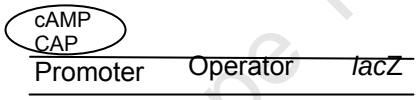


Figure 2.3: Schematic diagram of the *lac* operon and its regulatory gene, adapted from Laffend and Shular (1993)

Transcription of the *lac* operon can increase by 1000 to 5000 fold with the presence of an inducer (lactose or galactosides, e.g. IPTG) (Brown, 1989; Donovan *et al.*, 1996). A certain amount of the lactose is converted to allolactose, an intermediate compound, by the β-galactosidase present at basal levels. The lactose repressor has two binding sites that enable it to bind to the operator and also a molecule of allolactose at the same time. This repressor-inducer complex causes a conformational change in the repressor protein, thereby reducing its affinity for the *lac* operator, thus shifting the equilibrium. Increased transcription of the *lac* genes occurs with no repressor bound at the operator region and expression is induced. These interactions are illustrated in Figure 2.3. The repressors can only bind to the operator again if the lactose or inducer is exhausted (Laffend and Shular, 1993). A decrease in free lactose concentration affects the repressor-inducer complex equilibrium. This results in free repressor molecules with restored conformation and binds to the operator once again, so that the *lac* operon is "switched off".

The expression of the *lac* operon is also affected by the presence of glucose (Brown, 1989; Donovan *et al.*, 1996). If the cell has an adequate concentration of glucose (one of the monomers of lactose) for its energy requirements, it will not utilise lactose, even if the latter is present in the medium. Hence, a system is required whereby glucose can reverse the lactose system and keep the operon switched off when necessary. This process is called catabolite repression. It involves a second regulatory protein called the catabolite activator protein (CAP). Transcription from the *lac* promoter is also regulated by the binding of CAP to the promoter region. The affinity of the promoter for RNA polymerase is increased when CAP binds to the promoter, thereby increasing transcription of the *lac* genes. The association of CAP with cyclic adenosine monophosphate (cAMP), a modified nucleotide derived from ATP by a reaction catalysed by adenyl cyclase, enhances its affinity for the promoter. The presence of glucose inhibits the activity of adenyl cyclase. Thus, a high cellular level of cAMP is only possible when the glucose concentration is low. When the operator is free from the repressor at low glucose concentration, high intracellular concentrations of cAMP lead to high levels of cAMP-CAP complexes at the promoter site and cause a high level of transcription of the *lac* genes. This is schematically presented in Table 2.4. The use of these two mechanisms, CAP and the *lac* repressor, allows for control of the extent of transcription of the *lac* operon for optimal gene expression.

Table 2.4: Effect of glucose and lactose concentration in the growth medium on the level of transcription from the *lac* promoter in *E. coli*, adapted from Donovan *et al.* (1996)

Concentration of			Lac Promoter-Operator Region	Level of Transcription
Glucose	Lactose	cAMP		
Low	Low	High		Low
High	Low	Low		Low
High	High	Low		Low
Low	High	High		High
				

2.3.2 LACTOSE AND IPTG AS AN INDUCER FOR THE *LAC* PROMOTER

IPTG is a non-metabolisable galactose analog that induces the expression of the *lac* operon in *E. coli* by binding to the *lacI* repressor and altering its conformation. This inactivation prevents the repression of the β-galactosidase encoded by the *lacZ* gene. Although lactose is the natural inducer of the *lac* operon, IPTG has been used as an alternative at small scale due to certain advantages, namely that it is not metabolised by the cells and not subject to inducer exclusion by glucose (Weng *et al.*, 2006; Menzella *et al.*, 2003; Kilikian *et al.*, 2000; Donovan *et al.*, 1996). Determining optimum induction conditions using lactose is complicated by catabolite repression which gives preference for glucose metabolism over lactose. Further, the use of lactose as the sole carbon source is often discouraged due to low growth rate in comparison to glucose and glycerol (Duan *et al.*, 2000). Some strains of *E. coli* do not possess β-galactosidase activity to break down lactose to allolactose. Such cells cannot utilise lactose as an inducer or a substrate, and this affects the induction of recombinant gene expression. In contrast, IPTG binds directly to the *lac* repressor without modification. These conditions make IPTG preferable for research on a small scale process.

2.3.3 OPTIMISATION OF RECOMBINANT GENE EXPRESSION IN *E. COLI* USING THE *LAC* PROMOTER

The economical feasibility of using heterologous protein production depends on the attainment of high level protein expression in the recombinant organisms. Levels of recombinant gene expression in *E. coli* are system-specific, depending on the biochemical properties of the recombinant protein, the gene induction mechanism, host strain and cultivation conditions (Lee *et al.*, 1997). These factors have been studied extensively with the aim of optimising protein expression through molecular methods or bioprocess strategies. Bioprocess-based approaches for maximal transcription and translation of recombinant proteins involve optimisation of cultivation conditions with attention to medium composition, and high cell density cultivation, optimising gene expression by manipulation of inducer concentration, time and duration of induction (Zhou *et al.*, 2004; Anderson and Krummen, 2002; Kilikian *et al.*, 2000; Kim and Oriel, 2000; Lee *et al.*, 1997; Weickert *et al.*, 1996; Hahm *et al.*, 1995).

2.4 THE EFFECT OF IPTG CONCENTRATION IN RECOMBINANT GENE EXPRESSION

Continuous high level protein expression after induction may cause a reduction in cell growth rate or inhibit cell growth completely due to changes in the cell's metabolic flux from cell growth to protein production (Donovan *et al.*, 1996, Kilikian *et al.*, 2000). This physiological change caused by high levels of protein expression has been described as metabolic load, metabolic stress or metabolic burden (Zheng *et al.*, 2005; Hahm *et al.*, 1995). To maximise recombinant protein production, it is necessary to optimise cultivation strategies, genetic manipulations and gene expression to ensure a process that is economically feasible.

Approaches that have been used for optimal production of recombinant protein include maximising biomass concentration and optimising specific activity of the target enzyme using the appropriate induction method. In most instances, induction at early exponential phase of cell cultivation leads to low specific growth rate or volumetric productivity due to low final biomass concentration, extracellular inducer concentration and the resultant cell metabolic stress. In contrast, induction at late exponential phase with a high biomass concentration leads to low specific productivity due to reduced cell activity (Bentley *et al.*, 1991; Miao and Kompala, 1992). This leads most researchers to choose the phase of

growth to induce protein expression using the appropriate inducer concentration, so as to balance increasing product concentration in the cell and decreasing biomass concentration or growth rate after induction (Donovan *et al.*, 1996; Lee *et al.*, 1997; Vidal *et al.*, 2005; Xu *et al.*, 2007).

Across recombinant systems, a range of inducer concentrations have been used without a clear motivation for such. A wide range of IPTG concentrations (0 to 10 mM) has been reported in the literature (Table 2.5). The optimal IPTG concentration for *lac* derived promoters for the expression of soluble cytoplasmic proteins has been widely reported to be around 1 mM (Donovan *et al.*, 1996), although this varies in recombinant systems. Fournand *et al.* (1998) reported that amidase activity was up to 30% of the total soluble protein using no inducer. Doran *et al.* (2005) showed that amidase activity did not vary using IPTG in the range of 0.1 to 2 mM. Other authors (presented in Table 2.2) have reported maximum protein production using IPTG in the range of 0.06 to 2 mM. Hence, Sivakesava *et al.* (1999) concluded that soluble expression of the target protein using varied inducer concentration is dependent on the protein characteristics, host cell and the culture conditions. This was supported by Durany *et al.* (2004) and Grabherr *et al.* (2002).

A typical case was presented by Bentley *et al.* (1991) in studying the dynamics of chemically induced chloramphenicol-acetyl-transferase (CAT) expression in a batch cultivation of *E. coli*. The two competing factors in maximum productivity of the CAT protein were balanced; increased rate of protein synthesis in cell after induction and its inhibitory effect on cell growth. Weak induction of the cloned gene was suspected at IPTG concentrations below 1.0 mM while higher IPTG concentrations (> 1.0 mM) suppressed cell growth. Hence, a moderate IPTG concentration of about 1.0 mM was required to balance cell productivity and growth. Beyond balancing the two major factors, the level of inducer concentration required for optimal expression of heterologous protein is also dependent on the soluble expression and the biochemical characteristics of the recombinant protein, mechanism of gene expression (Wood and Peretti, 1991), and host strains (Chou *et al.*, 1996; Xu *et al.*, 2006; Mattanovich *et al.*, 1997; Jeong *et al.*, 2004).

Table 2.5**:** Production of recombinant proteins by high cell density culture of *E. coli* using IPTG inducer

Host system (*E. coli)*	Products	Inducer, concentration used	Culture condition (OD and time of induction)	Biomass (DCW) g/L and activity yield	Comments	Reference
BL21(DE3)pLysS (*Lac* promoter)	Amidase	IPTG 2 mM	OD_{600}= 1.0,	n/a, 0.4 µmol/min/ml/	Induction level were same from 0.1–2 mM IPTG	Doran *et al.*, 2005
BL21(DE3) (*Lac* promoter)	Amidase	No inducer	n/a	OD_{600} 30% of soluble proteins	Inducer gave no significant effect	Fournand *et al.*, 1998
M15	Glutamyltranspeptidase	IPTG, 10 mM	Varied temp., inducer conc. and induction time	n/a, 0.12 U/mg	Maximum expression was at 20 ºC, 0.1 mM IPTG & 12-h induction.	Yao *et al.*, 2006
BL21(DE3)	factor -2 (IGF-2)	IPTG, 0.06mM	OD_{600}= A).140(9hrs), B).210(12hrs), C).310(18hrs)	A). 92, 15% B). 125, 13% C). 156, 11.3%	No large variation of IGF-2 when IPTG was induced at different cell concentration.	Hu *et al.*, 2004
M15(pREP4)	Human galectin-1	IPTG, 2mM	OD_{600}=30, exponential phase	28, 450mg/L(purified)	Induction at late exponential phase	Lutomski *et al.*, 2004
K207-3/pKOS207-129/pBP130	6-deoxyerythronolide B (dEB)	IPTG 0.1mM	OD_{600}=52, at 28-30h	30 , 1.1g/L	Late induction	Lau *et al.*, 2004
BLR(DE3)	L-asparaginase 2	IPTG, 0.1mM	OD_{600}= A). 0.6 B). 2.0 C). 4.5	A).1315UI B).1600UI C).2095UI	Late induction gave a higher volumetric activity	Khushoo *et al.*, 2004
JM101(*Lac*UVpar8EGF) JM101(pWKW2)	Human epidermal growth factor (hEGF)	IPTG 0.2mM 0.1mM	OD_{550}= A). 4.0 B). 8.0 C).12.0	Not available	Maximum hEGF production was observed at mid log. IPTG conc. dependent on protein characteristics, host cell and culture conditions	Sivakesava *et al.*, 1999
TG1-pLACFGFB	Basic fibroblast growth factor (bFGF)	IPTG 1mM	Induction at DCW of 41g/L	Final DCW of 135 and bFBF conc. of 1.1g/L		Seeger *et al.*, 1995

In other investigations on recombinant protein expression, the significance of host cell and growth medium constituents have been reported. Xu *et al*. (2006) characterised the T7 promoter system (a *lac* derivative) for the expression of penicillin acylase (PAC) in *E. coli*. PAC expression was strongly induced by IPTG in *E. coli* JM109 (DE3) as the host. Most of the gene products were produced in the insoluble form and cell growth was severely inhibited, leading to cell lysis, with most of the PAC activity detected extracellularly. In contrast, over expression using recombinant *E. coli* BL21 (DE3) at an IPTG concentration of 0.5 mM did not inhibit growth or cause cell lysis. A similar study by Yildirim *et al.* (2007) shows that biomass (*E. coli*) concentration was affected at an IPTG concentration of 0.5 mM, and severely affected beyond this concentration.

In studying the influence of growth medium on the expression of recombinant fuculose-1-phosphatealdolase in *E. coli*, Durany *et al.* (2004) used IPTG concentrations up to 500 μM for maximum expression of the recombinant protein. For the four different growth media investigated, increasing the IPTG concentration beyond 100 μM did not increase aldolase activity. The authors observed that IPTG concentration of the range 50 to 500 μM did not affect cell growth. The same maximum specific growth rate was reported for the recombinant strain cultures and the plasmid free wild type strain, showing that the maintenance of the antibiotic-selection plasmid and over-expression of the target protein did not have any significant effect on the growth profile of the cells.

To understand the limitations to increased levels of production of the cloned gene, β-galactosidase, under the *tac* promoter system, Wood and Peretti (1991) studied the translation and transcription machinery using *E. coli* cells under steady state response. IPTG at a concentration range of 0.0 to 7.5 mM was used. Increases in the synthesis rate of β-galactosidase mRNA and enzyme activity were proportional to the IPTG concentration up to 1.0 mM, such that a 100 fold increase in IPTG concentration resulted in a 102–fold increase in enzyme activity per cell. This linear trend did not hold on further increasing the IPTG concentrations from 1.0 to 7.5 mM, as a 7.5 fold increase in IPTG concentration only resulted in a 23% increase in enzyme activity. The complete titration of the *lac*I repressor protein by IPTG was postulated, such that additional IPTG will not increase the transcription of the *lac*Z gene significantly.

Other contributing factors for optimal gene expressions are the promoter system (Weickert *et al.*, 1996; Terpe, 2006), cellular location of product expression (Choi *et al.*, 2006; Donovan *et al.*, 1996) and the cell cultivation conditions (Lee *et al.*, 1997).

2.5 EFFECT OF INDUCTION TIME ON RECOMBINANT GENE EXPRESSION

Cell response to induction of recombinant protein synthesis, in terms of metabolic stress, makes it necessary to induce protein expression at a period during cell cultivation that will favour achieving maximum final biomass concentration and optimal protein specific productivity. This requires consideration of decreases in growth rate, cell yields, product expression and plasmid stability which may arise from metabolic stress (Lee *et al.*, 1997). Metabolic load arising from induction may lead to reduction or inhibition of cell growth or maximum specific uptake capacities for glucose and the respiration capacity (Neubauer *et al.*, 2003). Such cells are preferably induced at late exponential phase or stationary phase due to the highly dense culture that would have accumulated. However, where the cell growth rate is not adversely affected by protein induction, induction can occur at early or mid exponential phase of growth (Donovan *et al.*, 1996).

Differing times of induction for maximum expression of recombinant protein have been reported (Table 2.5). Although Lutomski *et al.* (2004), Lau *et al.* (2004) and Khushoo *et al.* (2004) reported maximum protein production with induction at late exponential phase of growth, Sivakesava *et al.* (1999) showed that human epidermal growth factor expression was maximal with induction at mid-exponential phase of cell cultivation. Meanwhile, Hu *et al*. (2004) observed similar insulin yield when 0.06 mM IPTG was added at different phases of cell cultivation (biomass accumulation). A similar conclusion was stated by Kim *et al.* (2007), that the change in IPTG addition time at different cell density using different *E. coli* strains did not affect resilin production noticeably.

Another typical example is the dynamics of CAT expression studied by Bentley *et al.* (1991). Depleted levels of nutrient in the stationary phase of cell growth resulted in a low growth rate and initiated protease activity. This, in turn, led to reduced protein yield. Hence, Bentley *et al.* (1991) proposed induction of protein expression during the mid-exponential phase of cell growth for maximum product yield at a high cell density. This is

in contrast to the result presented by Xu *et al*. (2007) where higher biomass and productivity were attained concurrently by inducing glucose dehydrogenase expression at late exponential phase of growth.

In another study, Lee *et al.* (1997) reported a delay in catabolic substrate switching as a timing strategy for optimal protein expression. In the process optimisation for large scale production of chimeric fusion protein, TGF-α-PE40, in recombinant *E. coli*, the effects of time of IPTG induction on a laboratory and pilot scale was studied. Two distinct catabolic phases were found based on the preferential utilisation of protein hydrolysate over glycerol in a complex growth medium during cell cultivation. The cells switched to glycerol utilisation after the depletion of the protein hydrolysate in the growth medium. This "switching over" was exploited to increase biomass accumulation prior to protein induction. The cell culture was induced at three different phases of growth; early, mid or late exponential phase, and it was found that the maximum specific and volumetric productivities were achieved with cell culture induction at mid exponential phase of growth. The same growth rate and final biomass concentration was observed in induced and non-induced cells, although glycerol utilisation was slower in the induced cells. This process was scaled up in 180 and 800 litres bioreactors, resulting in increased productivity.

Since the optimisation of recombinant protein expression in *E. coli* is system specific, key factors such as the biochemical properties of the recombinant protein, the induction mechanism of gene expression, host strains and cultivation conditions must be noted as important factors for consideration prior to optimising protein productivity.

2.6 HIGH DENSITY CELL CULTURE

One of the bioprocess-based approaches for maximal volumetric productivities of recombinant proteins is high cell density cultivation (HCDC), specifically where the volumetric productivity of recombinant product is a function of the biomass concentration and specific productivity per cell (Equation 2.4). Moreover, HCDC has the advantage of increased cost effectiveness, improved space-time utilisation, enhanced downstream processing and minimised waste water generation (Choi *et al.*, 2006; Lee, 1996). Different approaches to HCDC of *E. coli* have been reviewed (Choi *et al.*, 2006; Shiloach

and Fass, 2005; Lee, 1996; Yee and Blanch, 1992). Fed batch cell cultivation has been identified as the best process for achieving a high density culture. In a fed batch process, an initial concentration of nutrients is added to the bioreactor with cell cultivation proceeding as a batch process. Following the lag phase, the cells grow at the maximum specific growth rate during this stage until the limiting substrate approaches depletion. Nutrient supplementation is initiated as a semi-batch approach. Finally, the product is harvested at the completion of the process. This process manipulates the feeding strategies to maximise high density cultivation by minimising the depletion of the limiting substrate, limitation in dissolved oxygen, accumulation of toxic compounds, substrate inhibition and catabolite repression (Shuler and Kargi, 2002; Narciandi, 1996). For example, to achieve high productivity in a process where the substrate is inhibitory, fed batch cultivation can be used to keep the substrate concentration low. Although fed batch cultivation is frequently designed to increase biomass, the cultivation conditions also impact on achieving maximum specific cellular product yield. Thus, both factors require consideration in HCDC. Factors impacting biomass and protein productivity in fed batch cultivation include the growth medium and the degree of growth limiting substrate addition (the carbon source, glucose, in most cases), feeding technique adopted (which influences cell growth rate, which in turn impacts on acetic acid accumulation and plasmid stability) (Choi *et al.*, 2006; Shiloach and Fass, 2005).

2.6.1 MEDIUM EFFECTS ON BIOMASS CONCENTRATION AND RECOMBINANT PRODUCT YIELD

Microbial growth media are classified as complex, semi-complex or defined. A complex medium contains nutrients that are not chemically definable such as yeast extract, peptone, tryptone and casamino acids, which can vary in composition and quality. This reduces reproducibility of cell cultivation (Lee, 1996). Complex media support higher specific growth rates than defined media due to the presence of biosynthetic intermediates and growth factors within the media which the cell no longer needs to synthesize, thus decreasing metabolic burden (Schmidt *et al.*, 1999). However, they are not used typically for HCDC (Durany *et al.*, 2004; Lutomski *et al.*, 2004). Further, they are less defined, so a controlled feeding system will be difficult.

The defined medium is a mineral salt solution supplemented with a chemically pure carbon source. It is usually used to obtain high cell density as the nutrients composition

can be varied precisely and controlled during cell cultivation. A well balanced nutrient medium composition is needed for supporting cell growth whilst avoiding inhibition and shift in the dominant metabolic pathways (Lee, 1996).

The semi-complex or semi-defined medium contains both complex and defined nutrients. This medium is used to tailor nutritional requirements and boost product formation (Vidal *et al.*, 2005). In a human leptin production study by Jeong *et al*. (2004), chemically defined, yeast extract containing, and casamino acid-containing solutions were compared for biomass concentration and leptin production. The defined medium resulted in an over 2 fold increase in biomass concentration (DCW) over the other types of medium. However, the use of casamino acid containing solution resulted into a 2.1 and 1.8 fold increment in volumetric productivity over values obtained with defined and yeast extract containing feeding solution respectively. This was attributed to the limitation in biosynthesis of the extra amino acids and other metabolites required for leptin production. A similar conclusion was presented by Vidal *et al*. (2005), in the optimal production of aldolase from a recombinant *E. coli* system.

Growth medium composition used for HCDC of recombinant *E. coli* is compared in Table 2.6. Essentially, a carbon (glucose or glycerol) and nitrogen source is common to all the media. Further, the media contain some salts (such as phosphate source, yeast extract and casamino acid) and trace elements to enhance metabolism. The authors (presented in Table 2.6) used fed batch process, feeding a defined media (with glucose and glycerol as the carbon source, $MgSO_4$ and some of the trace elements) to achieve a high biomass concentration. Various approaches such as use of pressurised culture to enhance oxygen transfer rate (Matsui *et al.*, 2006) and feeding strategy (Babaeipour *et al.*, 2007; Hu *et al.*, 2004; Lau *et al.*, 2004) were used to achieve high biomass concentration up to 183 g l^{-1} (DCW) (Table 2.6). Therefore, the choice of nutrient feeding strategy is important for effective high cell density cultivation.

Table 2.6: Media composition used for HCDC of recombinant *E. coli* strains in batch processes (final biomass value is from fed batch cultivation)

Reference	Korz *et al.*, 1995	Seeger *et al.*, 1995	Babaeipour *et al.*, 2007	Lau *et al.*, 2004	Kweon *et al.*, 2001	Jeong *et al.*, 2004	Hu *et al.*, 2004
Carbon Source (in g per litre):							
Glucose	25.0	27.5	10	7.5	20	20	20
Glycerol	30.0						
Nitrogen Source (in g per litre):							
$(NH_4)_2HPO_4$	4.0	4.0			4.0	2.0	
NH_4Cl							0.1
$(NH_4)_2SO_4$			2.5	0.6			5
$(NH_4)_6HPO_4$							
Yeast extract							20
Casamino acid							
N-Z- Amine AS							30
Phosphate(in g per litre):							
KH_2PO_4		13.3	7.5			6.75	6.75
K_2HPO_4	13.3		15	6.3	13.3		
$Na_2HPO_4.12H_2O$				18.1			3
Other salts (in g per litre):							
$MgSO_4.7H_2O$	1.2	1.2	2			0.7	
Citric acid	1.7	1.7	2	0.94	1.2	0.85	3
EDTA	0.0084	0.014			1.7		
Thiamine.HCl	0.0045	0.0045					0.02
$CaCl_2$					0.005		
Chloramphenicol($\mu g.ml^{-1}$)						5	
Trace element($ml.l^{-1}$)			1.0				6
Vitamin solution($ml.L^{-1}$)				3.8	10		
Na_2SO_4				3.8			
Ampicillin		50					
$MgCl_2$							1.5
pH control:	NH_3(25% w/w)	NH_3(25% w/w)	25% (w/v), NH_4OH or 3M H_3PO_4	17M NH_4OH, 2.5N H_2SO_4	NH_4OH HCl	28 % Ammonia water	28 % Ammonia water
Antifoam	SP1 0.1ml	SP1 0.1ml	Silicon antifoaming	50% antifoam B			Sigma A-5758
Temperature (^oC)	28	30		37	28	37	
pH	6.7	6.9	7	7.0	6.7	6.8	7.0
N (rpm)	500	500	50-1200	600		1000	1000
Time(hrs)			16.5±5	45	25	36	30
Final Biomass X, DCW(g/L)	128(Glucose), 148(Glycerol)	135	115±5	OD_{600}=115	60	120	183

2.6.2 FEEDING STRATEGIES IN FED BATCH PROCESSES

Substrate limitation inhibits growth or causes growth rate to decrease, resulting in productivity loss. Excess substrate can lead to the accumulation of the carbon source or by-product formation (Choi *et al.*, 2006). This affects the metabolic pathway flux distribution which, in turn, affects the maximum attainable cell concentration, specific productivity of recombinant proteins, and by product formation and yield of both biomass and products. Hence, feeding strategies are critical for *E. coli* cultivation (Lee, 1996; Li *et al.*, 1998).

Feeding methods in fed batch cell culture can be summarised as "without feedback control" and "with feedback control" (Lee, 1996; Yee and Blanch, 1992). In the feedback control system, the bioreactor online monitoring sensors respond to changes in the culture medium through a negative feedback mechanism, and nutrient medium is fed accordingly. A change in cultivation conditions such as pH (Hu *et al.*, 2004; Jeong *et al.*, 2004) and dissolved oxygen (Lim *et al.*, 2000) when the limiting substrate is depleted lead to a pre-determined volume of medium being fed into the bioreactor. These feedback control mechanisms have the advantage of preventing the overfeeding of nutrients into the bioreactor while maintaining critical levels of limiting factors during cell cultivation (Choi *et al.*, 2006).

The "without feedback control" mechanisms utilise "constant" or "exponential" rates of feeding. The "constant feeding rate" has been described as the simplest pre-determined feeding profile. In this system, the feed containing the limiting substrate is fed continually into the bioreactor at a pre-set rate. With time, the feed addition relative to the biomass concentration decreases due to an increase in biomass concentration.

The biomass concentration increases until the limiting substrate becomes inadequate for the growing cells, thereby resulting in a reduction in specific growth rate (Lee, 1996). The equations governing constant feeding have been proposed by Yee and Blanch (1992). At constant feeding, a quasi-steady state exists for substrate concentration when the growth limiting substrate is consumed rapidly. The specific growth rate of the organism then becomes

$$\mu = \frac{Y_{x/s} C_s F}{C_x V} \tag{2.5}$$

where V is the working volume of the bioreactor, C_x is the biomass concentration in the bioreactor, C_s is the substrate concentration in feed, $Y_{x/s}$ is the biomass yield on carbon substrate and F is the feed flow rate. Assuming $Y_{x/s}$, C_s and F are constant, the cell mass balance can be integrated to give:

$$\begin{aligned} \frac{d}{dt}(VC_x) &= V\mu C_x \\ VC_x &= C_1 t + C_2 \end{aligned} \tag{2.6}$$

where the two constants are $C_1 = Y_{x/s} C_s F$ and $C_2 = (V_o C_{xo})$. Equation 2.6 shows the linear relationship between total biomass and time (Yee and Blanch, 1992).

A situation where the limiting substrate is fed at an increasing rate such that the specific rate of substrate provided is constant is referred to as exponential feeding. Biomass concentration increases with an exponential feed rate. This type of feeding is also used to control the specific growth rate at a constant value below the critical value of acetate formation by using the carbon source (such as glucose) as a growth limiting nutrient (Suarez and Kilikian, 2000; Babaeipour *et al.*, 2007; Li *et al.*, 1998; Lee, 1996).

Given that $t = 0$, $V = V_o$, and $C_x = C_{xo}$, the cell mass balance equation can be integrated (Yee and Blanch, 1992) and written as

$$C_x V = C_{xo} V_o \exp(\mu t) \tag{2.7}$$

If a quasi-steady state exists for the substrate concentration, then $\frac{ds}{dt} = 0$. Assuming that $Y_{x/s}$ is constant and substituting Equation 2.7 into the substrate mass balance, an exponentially increasing profile is obtained:

$$F = \frac{\mu C_x V}{C_{so} Y_{x/s}} = \frac{\mu C_{xo} V_o \exp(\mu t)}{C_{so} Y_{x/s}} \tag{2.8}$$

Substituting Equation (2.8) into the overall mass balance $\frac{dV}{dt} = F$

$$V = \frac{V_o}{\mu}(\mu + AC_{x\,o}\exp(\mu t) - AC_{x\,o}) \tag{2.9}$$

where $A = \frac{\mu}{Y_{x/s}C_{s\,o}}$

Substituting Equation 2.9 into 2.7, an expression for change in cell concentration with time is obtained:

$$X = \frac{\mu C_{x\,o}\exp(\mu t)}{(\mu + AC_{x\,o}\exp(\mu t) - AC_{x\,o})} \tag{2.10}$$

Korz *et al.* (1995) derived Equation (2.12) using mass balance, assuming constant cell yield on substrate. The equation was proposed for calculating the feed rate that allows for the exponential growth of cells.

$$M_s(t) = F(t)C_{s\,o}(t) = \left[\frac{\mu}{Y_{x/s}} + m\right]C_x(t)V(t) = \left[\frac{\mu}{Y_{x/s}} + m\right]C_x(t_o)V(t_o)\exp\left[\mu(t - t_o)\right] \tag{2.11}$$

where M_s is the mass flow rate of the carbon source (g.h^{-1}), m is the specific maintenance coefficient [g g^{-1} (DCW) h^{-1}] and t_o is the time at which feeding is started.

Exponential feeding has the advantage of maintaining a desired specific cell growth rate in the bioreactor, thereby controlling critical conditions in the bioreactor such as acetate formation (Babaeipour *et al.*, 2007). Further, process time can be reduced with a corresponding increase in biomass concentration, productivity and biomass yield compared with other methods of feeding (Li *et al.*, 1998).

Preventing the accumulation of toxic levels of acetic acid (a by-product of the *E. coli* metabolism of glucose in the presence of excess glucose, typically caused by oxygen limitation) is one of the major factors in achieving high cell concentrations and protein productivity in the bioreactor (Suarez and Kilikian, 2000; Lee, 1996; Korz *et al.*, 1995). Acetic acid accumulation is postulated to inhibit both cell growth and recombinant protein expression (Vidal *et al.*, 2005). The correlation between specific acetic acid accumulation (q_{Hac}), and specific growth rate (μ) of *E. coli* was studied by Suarez and Kilikian (2000) in a fed batch process using an oxygen saturated semi-synthetic medium.

The authors observed an exponential relationship between q_{Hac} and μ with major increments in q_{Hac} for μ values higher than 0.30 h^{-1}. Hence, it was necessary to grow *E. coli* at a rate between 0.20 and 0.30 h^{-1} to reduce acetic acid formation and accumulation in a fed-batch process. The use of pre-determined exponential feeding for maximum biomass concentration was reported by Korz *et al.* (1995) where cells were grown at the critical desired growth rate, below 0.17 h^{-1}, with no accumulation of acetic acid. Cell concentrations increased to 128 and 148 g l^{-1} DCW respectively when glucose and glycerol were used as the carbon sources to maintain a carbon limited growth during the fed batch process (Table 2.6). Babaeipour *et al.* (2007) and Vidal *et al.* (2005) exploited methods of exponential feeding to control acetic acid formation with the simultaneous improvement in heterologous protein productivity. Babaeipour *et al.* (2007) studied the effects of feeding strategy on medium constituents, accumulation of acetic acid, human interferon gamma (rhIFN-γ) production and recombinant protein expression in a fed batch process. The authors observed reduced cultivation time, a DCW of up to 120 g l^{-1}, a 2.5 fold increase in overall productivity of the rhIFN-γ and an acetate concentration lower than the inhibitory concentration. A similar study by Vidal *et al.* (2005) showed that a lower growth rate (controlled by feeding) led to a reduction in acetic acid formation, an exponential increase in biomass concentration and an accompanying increase in enzyme concentration and volumetric productivity compared to batch mode.

Another factor affected by feeding approach in recombinant protein production is maintenance of the plasmid, especially in a highly dense culture. Several approaches such as optimisation of cultivation media and conditions and maintenance of selective pressure are used to enhance plasmid stability (Zhao *et al.*, 2007). Most often, on a small scale, plasmid stability is ensured during cell cultivation by applying selection pressure through the use of antibiotics, because the recombinant strains have antibiotic resistant gene encoded in their plasmids (Korpimaki *et al.*, 2003). Plasmid instability can be characterised as structural, segregational and competitive instability, though the last two are most common in recombinant gene expression (Wang *et al.*, 2001; Zhao *et al.*, 2007). Defective partitioning of the plasmid DNA between daughter cells during cell division resulting into loss of plasmid from one cell of the daughter cells is termed segregational, while instability resulting from change in plasmid base sequence due to insertion, deletions or mutations is referred to as structural plasmid instability.

Meanwhile, competitive instability is due to outgrowing of the plasmid bearing cells by plasmid free cells under nonselective conditions.

The change in growth rate, which is affected by the choice of feeding method, impacts plasmid stability (Nayak and Vyas, 1999). Plasmid stability has been reported to be enhanced at lower growth rate. Gupta *et al*. (1995) suggested that this might be due to either inability of the plasmid free cells to outgrow recombinant cells at such a low growth rate or the non synchronisation of the rate of plasmid replication with that of the host. Meanwhile, Nayak and Vyas (1999) hypothesised that there is an optimum growth rate at which recombinant plasmid is maintained for the most time.

Nayak and Vyas (1999), demonstrated the effects of feeding regimes (constant, intermittent, linear and exponential) on plasmid stability and expression in *E. coli* in a fed batch process, and observed a higher plasmid stability of 45 to 60% using linear and exponential feeding. However, a higher biomass concentration using exponential feeding approach gave a corresponding increase in recombinant carboxymethylcellulase activity.

The influence of feeding strategy on protein expression is important as this not only affects biomass and by-product accumulation, but also plasmid stability in recombinant gene expression. In general, the choice of feeding approach is important as this impacts on the total output of the desired product, which affects bioprocess economics.

2.7 PROTEIN RECOVERY

Downstream processing for the recovery of proteins from cells after cultivation involves the separation and purification of the biological product into a form suitable for its intended use. The stages usually involved in downstream processing are solid-liquid separation i.e. biomass concentration, the isolation of the product, purification and polishing or formulation (Hatti-Kaul and Mattiasson, 2001; Harrison *et al.*, 2003).

Bacteria produce biological compounds extracellularly (by excreting them into the surrounding growth medium) or intracellularly (by retaining them within the cell membrane or cytoplasm). A large proportion of potentially useful microbial products of recombinant DNA technology are retained within the cell membrane (Balasundaram and

Pandit, 2001b; Middelberg, 1995; Harrison, 1991; Schutte and Kula, 1990). The isolation of these intracellular products requires either that the cells are genetically engineered so that intracellular products can be excreted into the surrounding medium or the cells must be disrupted or permeabilised by physical, chemical or biological means to release their periplasmic or cytoplasmic content. Thus, cell disruption is the first step in separation of cell intracellular components. This is a critical stage as the choice of cell disruption process may impact the extent of protein recovery, the ease of the following purification steps, the type of the suspensions to be processed and the form and quality of the final product (Keshavarz *et al.*, 1987). These combined recovery processes are significant in overall cost effective recombinant protein production. The cell disruption process is a function of factors such as physical strength of the cell wall of the micro-organism and the intracellular location of the enzyme (Harrison, 1991). In order to propose a suitable approach to product release, it is necessary to consider the nature of the cell envelope to be disrupted or permeabilised, the location of the product within the cell and the methods of cell disruption.

2.7.1 CELL STRUCTURE OF GRAM NEGATIVE BACTERIA

Gram negative bacteria have a semi-rigid cell envelope that provides essential strength to protect the cell from lysis arising from osmotic pressure. It also acts as a biologically active boundary between the organism and its surrounding medium. The cell envelope of Gram negative bacteria (including *E. coli*) consists of a lipo-protein outer membrane layer, a thin rigid peptidoglycan layer that is sandwiched between the outer and inner membrane, the periplasmic space and an inner semi-permeable cytoplasmic membrane (Harrison, 1991; Engler, 1985). This is shown diagrammatically in Figure 2.4

The outer membrane is comprised of lipopolysaccharides (LPS), phospholipids and protein. It separates the thin rigid peptidoglycan layer from the medium environment, thereby acting as a barrier to external interaction. The lipoprotein complex connects the lower portion of the phospholipid bi-layer to the peptidoglycan layer (Middelberg, 1995). Divalent cations, such as Ca^{2+} and Mg^{2+}, stabilise the outer membrane structure by binding LPS molecules to each other as well as to outer membrane proteins. The rigid cross-linked peptidoglycan layer forms the fundamental structural component of the cell envelope, providing mechanical strength to the cell. Together with the cell membrane which is comprised mainly of phospholipids, the peptidoglycan layer also helps in

maintaining concentration gradients between the cell and the external environment (Middelberg, 1995). The peptidoglycan layer is comprised of linear polysaccharide chains of alternating *N*-acetyl-D-glucosamine (NAG) and *N*-acetyl-muramic acid (NAM) residues joined by β-(1-4) glycosidic bonds. The chains are cross linked by a tetra peptide of the basic structure L-alanyl-D-glutamyl-L-R3-D-alanine attached to the NAM residues and the peptide branches of the parallel chains are further cross-linked. This rigid structure acts as a single molecular network to give the cell shape and tensile strength and to osmotically protect the cell envelope from the external environment. The cytoplasmic membrane serves as the interactive layer between the internal cell environment and the external environmental. This layer consists of phospholipids and proteins, which help in the maintenance of the cell concentration gradient. It also houses the transport system that is involved in the generation of adenosine triphosphate (ATP). The cell membrane has little mechanical strength. Cells are therefore easily disrupted to release their intracellular contents following breakage or removal of the strength-giving peptidoglycan in the cell wall, unless they are stabilised osmotically (Middelberg, 1995; Harrison, 1991; Engler, 1985).

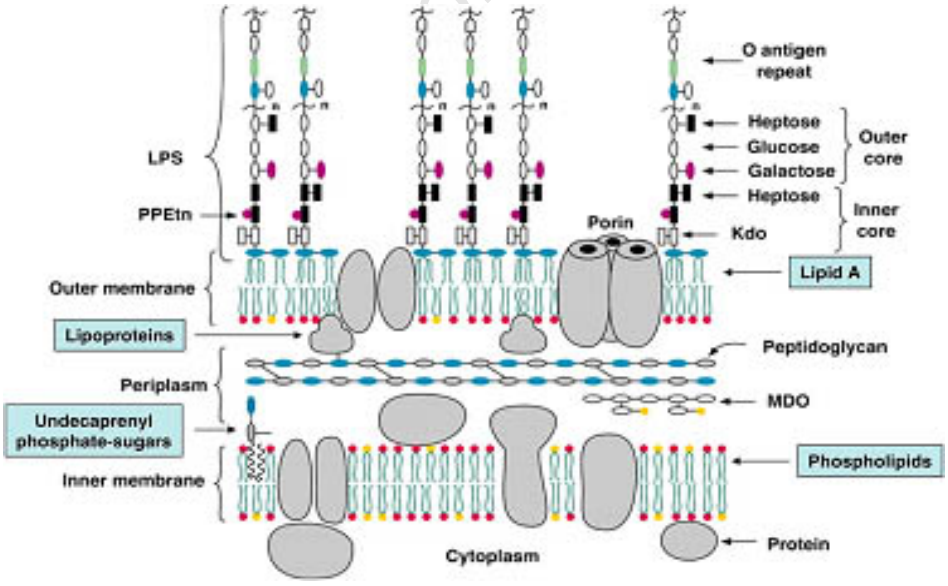


Figure 2.4: Cell envelope of Gram negative bacteria (Anonymous, 2006)

Overall, the outer membrane protects the inner layer from direct chemical attack, the peptidoglycan layer gives the mechanical strength and the cytoplasmic membrane plays

a major role in cell permeability and controls the active transport of compounds into and out of the cell (Harrison, 1991).

2.7.2 INFLUENCE OF PROTEIN LOCATION AND SELECTIVE PRODUCT RELEASE

The release of intracellular components of microbial cells is typically achieved by disruption and disintegration of the strength-providing components of the wall, i.e. the peptidoglycan layer in Gram negative bacteria (Harrison, 1991). Microbial cells contain a range of protein, nucleic acid molecules and other bio-compounds. Complete cell disruption releases the target product into an environment consisting of complex bio-molecules. This, and micronisation of cell debris resulting from mechanical disruption of cells, further complicates the downstream processing of the target protein (Balasundaram and Harrison, 2008b). This may reduce the final yield of the target protein from the process, while enzyme activity may be affected by exposure to inhibiting substances in the environment or the presence of proteases (Hatti-Kaul and Mattiasson, 2001). Hence, the idea of selective release of the target protein while avoiding cell debris micronisation may improve overall yield and productivity of the desired protein.

In microbial cells such as *E. coli*, protein production is often limited to a particular compartment of the cell, for example the cell periplasm or cytoplasm. Total cell disruption is not required for release of periplasmic protein, but may be needed for the release of enzymes produced in the cytoplasm. This difference can be utilised for selective protein release, such that periplasmic proteins are released prior to proteins in the cytoplasm. This dependence of protein release rate on intracellular location has been exploited by different authors for *E. coli* and *Saccharomyces cerevisiae* systems (Balasundaram and Pandit, 2001b; Balasundaram and Pandit, 2001a; Kuboi *et al.*, 1995; Huang *et al.*, 1991; Follows *et al.*, 1971). Selective release of proteins from the microbial cells can simplify further downstream processing of the proteins, in turn impacting on productivity and protein purification.

2.8 METHODS OF CELL DISRUPTION

The various methods for cell disruption have been reviewed (Middelberg, 1995; Harrison, 1991; Keshavarz *et al.*, 1987; Chisti and Moo-Young, 1986). Methods for recovery of intracellular products through cell disruption can be broadly classified into

mechanical and non-mechanical methods. As shown in Figure 2.5, mechanical methods include the use of a bead mill, high pressure homogenisation and hydrodynamic cavitation. Chemical treatment, enzymatic lysis, and physical methods such as osmotic shock are examples of non-mechanical methods. Mechanical methods such as high pressure homogenisation are typically utilised on an industrial scale for complete disruption of cells and are easily scaled up (Hetherington *et al.*, 1971; Harrison, 1991; Middelberg, 1995; Schutte and Kula, 1990). Chemical and enzymatic methods are generally restricted to small scale processes or low volume processes of high value, owing to the added requirement of chemicals or enzyme separation during the downstream processes and the cost of chemical and enzyme addition (Schutte and Kula, 1990). In other instances, mechanical and non-mechanical methods can be combined to improve the disruption process by avoiding high energy requirement and micronisation of cell debris that arises using mechanical cell disruption (Harrison *et al.*, 1991; Anand *et al.*, 2007).

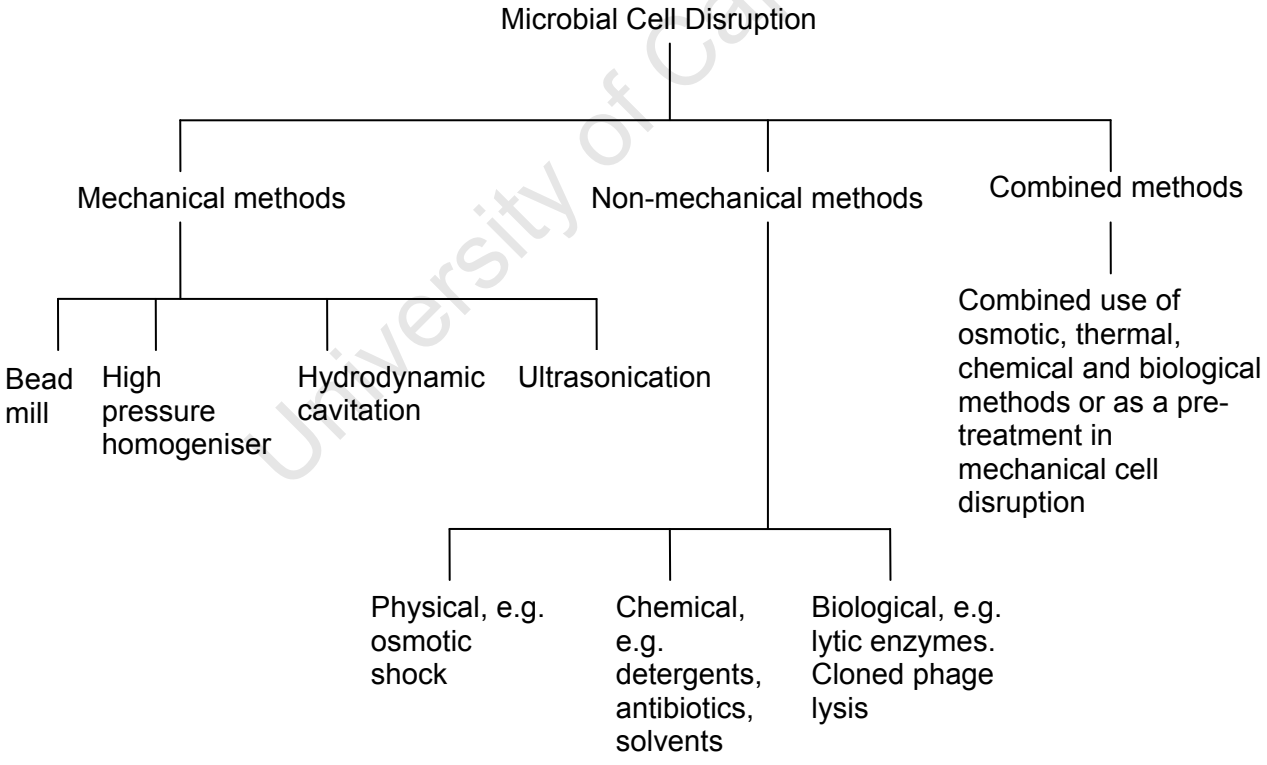


Figure 2.5: Methods applicable for microbial cell disruption, adapted from Harrison (1991)

2.9 DESIGNING THE DOWNSTREAM PROCESS

Steps subsequent to cell disruption involve recovery and purification of the protein of interest through a number of downstream processing steps. Different separation principles, based on the biochemical characteristics of desired proteins, are exploited in their purification processes. The complexity of the downstream process is determined by the required purity of the product, dependent on its intended use. Therefore, an increasing number of purification steps is needed for a product requiring high purity. For example, protein recovered in the form of inclusion bodies needs to be recovered from the cell debris and renatured prior to standard purification (Harrison *et al.*, 2003). The standard approach and general unit operations utilised in downstream processing of proteins are presented Figure 2.6.

The microbial cells produced at the bioreactor stage are concentrated in a solid-liquid separation process and subjected to cell disruption for the release of intracellular products. The soluble protein is separated from the cell debris in another solid-liquid operation, using centrifugation or filtration. In some instances, both methods are combined or the centrifugation step precedes, with the latter following. The desired protein is separated from the crude protein mixture in a series of purification steps by exploiting its biochemical properties. Each of these steps in the recovery scheme will impact on the overall process economy by increasing capital and operational cost and process time, while reducing the overall yield achievable. In addition, the total achievable yield may be reduced at increased contact period between crude lysate and desired protein, due to proteolytic degradation (Barnfield-Frej *et al.*, 1994a). Thus, integration of the functions of several unit operations into one step is desirable to reduce the processing steps and hence offer an efficient way of reaching high process economy in the general production process. For instance, cell debris has to be removed using centrifugation or micro-filtration prior to use of packed bed adsorption chromatography to avoid bed clogging. The efficiency of the centrifugation step depends on particle size, density difference between particles, the surrounding liquid and the feedstock (Hatti-Kaul and Mattiasson, 2001; Harrison *et al.*, 2003).

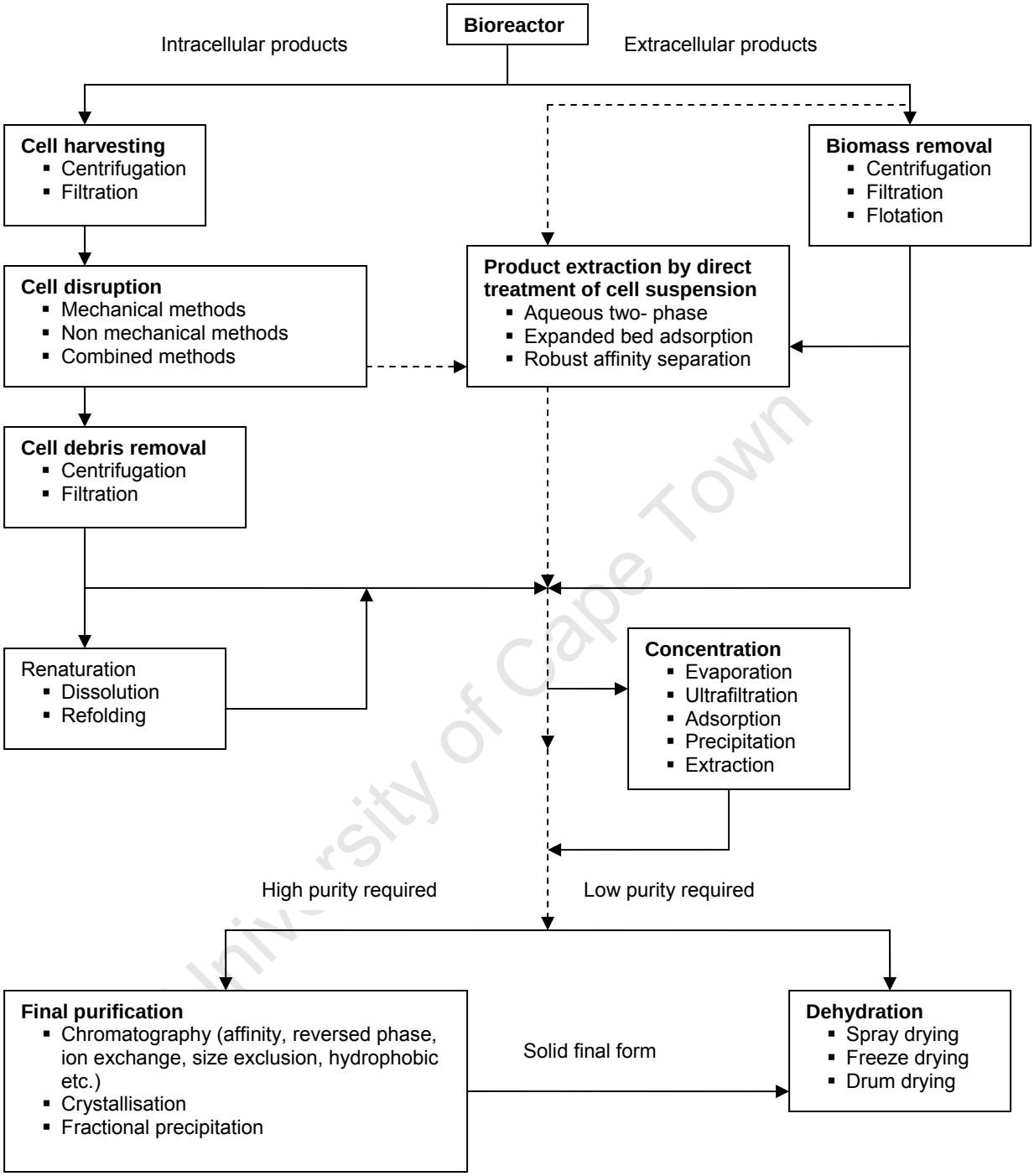


Figure 2.6: Generic block diagram of downstream processing, adapted from Harrison *et al.* (2003)

Mechanical cell disruption often results in cell debris micronisation, increasing difficulty of separation. Therefore, centrifugation may be combined with micro-filtration to obtain a clear and complete particle free supernatant. The latter has disadvantages which include membrane fouling and the reduction of flux of liquid per unit membrane area during filtration which eventually leads to a partial rejection of proteins. Furthermore, higher energy inputs are needed and higher shearer forces develop, which may affect shear sensitive cells (Harrison *et al.*, 2003). The use of membrane ultra-filtration steps between chromatography steps to exchange buffers and concentrate dilute product add further to cost. Furthermore, the combined use of both biomass concentration methods add to processing time, capital and maintenance costs. This is a disadvantage of the packed bed purification process (Fahrner *et al.*, 1999). There is a need to process cell suspension directly from crude lysate using a robust affinity separation to avoid capital expenditure, recurrent maintenance cost, time of processing steps and increased yield. An integrated process where centrifugation, micro-filtration and adsorption steps are merged into one unit operation may help achieve this. Hence, integration of downstream processing steps while maintaining or enhancing product quality, may result in an economical process design. This is illustrated in Figure 2.6 where solid-liquid separation, product concentration and adsorption can be achieved using one single unit operation. Two commonly employed methods are aqueous two-phase systems and expanded bed adsorption (EBA).

2.9.1 AQUEOUS TWO PHASE SEPARATION

Aqueous two phase extraction is a liquid-liquid extraction method exploiting partitioning of biomolecules such as proteins, cells and cell organelles, between the liquid phases used for separation. This method is a non-denaturing and non-degrading technique. Two phase aqueous systems are prepared by mixing two incompatible water soluble polymers such as polyethylene glycol (PEG) and dextran, or a polymer and a salt above a certain limiting concentration, with water as a major component (Hatti-Kaul and Mattiasson, 2001). These systems separate into two immiscible liquids layers with each layer enriched in respective phase components, i.e., PEG as major constituent on the top phase, while dextran or salt is the key component in the bottom phase. The composition and volume of the phases are dependent on the molecular weight of the polymer, polymer concentration and also salt concentration. Soluble materials such as proteins and particulates (including living cells) added to the system will partition

between the two phases based on their affinity for each phase which selectively separates the product from the crude mixture.

A disrupted suspension with high viscosity containing various sizes of cell debris can be used in aqueous two-phase extraction. The product is extracted directly from the cell lysate, thus eliminating the solid-liquid separation stage (Figure 2.7). The mild environment of the aqueous two-phase system and low interfacial tension, in addition to the ease of scale up and continuous operation, makes two-phase aqueous systems suitable for the large scale purification and production of proteins. Phase component recycling minimises material costs (Hatti-Kaul and Mattiasson, 2001). This approach has been utilised successfully by many research groups (Xue-Jun *et al.*, 2004; Gavasane and Gaikar, 2003; Rito-Palomares and Lyddiatt, 2002; Su and Feng, 1999).

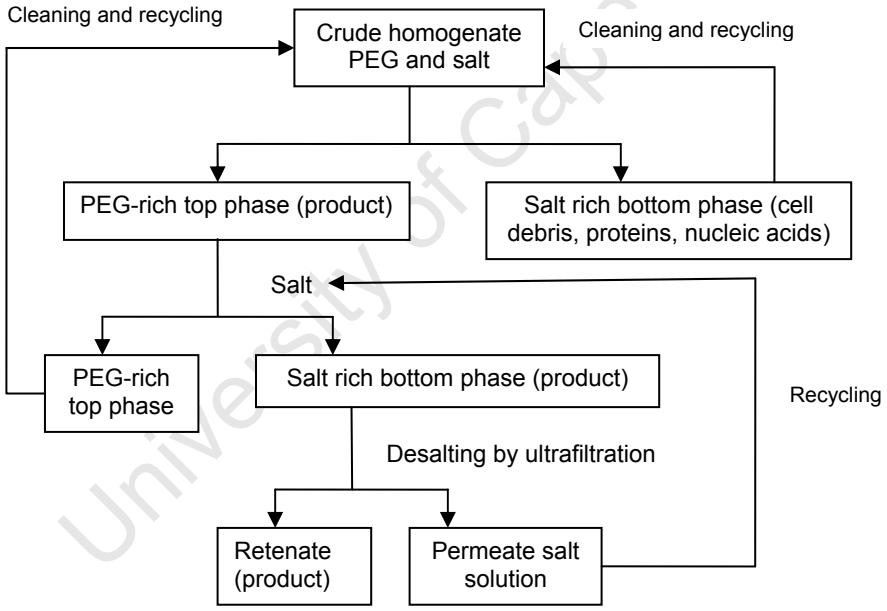


Figure 2. 7: Simplified representation of a two- phase stage aqueous process (redrawn from Hatti-Kaul and Mattiasson (2001)

2.9.2 EXPANDED BED ADSORPTION

The complexity of downstream processing can be reduced by eliminating certain filtration, centrifugation and concentration steps using expanded bed adsorption (EBA). EBA can be described as an integrated technology for the primary capture of proteins from an unclarified cell broth or lysate (Lin *et al.*, 2004). This technology integrates solid-

liquid separation, volume reduction and partial purification by protein adsorption into one unit operation without compromising on separation efficiency. The capture objectives using EBA are to stabilise the product, remove proteases, solids, cells, water and bulk quantities of proteins, nucleic acids and carbohydrates, as well as to prepare the product for further chromatography (Bierau *et al.*, 1999; Feuser *et al.*, 1999; Anspach *et al.*, 1999; Chang and Chase, 1996b; Chase, 1994). This technology has been investigated on laboratory scale and used on pilot (Barnfield-Frej *et al.*, 1994a; Brobjer, 1999) and industrial scale (Shepard *et al.*, 2001).

2.9.2.1 Principles of expanded bed adsorption

The adsorbent bead bed is expanded by applying an upward buffer solution to the column prior to feeding the crude cell lysate. During equilibration, the bed is usually expanded by a factor of 2 or 3, thereby increasing the voids between the adsorbent particles and the bed porosity. The voids created enable the particles in the crude feedstock to pass through the bed. This gives the EBA its unique properties compared with the conventional packed bed (Chang and Chase, 1996b; Fernandez-Lahore *et al.*, 2000; Lin *et al.*, 2004).

A stable fluidised bed is formed when the adsorbent particles are suspended in equilibrium due to the balance between particle sedimentation velocity and upward liquid velocity (Anspach *et al.*, 1999). The crude unclarified feed containing the cells, cell debris and other particulate matter is introduced into the column. Due to the higher viscosity and other physical conditions of the homogenate, the bed expands further if the flow rate is maintained. This leads to an increase in void space between the adsorbent thereby allowing passage of particulate contaminants and weakly bound particles such as residual cells and cell debris, while target proteins adsorb onto the adsorbent beads in the column (Fernandez-Lahore *et al.*, 2000; Fernandez-Lahore *et al.*, 1999; Lin *et al.*, 2003). Provided that the settling velocity of the particulates in the feedstock is much lower than that of the adsorbent particles, weakly retained particles in the voids of the bed can be washed away after the adsorption phase, and the protein attached to the beads can be eluted from the bed through downward flow in the packed bed mode (Anspach *et al.*, 1999). The eluate contains the target protein at an increased concentration, clarified, partly purified and ready for further purification in a high resolution chromatographic step. After elution, the beads can be regenerated in a clean-

in-place (CIP) procedure using buffers or solutions specific for the type of chromatographic principle applied, and re-used (Chase, 1994).

The efficient use of expanded bed for protein adsorption depends on less pronounced axial dispersion and absence of flow channels or dead zones within the bed, in the presence of viscous crude feedstock (Shepard *et al.*, 2001; Lin *et al.*, 2001a; Lin *et al.*, 2004). This allows the formation of several mass transfer units thereby imitating operation using the conventional packed chromatography column (Fernandez-Lahore *et al.*, 2000). The process design variables for optimising expanded bed chromatographic separation include chemical and physical properties of the adsorbent and the feedstock. A chromatographic adsorbent with appropriate properties such as density, particle size and mechanical stability is required for an optimal expanded bed process. Other important factors are the physico-chemical properties of the feed such as pH, viscosity, ionic strength and the type of buffer used. These affect selectivity and capacity of the process, which is similar to a packed bed operation (Chang and Chase, 1996b).

2.9.2.2 Design features for expanded bed adsorption

Although the expanded bed adsorption process works in a similar way to the conventional packed bed chromatography system, its fluidisation properties require special features to enable an efficient operation. Such features include the type of column used, the choice of adsorbent for bed stability and optimal protein adsorption. These are all critical to formulating an optimal protein recovery process using EBA.

Column requirements

As with packed bed chromatography system, expanded bed operation requires a column, pump, online spectrometer and fraction collector. The major difference in column design is the inclusion of two adapters. The adapter located at the top of the expanded bed is used to reduce the dead volume of liquid in the column, and to ensure the proper washing and elution when the bed is operated in a packed mode (Ng *et al.*, 2007; Fernandez-Lahore *et al.*, 1999). The lower adapter holds a flow distributor that ensures even flow distribution in the column through an even pressure drop (Anspach *et al.*, 1999). While the distributor enables the passage of particles without blockage, the

additional screen above it helps to retain the adsorbent in the column when the flow is stopped or reversed (Bierau *et al.*, 1999).

Adsorbents in expanded bed

The choice of adsorbent is a vital step in the efficient recovery of protein using an EBA process. The adsorbents used in packed bed systems are not optimal for use in expanded beds due to mass transport, axial mixing and the influence of biomass on equilibrium and breakthrough (Xiu and Rodrigues, 2005; Chase, 1994; Chase and Draeger, 1992; Karau *et al.*, 1997). In addition, the small size and low specific density of the adsorbent beads makes it unsuitable for use in fluidised systems (Barnfield-Frej *et al.*, 1994a). The use of these adsorbents for fluidised systems results in bed expansion to an unsuitably high degree at low feed velocities. This results into bed instability when operated with the use of crude homogenate as feedstock (Chang and Chase, 1996b). Since a larger bead size is undesirable due to diffusion limitation, a way to overcome this problem is to use small, highly dense particles which will allow high fluidisation velocities coupled with an increase in mass transfer efficiency (Gondkar *et al.*, 2001; Dong and Sun, 2001; Voute and Boschetti, 1999; Karau *et al.*, 1997). Tong *et al*. (2002) and Voute and Boschetti (1999) designed and reported adsorbents exhibiting high levels of dynamic binding capacities at elevated velocities. Moreover, the small-sized dense adsorbent showed less axial dispersion in comparison to the streamline gel.

In addition, adsorbents for use in expanded bed are expected to be resistant to fouling, as the crude feedstock contains complex bio-compounds such as biomass, nucleic acids, lipids and other medium components. For a cost effective operation of the expanded bed adsorption, the adsorbents should be easily and cheaply regenerated by a CIP procedure that will not affect the adsorption and fluidisation properties of the adsorbents (Chase, 1994).

Bed expansion characteristics and assessment of bed stability

Bed fluidisation occurs when the upward velocity of particles in a column corresponds to their sedimentation velocity. The extent of bed expansion is a function of the size and density of the adsorbent beads, the linear flow velocity and viscosity of the mobile phase (Chang and Chase, 1996a; Ng *et al.*, 2007). While a wide range of flow velocity is needed in a packed bed operation to enhance protein adsorption or productivity, a

reduced feed flow in an expanded bed is mainly used to maintain the required extent of bed expansion. This is advantageous in an expanded bed process, as costs associated with pump selection and operation can be reduced. An expanded bed height corresponding to a 3 fold increase in volume, equivalent to a voidage of 0.8, is needed to reduce dispersion and enhance protein adsorption by allowing higher breakthrough capacities over a range of flow rate. Excessive bed expansion up to four fold (above voidage of 0.85) is often discouraged as this may lead to an axial spread in the protein front passing through the bed. The pressure drop across an expanded bed will be reduced compared to a packed bed irrigated at the same flow velocity (Thommes *et al.*, 1995; Karau *et al.*, 1997; Chang and Chase, 1996a). Specifically, Chang and Chase (1996a) demonstrated less efficient protein adsorption at increased bed height, as a result of a highly viscous solution and increased feed velocity. Sharp breakthrough was observed for the model protein used, lysozyme.

Bed stability can be characterised by visual assessment and the degree of bed expansion, i.e. the ratio of expanded bed height to sedimented bed height. Reduced expansion over that expected might indicate poor stability or channeling due to trapped air under the distributor plate, contamination or fouling of the adsorbent, the column not being in a vertical position or a blocked distributor plate (Bruce *et al.*, 1999). In addition, bed stability can be directly assessed through visual examination of particle movement in expanded beds. Small circulatory movements of the adsorbent beads are observed in stable beds, while turbulent flow and channeling are common features of unstable beds, which lead to less efficient protein adsorption to the beads.

Bed stability has been shown to be maintained with the introduction of crude feedstock. Lin *et al.* (2003) investigated stability of expanded bed during application of crude feedstock using yeast cells and STREAMLINE AC (a stationary base for EBA with no ligand attached to it). The authors observed that classified expanded bed was not affected when crude feed was introduced into the column, provided there was no interaction between the cells and the adsorbent. An ordered additional bed expansion was observed with corresponding bed stability.

2.9.2.3 Break through curve

In a flow through column, some conditions are required to achieve an ideal breakthrough curve (Seader and Henley, 1998). These include (1) external and internal mass transfer resistances are very small; (2) plug flow is achieved; (3) axial dispersion is negligible; (4) the adsorbent is initially free of adsorbate; and (5) the adsorption isotherm begins at the origin and equilibrium between the fluid and the adsorbent is instantly achieved. If these hold, a shock-like wave, called a stoichiometric front, moves as a sharp concentration band through the bed and the breakthrough curve shown in Figure 2.8 is observed.

At the start of feeding (t=0), the sample with a concentration C_o is pumped into the equilibrated bed. At the upstream region of the column (between t=0 and $t=t_s$), there exists an equilibrium between the binding biomolecules in the feedstock and the adsorbent beads, thereby making the adsorbent spent.

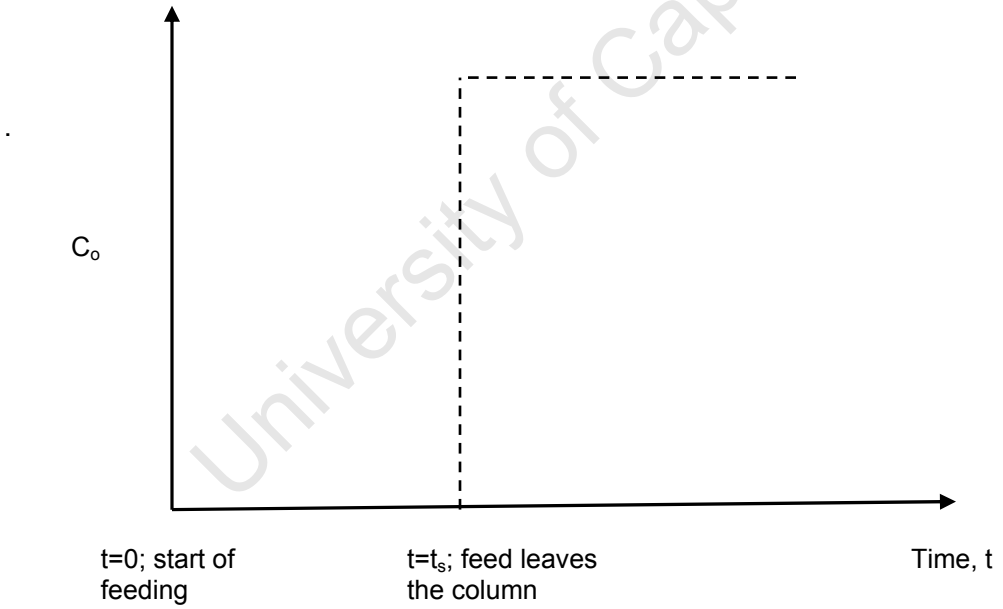


Figure 2.8: An ideal breakthrough curve as adapted from Seader and Henley (1998)

Meanwhile, the adsorbent is unused at the downstream region of the column. This leaves the concentration of the exit fluid to be zero. The adsorbent becomes saturated over a period of time ($t=t_s$), hence the concentration of the solute in the outlet fluid rises to the inlet value, C_o, until no further adsorption is possible, and adsorption is terminated.

This point is referred to as the breakpoint, and the stoichiometric wave front becomes the ideal breakthrough curve (Seader and Henley, 1998).

In a practical process, the conditions leading to the profile in Figure 2.8 are not entirely valid. Internal and external transport resistances are finite. Also, solid-liquid phase axial dispersion can be significant, particularly with beds irrigated at low flow rates such as expanded beds (Chang and Chase, 1996a; Seader and Henley, 1998). These factors contribute to the development of broader concentration fronts as depicted in Figure 2.9, in which the ratio of outlet to inlet solute concentration in the fluid is given as a function of time from the start of feeding. The S-shaped curve is referred to as the breakthrough curve. Between t=0 and t=a, the outlet solute concentration is very minimal owing to ample capacity for adsorption in the bed. At t=b, the outlet solute concentration is observed to rise sharply eventually reaching the inlet concentration at t=c, owing to the adsorbents being saturated. The slope at the beginning of the breakthrough curve is influenced mainly by film transfer, whereas pore diffusion affects the mid-point slope. The capacity of an adsorbent bed that can be utilised is determined by the steepness of the breakthrough curve (Karau *et al.*, 1997), thus making the shape an important factor in determining the length of the adsorption bed.

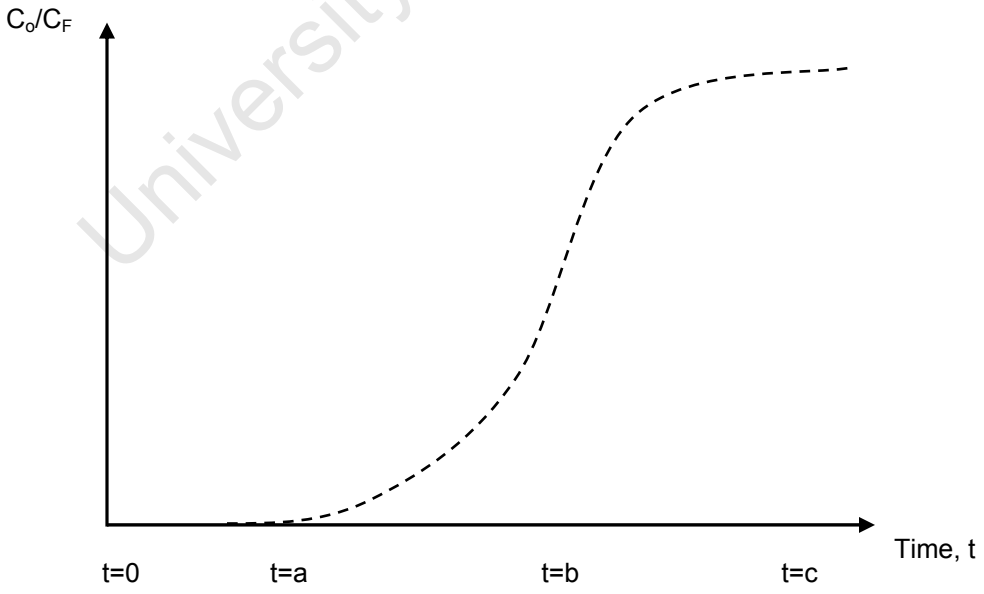


Figure 2.9: The practical breakthrough curve protein release from a column

2.9.2.4 Dynamic binding capacity

Dynamic binding capacity (DBC) is a measure of the amount of protein that can be loaded onto the bed under conditions of operation. DBC can be defined as the quantity of protein in the column per unit adsorbent volume, excluding the amount in the dead space i.e. the space between the height of the bed and the upper adaptor (Dong and Sun, 2001). The DBC is dependent on the feed flow velocity through the expanded bed, and the uptake rate of the desired protein under defined processing conditions such as pH, viscosity and conductivity (Chang and Chase, 1996b). The available binding capacity of the protein is also a function of molecular weight of the target substance and binding strength to the ligands on the matrix. The DBC decreases with an increase in flow velocity, ionic strength and with an increase in the viscosity of the feed due to the impact of mass transfer resistance created in the system (Fahrner *et al.*, 1999; Chang and Chase, 1996b; Amersham-Biosciences, 1997). The presence of cells, cell debris and other particulates with charged surfaces may also affect the DBC due to interaction that might occur with the adsorbent. The DBC can be determined from the breakthrough profile of the protein occurring at a certain percentage of the inlet concentration of protein, usually between 5 to 15% (Fernandez-Lahore *et al.*, 2000; Dong and Sun, 2001; Ng *et al.*, 2007).

2.9.2.5 Feedstocks characteristics in expanded beds

The properties of the starting materials are a major factor to be considered in an expanded bed operation since the interaction of the raw feed with an adsorbent is more complex than the application of clarified feedstock to a packed bed adsorbent (Fernandez-Lahore *et al.*, 1999). The composition of the feedstock depends on the media used for cultivation, organism type, cell pre-treatment and location of the accumulated product in the producing organism. Beyond the feed composition, the high fluid viscosity of the homogenate (due to biomass and biomolecules such as nucleic acid) is expected to be a major issue. Clarified samples, used in packed bed operation are less viscous (Barnfield-Frej *et al.*, 1994a; Lin *et al.*, 2003). Having knowledge about these feedstock characteristics will assist development and optimisation of the process.

Fluid viscosity is an important factor in an expanded bed. High fluid viscosity reduces the mass transfer coefficients between the adsorbent beads and the liquid (Chang and Chase, 1996a). The feed viscosity for an expanded bed is dependent on whether the

desired product is produced extracellularly or accumulated intracellularly. Undisrupted cell suspensions that do not contain extracellular polymers are typically of low viscosity, containing diluted low protein concentrations and contaminants, unless very high cell concentrations are used (~ 100 g l^{-1} DCW) or extracellular polysaccharides are produced. This simplifies the downstream processing. On the other hand, disruption of cells to release intracellular products is typically accompanied by increased viscosity due to the components released from the cell, particularly the nucleic acids, and the presence of fine particles in suspension (Chang and Chase, 1996b). A typical composition of a recombinant *E. coli* cell, relative to the location of desired product is shown in Table 2.5 and Figure 2.4. Highly viscous feedstocks may result in bed instability, thereby affecting adsorption performance in expanded beds. Therefore, it may be necessary to remove nucleic acids by precipitation or treat feedstock with nucleases prior to the application of the feedstock to the expanded bed (Chang and Chase, 1996a). Other contaminants may include charged particulates that act as ion exchangers and bind to the adsorbent, thereby fouling the adsorbent. Hence, it may be necessary to adopt an efficient CIP in expanded bed adsorption after each run (Chang and Chase, 1996b).

Table 2.7: Characteristics of a feedstock according to the location of the product in a recombinant *E. coli* (Amersham-Biosciences, 1997)

Cell component	Likely Feed components
Secreted	Dilute, low viscosity feed containing low amount of protein. Presence of proteases, bacterial cells and endotoxins. Likely cell lysis at low pH. DNA can be released and cause high viscosity
Cytoplasmic	Cell debris, high content of protein, lipid, DNA and proteases are present. Very thick feedstock. Intact bacterial cells and endotoxins are present
Periplasmic	Cell debris, high content of protein, lipid and proteases present. Feedstock may be viscous. DNA is present if cytoplasmic membrane is broken. Intact bacterial cells and endotoxins are present
Inclusion bodies	Cell debris, high content of protein, lipid and proteases are present. Very diluted solutions after renaturation. DNA and endotoxins are present. Precipitation of misfolded variants occurs in a time dependent manner

2.9.2.6 Influence of cells and cell debris on column performance

In a bacterial system, the liberation of accumulated products from the cytoplasm or periplasm by cell disruption results in a suspension containing cells, cell debris and other intracellular components such as nucleic acids and proteins (Table 2.7). Interactions of components of the crude feedstock with each other and adsorbents in the expanded bed system are very complex. Biomass interaction with an adsorbent matrix (through electrostatic or hydrophobic interaction and particulate size influencing collision) can affect the binding capacity of the target protein, as adsorbent particles with cells adsorbed to their surface vary in diameter and density, which then alters the pattern of fluidisation. This can result in bed instability, deviation from plug flow and reduced adsorption performance (Hubbuch *et al.*, 2006; Fernandez-Lahore *et al.*, 1999). Besides, a thorough cleaning in place condition will be needed to regenerate the adsorbent (Vennapusa *et al.*, 2008). These all affect the overall process efficiency and economics of the use of expanded bed (Feuser *et al.*, 1999; Lin *et al.*, 2003). Hence, it is necessary to control and reduce the interaction between particles in the feed and expanded adsorbent.

Several authors have reported the impact of cell-adsorbent interactions on bed stability and protein adsorption in expanded bed adsorption processes (Hubbuch *et al.*, 2006; Vergnault *et al.*, 2004; Lin *et al.*, 2004; Fernandez-Lahore *et al.*, 2000; Fernandez-Lahore *et al.*, 1999; Chang and Chase, 1996b; Barnfield-Frej *et al.*, 1994b; Chase and Draeger, 1992). Methods such as finite bath adsorption, biomass pulse response and residence time distribution (RTD) have been used to examine biomass-adsorbent interactions (Fernandez-Lahore *et al.*, 2000; Feuser *et al.*, 1999; Lin *et al.*, 2004). Lin *et al.* (2001a) characterised the methods and arranged them in a sequence for evaluation of bed stability in EBA operation. For the initial screening, the use of finite bath experiment was adopted to identify strongly interacting systems, while the pulse response method was used to define optimal operating conditions. The RTD measurement was presented as a tool for proving bed stability.

Most authors have studied the influence of biomass on adsorbent using *Saccharomyces* with few reporting the use of *E. coli* systems. Selected results from the literature are summarised in Table 2.8. Feuser *et al.* (1999) reported cell-adsorbent interactions in expanded bed adsorption of protein using a pulse response technique. The authors

investigated various systems such as bacteria, yeast and mammalian cells using a range of adsorbents (STREAMLINE AC, DEAE, phenyl and rProtein A). The authors reported that *E. coli* cells had the lowest binding tendency to all matrices. Specifically, only about 15% of *E. coli* biomass was retained within the fluidised anionic streamline DEAE bed, while a relative higher amount (about 50 to 75%) of *S. cerevisiae* and yeast homogenate biomolecules bound to the adsorbent (Table 2.8). In another study, Fernandez-Lahore *et al.* (1999) investigated the influence of whole yeast cells, yeast homogenate and bacterial homogenate on bed stability in an expanded bed. At long operational time, less than 2% *E. coli* led to extreme channeling, which in turn resulted into collapse of the expanded bed. The presence of nucleic acid (its charge and size) was suspected to be a major contributory factor.

Hence, the system was improved using a nuclease-treated feed to reduce the bridging effects within the fluidised system. This improved the hydrodynamic stability of the bed, and thus, up to 10% *E. coli* homogenate was applied to the fluidised bed. The nuclease-treated *E. coli* homogenate showed that the SP matrix was affected by the presence of biomass than the DEAE adsorbent (Table 2.8). Both authors attributed the relationship between the biomass and adsorbents to electrostatic interactions. Feuser *et al.* (1999) proposed and showed that increase in ionic strength can be used to reduce such electrostatic interaction. However, this reduces the binding capacity for target protein and often leads to a compromise between maximising protein adsorption and reducing cell-adsorbent interaction (Lin *et al.*, 2004). Fernandez-Lahore *et al.* (1999) and Lin *et al.* (2001a) also showed that the interaction between cells and adsorbents may also increase with increasing contact time. This interaction was considered irreversible, due to fouling resulting from cell to cell layering and adsorption of biomolecules in the feedstock.

Previous findings by Barnfield-Frej *et al.* (1994a) showed the impact of *E. coli* homogenate concentration in the feedstock on bed stability. The authors observed bed instability at a biomass concentration of over 8% DCW. This was associated with increased feed viscosity reducing the settling velocity of beads, resulting in the transport of adsorbent beads out of the column. Formation of flow channels was observed in the bed at the same biomass concentration. Biomass concentration therefore had to be limited.

Table 2.8: Comparative adsorption of biomass and protein by anion and cation exchangers

Adsorbent	Whole yeast suspension	Yeast Homogenate	*E. coli* homogenate	Comments	Reference
SP DEAE	0% 72%	0% 45%	15% 15%	Cell surface is negatively polarised at neutral pH. So, cells repel from cationic exchanger, while they attach to anionic resin through electrostatic effects between cells and adsorbent	Feuser *et al.*, 1999
SP DEAE	Reversible interaction between yeast and both adsorbent particles	Pronounced effect of biomass interaction with SP resin. Elutriation of biomass observed at high biomass. Modest effect on DEAE system hydrodynamics, though pronounced at high biomass concentration	SP more affected due to ligand effects. DEAE slightly affected	Fluidisation properties of matrix was studied in the presence of biomass	Fernandez-Lahore *et al.*, 1999
SP DEAE	< 15% 77%			Used hen egg white lysozyme and BSA as standard protein. Electrostatic interaction noted	Fernandez-Lahore *et al.*, 2000
DEAE	Increase in biomass concentration in the feed to 5% and 10% decreased the dynamic binding capacity of desired protein by 2.2 and 3.1 fold respectively.			Biomass/adsorbent interaction, biomass concentration and flow rate are major factors in maintaining bed stability.	Lin *et al.*, 2004
DEAE			Feedstock with biomass above 8% DCW resulted into channeling, while the expanded bed collapsed at DCW of 9.2%	Biomass/adsorbent interaction and high fluid viscosity likely to be responsible	Barnfield-Frej *et al.*, 1994a

Beyond affecting bed stability, the cell-adsorbent interaction may reduce the binding capacity of the target protein. This was reported by Chase and Draeger (1992). The authors reported the influence of cells on BSA adsorption to matrices, using various adsorbents. The charge interaction exhibited between the adsorbent and cells reduced the protein binding capacity. This was more pronounced with small organisms due to the presence of cells in the porous adsorbent structure. Fernandez-Lahore *et al.* (2000) showed a direct correlation between presence of cells in the feedstock and bed stability coupled with efficient protein adsorption.

Based on these reports from the literature, some consequences of cell-adsorbent interaction during EBA can be expected. Channeling and formation of stagnant zones arising from sample application may result in bed instability which may hamper protein binding capacity. The poor packing quality resulting from cell-adsorbent interaction may affect the elution mode and aggravate axial dispersion, which may result in distorted elution peaks. Harsh regeneration conditions may be needed to release tightly bound cellular materials. This reduces the life expectancy of the adsorbent as a result of irreversible fouling effects. These factors will in turn add to processing cost. Thus, it is necessary to assess the extent to which solid components (cells or cell debris and nucleic acids) from the feedstock bind to the adsorbent under process conditions and whether the interaction affects bed stability and protein binding during fluidisation of the resins in the feedstock. This will determine the relationship between adsorption efficiency and bed stability under process conditions. With this information, the process conditions can be optimised with regard to minimised interference of solid feed components with process performance.

2.10 RESEARCH MOTIVATION

This literature review has described the importance of biocatalysis in industrial processes and its advantages over the traditional chemical processes. The use of recombinant DNA technologies in enhancing enzyme potential improves biocatalysis attractiveness on an industrial scale. The classification of amidases as reviewed in the literature shows that these enzymes exhibit broad substrate specificities, ranging from aliphatic to aromatic amides, while some are even regarded as wide spectrum amidases. Some amidases display enantioselectivity and can be used to produce chiral compounds, others have thermostable properties, thereby enabling them to function

under high temperature conditions. This is a great advantage considering that high reaction rates can be achieved at such temperatures.

Based on the success of the few amidases already in use in industry and the potential of others, there is a need to develop an innovative, industrially applicable method for optimising total achievable protein yield through HCDC, the effective transcription and translation of amidase gene in recombinant *E. coli* and its subsequent recovery using an integrated protein purification process that will be applicable to general protein recovery. This will result in an economic process design which can impact on production cost.

2.11 RESEARCH OBJECTIVES AND KEY QUESTIONS

Based on the studies reviewed in the preceding sections and the motivation described above, the following objectives were developed for this project:

i. To maximise biomass productivity of amidase and enzyme expression by recombinant *E. coli* through study of cultivation conditions and enzyme induction.
ii. To investigate the influence of high biomass concentration on amidase expression using a fed batch process
iii. To design a process for reducing the total number of steps in amidase recovery from recombinant *E. coli* using expanded bed adsorption. The choice of adsorbent ligand and the impact of biomass-adsorbent interaction were identified as important factors to be considered.

While IPTG is used routinely to induce the expression of genes under the control of the *lac* promoter, the impact of high biomass concentration on IPTG induction has not been rigorously investigated previously. A key question unanswered to date is: Is the inducer used effective on a protein-biomass or volumetric concentration basis?

Chapter 3

Materials and Methods

3.0 INTRODUCTION

Chapter 3 describes the detailed experimental methodologies and materials used in this study. The experimental approach includes bioreactor optimisation of amidase expression in batch and fed batch studies and the subsequent enzyme recovery in one purification step using expanded bed adsorption as an integrated technology.

3.1 BACTERIAL STRAIN AND STOCK MAINTENANCE

A recombinant strain of *Escherichia coli* BL21 (DE3) was obtained by kind donation of Professor D.A. Cowan of the Institute of Microbial Biotechnology and Metagenomics, University of Western Cape, South Africa. The amidase gene from *Geobacillus pallidus* RAPc8 had been inserted into an expression vector, PNH223 and functionally expressed in *E. coli* BL21 (DE3) under an isopropyl β-D-thiogalactopyranoside (IPTG) inducer (Cameron *et al.*, 2005). The *E. coli* was sub-cultured every two weeks. The stock culture was maintained in glycerol at -70 ºC.

3.2 MEDIUM PREPARATION

At the initial stage of this study, three types of growth media were considered for high biomass concentration. Luria broth (LB) medium (tryptone 10 g l^{-1}, yeast extract 5 g l^{-1} and NaCl 10 g l^{-1}) was used as the complex medium, while the defined medium used was adapted from Korz *et al.* (1995) and is shown in Table 3.1. LB was supplemented with glucose as the carbon source to make the third growth medium. The initial concentration of glucose used for pre-cultures was 15 g l^{-1}. Preliminary shake flask

experiments were carried out using the three types of growth media to determine the growth rate and biomass concentration of the recombinant *E. coli.*

Table 3.1: Comparison of growth media for high biomass concentration

Medium composition	Defined medium (l^{-1})	Complex LB medium (l^{-1})	Supplemented complex LB medium (l^{-1})	Feeding solution for fed batch (l^{-1})
Glucose	25.0 g		25 g	250 g
KH_2PO_4	13.3 g			
$(NH_4)SO_4$	4.0 g			
$MgSO_4.7H_2O$	1.2 g			9 g
Citric acid	1.7 g			
EDTA	8.4 mg			13.0 mg
$CoCl_2.6H_2O$	2.5 mg			4.0 mg
$MnCl_2.4H_2O$	15.0 mg			23.5 mg
$CuCl_2.2H_2O$	1.5 mg			2.5 mg
H_3BO_3	3.0 mg			5.0 mg
$Na_2MoO_4.2H_2O$	2.5 mg			4.0 mg
$Zn(CH_3COO)_2.2H_2O$	13.0 mg			16.0 mg
$FeCl_3$	110.0 mg			40.0 mg
Antifoam	0.1 ml	0.1 ml *		0.1 ml
Tryptone		10 g	10 g	
Yeast extract		5 g	5 g	
NaCl		5 g	5 g	
Ampicillin	100 mg	100 mg	100 mg	100 mg

* Antifoam only added in bioreactor studies, not shake flask studies.

The LB medium was supplemented with 25 g l^{-1} glucose to determine whether this can support equivalent biomass production to the defined medium. The complex medium and the defined medium were compared under batch bioreactor cultivation (New Brunswick bioreactor). The growth medium that gave the highest biomass concentration was chosen for study of the enzyme induction in the Sixfors bioreactors and fed-batch studies in the New Brunswick bioreactor as described in Sections 3.3.1 and 3.3.2.

3.3 EQUIPMENT

3.3.1 NEW BRUNSWICK BIOREACTOR

The New Brunswick Scientific BIOFLO 110 series bioreactor system (USA) has a total internal volume of 7 litres. It is fitted with online monitoring of temperature, pH and dissolved oxygen to ensure preferred growth conditions during cell cultivation. The equipment is shown in Figure 3.1. The pH of the medium was measured with a pressurised gel-filled pH probe (Mettler Toledo). The pre-set value was maintained by the automatic titration of 5M NaOH and 5M H_3PO_4 for LB, while aqueous ammonia (25% w/v) was used for the defined medium. Dissolved oxygen was measured with a polarographic dissolved oxygen probe (Mettler Toledo) fitted with a Teflon membrane. Temperature was measured by a probe and maintained by the use of a heating jacket and cold water circulation through the internal cooling coil. Compressed filter-sterilised air was added through a ring sparger beneath the bottom impeller. Adequate mixing was ensured through the use of two, six blade Rushton impellers and four baffles.

Figure 3.1: The New Brunswick Scientific BIOFLO 110 bioreactor

3.3.2 THE SIXFORS BIOREACTOR

The Sixfors bioreactor system (Infors bioreactor, version 3.01, Switzerland) has six independent bioreactor units, each having a total internal volume of 350 ml (Figure 3.2). Dissolved oxygen and pH were monitored independently in each bioreactor and controlled to specified set-points. The pre-set temperature was maintained with heating and cooling supplied through the black heater block in which each vessel stands, shown in Figure 3.2. Aeration was achieved by sparging compressed filter-sterilised air through a sparger beneath the lower impeller. Adequate mixing was ensured through the use of a magnetically rotated stirrer shaft, fitted with two three-blade marine propellers and the presence of baffles.

Figure 3.2: The Sixfors bioreactor design (Infors, version 3.01, Switzerland)

3.4 METHODOLOGY

3.4.1 STERILISATION

The growth medium used for cell cultivation was sterilised by autoclaving at 121ºC for 20 minutes. For the defined growth medium, glucose and $MgSO_4$ were autoclaved separately to prevent their reaction with other salts. The components were combined after cooling and the pH was adjusted to pH 6.7 using 25% aqueous ammonia solution

(w/v). Ampicillin was filter-sterilised (Express® PES membrane, Millipore, Corp.) and added to all growth media used at a final concentration of 100 μg ml^{-1} to maintain plasmid selectivity and prevent the growth of non-recombinant *E. coli* strains.

3.4.2 CULTURE CONDITIONS

3.4.2.1 Precultures

LB was used to cultivate the pre-cultures to be used as inocula for the complex, semi-complex and defined medium experiments. The pre-inoculum of 30 ml was inoculated from an agar plate culture of *E. coli* BL21 (DE3) and incubated in a 250 ml Erlenmeyer flask for 12 h at 30°C with agitation at 180 rpm.

3.4.2.2 Batch shake flask experiments

At the preliminary stage of this study, shake flask experiments were conducted on the three types of growth media (complex, defined and glucose-supplemented complex) to determine the growth rate and biomass concentration. The pre-inoculum was transferred into a 270 ml culture medium in a 2000 ml shake flask (10%, v/v), and incubated for 11 h at 30°C and an agitation rate of 180 rpm on an orbital shaker. A total working volume of 300 ml was used to ensure adequate aeration for the cells.

3.4.2.3 Batch bioreactor cultivation and control

Typically, an inoculum volume of 1 to 20% (v/v) of the total working volume of the bioreactor is sufficient to ensure a dense biomass culture and shorten the lag phase (Bailey and Ollis, 1986). A three-step inoculation process was adopted to inoculate the working volume of 3 l and 300 ml used in the New Brunswick and Sixfors bioreactors respectively. A 10% inoculum (by volume) was used. The pre-inoculum (as described in Section 3.4.2.1) was transferred aseptically to a 270 ml growth medium in a 2000 ml Erlenmeyer flask and incubated for 12 h at 30°C with agitation at 180 rpm. Thereafter, the final inoculum was transferred into 2.7 l of either the complex or defined growth medium in the New Brunswick bioreactor or the 270 ml defined medium in the Infors bioreactors under aseptic conditions to provide a 10% inoculum. The process operation is summarised in Table 3.2. A pre-set value of pH 6.7 was maintained for LB and the defined medium.

Table 3.2: Batch operation conditions of New Brunswick and Sixfors bioreactors

	New Brunswick		Sixfors
	Complex	**Defined**	**Defined**
Working volume (l)	3	3	0.3
Temperature (ºC)	30	30	30
Aeration (vvm)	1	1	1
Agitation (rpm)	600	600	700
pH	6.7	6.7	6.7
Alkali	5M NaOH	25% NH_3 solution	25% NH_3 solution
Acid	5M H_3PO_4	None	None

The dissolved oxygen concentration was maintained at a minimum of 5% of air saturation by either increasing the agitation speed or air flow rate or both. Temperature was maintained at 30°C for the defined medium and LB respectively. Initial induction studies (such as time of induction) were carried out using the New Brunswick bioreactor, while the Sixfors bioreactor system (Figure 3.2) consisting of 6 independent bioreactors (300 ml) was used for the later IPTG induction studies to allow the use of identical inoculum across the experiments and broaden the range of inducer concentration studied for this research. The optimal IPTG concentration for amidase expression was determined by varying IPTG concentration from 0 to 1000 µM on a volumetric basis or 0 to 200 µmol g^{-1} biomass on a biomass basis. The IPTG concentration range was calculated based on the ratio of IPTG to cell concentration at the time of induction reported previously (Table 2.5).

3.4.2.4 Fed batch bioreactor cultivation

The fed batch process was carried out to increase biomass concentration. A New Brunswick Scientific BIOFLO 110 bioreactor (Figure 3.1) equipped with online pH, dissolved oxygen and temperature measurement and control was used. The cultivation was initiated in batch operation for 10 hrs. A simple exponential feeding strategy using defined medium (Table 3.1) was developed. Addition of glucose-rich feed (Table 3.1) started on depletion of the carbon source (glucose) in batch cultivation. The feed solution and the aqueous NH_3 (25% w/v) were each placed on a balance in order to allow the determination of the time-dependent volume of the culture broth, addition of

glucose, formation of acid and other process parameters. The dissolved oxygen concentration was maintained at 10% of air saturation by increasing the stirrer speed or blending air with pure oxygen or both. Foam was suppressed by adding an antifoaming agent (Sigma antifoam 204). Temperature and pH were maintained at 30°C and pH 6.7 respectively. Mixing was achieved at an agitation rate of 700 rpm and aeration rate of 1 vvm. Acetate concentration, plasmid stability and antibiotic potency were determined over the duration of the experiment (see Section 3.5 below).

3.5 ANALYTICAL METHODS

3.5.1 BIOMASS DETERMINATION

Biomass concentration was measured gravimetrically as cell dry weight and by absorbance at A_{660}. Samples of 1.5 ml were centrifuged (13 000 rpm, 10 min) in pre-dried (80 °C, 48 h) and pre-weighed microfuge tubes using an Eppendorf® centrifuge (model 5415 D). The supernatant was decanted and the cell pellet washed twice with distilled water. The washed sample pellets were dried at 80°C for 24 h and cooled in a desiccator prior to weighing to 4 decimal places to determine the dried biomass. Turbidimetric measurement at a wavelength of 660 nm (UNICAM Helios α, UV-Vis spectrophotometer) was used as an alternative to determine biomass concentration. To obey the Beer Lambert's law, samples were diluted until the absorbance reading was below an absorbance of 0.8. The culture absorbance was determined as the product of absorbance and the dilution factor.

3.5.2 GLUCOSE ANALYSIS

Residual glucose concentration in the culture supernatant was determined by a modified dinitrosalicylic acid colorimetric method (Miller, 1959). This method is based on the principle of conversion of dinitrosalicylic acid to form a reddish-brown colour due to the oxidation of the aldehyde group present in glucose to form a carboxyl group.

Clarified culture samples were diluted to obey Beer Lambert's law and subjected to the glucose assay using the DNS reagent described by Miller (1959). A 3 ml volume of diluted supernatant was added to 3 ml of DNS reagent in lightly capped test tubes. The mixture was heated in a water bath for 10 min at 90°C. Thereafter, 1 ml 40% (w/v) sodium tartate solution was added to the mixture, mixed and subsequently cooled. The

absorbance was read at a wavelength of 575 nm using a UNICAM Helious α, UV-Vis spectrophotometer. Standard glucose solutions (0.25 to 1.00 g l^{-1}) were subjected to the same treatment as the samples. The average coefficient of variance was calculated to be 3.9%. The standard curve and reagent preparation are given in *Appendix A1.*

3.5.3 ACETATE DETERMINATION

The concentration of acetate in clarified culture medium was estimated by high performance liquid chromatography (HPLC, Beckman, System Gold) using a glass lined Wakosil column, as modified from Moosa *et al.* (2002), with a UV detector (Detector no. 168), Phosphoric acid (20 mM, pH 2.5) was used as the mobile phase at a flow rate of 0.5 ml min^{-1}. Samples were diluted and filter-sterilised (using 0.22 µm Express® PES membrane, Millipore, Corp.) prior to analysis. Standard solutions (0.05, 0.1, 0.5 and 1.0 g l^{-1}) were prepared by performing serial dilutions on HPLC grade acetate using deionised water.

3.5.4 CELL DISRUPTION USING ULTRASONICATION FOR ANALYSIS

Biomass was harvested from cultures by centrifugation at 13,000 g for 10 minutes in an Eppendorf tube, washed and re-suspended in potassium phosphate buffer solution (50 mM, pH 7), prior to disruption by sonication on ice (Virsonic ultrasonic 100 cell disrupter, 50 W, 22.5 kHz). A total sonication time of 2 min was divided into 15 s treatments followed by 30 s rest time. A separate experiment in which cells were sonicated revealed this sonication interval and intensity released the bulk of the amidase activity from the cells. Complete disruption was ensured by viewing cells under the light microscope (Olympus model BX40). The crude extract was fractionated into supernatant and cell debris, and subjected to protein and amidase assays.

3.5.5 TOTAL SOLUBLE PROTEIN DETERMINATION ASSAY

The soluble protein concentration was determined by analysis of the supernatant following disruption using the spectrophotometric Bradford assay using Coomasie Brilliant Blue G – 250 (Bradford, 1976). A 100 µl aliquot of clarified cell lysate was added to 1 ml of Bradford solution in a 1.5 ml cuvette and vortexed thoroughly. The protein concentration was determined spectrophotometrically at 595 nm after 5 minutes of incubation at room temperature. Protein standards (10 to 100 µg ml^{-1}) were prepared using bovine serum albumin and treated as the samples. The standard curve, error

analysis and reagent preparation are detailed in *Appendix A2.* The average coefficient of variance was calculated to be 7%.

3.5.6 AMIDASE ACTIVITY DETERMINATION ASSAY

Amidase activity was determined using a modified phenol-hypochlorite ammonia detection method developed by Weatherburn (1967). This method is based on the conversion of amides to their corresponding acids and ammonia with subsequent quantification of the ammonia. The ammonia reacts with hypochlorite and phenol, catalysed by sodium nitroprusside, to form indophenol which yields an intense blue compound.

Amidase activity was determined in the clarified cell lysate. A 100 µl aliquot of reaction mixture (clarified lysate and 25 mM acetamide) was mixed with 500 µl reagent A (10 g l^{-1} phenol and 0.05 g l^{-1} sodium nitroprusside) and 500 µl reagent B (5 g l^{-1} sodium hydroxide and 0.0084 g l^{-1} sodium hypochlorite). The samples were vortexed and allowed to incubate at room temperature for 30 minutes. Absorbance was read at 625 nm using a UNICAM Helious α, UV-Vis spectrophotometer. Activity standards were prepared using ammonium chloride solution (0.1 to 0.5 mM) treated in the same manner as the samples. One unit of activity is defined as the amount of enzyme required to produce one µmol of ammonia within one minute under standard assay conditions (pH 7 and 50ºC). The average coefficient of variance was calculated to be 7.4% for ammonia assay. The standard curve and reproducibility data for quantification of ammonia is presented in *Appendix A3.*

3.5.7 SDS-PAGE GEL ELECTROPHORESIS

SDS-PAGE gel electrophoresis was used to determine the presence of amidase protein (in either active or inactive form) on the sample fractionation that followed cell disruption by sonication. The method, adapted from Laemmli (1970), used a 20% acrylamide gel in the presence of 0.1% SDS at 50 mA and 120V. Denatured samples were prepared by heating 200 µl of protein and 50 µl SDS sample buffer at 80°C for 5 minutes. The heated mixture was centrifuged at 13, 000 rpm for 1 minute to remove cell debris, and 100 µl of the supernatant was mixed with 20 µl of SAB and loaded onto the gel. The gel was stained with 0.25% Coomasie Brilliant Blue G-250 containing 10% glacial acetic acid and

50% absolute methanol. Excess stain was removed using a destaining solution of 10% glacial acetic acid.

3.5.8 ANTIBIOTIC DETECTION ASSAY

A simple agar disc diffusion method adapted from Davis and Stout (1971) and Domig *et al.* (2007) was used to detect ampicillin potency in clarified samples of culture medium in the fed batch operation. The diffusion assay for ampicillin employs a convenient and efficient agar disc plate method based on the diameter of inhibition zones to concentration of antibiotic in the culture medium. The agar medium made up of 30 g l^{-1} nutrient agar and LB (described in Table 3.1) was autoclaved (121°C, 15 min), poured into petri-dishes (at an approximate volume of 25 ml/plate) and allowed to cool down. The remaining agar was cooled down to about 45°C and inoculated with the wild-type strain of *E. coli* BL21 (DE3). This was poured onto the prepared agar plate as a upper layer. On solidification, circular wells of 6 mm in diameter were made aseptically in the agar plates, and 250 µl of the prepared supernatant from the culture medium was pipetted into the wells under aseptic conditions. The agar plates were incubated overnight at 37°C. The active antibiotic concentration present in the supernatant was correlated to the diameter of the inhibition zones (mm).

3.5.9 PLASMID STABILITY DETECTION

The stability of the plasmid in the recombinant *E. coli* strain was determined by a method adapted from Babaeipour *et al.* (2007). Samples taken from the bioreactor during cell cultivation were diluted using sterile distilled water and plated onto selective (200 mg l^{-1} ampicillin, 30 g l^{-1} agar) and non-selective (no ampicillin) LB agar plates to yield a nominal count of 30 to 300 cells per plate in the absence of ampicillin. This was done in triplicate. The fraction of plasmid-containing cells was calculated as the average ratio of viable colonies on LB with ampicillin to those on LB plates without the antibiotic.

3.5.10 ELECTRON MICROSCOPY

Disrupted *E. coli* cells were subjected to transmission electron microscopy to look for formation of inclusion bodies in the cells after induction with IPTG. Viewed samples were prepared by negative staining using 2% uranyl acetate. Approximately 3 µl of the cell lysate was applied to a carbon coated copper grid for 30 secs. Thereafter, the excess

solution was removed with filter paper. The grid was washed with a drop of distilled water and 5 drops of 2% uranyl acetate was dropped onto the grid. Excess stain was removed with filter paper and the grid was left to dry at room temperature. The sample was viewed on a Transmission Electron Microscope (TEM) LEO 912 omega (Germany). Photographs were captured using a 2k x 2k CCD camera.

3.6 AMIDASE RECOVERY USING EXPANDED BED ADSORPTION

3.6.1 CELL DISRUPTION

Cell disruption for the EBA operation was performed using high pressure homogenisation. The cells were recovered by centrifugation (10,000g for 10 minutes), weighed and 1% wet weight (w/v) suspended in 50 mM potassium phosphate buffer (pH 7) prior to disruption. Cell disruption was carried out using an APV Rannie AS high pressure homogeniser. Complete disruption was carried out by 5 passes at 41.4 MPa (6000 psi) and partial cell disruption was achieved by 2 passes at 13.8 MPa (2000 psi). Complete and partial cell disruption were confirmed by viewing the cells under an Olympus model BX40 light microscope. Low biomass concentration was used to avoid bed instability that might arise in the subsequent EBA study due to biomass-adsorbent interaction at high cell concentration (Lin *et al.*, 2004). The disruption conditions chosen are based on the results presented by Balasundaram (2004) and Balasundaram and Harrison (2006b) in which the effect of varying operating conditions of *E. coli* disruption on the extent of disruption was reported.

3.6.2 BATCH EXPERIMENTS TO DETERMINE OPTIMAL BINDING CONDITIONS FOR AMIDASE ENZYME TO ADSORBENTS

Two types of adsorbents were used in this study: anionic DEAE and hydrophobic SP beads. Shake flask experiments were conducted in a batch run to determine the optimal conditions for amidase adsorption to the adsorbent both in terms of protein recovery and selectivity. Initial studies were carried out on by varying the pH and conductivity. The solution pH was varied across the range using 0.1 M potassium phosphate buffers. Conductivity was investigated by varying the molar concentration of the potassium phosphate buffer solution across the range 25 to 100 mM at pH 7.7. The beads were washed with buffer solution three times prior to the start of experiment. Clarified cell lysate was added to the beads after centrifugation (13,000 × g, 4ºC, 10 mins), such that

500 µl beads, 500 µl buffer and 1 ml of enzyme solution were mixed gently on a platform shaker (about 50 rpm) for about 5 hours at room temperature. Total enzyme and protein adsorption to the adsorbent were calculated from the residual value, i.e. before and after the experiment. Enzyme loading per bead was also investigated by decreasing the bead concentration in the flask, while other factors such as the amount of enzyme suspension and buffer pipetted remained constant. The degree of protein binding to the beads was determined by comparing the experimental samples to the control (without adsorbent).

3.6.3 EXPANDED BED ADSORPTION IN THE COLUMN

The impact of biomass-adsorbent interaction was studied in an expanded bed column. The column used for the EBA studies was purchased from Soham Scientific Ltd. (Ely, UK). It had a diameter of 1.0 cm, with a total length of 45 cm. The column had a porous glass frit fitted to the lower end to act as a support for the bed and also as a flow distributor. The fitted glass frit was 2 mm in thickness, with a nominal pore size of 100 to 160 µm. The bed was irrigated by pumping liquid through it at a flow rate of 0.65 ml min^{-1} using an Ismatec peristaltic pump (Switzerland). The outlet of the column had an adaptor which can be adjusted manually to any point within the column during bed expansion. The liquid from the outlet of the column was transferred through silicon tubing to the flow cell, of a UV–visible spectrophotometer (Analytikjena, Specord 40) to the fraction collector. The proper vertical alignment of the column was ensured. A magnetic stirrer was used to ensure adequate mixing of the influent and hence reproducible conditions for the duration of the experiment. All experiments were performed at room temperature (approximately 22ºC). The column was filled with 10 ml adsorbent and equilibrated with phosphate buffer (minimum of 10 times the column volume) at the desired pH prior to the start of each run. Bed stability was ensured by using a bed expansion factor of 2.5 to 3 and the absence of flow channels and stagnant zones in the bed by visual inspection.

The impact of whole cells on protein adsorption to the bed was studied by using BSA as a standard protein. The BSA concentration of the 1% *E. coli* suspension was varied. The suspension was fed into the expanded bed column at a flow rate of 0.65 ml min^{-1}. The influence of partially and fully disrupted cell debris on protein adsorption to adsorbent was investigated. After each run, the beads were washed in the expanded bed mode with phosphate buffer to remove any residual cell debris. Protein was eluted using 1M NaCl and the adsorbent regenerated using 1M NaOH and stored in 20% ethanol for

further use. The re-use of the adsorbent beads, STREAMLINE DEAE and SP, as described for this study have been shown to exhibit identical characteristics to adsorbents used for the first time (Chang and Chase, 1996b).

3.7 EXPERIMENTAL PLAN

The aim of this study was to design a process for optimal production of amidase from recombinant *E. coli*. The optimisation study was divided into two portions: the bioreactor enzyme production stage and the integration of processes in the downstream sector through the use of expanded bed adsorption.

In the upstream stage (bioreactor), studies were carried out to both maximise biomass productivity and induction of the amidase enzyme. The composition and choice of culture medium was studied. Since protein production is dependent on biomass concentration, the aim of this approach was to choose a growth medium that will support high cell density cultivation. Induction studies using IPTG were carried out in the bioreactor to optimise amidase specific production in terms of inducer concentration and time of addition. Finally, fed batch studies were conducted to investigate further enhancement of biomass productivity with concomitant enzyme induction.

Further, the use of expanded bed adsorption was investigated in the downstream process train for amidase purification directly from crude cell homogenate to reduce unit operations involved in amidase purification. Reduced unit operations and affinity separation processes are expected to enhance recovery and yield, thereby enhancing overall productivity.

Thus, it was anticipated that the overall design of optimised bioreactor stage, coupled with the use of EBA, may be effective in optimising the production of thermostable amidase from recombinant *E. coli.* Further, it was expected that the optimised design may have elements of general applicability for optimal recovery of a variety of recombinant proteins from microbial sources.

Chapter 4

Bioreactor optimisation studies

4.0 INTRODUCTION

Cost-effective production of heterologous protein can be achieved by optimisation of protein expression through molecular methods or bioprocess strategies (Weickert *et al.*, 1996; Donovan *et al.*, 1996). In the best case, both methods are combined to enhance maximal production of the recombinant protein. In this study, a recombinant *E. coli* strain genetically modified to produce a thermostable amidase enzyme was used (Cameron *et al.*, 2005). To enhance production further, a bioprocess-based approach was adopted. This is focused on growing *E. coli* to high cell concentration and also the manipulation of induction conditions for effective transcription and translation of the amidase gene in recombinant *E. coli*. Both batch and fed batch processes are considered.

4.1 EXPERIMENTAL APPROACH

The influence of medium composition on biomass concentration was investigated by comparing growth on complex LB medium, glucose supplemented LB medium and a glucose-based defined medium. Initial experimental studies for the choice of growth medium were carried out in shake flasks. Thereafter, the two most promising growth media were compared at the bioreactor scale in the 7-L New Brunswick bioreactor. This afforded the opportunity to control the growth conditions such as aeration and pH. The medium with the most favourable result was chosen for further induction studies.

IPTG was used as the inducer of expression of the recombinant amidase enzyme. The optimal time of induction in the growth cycle for maximal expression was studied using

the 7-L New Brunswick bioreactor. The effect on inducer concentration was studied by comparing six concentrations of IPTG in the range 0 to 1000 μM IPTG on a volumetric basis and 0 to 200 μmol g^{-1} on a biomass basis. Thereafter, a fed batch process was developed through exponential feeding of a glucose-based medium and use of oxygen enriched air. The objective of this approach was to increase biomass concentration thereby increasing volumetric amidase expression, provided specific amidase expression was maintained. Further increase in volumetric expression would be augmented by increased specific amidase expression. However, enhanced amidase production depends on other factors such as partitioning of available carbon during cell cultivation, plasmid stability and the maintenance of the selective pressure of the recombinant gene in the dense *E. coli* culture. These factors were investigated in the fed batch process.

4.2 REPRODUCIBILITY OF EXPERIMENT

Reproducibility of the biomass cultivation and amidase induction studies was evaluated by repeating the experiments at least twice under identical conditions. Results from the fed batch study (Figure 4.1) in which induction was initiated at early exponential phase (3^{rd} hour, using 400 μM IPTG) in batch culture with continuous addition of inducer from the start of feeding (10^{th} hour, 400 μM) was used to demonstrate reproducibility. For each of the experimental runs, the same *E. coli* strain was used, but one was from the sub-cultured set (sub-cultured fortnightly from the stock over 6 months),run 1, while the other was directly from the stock culture, run 2 (Section 3.1). This was to confirm the integrity of the cells in terms of growth parameters and amidase expression over a period of six months.

Reproducibility was expressed in terms of the coefficient of variance (CV), that is, the standard deviation expressed as a percentage of the mean. The average CV values for biomass and specific amidase activity data were determined to be 9.6 and 23% respectively. The standard deviations of duplicate experimental analysis for both parameters were less than 15%. This shows acceptable reproducibility and hence confirms that the cells were unchanged over the duration examined.

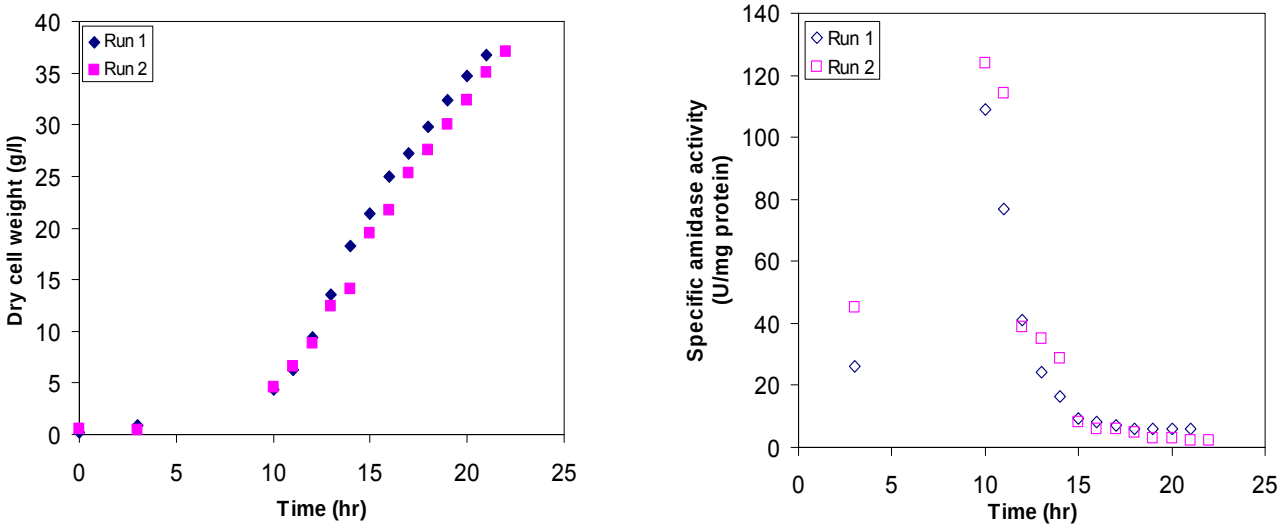


Figure 4.1: Fed batch studies of amidase expression (with a final IPTG concentration of 400 µM) from recombinant *E. coli*, showing dry cell weight and specific amidase activity

4.3 EFFECT OF MEDIUM COMPOSITION ON BIOMASS YIELD

To compare biomass accumulation as a function of medium, both shake flask and 7 litre batch bioreactor experiments were used. Cell growth and biomass accumulation were monitored over a period of 10 hours through measuring culture absorbance at 660 nm and determination of dry cell weight. The growth profiles generated using the bioreactor are presented in Figure 4.2. The specific growth rates and maximum biomass concentrations are given in Table 4.1. The correlation of dry cell weight and absorbance data gave a regression value of 0.98, allowing absorbance to be used as a rapid measure of biomass concentration.

The specific growth rate achieved in the LB and glucose supplemented LB medium of 0.45 h^{-1} was 1.5 fold that achieved with defined medium in the shake flask (0.30 h^{-1}). Similarly, an increase of about 1.8 fold was observed with the complex LB (0.69 h^{-1}) over the defined medium (0.38 h^{-1}) in the bioreactor experiment (Table 4.1). Complex media have been reported to support higher specific growth rates than defined media. This is due to the presence of biosynthetic intermediates and growth factors (Yee and Blanch, 1992; Durany *et al.*, 2004; Lutomski *et al.*, 2004). The presence of these intermediate metabolites usually leads to a reduction in cell metabolic burden, as the cells do not have to synthesize intermediates *de novo* (Yee and Blanch, 1992).

The defined medium supported the cell growth over an extended period, leading to a 5 fold increase in cell concentration as dry biomass over complex medium in the bioreactor (Figure 4.2). A lower increase of approximately 2 fold was reported using glucose supplemented medium. The addition of glucose to the LB medium did not result in further increase in the biomass concentration or the specific growth rate. Glucose utilisation remained low, suggesting another limiting agent in this medium (Table 4.1). This was also concluded by Hoffmann *et al.* (2004) and Kweon *et al.* (2001), who added concentrated glucose solution to the complex medium in *E. coli* cultivation to increase specific product concentration.

In the absence of complex media components, the overall yield coefficient ($Y_{x/s}$) of biomass on glucose in defined medium was 0.14 g g^{-1} in shake flasks and 0.33 g g^{-1} in the bioreactor.

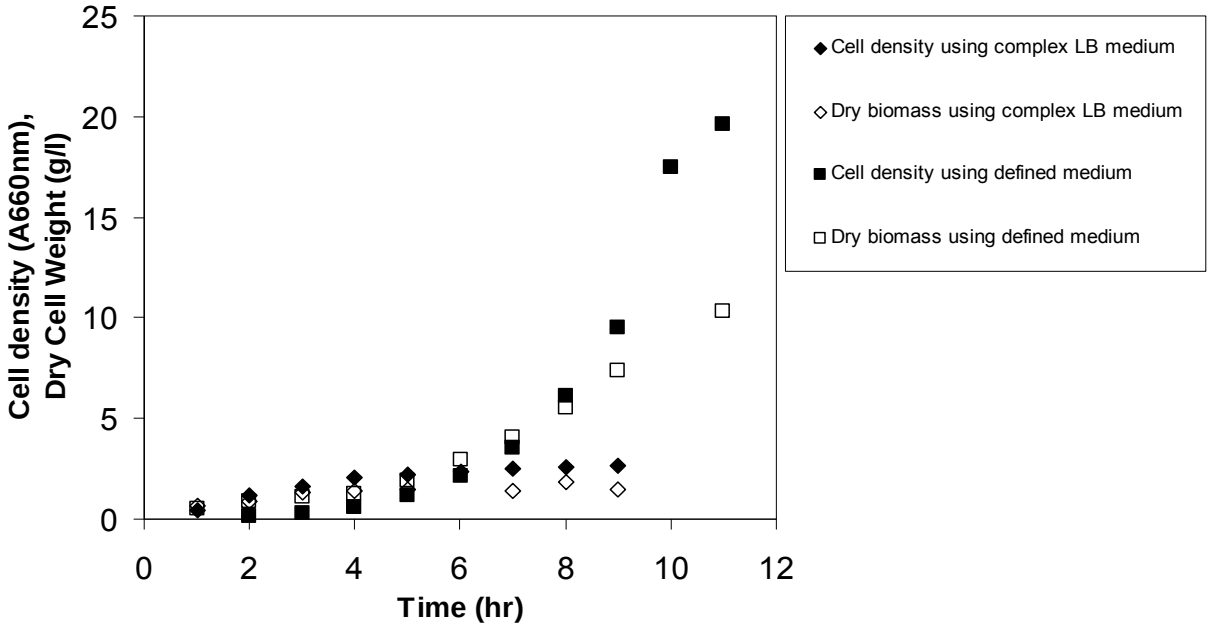


Figure 4.2: Effect of growth medium on the growth of *E. coli* BL21 (DE3) measured as absorbance and dry weight on cultivation in the New Brunswick Bioflo bioreactor at 30ºC and pH 6.7

Table 4.1: Effect of medium composition on growth rate and biomass concentration in shake flask culture and in the New Brunswick Bioflo bioreactor at 30ºC and pH 6.7

Parameters	Shake Flask			Bioreactor	
	Complex Luria Broth	Complex Luria Broth with 25 g l^{-1} glucose	Defined medium with 25 g l^{-1} glucose	Complex Luria Broth	Defined medium with 25 g l^{-1} glucose
Specific growth rate, $\mu_{max}(h^{-1})$	0.45	0.46	0.30	0.69	0.38
Correlation coefficient for growth rate determination	-	-	-	0.90	0.99
Maximum Biomass density (A_{660nm})	1.80	1.60	2.99	2.60	19.64
Final biomass C_x (g l^{-1})	1.02	0.91	1.70	1.80	9.40

Similar studies of recombinant protein production by Srivastava and Mukherjee (2005), Durany *et al.* (2004) and Kweon *et al.* (2001) have suggested that the nutrient content of LB is insufficient to support cell growth over a longer period.

Srivastava and Mukherjee (2005) reported approximately 5 - and 2 - fold increases in biomass and specific human interferon alpha yield respectively on using a glycerol-based defined culture medium in place of complex LB medium. Durany *et al.* (2004) observed a two fold increase in biomass concentration was observed using a glucose-based defined medium over LB medium. Further, the authors showed that proper folding and accumulation of the recombinant proteins is enhanced using defined medium in preference to complex medium. These authors reported a 4 fold increase in volumetric aldolase activity using defined media over complex medium at same cell concentration. Therefore, defined medium is preferred for higher biomass.

Furthermore, the use of a defined medium ensures reproducibility, enhanced plasmid stability, manipulation of growth strategies and easier control of growth culture such as limiting substrate availability (Hoffmann *et al.*, 2004; Yee and Blanch, 1992). Since

product yield is a function of biomass concentration, further experimental studies in this thesis were conducted using the glucose-based defined medium.

4.4 EFFECT OF TIME OF INDUCTION ON CELL GROWTH AND PROTEIN EXPRESSION IN BATCH PROCESS

Cell growth in a batch process may be considered in three stages: lag, exponential and the stationary phase. In order to maximise amidase production, the optimal growth phase for inducing recombinant protein expression was determined.

Induction of protein expression during the early growth phase may be expected to provide maximal opportunity for protein expression while induction at the later growth phase avoids negative impact on growth. The dominant effect was determined by inducing amidase expression with 400 µM IPTG at the 5^{th} and 8^{th} hr of cell cultivation, representing the early and mid exponential phase of growth respectively. The data collected are presented in Figure 4.3 and Table 4.2 respectively. Further, these are compared to the control in the absence of IPTG induction in Table 4.2. Induction at early and mid exponential phase gave similar profiles of dry biomass concentration (Figure 4.3). Soluble protein concentration was found to increase by a factor of approximately two following IPTG addition, compared to the process without inducer (Table 4.2). Little difference was observed in protein concentration as a function of time of induction. Without induction, the volumetric amidase activity and specific amidase activity achieved were 9.51 U ml^{-1} and 1.66 U mg^{-1} protein respectively. These were improved when amidase was induced at either early or mid phase of cell growth. The volumetric activity achieved when amidase was induced at the early (664 U ml^{-1}) and mid (207 U mg^{-1} protein) exponential phase of growth was 70 and 22 fold respectively to that achieved without inducer. Similarly, an increment of approximately 45 and 12 fold in specific amidase activity was observed with induction at early and mid exponential phase of growth respectively (Figure 4.3; Table 4.2).

Table 4.2: The effect of induction time on amidase expression by recombinant *E. coli* in a batch process (New Brunswick Bioflo bioreactor) using 400 µM IPTG

	No Induction	Induction at early exponential phase (5^{th} hr)	Induction at mid exponential phase (8^{th} hr)
Biomass (g l^{-1})	9.40	9.40	10.03
Protein concentration (mg ml^{-1})	5.73	9.02	9.65
Volumetric amidase activity (Units ml^{-1})	9.51	663.69	206.97
Specific amidase activity (Units mg $protein^{-1}$)	1.66	74.96	19.82

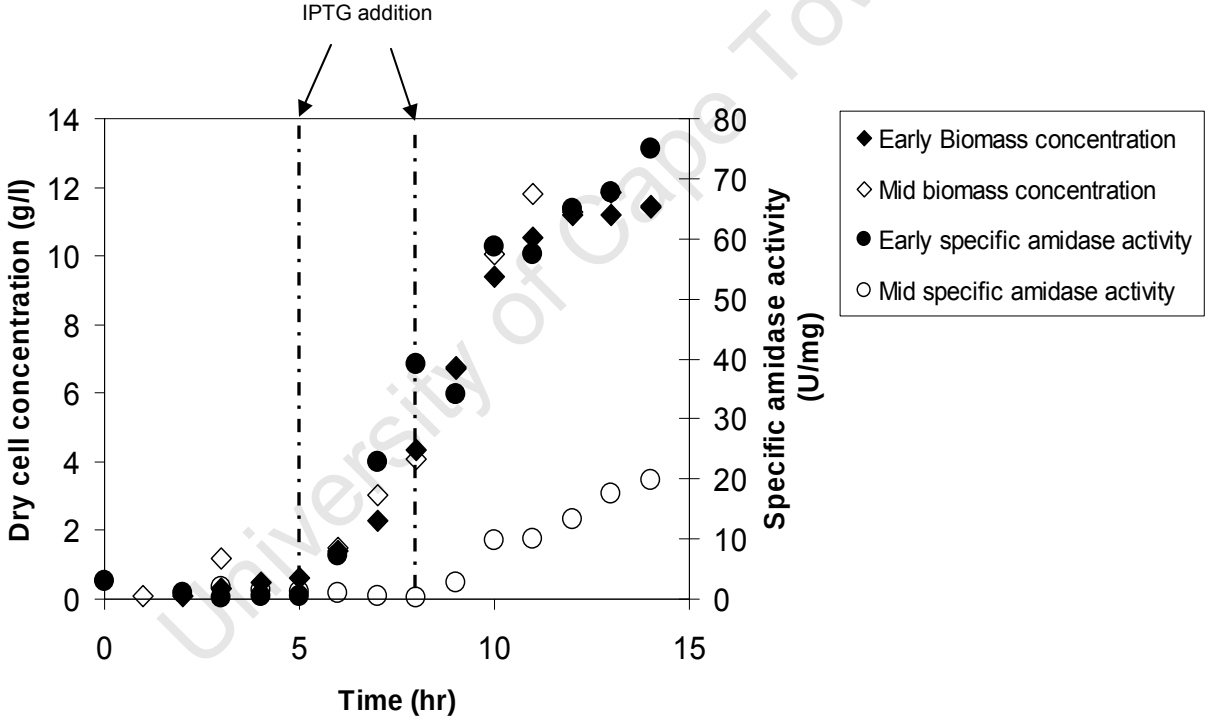


Figure 4.3: Effect of time of inducer addition on formation of biomass, total soluble protein and volumetric amidase activity comparing the early (5^{th} hr) and mid exponential phase (8^{th} hr) using 400 µM IPTG in the New Brunswick Bioflo reactor

Low productivity that accompanies recombinant protein induction at the later stage of exponential or stationary phase of cell growth has been attributed to a decrease in carbon source and other nutrients (Duan *et al.*, 2000) and impact of extracellular inducer concentration (Miao and Kompala, 1992). This, in turn, results in reduced cell activity during this phase of growth. Since no significant difference was observed in biomass

concentration, reduced cell activity at mid exponential growth phase may explain the results presented in Figure 4.3 and Table 4.2. Similar trend of results attributed to reduced cell activity at late log phase of growth have been reported by several authors. Chen *et al.* (1997) investigated the timing of IPTG addition in the production of chitinase by recombinant *E.coli*, and observed that chitinase production was maximum following induction at the initial exponential phase of growth and decreased rapidly after the third hour. A similar report was presented by Durany *et al.* (2004) and Lim *et al.* (2000) for maximum production of recombinant aldolase and interferon-α respectively. A study on time of induction by Bentley *et al.* (1991) indicated that a depleted level of nutrient in the stationary phase of cell cultivation resulted in low growth rate and initiated protease activity. This, in turn, led to reduced protein yield. Therefore, the authors induced protein expression during the mid-exponential phase of cell growth for maximum product yield while also achieving a high cell density. Kim *et al.* (2007) varied induction conditions such as time of IPTG addition, cell densities, duration of induction (3 to 6 hrs) and concentrations of IPTG (up to 1 mM), using different strains of *E.coli*. They observed that resilin production was not affected noticeably. These authors concluded that recombinant protein expression at different times of the growth phase is dependent on the effects of the inducer on the cells and host-target gene relationship.

Hence, results from the present study (Table 4.2 and Figure 4.3) suggest that reduced cell activity at the mid exponential phase of growth contributed to an observed decrease in amidase activity. In this system, IPTG did not affect biomass formation significantly, hence early induction was favoured.

4.5 EFFECT OF INDUCER CONCENTRATION

4.5.1 EFFECT ON CELL GROWTH AND PROTEIN PRODUCTION

It was necessary to study the effect of IPTG concentration on cell growth as the overall volumetric yield of amidase is a function of both the cell yield and the yield of amidase per cell. The *E. coli* growth profile under IPTG induction of recombinant amidase expression across the concentration range 0 to 1000 μM is presented in Figure 4.4a. The inducer concentration in the range 0 to 1000 μM did not significantly affect cell growth when induction was carried out at the early exponential phase of growth. The cell specific growth rate without induction (0.5 h^{-1}) was little affected when amidase was induced with IPTG up to 1000 μM. The dry biomass data were subjected to inference

statistical analysis using the t test at 95% confidence limit. No significant difference ($P < 0.05$) in growth was noticed comparing the control (no induction) and induction at IPTG concentration over the range 40 to 1000 μM. This indicates that the consequent metabolic response after cell induction did not result in limitation of growth.

This agrees with observations reported by Durany *et al.* (2004) and Dedhia *et al.* (1994), that neither the maintenance of antibiotic selection plasmid in a recombinant *E. coli* nor the extra expression of the target protein when induced (50 – 500 μM IPTG) caused a metabolic load sufficient to influence the maximal growth rate or the growth profile. The authors reported that the recombinant strain cultures showed a maximum specific growth rate equivalent to the plasmid-free wild type. In another study by Srivastava and Mukherjee (2005), the authors reported negligible decline in cell growth rate following induction with IPTG up to 1000 μM. This was attributed to the presence of exogenous metabolites to support cell growth.

In contrast, some authors have reported that the induction of expression diverts metabolic fluxes from biomass production towards recombinant protein synthesis. Wood and Peretti (1991) studied the impact of recombinant mRNA synthesis on cellular activities of *E. coli* following induction. At inducer concentration below 0.1 mM IPTG, the synthesis rate and steady state levels of ribosomal RNA were increased, while a decrease was noticed at higher inducer concentration. The authors attributed the observed reduction in growth rate and plasmid stability at high inducer concentration (> 0.1 mM IPTG) to energy diversion for protein production as the cells tend to balance the metabolic load of gene expression and cell growth.

Similar observations have been noted by Chen *et al.* (1997) who showed reduction in specific cell growth after induction. They credited it to deviation of metabolic flux away from biomass production towards recombinant protein synthesis. Further, Teich *et al* (1998) suggested that plasmid amplification following cell induction impacts biomass accumulation.

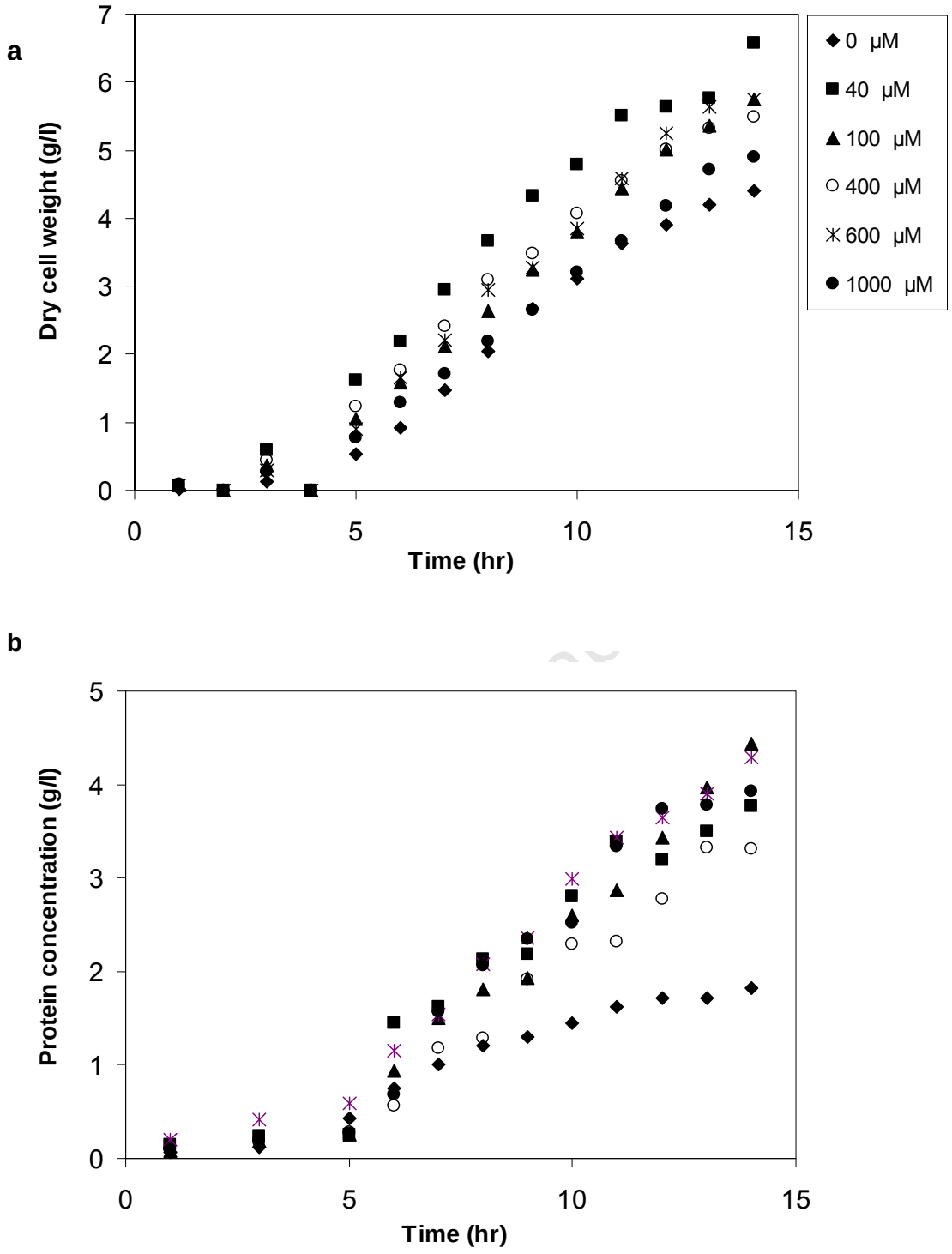


Figure 4.4: Effect of IPTG concentration, 0 – 1000 µM, on (a) biomass accumulation; (b) total soluble protein, following induction of the *E. coli* culture at early exponential phase in the Infors bioreactor at pH 6.7 and temperature of 30ºC

The results presented in this study (Figure 4.4a), coupled with previous investigation by various authors, emphasise that the effect of recombinant protein expression on cell growth and biomass accumulation varies between systems. This could be due to system specificity, properties of the recombinant protein and the host strain.

Further, in this investigation of amidase expression, the effect of IPTG concentration on the total soluble protein in the crude extract was determined and is shown in Figure 4.4b. Protein concentration increased up to two fold on adding IPTG at 40 µM. No significant further increase was noticed on increasing the IPTG concentration beyond 40 µM. This is comparable to the study by Yildir *et al.* (1998). The authors observed a two fold increase in total protein concentration at induction, with no further improvement following increase in IPTG concentration from 0.1 to 1.0 mM. Further, the ratio of protein to biomass increased from the typical 0.5 (prior to induction) to a range of 0.7 to 0.9 (following induction).

Table 4.2 and Figure 4.4 show that biomass concentration is one of the factors determining protein concentration. A higher biomass concentration of about 10 g l^{-1} DCW was achieved using the New Brunswick bioreactor (Figure 4.2), while about half of this was harvested using the Infors bioreactor (Figure 4.4). This was reflected in the total protein concentrations of 9 g l^{-} and 4 g l^{-1} determined, as presented respectively in Table 4.2 and Figure 4.4.

4.5.2 EFFECT OF INDUCER CONCENTRATION ON AMIDASE ACTIVITY

For large scale processes, it is necessary to reduce inducer concentration to the effective minimum to ensure economic feasibility. In the present study, amidase production was induced by IPTG concentration in the range 0 to 1000 µM using the Sixfors bioreactor. The result is expressed in terms of specific activity, both as amidase activity per unit biomass and per unit protein.

As presented in Figure 4.5, specific amidase activity in the presence of IPTG at 40 µM was approximately 50 fold higher than in the absence of the inducer. Although it has been suggested that the optimal IPTG concentration using *tac*- or other *lac*-derived promoters for the expression of soluble cytoplasmic recombinant proteins is about 1mM (Donovan *et al.*, 1996), results presented in Figure 4.5 show that maximum specific

amidase activity was obtained with induction using 400 μM IPTG concentration. The value of 180 U mg^{-1} protein represents a 9 and 6 fold improvement over the value reported by Makhongela *et al.* (2007) and Cameron *et al.* (2005) respectively. Both authors previously purified *G. pallidus* RAPc8 amidase from recombinant *E. coli* using LB as growth medium and an IPTG concentration of 400 μM. No improvement in specific amidase activity was observed above this concentration. The specific amidase production reached a maximum level 6 hours post-induction, corresponding to 11 hours post inoculation (Figure 4.5). There was no observable increase in amidase activity beyond this time.

Since no cell growth retardation or expression in insoluble inclusion bodies was observed (discussed in section 4.6), results shown in Figure 4.5 could be explained on the basis of probable complete titration of the repressor gene by IPTG, such that additional IPTG did not further increase transcription significantly. This can be further supported by examining amidase activity per unit biomass as a function of IPTG concentration in Figure 4.6.

In Figure 4.6, it is seen that the maximum specific activity was reached at an IPTG concentration of 80 μmol IPTG g^{-1} biomass (corresponding to a volumetric concentration of 400 μM), with no further improvement being attained on increasing IPTG concentration. Varied reports on the optimal inducer concentration for protein expression in recombinant systems are summarised in Table 4.3. Researchers have used IPTG concentrations in the range of 0 to 10000 μmol l^{-1}, with the optimal concentrations reported varying between 30 and 1000 μM across the recombinant batch systems.

Typically, IPTG concentration in the range of 100 to 1000 μmol l^{-1} has been reported for achieving maximum protein expression. On considering amidase expression specifically, Fournand *et al.* (1998) reported that the addition of IPTG to recombinant *E. coli* cells did not increase amidase biosynthesis while Doran *et al.* (2005) observed that amidase expression was the same over a range of IPTG concentrations 0.1 to 2.0 mM. Neither study is in agreement with the dependence on IPTG concentration reported here.

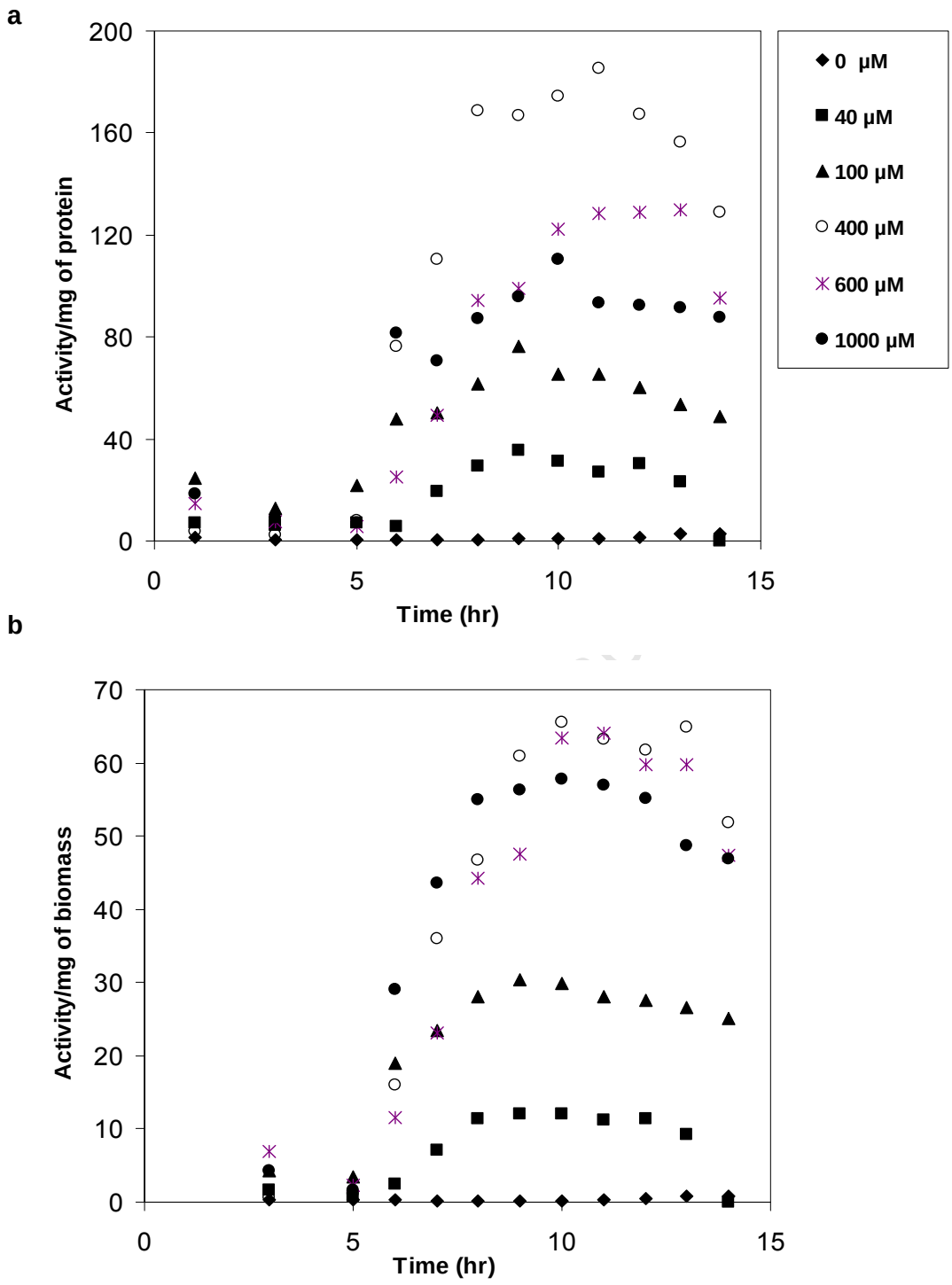


Figure 4.5: Amidase production expressed as (a) Amidase activity per mg of protein; (b) Amidase activity per g of DCW, with IPTG concentration in the range 0 to 1000 μM and induction at early exponential phase (5th hour) using the Sixfors bioreactor

Table 4.3: Comparison of optimal IPTG concentration for maximum protein expression in various recombinant systems

System studies	Range of IPTG concentration (μmol L^{-1})	IPTG Concentration achieving maximum enzyme activity		Reference
		Volumetric (μmol l^{-1})	Biomass (μmol g^{-1})	
E. coli and *Phytolacca Insularis* protein	0–1000 (batch), 3000–6000 (Fed-batch)	1000 (Batch) 6000 (Fed-batch)	68.5 (Batch) 101.7 (Fed batch)	Kweon *et al.*, 2001
E. coli regulated by T7 promoter	0 - 0.05 g L^{-1}	210	105	Miao and Kompala, 1992
E. coli and Chitinase	0 – 2000	500		Chen *et al.,* 1997
E. coli XL1 Blue MRF' and aldolase	0 – 500	100	41	Durany *et al.,* 2004
E. coli BL21(DE3)	100 – 1000	1000		Srivastava and Mukherjee, 2005
E. coli and *Vitreoscilla* Haemoglobin	0 – 500	500	100	Tsai *et al.*, 1996
E. coli BL21(DE3)pLysS and amidase	0 – 2000	100		Doran *et al.,* 2005
E. coli and Glutamyltranspeptidase	0 – 10000	100		Yao *et al.,* 2006
E. coli BK6 and β-galactosidase	0 – 7500	1000		Wood and Peretti, 1991
E.coli M15 and aldolase	0 - 1000	30	20	Vidal *et al.*, 2005
***E.coli* BL21(DE3) and amidase**	**0 – 1000**	**400**	**80**	**This study**

In a study by Wood and Peretti (1991) to understand limitations to increased levels of cloned gene expression of β-galactosidase, IPTG concentrations were varied from 0.0 to 7.5 mM. The authors observed a linear trend for increase in enzyme activity up to IPTG concentration of 1.0 mM, beyond which a plateau was formed. This was attributed to the likely complete titration of the *lac*I repressor protein by IPTG, such that additional IPTG did not further increase transcription of the *lac*Z gene significantly. Meanwhile, other

studies have reported formation of inclusion bodies at higher inducer concentration (Lim *et al.*, 2000), as well as retardation in cell growth at IPTG concentrations over 0.5 mM (Chen *et al.*, 1997). As suggested by Sivakesava *et al.* (1999), Teich *et al.* (1998) and Lee *et al.* (1997), the concentration of an inducer required for optimal protein expression depends on several factors and features of the host-vector system. Such factors could include complete titration of the repressor gene by the inducer, inhibition of ribosomal RNA synthesis and degradation of ribosomes after induction

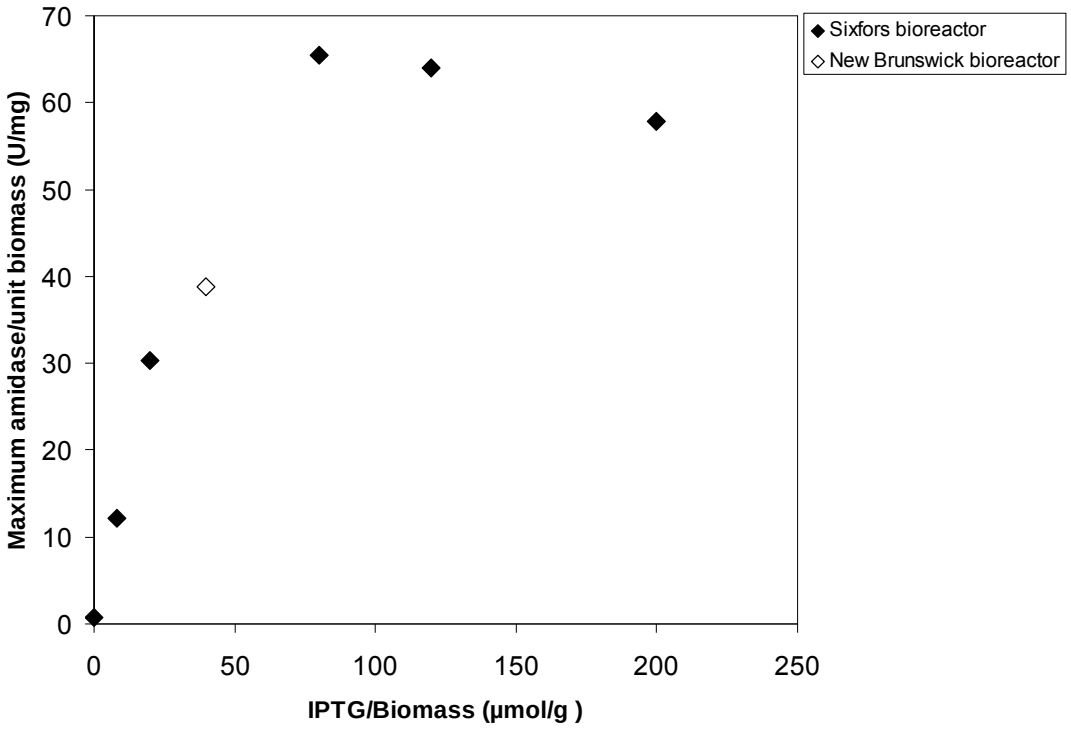


Figure 4.6: Impact of IPTG per biomass concentration on specific amidase expression

4.5.3 BIOMASS AND VOLUME AS BASIS OF INDUCTION

Many researchers induce expression of recombinant genes with varying the volumetric inducer concentration without the justification for such. From Table 4.3, IPTG concentrations in the range of 0.1 to 10.0 mM were used by the authors without reference to the biomass concentration. The *lac* operon, illustrated in Figure 2.3 is controlled by a repressor which prevents transcription or can allow transcription by conformational change upon binding with an inducer such as IPTG. Thus, it may be expected theoretically that the amount of inducer required to suppress the repressor is

proportional to the product of the cell concentration and frequency of the *lac* operon per cell. Hence, a simple comparison was made using data from the New Brunswick cultivation experiment (Figure 4.3) and the Sixfors cultivation process (Figure 4.4). An IPTG concentration of 400 μM was used in both experiments while the biomass concentration differed. The New Brunswick and Sixfors experiments yielded final biomass concentrations of approximately 10 and 5 g l^{-1} respectively, thus resulting in 40 and 80 μmol IPTG g^{-1} cell on biomass basis.

The result presented in Figure 4.7 is expressed in terms of specific activity, both as amidase activity per unit biomass and per unit protein. In both bioreactor systems, maximum specific amidase activity was observed at the 10th hour of cell cultivation. At this point, the specific amidase activity in the New Brunswick system (34 U g^{-1} cell) was about half the observed value in the Sixfors (65 U g^{-1} cell), while the biomass concentration in the former was higher. It can be concluded that the IPTG concentration present in the more dense culture at 10 g l^{-1} (40 μmol IPTG g^{-1} cell) was insufficient to fully titrate the cells, thereby resulting in approximately half the activity observed in the less dense culture of 5 g l^{-1} (80 μmol IPTG g^{-1} cell). Further, the influence of biomass can be emphasised as shown in Figure 4.6. The maximum amidase per unit biomass attained in the New Brunswick system using 40 μmol IPTG g^{-1} cell (34 U g^{-1} biomass), falls almost on the same trend when compared with values from that Sixfors bioreactor experiments. However, the trend differs largely when inducer concentration is compared on a volumetric basis (Figure 4.7), thus suggesting the importance of biomass as basis for induction of protein expression.

In studies involving high cell density cultures, Vidal *et al.* (2005), Durany *et al.* (2004) and Kweon *et al.* (2001) have suggested that the amount of inducer needed to fully induce protein expression ought to be in accordance with the expected biomass accumulation (Table 4.3). Hence, although it has not been practiced routinely in literature studies, it is apparent from this study and those of Vidal *et al.* (2005), Durany *et al.* (2004) and Kweon *et al.* (2001) that the expected biomass concentration should be taken into account when inducing recombinant protein expression.

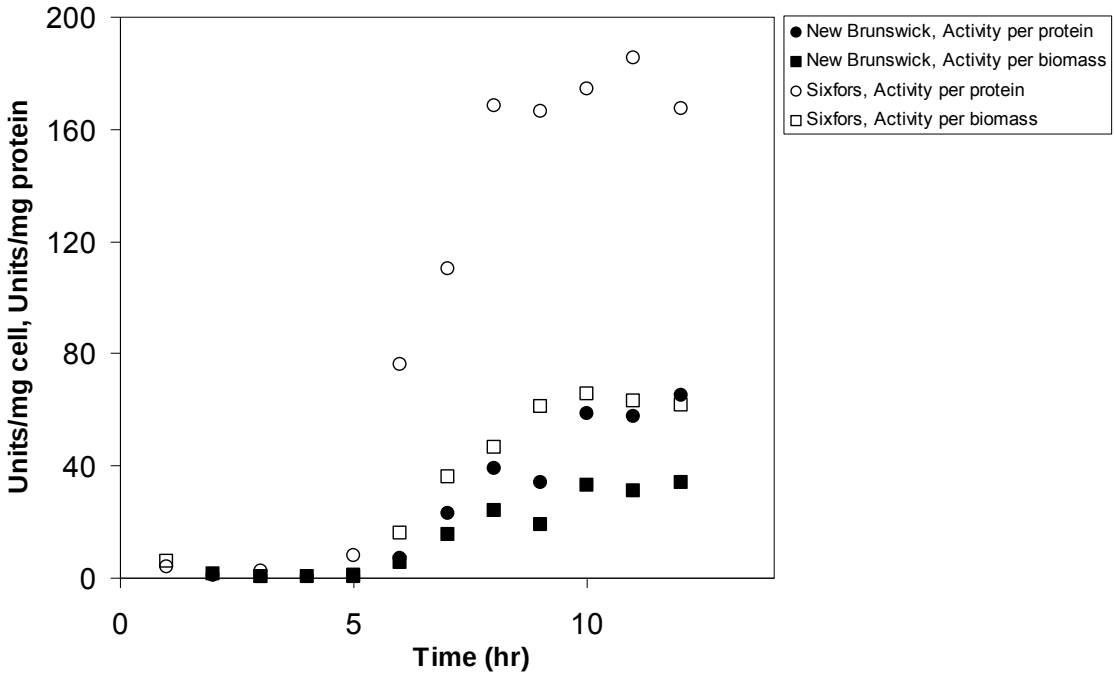


Figure 4.7: Comparison of amidase induction on a volumetric and biomass basis. Amidase expression was induced in New Brunswick (biomass yield of approximately 10 g l^{-1}) and Sixfors bioreactor systems (Biomass yield of approximately 5 g l^{-1}) using 400 µM IPTG concentration (volumetric concentration). This represents specific IPTG concentrations of 40 (closed symbols) and 80 (open symbols) µmol IPTG g^{-1} biomass respectively in both bioreactor systems.

4.6 AMIDASE ACTIVITY IN THE CELL DEBRIS

The enzyme accumulation, in terms of volumetric amidase activity, in the soluble intracellular fraction and the insoluble debris aggregate, was determined to confirm amidase as a cytoplasmic enzyme, and to investigate the possible formation of insoluble aggregates in the form of inclusion bodies on increased expression. *E. coli* cells were disrupted and the extract fractionated. The debris was treated with the detergent, Triton X-100, for solubilisation of the lipid-bound components of the debris aggregate. The effect of Triton X-100 and other detergents on amidase activity and protein solubility was studied to ensure minimal interference. Triton X-100 was chosen because an almost identical volumetric amidase activity and protein concentration was found in its absence and presence at 0.1% concentration (v/v).

In Figure 4.8, it is seen that over 95% of amidase activity was found in the soluble cell fraction prior to detergent treatment. The target protein was accumulated as intracellular active protein during the growth phase in the batch process. This was confirmed by the

SDS PAGE electrophoresis (Figure 4.9).The supernatant from study using 400 µM IPTG concentration exhibited darker bands when compared with bands of lower inducer concentration. Hence, this shows increased dominance over lower IPTG concentration shown on the band. No visible amidase band was observed in the lanes loaded with extracts of cell debris after solubilisation with detergent (Figure 4.9; lanes b, d and f). This is supported by the result presented by Doran *et al.* (2005), that about 91% of amidase from *Microbacterium sp.* AJ115 (Table 2.1) was present in the soluble fraction when recombinant *E. coli* cells were grown at 30ºC.

The non formation of inclusion bodies was further confirmed by viewing the cell debris under the transmission electron microscope (as described in Section 3.5.10) and presented in Figure 4.10. The electron microscopy of the cell debris component (Figure 4.10) showed no visible aggregation of proteins in the form of inclusion bodies. Similar pictures to those presented in Figure 4.10 have been reported by Makhongela *et al.* (2007), where purified *G. pallidus* RAPc8 amidase appeared as homogenous particles of about 10 nm in dimension. Hence, the expression of this thermostable amidase from *G. pallidus* as a soluble cytoplasmic enzyme enhances its potential for use on a process scale.

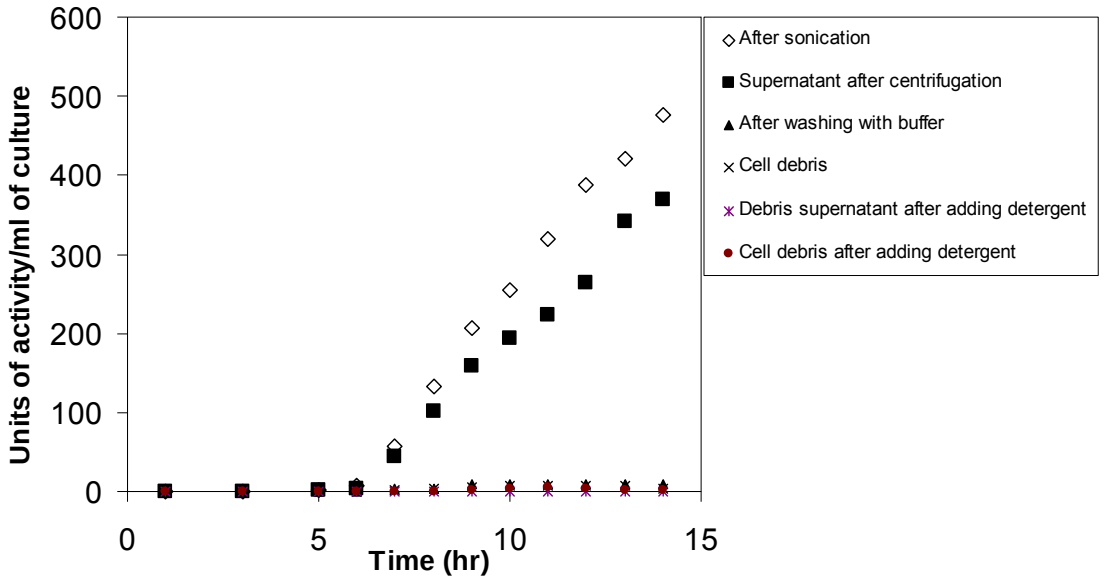


Figure 4.8: Fractionation of the disrupted *E. coli* extract following induction of amidase expression using 400 µM IPTG to demonstrate the location of amidase activity. Amidase activity was quantified after each treatment as indicated in the legend.

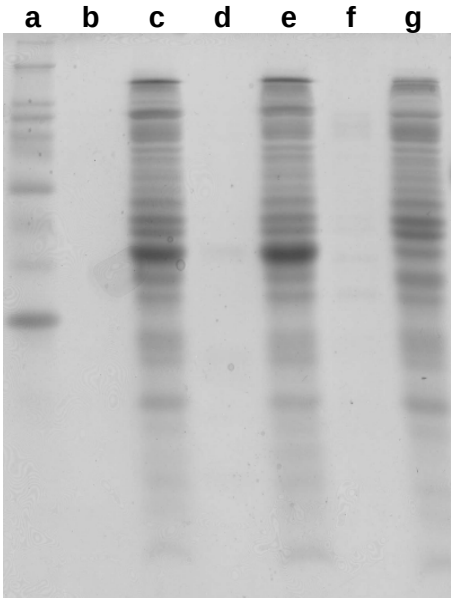


Figure 4.9: SDS-PAGE gel electrophoresis showing (a) Molecular weight marker (b) Cell debris sample from experiment using 100 µM IPTG (c) Supernatant from experiment using 100 µM IPTG (d) Cell debris from experiment after induction with 400 µM IPTG (e) Supernatant from induction study with 400 µM IPTG (f) Cell debris from experiment without inducer (g) supernatant from experiment with no IPTG

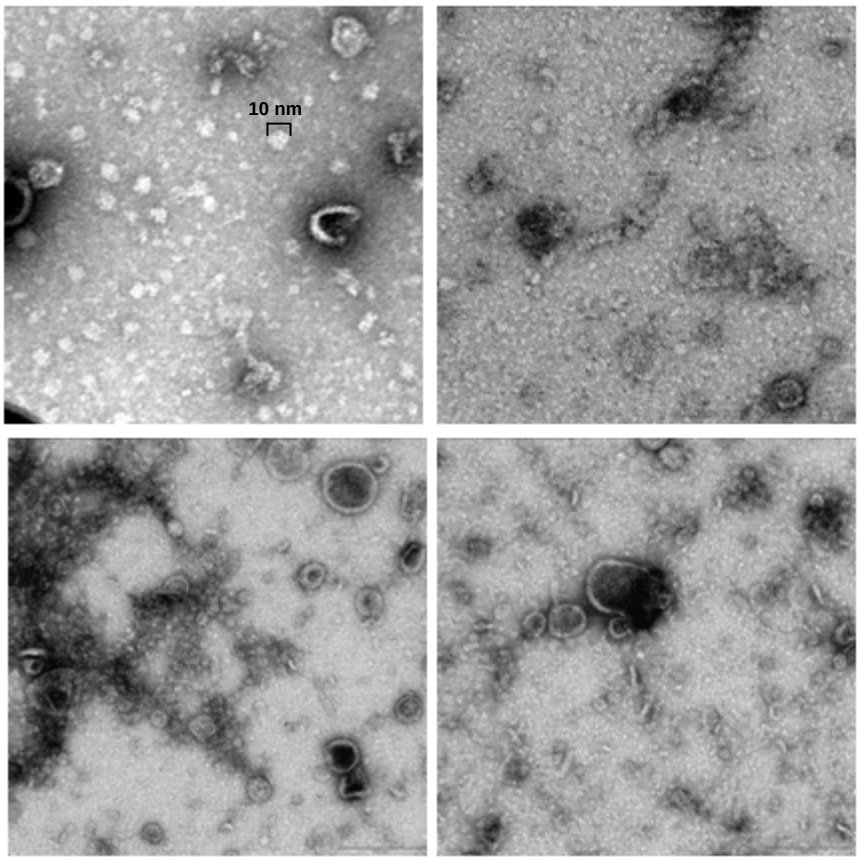


Figure 4. 10: Electronic microscopic (× 10,000) visualisation of debris following cell sonication

4.7 AMIDASE EXPRESSION STUDIES IN FED BATCH CULTIVATION

To maximise volumetric productivity of the thermostable amidase, its specific activity, the biomass concentration, and the growth rate need to be optimised simultaneously. While IPTG is used to enhance specific activity, fed batch culture is well recognised to enhance biomass concentration and biomass productivity. Hence, fed batch production of amidase was studied with a view to increasing the volumetric amidase activity. The exponential feeding of the glucose-based feed medium started after the depletion of the carbon source in the batch process. Oxygen-enriched air was used for these experiments.

4.7.1 CELL GROWTH RATE AND BIOMASS YIELD

Amidase expression was induced at different times during fed-batch cell cultivation: start of feeding (at a final IPTG concentration of 400 μM), continuous induction (i.e. IPTG included in feeding solution at a final concentration of 400 μM) following induction at early exponential phase of growth in the batch process, and intermittent addition of IPTG during feeding to a final concentration of 1.6 mM. At all induction conditions, an initial feed rate of 1.2 ml min^{-1} corresponding to specific growth rate of about 0.2 h^{-1} was used to discourage the accumulation of acetic acid (Suarez and Kilikian, 2000). The feed rate was increased every two hours to cater for increase in biomass by mimicking an exponential feeding strategy. The specific growth rate decreased to approximately 0.05 to 0.1 h^{-1} at the 24^{th} hour of the experiment (Figure 4.11 a, c and e). The final biomass concentration was observed to average 40 g l^{-1} DCW (absorbance value of 80–100 at 660 nm) for all experiments, representing a four fold increase over maximum cell concentration in batch cultivation (Figures 4.11 a, c and e). This supports reports by Sanden *et al.* (2003) that biomass is densely accumulated at low feed rate using an exponential process. The average yield of biomass on glucose across the fed-batch experiments (Figures 4.11 a, c and e) was 0.3 g g^{-1} at the end of batch cultivation, but this decreased slowly about the 17^{th} hour of cell cultivation. This may be due to the inability of the feed rate to maintain a specific growth rate of 0.2 h^{-1}, thus reducing to 0.05 h^{-1}, at which stage, a large fraction of the glucose fed was expected to be utilised for cell maintenance.

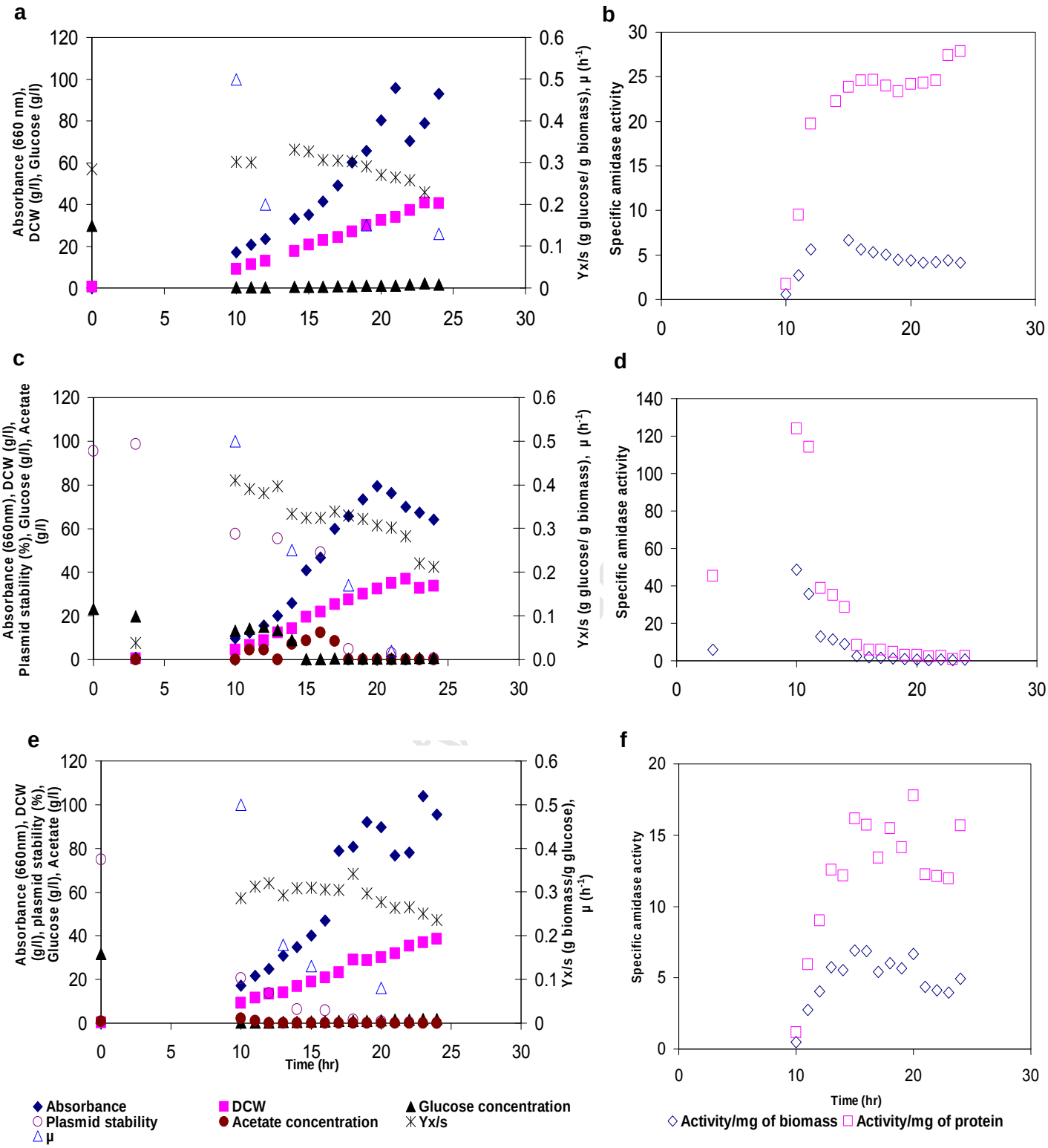


Figure 4.11: Fed batch studies of amidase expression (a, b) start of feed and induction at 10th hr to result in a final IPTG concentration of 400 μM, final concentration of ampicillin, 100 μg/ml (c, d) Induced amidase expression with 400 μM IPTG at the 3rd hr in batch phase with continuous induction and feeding (with feed containing 400 μM IPTG) commencing at the 10th hr, final concentration of ampicillin, 100 μg/ml (e, f) Feeding starts at the 10th hr, induced with a final IPTG concentration of 1.6 mM at 14th hr, final concentration of ampicillin, 400 μg/ml (intermittent addition of IPTG and ampicillin over the duration of experiment)

4.7.2 ACETATE ACCUMULATION DURING FED BATCH

Acetic acid formation is an indicator of imbalance in carbon metabolic flux. It often results from overflow of glucose metabolism, oxygen limitation or addition of inducer as single dose compared to feeding same amount (Sanden *et al.*, 2003; Lin *et al.*, 2001b). Accumulation of acetate could result in reduced cell yield as well as unwanted side effects on the product protein formation (Shiloach and Fass, 2005).

An initial build up of acetate up to 2.0 g l^{-1} was observed at the end of batch process, when amidase was induced either continuously or intermittently (Figure 4.11c and e). However, this is less than the reported inhibitory concentration of between 5 to 10 g l^{-1} (Babaeipour *et al.*, 2007; Rothen *et al.*, 1998; Lee, 1996; Yee and Blanch, 1992). In fed-batch studies, acetate concentration increased to a maximum of approximately 10 g l^{-1} after 15 hrs of cell cultivation in the continuously induced system (Figure 4.11c). Otherwise, acetate concentration for both induction methods was maintained at a nearly zero concentration for most of the duration of the fed batch process. This was achieved by controlling the feed rate of the glucose based medium to keep a low growth rate (0.05 to 0.2 h^{-1}) and to prevent excess glucose concentration in the bioreactor. The slight increment observed at the 13th hour in the continuously induced system (Figure 4.11c) is suspected to be due to metabolite overflow. The uptake rate of the carbon source was reduced on induction at the 3rd hour compared with the 10th hour in the batch phase. This must have led to the increased glucose concentration observed at the start of feeding, 10 g l^{-1}, (Figure 4.11c), which in turn resulted into metabolite overflow and is postulated to have led to the formation of acetate.

4.7.3 PLASMID STABILITY AND AMIDASE ACTIVITY IN A HIGHLY DENSE CELL CULTURE

Preserving the selection pressure of recombinant organisms in a culture is important to prevent the mutant strain from being out-competed by more rapidly growing cells or the wild-type strain. Hence, the maintenance of plasmid stability in the cultivation of recombinant strain is necessary to maximise production and prevent increased production cost (Nayak and Vyas, 1999).

Plasmid stability was determined in the fed batch cultivation by determining ampicillin resistant cell number (Section 3.5.8). The results are presented in Figures 4.11 c and e.

A decrease was observed in plasmid stability (dominance of non producing strain) at the end of batch cultivation. This became increasingly pronounced at the start of feeding. Complete loss of recombinant population was experienced between 20 to 22 hours of cell cultivation, when amidase expression was induced continuously (Figure 4.11c) and intermittently (Figure 4.11e). This loss could be attributed to the likely ineffectiveness of the agent (ampicillin) responsible for maintaining the selective pressure, since biomass formation and accumulation were not affected adversely. The loss of plasmid carrying cells accounts for the reduced specific amidase activity presented in Figures 4.11 b, d and f, compared to results from batch cultivation presented in Figure 4.5. The low specific amidase activity experienced prior to induction (Figures 4.11 b and f) increased initially for the first 2 hours after induction and did not improve further. Rather, specific activity decreased over the extended duration of the fed batch process. This cannot be attributed to inducer concentration as the same trend was observed for amidase expression induced with a final IPTG concentration of 400 and 1600 μM as shown in Figures 4.11 b and f respectively.

Due to increased biomass concentration in the fed batch process, amidase activity was determined in the insoluble cell fraction to check for protein accumulation in form of inclusion bodies. Following sonication, the cell lysate was fractionated and assayed for amidase activity in the insoluble cell debris. Less than 4% of total amidase activity was determined to be associated with cell debris, using treatment with detergents, showing that amidase was dominantly expressed as soluble protein (Figure 4.12). Similar findings were reported in batch cultivation (Figure 4.8).

In Figure 4.11d, amidase expression was induced at early exponential phase of growth in the batch phase (3rd hour) and IPTG was added to the feed medium (to a combined final concentration of 400 μM) from initiation of feeding at the 10th hour onwards to induce expression continuously for improvement in volumetric amidase activity. A specific amidase activity of 48 U mg^{-1} of biomass and 130 U mg^{-1} of protein were observed at the end of batch phase, similar to the data presented in Figure 4.5.

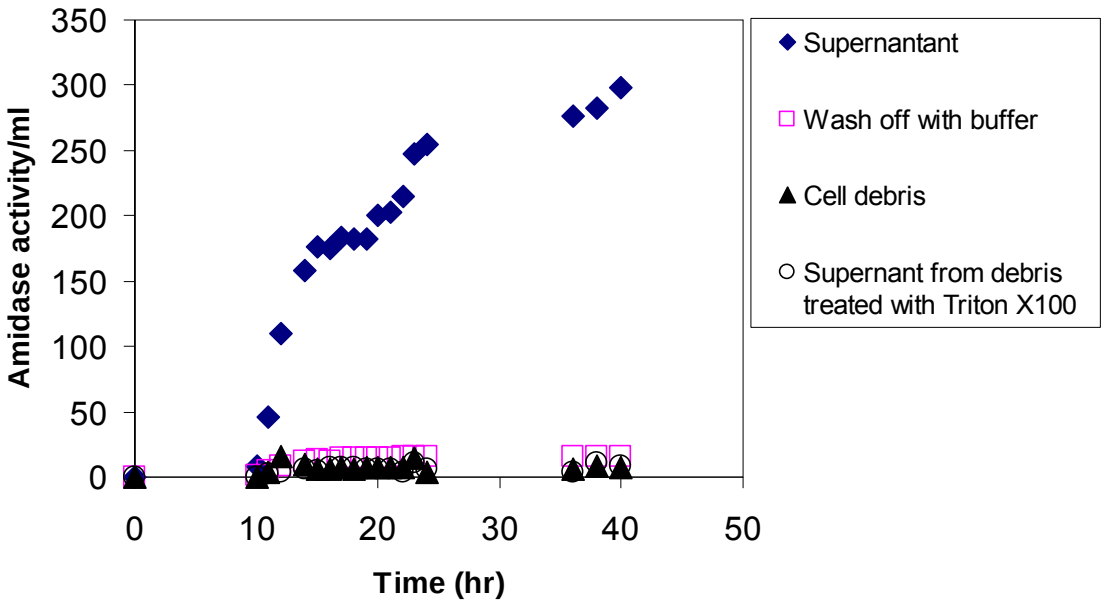


Figure 4.12: Cell fractionation from samples taken at start of feed and induction with final IPTG concentration of 400 µM at 10th hr and final concentration of ampicillin, 100 µg/ml. The supernatant and the cell debris was separated after sonication and assayed for amidase activities. The "washing buffer" as well as the supernatant from solubilised debris (detergent treated) were also assayed for amidase activities.

Although continuous addition of inducer was expected to improve the rate of expression of the recombinant gene over a prolonged period of time (Puschmann *et al.*, 2002), a rapid and consistent decrease in specific amidase activity and plasmid stability was noticed immediately at the start of feeding the glucose based medium containing the inducer (Figure 4.11d).

From these results (non-accumulation of acetate and loss of cell's recombinant population), there is an obvious relationship between the observed plasmid instability and reduction in specific activities. Non-maintenance of selection pressure by ampicillin is postulated to have resulted in plasmid instability (Korpimaki *et al.*, 2003). This can be due to change in plasmid structure owing to re-arrangement of DNA (structural instability), defective partitioning during cell division (segregational instability) or the growth advantage of non-plasmid bearing cells over the recombinant strain (competitive instability) (Zhao *et al.*, 2007; Zheng *et al.*, 2005; Nayak and Vyas, 1999).

4.7.4 ANTIBIOTIC EFFECTIVENESS IN MAINTAINING SELECTIVE PRESSURE OF PLASMID

A simple plate well experiment (described in Section 3.5.7) was conducted to study the potency of ampicillin in maintaining the selection pressure in cultures of recombinant *E.coli* BL21 (DE3) cells. While the control (ampicillin controlling medium from the bioreactor before inoculation) inhibited growth of the wild type *E. coli* around its well across a radius of up to 10 mm from the outer ring of the well (Figure 4.13a), no inhibition was observed around the wells into which supernatant of the samples taken at 0, 10 and 24 hrs was added (Figure 4.13b). This suggests that ampicillin at the initial concentration of 100 µg/ml was degraded at the early stage of batch phase of cell growth removing the selective advantage of the recombinant strain and allowing competition by wild type strain, thereby leading to the total loss of non-plasmid carrying population at the 22nd hour (Figures 4.11c and e).

To further understand if antibiotic degradation was due to increased biomass in the high cell dense culture, a simple experiment was designed in which biomass concentration was varied and selective pressure was maintained by adding the same concentration of ampicillin. *E. coli* suspension was pipetted in the ratio 2:5:10 into three shake flasks containing sterilised defined medium and filter sterilised 100 µg ml^{-1} ampicillin was added. A clarified supernatant from the *E. coli* suspension (equivalent to the highest ratio of cell suspension used) was pipetted into a fourth flask and 100 µg ml^{-1} ampicillin was added. The fifth flask served as the control (ampicillin and growth medium only). The samples taken from these flasks prior to inoculation all inhibited the growth of wild type *E. coli* strain up in a zone with a radius of up to 10 mm from the outer circle of the well (Figure 4.13c). Meanwhile, only the samples from the supernatant flask and the control inhibited growth of the wild type strain to the same extent after incubation for 2 hours (Figure 4.13d), meaning the antibiotic present in the flasks with cell suspension (irrespective of biomass concentration) had been degraded.

Antibiotic degradation is due to non-biological and biological factors (Kakimoto and Funamizu, 2007; Basaez and Vanysek, 1999). Non-biological factors include increased concentration of phosphate and ammonia (up to 50 g l^{-1}) and acidic or basic hydrolysis of antibiotic, while biological factors include the secretion of β-lactamase into the culture medium.

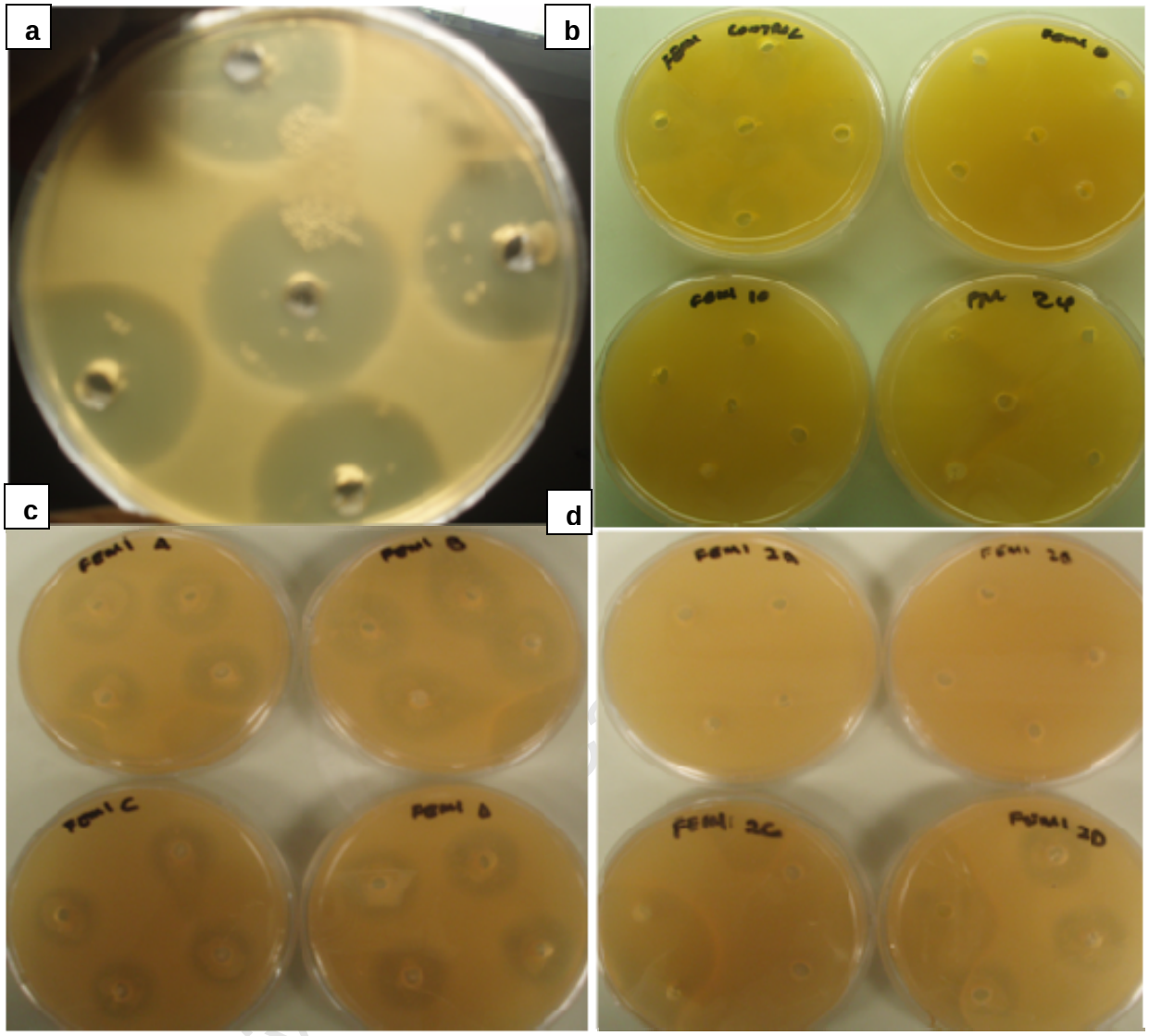


Figure 4.13: Disc plate diffusion assay showing (a) Control (b) Samples taken from fed batch cultivation; control, immediately after inoculation, 10th and 24th hour. (c) Disc plate antibiotic test using different concentration in ratio 2:5:10 marked A, B and C respectively and cell supernatant labelled D at t=0 hr (d) condition "C" after 2 hours of culture incubation at 30ºC and agitation of 180 rpm.

Since this study was carried out at an almost neutral pH using phosphate and ammonia concentration much below the inhibitory concentration (Table 3.1), biological factors are suspected to be responsible for the observed ampicillin degradation. β-lactamase is a by-product of *E. coli* cultivation which acts on penicillin derivatives such as ampicillin (Korpimaki *et al.*, 2003). The recombinant bacteria carrying the antibiotic resistant gene may produce extracellular β-lactamase which, in turn, degrades the antibiotic, thereby

making the culture susceptible to external and internal contamination (facilitating loss of mutant strain). This confirms previous studies (Korpimaki *et al.*, 2003; Wulfing and Pluckthun, 1993) that have suggested that β-lactam resistance is less than ideal for maintaining selective pressure in cultivation of recombinant *E. coli* cells due to the cleavage of the β-lactam ring by the action of β-lactamase.

4.8 SUMMARY

The results presented in this study emphasise the importance of growth medium in achieving high biomass concentration and the subsequent impact on total achievable soluble protein concentration. Although complex LB and glucose-supplemented LB medium gave a higher growth rate, the nutrient content did not support a high biomass concentration, even following supplementation with glucose. Defined medium enhanced biomass concentration attained at a lower growth rate. This increases the probability of proper folding of proteins. Further, the importance of selection of an appropriate induction time for maximum enzyme activity was demonstrated using the appropriate inducer concentration calculated based on the ratio of IPTG to biomass concentration at the end of batch cultivation. A 5 fold increase in volumetric amidase activity was observed when expression was induced at early exponential phase compared to induction at mid exponential phase. While protein expression increased 2 fold in the presence of inducer, similar protein concentration was maintained over the range of inducer concentration from 40 to 1000 μM. No inhibition of biomass production was observed across the IPTG concentration range. The maximum specific activity of amidase was observed at 80 μmol IPTG g^{-1} biomass, corresponding to a volumetric concentration of 400 μM. Volumetric and biomass based induction was studied by comparing data from two bioreactor systems, New Brunswick (10 g l^{-1} dry biomass) and Sixfors (5 g l^{-1} dry biomass). An IPTG concentration of 400 μM (volumetric concentration) was used, resulting in 40 and 80 μmol g^{-1} cell for the respective bioreactor systems. Approximately, 2 fold increase in amidase expression was observed using the Sixfors bioreactor, suggesting that biomass concentration ought to be taken into consideration prior to induction.

This thermostable amidase was produced as intracellular, soluble and cytoplasmic enzyme, thereby enhancing its potential for application on an industrial scale. Less than 4% of the amidase activity was lost with the cell debris.

Fed-batch cultivation was shown to provide high density cell culture of some 40 g l^{-1} (DCW), representing a 4 fold increase over the batch system. Further, biomass productivity was increased by 2 fold. However, the maximal production of this enzyme in a fed batch system was unsuccessful as the selection pressure could not be maintained, probably due to the degradation of ampicillin (a β-lactam antibiotic) by β-lactamase. This led to plasmid instability, which in turn contributed to reduced specific amidase activities. This was not resolved by increasing either ampicillin or IPTG concentration or supplying this continuously.

In summary, it can be concluded that levels of recombinant gene expression are system-specific, depending on the biochemical properties of the recombinant protein, the induction mechanism for gene expression, the host strain and cultivation conditions.

Chapter 5

Expanded bed adsorption

5.0 INTRODUCTION

Expanded bed adsorption (EBA) integrate the functions of up to three operation units (centrifugation, filtration and initial adsorption) in the downstream processing of protein. The cell homogenate obtained after cell disruption, is passed through a stabilised expanded bed in a column where solid-liquid separation is achieved. The desired protein is purified directly from the complex mixture of biomolecules present in cell homogenate. This method allows for integration of the bioprocesses and hence increases the total protein yield achievable with a concurrent reduction in equipment, maintenance and production cost. The efficient operation of the EBA process depends on a large number of factors including choice of adsorbent, operating parameters (pH, solution ionic strength), physical characteristics of homogenate and feedstock composition. This chapter presents the study of EBA for use in amidase recovery from recombinant *E. coli.*

5.1 EXPERIMENTAL APPROACH

Initial studies were carried out in shake flasks to determine the choice of beads for optimal amidase adsorption. The STREAMLINE sulfopropyl (SP) bead exhibiting cationic properties and the STREAMLINE anionic diethylaminoethyl (DEAE) adsorbent (Amersham Biosciences, Uppsala, Sweden) were compared for maximum amidase adsorption. In addition, the optimal pH and ionic strength for enhanced amidase adsorption were determined, as well as the maximum adsorbent capacity of the enzyme.

A major problem with the use of an ion-exchanger is the electrostatic interaction that results from the reaction of the cell surfaces and the ionic charge on the resin (Anspach *et al.*, 1999; Lin *et al.*, 2001a). Hence, the impact of whole *E. coli* cells, partly and

completed disrupted cells on protein adsorption to the beads was investigated in a shake flask and subsequently in a stable expanded bed in a column (Sections 3.6.1 to 3.6.3). Previous reports by Balasundaram and Harrison (2006a,b) have suggested that the extent of cell disruption and the particle size of the debris is a function of the intensity of disruption. Therefore, the intensity of cell disruption using a high pressure homogeniser was varied in this study. This resulted in partly broken and fully disrupted cells which were used in this study. Knowledge of the extent of biomass-adsorbent interaction will give an insight into means of optimising amidase recovery using the EBA process.

5.2 CONDITIONS FOR FAVOURABLE RETENTION OF AMIDASE OVER PROTEIN

Proteins are purified by exploiting their specific properties such as differences in their net surface charge in ion exchange chromatography (Hatti-Kaul and Mattiasson, 2001). Generally, amidases have been purified through their hydrophobic and anionic properties (Table 2.2). This study was only able to exploit the use of ion exchange due to non-availability of hydrophobic adsorbents for EBA at this time. Moreover, ion exchange incorporates two unique properties: high capacities and moderate selectivity with rational cost (Hubbuch *et al.*, 2006). Hence, STREAMLINE cationic SP and anionic DEAE adsorbent beads were examined for maximum amidase adsorption in a batch shake flask experiment. To maximise amidase binding to the chosen adsorbents, the pH and ionic strength of the solution were varied. Since the proposed pI values for amidase range from 4.0 to 5.7 (Table 2.1), slightly acidic pH values from 4.5 to 7.0 were examined for SP beads (amidase will be expected to absorb to a cationic matrix at a pH lower than the pI), while values ranging from 6.9 to 8.0 were investigated for DEAE beads. A cell concentration of 0.5% (w/v, wet weight) was used for the experiments to prevent highly viscous conditions. Amidase activity and protein concentration were determined in the supernatant before and after addition of clarified homogenate to the beads. The amount of protein and amidase adsorbed to the beads was determined from the residual concentration using a simple mass balance. The results from the studies are presented in Tables 5.1 and 5.2.

The SP beads adsorbed only 5 to 8% protein, while approximately 25 to 28% of amidase enzyme was determined to be adhered to the beads under conditions used. The degree of amidase adsorption to DEAE adsorbent is considerably higher, with a maximum

adsorption of 82% detected at a pH of 7.7. In contrast to SP beads, a large protein retention (50 to 70%) was found with the DEAE beads. The reduced amidase binding to the SP adsorbent could be due to its cationic properties, while the relative high adsorption of protein to the DEAE adsorbent beads may be due to the non-specific nature of the adsorbent. Similar observations regarding non-specific adsorption have been noted by Chase (1994).

Table 5.1: Impact of pH on adsorption of amidase (A, Units/ml) and protein (P, mg/ml) to cationic-hydrophobic SP adsorbent

pH	4.5		5.7		6.5		7.0	
	P	A	P	A	P	A	P	A
Solution prior to addition of beads	2.06	41.68	4.58	43.60	2.35	37.00	2.25	33.88
Solution in the presence of adsorbent	1.92	31.48	3.24	33.04	2.24	26.80	2.14	25.24
Absorbed (%)	6.80	25.00	30.00	25.00	5.00	28.00	5.00	25.00
Purification factor		1.23		0.93		1.31		1.27

Table 5.2: Influence of pH on amidase (A, Units/ml) separation from crude protein solution (P, mg/ml) using anionic DEAE adsorbent

pH	6.9		7.2		7.4		7.7		8.0	
	P	A	P	A	P	A	P	A	P	A
Solution prior to addition of beads	1.55	27.51	1.80	39.53	0.58	32.75	1.82	62.03	5.40	31.60
Solution in the presence of adsorbent	0.47	9.60	0.55	10.65	0.17	8.87	0.80	11.37	2.20	26.00
Absorbed (%)	69.92	65.10	71.04	73.06	70.10	72.91	55.81	81.66	60.00	18.00
Purification factor		0.87		1.13		1.08		2.39		0.50

*** Potassium phosphate buffer solution (50 mM) was used for both experiments. Adsorption (%) was calculated from the residual concentration.

From the results presented in Table 5.1 and 5.2, the desired condition of low protein adsorption concurrently with high amidase retention to the adsorbent was found when amidase was captured from the crude homogenate using DEAE beads at a pH of 7.7.

Further, the highest purification factor was observed at this pH. Thus, the use of DEAE adsorbent at a pH of 7.7 was favoured for further use in this study.

To further optimise amidase binding to the DEAE adsorbent, an experiment was conducted to vary the solution ionic strength using phosphate buffer solutions in the range 25 to 100 mM (conductivity reading from 7.8 to 31.2 mS). Results presented in Table 5.3 showed a strong dependence on ionic strength. Amidase was adsorbed maximally using 50 mM potassium phosphate buffer. These optimised conditions of solution pH and ionic strength were utilised for further experiments in this study.

Table 5. 3: Influence of medium ionic strength on amidase adsorption to DEAE adsorbent using potassium phosphate buffer at a pH of 7.6

Conductivity, mS (Phosphate buffer)	**7.80 (25 mM)**		**15.60 (50 mM)**		**31.20 (100 mM)**	
	Protein (mg ml^{-1})	**Amidase (U ml^{-1})**	**Protein (mg ml^{-1})**	**Amidase (U ml^{-1})**	**Protein (mg ml^{-1})**	**Amidase (U ml^{-1})**
Solution prior to addition of beads	1.00	77.56	1.00	77.56	1.00	77.56
Solution in the presence of adsorbent	0.74	43.54	0.63	15.27	0.74	25.57
Absorbed (%)	26.04	43.86	37.54	80.32	25.74	67.03
Purification factor		1.32		3.20		2.24

***Adsorption (%) was calculated from the residual concentration.

5.3 ADSORBENT LOADING CAPACITY

One of the major factors needed to assess the EBA process performance is knowing the capacity for a target protein as this helps in investigation of ways to increase the capacity (Brobjer, 1999; Lin *et al.*, 2004). Consequently, this experiment was done to determine the enzyme loading obtainable per mass adsorbent. The amount of enzyme solution used was the same (0.5 ml enzyme solution), while the settled bead volume was varied from 0.1 to 0.5 ml. The result presented in Table 5.4 shows maximum protein (~ 70%) and amidase (90%) binding using 0.5 ml of beads. However, the amount of protein and enzyme adsorbed per bead decreased with increasing adsorbent volume. From the data presented in Table 5.4, the maximum load was determined to be 18 U ml^{-1} of adsorbent. At this enzyme loading, 100% adsorption was attained. This decreased

gradually with an increase in adsorbent loading (Figure 5.1). Further increment in enzyme loading per adsorbent led to a reduction in the amount of amidase adsorbed to the adsorbent.

Table 5.4: Bead saturation experiment to determine maximum protein (P, mg ml^{-1}) and amidase (A, U ml^{-1}) load per bead at pH 7.7, using 50 mM buffer concentration

Volume of bead (µl)	**100**		**200**		**300**		**500**	
	P	**A**	**P**	**A**	**P**	**A**	**P**	**A**
Solution prior to addition of beads	0.13	16.00	0.13	16.00	0.13	16.00	0.13	16.00
Solution in the presence of beads	0.06	3.81	0.05	2.82	0.05	2.36	0.04	1.53
Amount adsorbed to beads	0.07	12.19	0.08	13.17	0.08	13.63	0.09	14.46
Amount of adsorbed/ml beads	0.67	121.89	0.38	65.86	0.28	45.44	0.18	28.93
% Adsorption	51.34	76.20	59.21	82.35	64.88	85.22	68.50	90.42

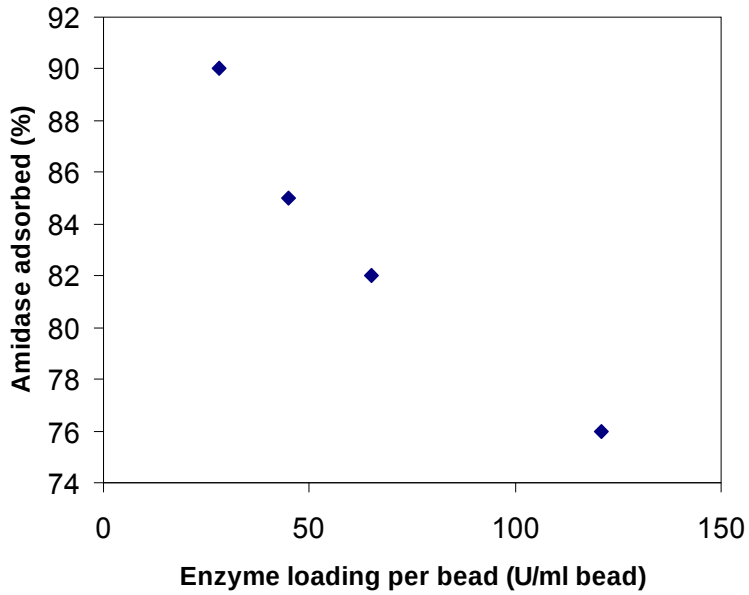


Figure 5.1: Amidase adsorption as a function of enzyme loading per bead

5.4 IMPACT OF CELLS AND DEBRIS ON PROTEIN ADSORPTION TO STREAMLINE DEAE ADSORBENT

The feed composition for the EBA process varies depending on protein location and extent of cell disruption (Table 2.6). In an EBA process where non-specific adsorbent such as DEAE is used, feed components with similar surface charge compete with the desired proteins for available binding spaces to the adsorbent, thus leading to reduced protein adsorption. In addition, this will affect the plug flow needed for bed stability (due to change in bead conformation) in an EBA process and thus impact the process efficiency. Although electrostatic attraction has been shown to be the major cause of interaction between biomass and adsorbent in ion exchange purification (Lin *et al.*, 2003), this study was conducted to know the extent of such interaction between *E. coli* (whole cells and broken debris) and DEAE adsorbent and its consequent influence on amidase adsorption to the beads. Interaction was characterised in terms of the bed stability, protein breakthrough and dynamic binding capacity.

Preliminary studies were carried out in shake flasks using clarified and unclarified protein samples. Subsequently, the experiment was repeated in the column, since the environment created in the expanded bed was different to the conditions used in the shake flasks.

Differences were observed in protein and amidase concentrations of the disrupted suspensions between sets of experiments. This could have been due to variation in process conditions, different storage, handling and growth conditions of the *E. coli* culture. Hence, data was compared within the same experiment (i.e. amount of protein adsorbed to the adsorbent beads). Where comparison across sets of experiments is required, normalised data are used.

Prior to protein separation in the EBA column, the DEAE adsorbent was equilibrated with potassium phosphate buffer solution. The vertical position of the column was ensured to prevent bed instability. Feed application was continued for a minimum duration of 3 hours to ensure that the level of protein and amidase in the eluent had risen above 10% of the inlet concentration to allow comparison of dynamic binding capacities for clarified and crude feedstock, thus giving an understanding into the influence of biomass. The breakthrough point in this study is defined as the corresponding volume of eluent at

which the first increase in C/Co from its baseline value was observed, while the dynamic binding capacity (DBC) of protein is also defined as the amount of the protein adsorbed per ml of adsorbent when the outlet concentration in the column was 10% of the inlet concentration:

$$DBC = \frac{C_o * V_b}{Vs} \tag{5.1}$$

where C_o is inlet concentration of protein (mg ml^{-1}) or enzyme activity (U ml^{-1}), V_b is volume (ml) at the breakthrough point ($C/C_o = 0.1$) and V_s is the volume of the settled adsorbent in the column (ml).

5.4.1 THE EFFECT OF CELL DEBRIS ON PROTEIN AND AMIDASE ADSORPTION DETERMINED IN BATCH EXPERIMENT.

A simple batch experiment was conducted in shake flasks to ascertain the extent of the presence of cell debris (after disruption) on protein adsorption to Streamline DEAE adsorbent beads. The initial biomass concentration of the suspension prior to disruption was maintained at 0.5% wet weight (w/v) for the experiments, while intensity of cell disruption was varied to achieve complete and partial cell disruption (Section 3.6.1). The degree of disruption was ensured by observing the cell lysate under the microscope. Samples of the clarified and unclarified cell lysate following partial and complete disruption were added to the DEAE adsorbent beads (for 5 hrs on a rotary shaker at 50 rpm) in a 1:1 ratio. Total soluble protein concentration and amidase activity were determined prior to addition of beads and following equilibration with the beads. The amount of protein and amidase adsorbed to the beads was determined from these concentrations using a mass balance.

The amount of protein and amidase activity in solution increased with increasing intensity of disruption (Table 5.5). Amidase activity released at complete cell disruption (9.30 U ml^{-1}) represented an increase of approximately 1.43 fold over that of partial cell disruption, 6.49 U ml^{-1}, while a 1.15 fold increase in total soluble protein concentration was found. The results presented in Table 5.5 show only minor differences in protein and amidase adsorption using clarified and unclarified samples from both partially and fully disrupted cell samples. In all cases, complete disruption resulted in a slightly

increased adsorption of some 0.3 to 2.7% over the amidase adsorption of 96.5% and protein adsorption of 71.1%. Use of the clarified homogenate resulted in an improvement of 1 to 1.7% for amidase and 1 to 2.7% for protein. This shows that cell debris from partially and fully disrupted samples does not have a pronounced effect on protein and amidase adsorption to the DEAE adsorbent in an equilibrated system.

Table 5.5: Shake flask experiment to determine the effects of cell debris on protein (P, mg ml^{-1}) and amidase (A, U ml^{-1}) adsorption to STREAMLINE DEAE adsorbent bead using clarified and unclarified samples from complete and partial cell disruption.

	Complete disruption (41.4 MPa, 5 passes)		Partial disruption (13.8 MPa, 2 passes)	
	P	A	P	A
Concentration of solution prior to addition of beads	0.15	9.30	0.13	6.49
Concentration of clarified solution in the presence of adsorbent (P mg ml^{-1}; A U ml^{-1})	0.04	0.21	0.04	0.22
Concentration of crude homogenate in the presence of adsorbent ((P mg ml^{-1}; A U ml^{-1})	0.04	0.37	0.04	0.28
% Adsorption in clarified solution	72.5	97.7	71.6	96.7
% Adsorption in crude solution	71.5	96.0	68.9	95.7

5.4.2 INFLUENCE OF WHOLE CELLS ON PROTEIN ADSORPTION

The effects of cell debris on protein adsorption was further studied in a column using clarified and unclarified protein solution, as effects such as the characteristic circulatory movement of the beads in a expanded bed and residence time effects were not considered in the shake flask. In addition, the effect of other biomolecules, majorly biomass and nucleic acid, on bed stability can be observed. The influence of whole cells on protein adsorption to DEAE adsorbent in an expanded bed was investigated using bovine serum albumin as the standard protein. Three concentrations of protein were chosen for this study: 0.24 (system A), 1.74 (system B) and 5.12 (system C) mg protein ml^{-1} feed solution. The feed rate was maintained at 0.65 ml min^{-1}, representing a superficial velocity of 0.82 m s^{-1}.

Bed stability was characterised by visual assessment and the degree of bed expansion. Prior to the start of feeding, only the characteristic small circulatory movement of the adsorbent beads was observed in the fluidised bed. The bed height expanded by approximately 2.5 fold with no observed channeling and back mixing of adsorbent beads. Bed stability was maintained when the feedstock was applied while bed expansion increased to a bed height of some 3 times the height of the settled bed. This can be attributed to the increase in fluid viscosity due to the presence of biomass (Chang and Chase, 1996a).

Figure 5.2a shows the breakthrough obtained for BSA on DEAE adsorbent as a function of protein concentration in the presence of 1% (w/v, wet weight) whole *E. coli* cells. The BSA protein breakthrough profiles show a slight delay in initial breakthrough for system C containing 5.12 mg ml^{-1} protein compared with the lower protein concentration of 1.74 mg ml^{-1}. However, a sharp increase at an outlet to inlet ratios of protein concentration (C/Co) value of 0.5 was observed after feeding about 12 mg of protein per ml adsorbent into the column (36 ml of eluent collected). The eluent protein concentration plateaud at a C/Co value of 0.93 after 18.6 mg protein per ml of adsorbent had entered the column (67 ml of eluent collected), while early initial breakthrough was observed for systems A and B after feeding approximately 0.68 and 4.78 mg ml^{-1} bead (about 30 ml of eluent collected), these breakthrough trends were non-ideal increasing gradually and reaching a plateau at a C/Co value of 0.78 and 0.88 respectively (90 ml of eluent collected), Deviation from an ideal step breakthrough increased with decreasing protein concentration.

The dynamic binding capacities of BSA were found to be 0.95 and 4.98 mg ml^{-1} adsorbent for feedstock A and B respectively. The DBC value for system C was so high. Owing to the sharp breakthrough observed for system C, the DBC was calculated by interpolation to find the eluent value corresponding to a C/Co of resulting in a DBC of 17.6 mg ml^{-1}. An increase in dynamic binding capacity was found on increasing the protein concentration per ml of adsorbent, illustrating increased adsorption performance. Further, at high BSA concentration, biomass breakthrough was more rapid than at lower protein concentrations (systems B and C) (Figure 5.2b). It is apparent that the high BSA concentration competed favourably with biomass, thus leading to the early biomass breakthrough observed. This is consistent with reports by Balasundaram and Harrison

(2008a) that higher concentration of total soluble protein reduces transportation limitations and thus enhances mass transfer and subsequent adsorption of the protein molecules. Further, the A/Ao value of almost 1 as shown by the biomass profile in Figure 5.2b suggests that the cells cannot bind when the beads are fully loaded with protein.

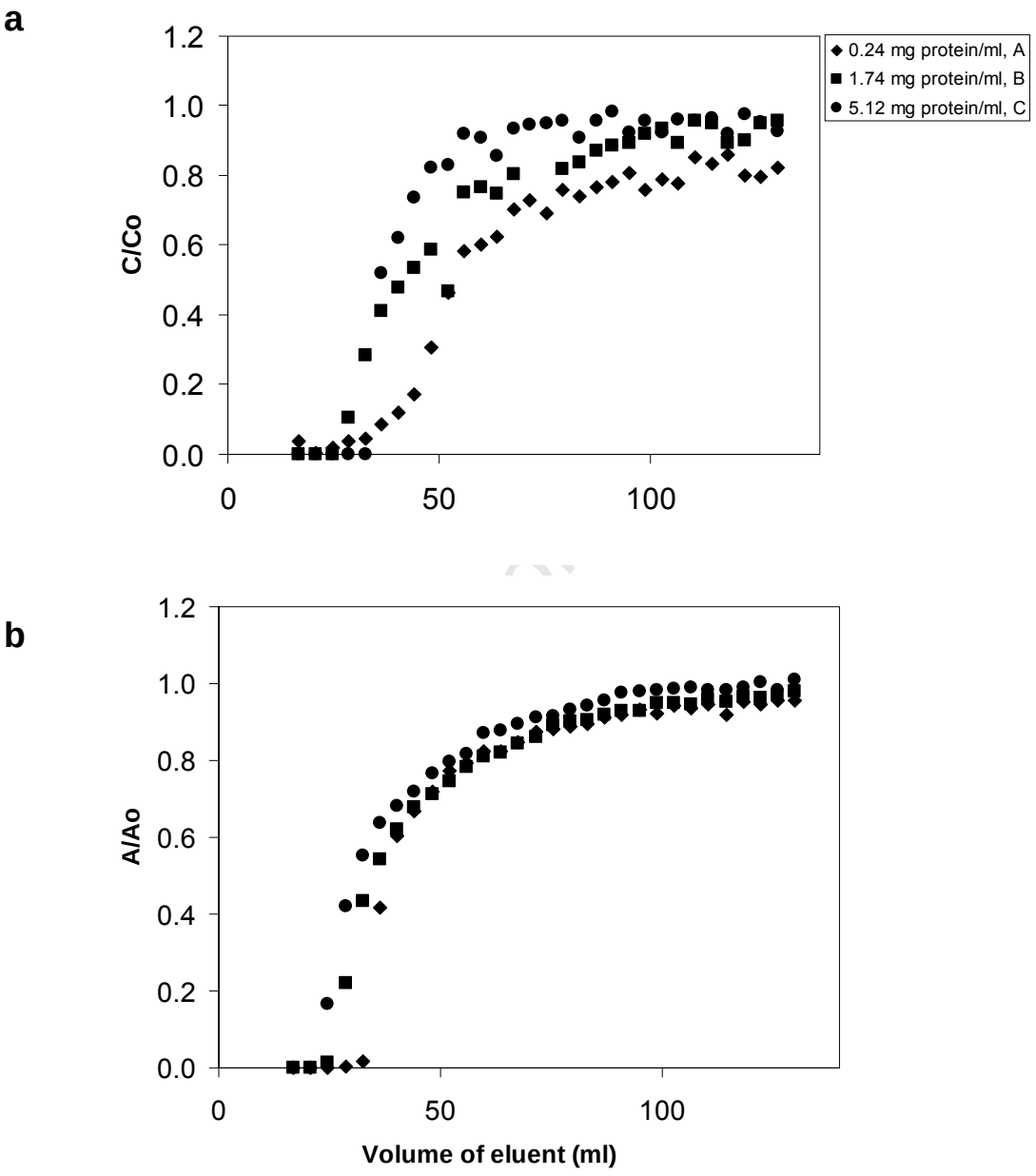


Figure 5. 2: The breakthrough profile of (a) total soluble protein and (b) cell concentration (absorbance), using Streamline DEAE adsorbent and BSA as the standard protein.

Generally, the influence of whole *E. coli* cells (at 1% w/v, wet weight) on protein adsorption reduced with increasing protein concentration. Further, the picture (taken under a light microscope at a magnification power of × 1000) in Figure 5.3 shows that biomass was apparently not adsorbed to the Streamline DEAE adsorbent beads after application of feed containing whole cells. This suggests that the influence of whole *E. coli* cell was minimal on protein adsorption to the adsorbent.

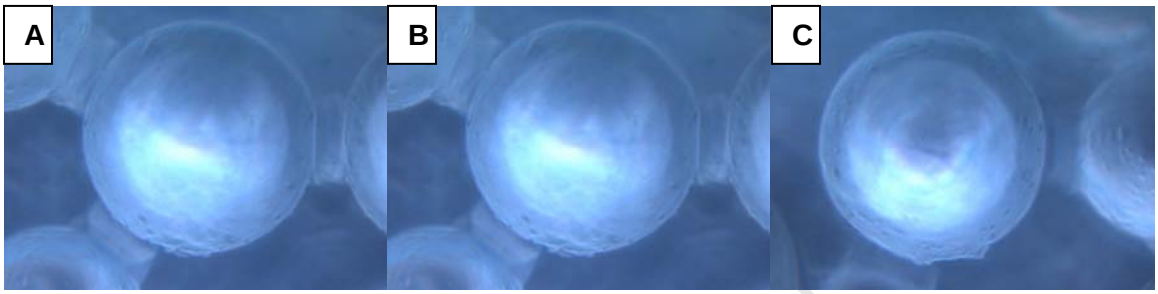


Figure 5.3: STREAMLINE DEAE adsorbent beads taken with Olympus model BX40 microscope, ×1000 (a) Prior to application of feed (b, c) following feed application (using system C).

5.4.3 AMIDASE SEPARATION FROM CLARIFIED CELL LYSATE IN AN EBA COLUMN

For this study, the low biomass concentration of 0.5% (w/v, wet weight) used for the disruption experiment yielded a low viscous suspension across all disruption intensities after clarification. The total soluble protein concentration and amidase activity were determined after subjecting the cells to partial and complete disruption (Section 3.6.1). The amount of protein and amidase activity released increased with intensity of disruption (Table 5.6). At complete cell disruption, an approximate increase of 1.35 fold was observed for protein release, while a slight increment of 0.72 U ml^{-1} was found for amidase activity over samples from partially broken cells.

The clarified feedstocks following both partial and complete cell disruption were passed through the stable expanded DEAE bed and bed stability was examined visually. The bed was observed to be stable as reported for the buffer-adsorbent system with no further increase in bed height (Section 5.4.2). This suggests that the fluid viscosity was low (similar to the buffer suspending medium) and thus insufficient to influence the bed height.

The breakthrough profiles for protein and amidase for clarified supernatants from complete and partial cell disruption show that early breakthrough was observed for protein and amidase from the complete cell disruption homogenate (Figure 5.4). While protein came through the column sharper, amidase breakthrough was gradual (higher residence time in the column) and eventually leveled off after feeding approximately 100 ml of clarified homogenate. Protein breakthrough profile was continuous and was yet to plateau out at the end of feeding. Amidase broke through after collecting about 28 to 32 ml of eluent using feedstock from both complete and partial cell disruption. Meanwhile protein came through after collecting 23 ml of eluent when feedstock from full cell disruption was used, and this value increased with 17% (27 ml of eluent collected) when feed from incomplete cell breakage was applied. The gradual amidase breakthrough may possibly be due to some interaction with the adsorbent. This, coupled with amidase breakthrough point indicates that there was higher selectivity of STREAMLINE DEAE for amidase over general protein in the clarified homogenate.

Further, the DBC values for protein and amidase were calculated and are shown in Table 5.6. Higher DBC values were observed for protein (2.01 mg ml^{-1} adsorbent) and amidase (115.64 U ml^{-1} adsorbent) from fully disrupted homogenate samples, showing enhanced adsorption performance at higher protein concentration.

Table 5.6: Results of protein concentration and amidase activity from clarified suspension after partial and full disruption

Relative Intensity of disruption	Operating Pressure (MPa)	Number of passes	Protein (mg ml^{-1})	Amidase (U ml^{-1})	DBC of protein (mg ml^{-1} adsorbent)	DBC of amidase (U ml^{-1} adsorbent)
Full disruption	41.4 (6000 psi)	5	0.42	11.80	2.01	115.64
Partial disruption	13.8 (2000 psi)	2	0.31	11.08	1.24	89.94

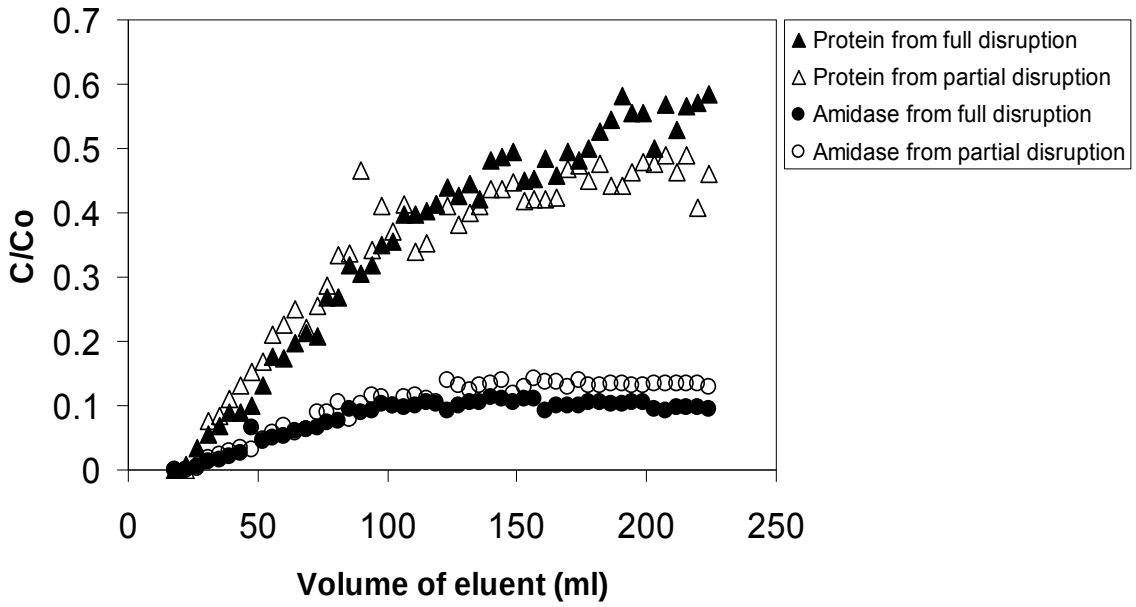


Figure 5.4: The breakthrough profile of total soluble protein and amidase activity from clarified feedstocks using Streamline DEAE adsorbent after subjecting cell suspension to partial and full disruption

5.4.4 IMPACT OF CELL DEBRIS ON PROTEIN AND AMIDASE ADSORPTION FROM UNCLARIFIED LYSATE IN AN EBA COLUMN

The unclarified feed following both partial and complete disruption characterised in Table 5.7, were passed through the stable expanded bed at a flow rate of 0.65 ml min^{-1}. The settled bed expanded approximately up to 2.5 fold prior to application of feedstock. Bed stability was maintained despite the slight increase (1.2 fold) observed in bed height due to the presence of cell debris in the feedstock. A 1.19 fold increase in release of both amidase and soluble protein on complete disruption over that released at partial disruption was observed.

Table 5.7: Results of protein concentration and amidase activity from unclarified cell suspension after partial and full disruption

Relative Intensity of disruption	Operating Pressure (MPa)	Number of passes	Protein (mg ml^{-1})	Amidase (U ml^{-1})	DBC of protein (mg ml^{-1} adsorbent)	DBC of amidase (U ml^{-1} adsorbent)
Full disruption	41.4 (6000 psi)	5	0.43	10.58	1.32	50.36
Partial disruption	13.8 (2000 psi)	2	0.36	8.91	1.26	49.86

Analysis of the collected fractions indicated that general protein and amidase started to breakthrough the bed when less than 18 ml of eluent had been collected (Figure 5.5a). Breakthrough was observed to be sharper for protein while amidase was gradually increased in the effluent reaching its peak after feeding about 100 ml. The slight retention of amidase over the general protein suggests that amidase was prefentially adsorbed by STREAMLINE DEAE despite the presence of cell debris, after either complete or partial disruption.

As with clarified feedstock, DBC values were higher for protein and amidase when feed from complete cell disruption (Table 5.7). However, the difference between DBC values of homogenate from full and incomplete cell disruption is small when compared with values determined from clarified samples. While a difference of 22 to 40% was observed with clarified feedstock, the DBC difference for crude homogenate differed by 0.1 to 5%. It is suspected that the finer cell debris generated at complete disruption affected the binding capacity. As presented in Figure 5.5b, the ratio of biomass absorbance at the outlet as a fraction of the inlet concentration (A/Ao), as a function of the volume of eluent collected shows that the debris from partial cell breakage came through earlier and sharper. The breakthrough curve levelled out after collecting 120 ml of eluent. Meanwhile, cell fragments from complete disruption (which are expected to be finer) increasingly and gradually broke through, suggesting an increase in residence time for finer cell debris (Figure 5.5b).

Since increase in residence time will enhance interaction between the adsorbent and the cell debris, the decreased DBC (between feedstock from full and incomplete cell disruption) can be attributed to the finer debris generated at increased intense in cell homogenisation. The impact of biomass and its effect on protein and amidase adsorption is discussed in detail in the subsequent section.

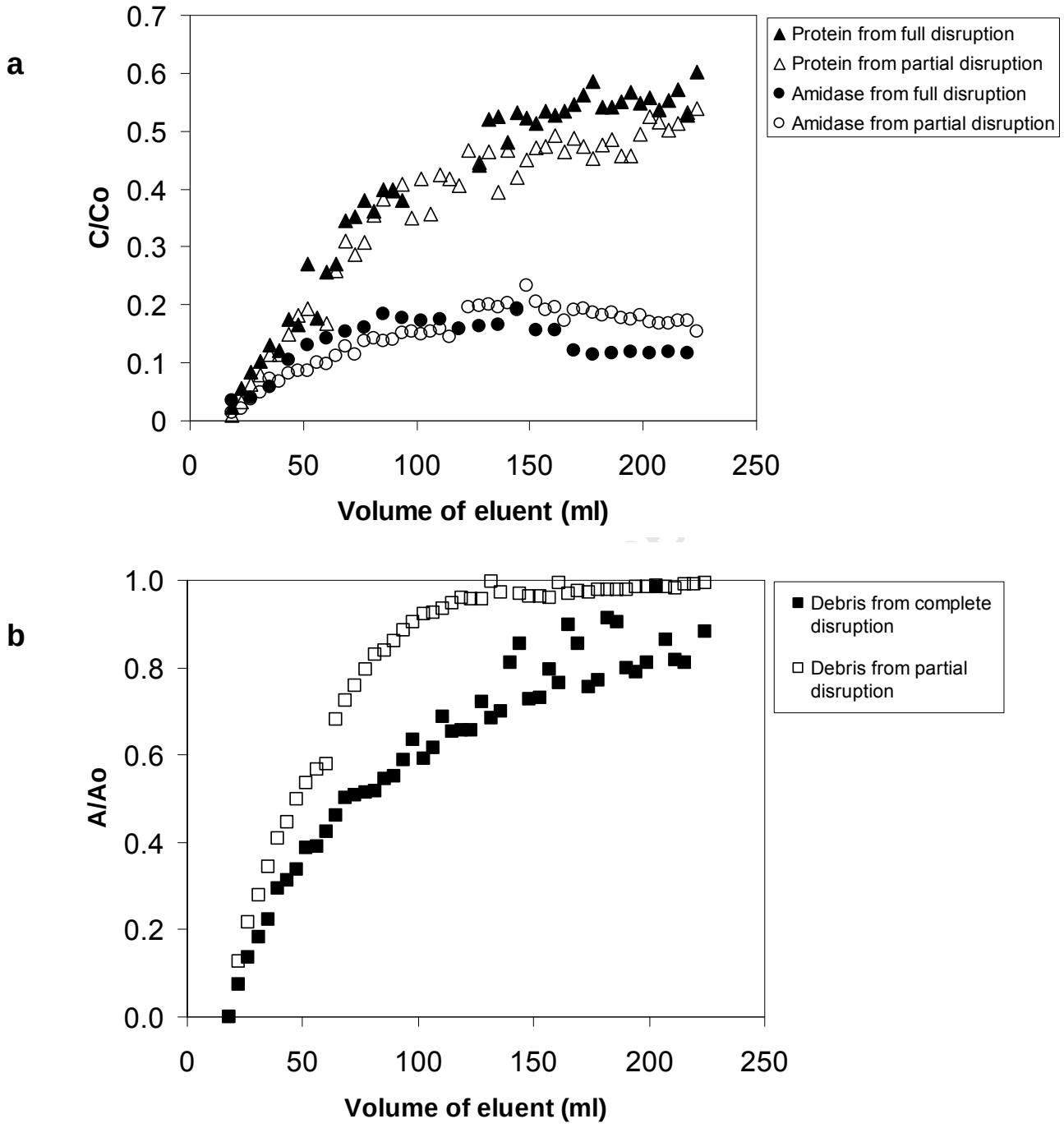


Figure 5.5: The breakthrough profile of (a) total soluble protein and amidase activity (b) cell debris, from unclarified feedstocks using Streamline DEAE adsorbent after subjecting cell suspension to partial and full disruption

5.5 GENERAL DISCUSSION ON INFLUENCE OF BIOMASS ON BED STABILITY AND PROTEIN-ADSORBENT ADSORPTION

In protein purification, the use of clarified cell lysates in EBA is expected to show similar bonding to packed bed while unclarified is typical of EBA. Lin *et al.* (2004) had previously noted that most cells are negatively charged under fluid phase conditions and hence react with positively charged anion exchange resin. Hence, the use of clarified and crude feedstock (whole cells and cell debris) is discussed in this section with a view to assessing the application of EBA for amidase separation.

Although some authors have reported bed channeling due to biomass aggregation and accumulation at the fluid distributor or in the column (Fernandez-Lahore *et al.*, 1999; Feuser *et al.*, 1999; Hubbuch *et al.*, 2006), the expanded bed investigated in this study was stable across all experimental conditions, irrespective of the feedstock composition. The use of clarified feed resulted in the same stability as use of the buffer solution, while the application of crude homogenate and whole cell suspension in BSA solution increased the bed height slightly. This can be attributed to an increase in fluid viscosity, as expected for feed containing biomass, either whole or broken.

The sigmoid shapes of the breakthrough curves were identical, irrespective of whether a clarified feed or crude homogenate was used. However, the breakthrough point was influenced by the presence of cell debris. A comparison of Figures 5.4 and 5.5a shows that early breakthrough was observed when crude feedstock was applied. For example, when clarified homogenate (after full disruption) was used, amidase activity was detected after collecting about 31 ml of eluent, meanwhile amidase broke through earlier (less than 20 ml of eluent collected) with crude homogenate. This corresponds to 36.5 U ml^{-1} and 23.6 U ml^{-1} adsorbed at breakthrough, which was also the maximum load capacity for amidase. This difference in the breakthrough point and the amount of amidase enzyme adsorbed shows that the presence of biomass and cell debris affected amidase adsorption to the STREAMLINE DEAE adsorbent. This was further confirmed by the dynamic binding capacity presented in Table 5.6 and 5.7. From the dynamic breakthrough capacities calculated (after full cell disruption), the application of the crude homogenate feedstock led to a 56% reduced capacity of the adsorbent, while a value of 45% was determined for homogenate from partial cell disruption for amidase at 10% breakthrough capacity. This difference in adsorption capacity (between feedstock from

partial or complete cell homogenisation) is postulated to be due to the increased residence time of finer debris from complete disruption as evident in Figure 5.5b. Although reports from Hubbuch *et al.* (2006) and Balasundaram and Harrison (2008a) reported enhanced adsorption performance with smaller cell fragments, Gondkar *et al*. (2001) suggested that finer debris (from micronisation of cells using homogenisation) can enter and block pores of adsorbent matrix and thus lead to diffusional limitation of protein. This in turn reduces binding capacity of the desired enzyme. Further, the plateauing of the amidase breakthrough profile at low C/Co in the range 0.1 to 0.2 suggests the continuous adsorption of the amidase enzyme. This could be due to competitive binding or non-specific retention in multilayer adsorption of the protein (Anand *et al.*, 2007).

From this study, protein adsorption capacity was reduced by the presence of biomass, either whole *E. coli* cells or its disrupted fragments (irrespective of the extent of homogenisation). This is consistent with reports by other authors. Hubbuch *et al.* (2006) studied the influence of homogenisation conditions on biomass-adsorbent interaction using ion exchange in EBA. The authors assessed protein adsorption by comparing breakthrough for crude homogenate and clarified feedstock and observed that the presence of cell debris led to 32% reduced capacity at 10% breakthrough. This was attributed to fluid viscosity and the adsorption of small debris particles to the adsorbent by electrostatic forces. In another study Feuser *et al.* (1999) showed that only 15% *E. coli* whole cell was retained within a DEAE expanded bed, while Fernandez-Lahore *et al.* (2000) showed that biomass from *E. coli* homogenate slightly interfered with protein adsorption to DEAE adsorbent.

Generally, the reduced binding capacity observed using crude homogenate as feedstock is attributed to factors such as electrostatic interaction between feed composition and adsorbent (Feuser *et al.*, 1999; Fernandez-Lahore *et al.*, 2000). This biomass-adsorbent bonding, as well as adsorbent interaction with other contaminating particulates (especially nucleic acids) leads to competition with the desired protein for binding spaces. In addition, increases in fluid viscosity (due to the presence of biomass and debris) can reduce inter-transport between the protein molecule and the adsorbent beads. However, *E. coli* interaction with ion exchange matrices, especially the anionic DEAE adsorbent varies. Barnfield-Frej et *al.* (1994a) noted that various *E. coli* host cells

differ in pH stability, which in turn impacts the degree of binding to beads. Other factors which impact on the surface charge of *E. coli* cells include medium composition, cultivation conditions, harvest time and storage. The use of fresh or frozen cells are also considered important, as the latter were found to be sticky and thus adsorb easily to matrices (Barnfield-Frej *et al.*, 1994a). Hence, biomass-adsorbent interactions occurring with use of *E. coli* as host cells can be regarded as system-specific.

5.6 SUMMARY

The aim of this study was to use an EBA process to capture the amidase from the crude homogenate in a single purification process step. Initial studies were carried out to choose the most appropriate adsorbent for maximum amidase separation and hence conditions for favourable retention of amidase over general protein were developed. An anionic DEAE adsorbent usually used in ion exchange purification was found to adsorb amidase prefentially (approximately 80% retained on adsorbents) over the use of cationic SP beads and maximally at pH 7.7 using a phosphate buffer with ionic strength of 50 mM. The maximum enzyme loading was calculated to be 18 U ml^{-1} adsorbent in the shake flask. In addition, the use of crude and clarified feedstock in the shake flask showed identical adsorption characteristics.

Further studies were carried out in an EBA column to investigate the influence of whole cells, as well as cell debris from partial and complete disruption, on protein adsorption to the DEAE adsorbent. Bed stability was ensured across all experiments using 0.5 to 1% biomass concentration (w/v, wet weight) irrespective of homogenisation treatment. Amidase enzyme was selectively adsorbed by the DEAE adsorbent over general protein present in the feedstock and the ongoing amidase adsorption suggests both specific and non-specific binding. The selective enzyme adsorption was evident in the delayed breakthrough exhibited by the enzyme. The maximum enzyme loading capacity increased by two fold in the column using clarified feedstock after complete cell disruption. There was a 27% reduction in enzyme loading capacity when crude homogenate was used, indicating the influence of biomass on protein retention to the beads. The early breakthrough and reduced dynamic binding capacity displayed by the use of crude homogenate as feedstock further confirms that biomass impacted amidase adsorption to the DEAE beads. This was more pronounced when completely disrupted samples were used as feedstock, suggesting that the finer debris particles interacted

more with the adsorbent. The gradual breakthrough of biomass particles when crude homogenate from full homogenised samples were used as feedstock confirms this. Beside the postulated electrostatic interaction between the biomass and adsorbent which led to reduction in binding capacity, it is suspected that the fluid viscosity and intraparticle diffusional limitation were other contributory factors.

Although further optimisation studies with crude homogenate need to be carried out, this study has demonstrated that EBA can be used for amidase capture from crude homogenate due to the higher affinity of the enzyme by anionic DEAE adsorbent beads over general proteins. Hence, the solid-liquid separation steps (centrifugation and microfiltration) can be merged with initial capture process to form a unit operation. This will enhance yield and impact on production cost.

Chapter 6

General conclusions and recommendations

6.0 INTRODUCTION

The use of enzymes as biocatalysts has shown immense potential for industrial syntheses due to numerous advantages, including mild operating conditions, improved specificity, wide substrate acceptability and reduction in the generation of by-products. These properties make biocatalysis attractive on comparison with conventional chemical processes over a wide range of applications. To ensure economical biocatalytic processes, high productivity of the enzymes is needed. In many cases, these enzymes are preferably expressed by recombinant organisms on a process scale. For the effective production of these recombinant enzymes, the cloned gene must be optimally expressed by the host organism and the enzyme subsequently recovered in a minimal number of steps. These two features are crucial for a high yield and the cost effective production of the recombinant protein.

The current study was carried out to optimise the production of a novel thermostable *Geobacillus pallidus* amidase enzyme from recombinant *E. coli*. The objectives of this study were to increase enzyme productivity by growing the host cell to a dense culture, choosing an induction method that favours maximum expression of the enzyme and also reducing the unit operations involved in downstream processing. These were investigated and the major findings are summarised in this chapter with a view to drawing out conclusions. Areas for further investigations are also presented.

6.1 CONCLUSIONS

6.1.1 OPTIMISATION OF AMIDASE EXPRESSION IN BATCH AND FED BATCH BIOREACTOR STUDIES

Preliminary studies in batch cultivation were carried out to determine the choice of growth medium for enhancement of biomass concentration and timing of induction to improve amidase expression. Further studies in fed batch process were carried out to improve volumetric amidase activity as it was envisaged that the expected increase in biomass concentration would result in a corresponding increase in volumetric amidase activity.

The biomass production was compared using a complex Luria Broth (LB) medium and glucose-supplemented LB growth medium and a glucose-based defined medium. Although the complex LB and glucose-supplemented LB media gave a higher growth rate (0.45 h^{-1}) in shake-flask culture, this was insufficient to improve biomass concentration beyond 1.0 g l^{-1} (DCW). However, the use of a defined medium led to a 1.7 fold increase in biomass concentration. Thereafter, the LB and glucose-based defined medium were compared using the 7 L New Brunswick bioreactor in batch cultivation. Growth conditions with both media were enhanced using the bioreactor. The defined medium resulted in a greater increase in biomass concentration at a lower growth rate, resulting in a biomass concentration of 9.40 g dry mass l^{-1}. This represented a 5 fold increment over the LB growth medium. This value was further increased up to 40 g l^{-1} in a fed batch process using an exponential feeding approach to support the increase in biomass accumulation while maintaining a pseudo-steady state growth rate. Hence, this study has emphasised the importance of choice of growth medium and cultivation process in biomass accumulation.

It was recognised that volumetric amidase production is a product of volumetric biomass production and specific amidase activity. Hence, the most favourable induction condition was sought by manipulating the time of inducer addition using the appropriate IPTG concentration for optimal enzyme expression. IPTG was added to the process in early, and mid exponential phase. The IPTG concentration range investigated of 0 to 1000 µM was selected based on the range of concentrations reported in the literature. This corresponded to a specific IPTG concentration of 0 to 200 µmol IPTG g^{-1} biomass, calculated based on the ratio of IPTG to biomass concentration at the end of batch

cultivation. A 4 fold increase in amidase per unit protein was observed when expression was induced at early exponential phase compared to induction at mid exponential phase. The inducer concentrations studied did not significantly affect biomass production and protein concentration was maintained over the range of inducer concentration 40 to 1000 μM, following a doubling of protein concentration on the addition of IPTG. The maximum amidase specific activity (180 U mg^{-1} protein) was observed at IPTG concentration of 400 μM, corresponding to a concentration of 80 μmol IPTG g^{-1} biomass. This represented a 6 fold increase over the highest value reported to date for this system (Cameron *et al.*, 2005; Makhongela *et al.*, 2007). No improvement was observed in the amidase activity on further increase in the IPTG concentration to 1000 μM on a volumetric basis and 200 μmol IPTG g^{-1} biomass on a biomass basis. Induction was further shown to be biomass-based. Therefore, the prospective concentration of biomass in a process should be taken into consideration prior to protein induction. Previous reports have suggested that protein expression is dependent on factors which include biochemical properties of the recombinant protein, induction mechanism of the gene expression, host strains and cultivation conditions. Hence, this study is specific for *G. pallidus* amidase from *E. coli* BL21 (DE3). However, the method adopted for optimising amidase in this study can be used for maximising recombinant protein production from micro-organism.

An analysis of the cell debris fraction using SDS-PAGE electrophoresis and electron microscope showed that the amidase was produced as intracellular, soluble and cytoplasmic enzyme, thereby enhancing its potential for application on a process scale over a mixed location or cell envelop-based location. To further improve amidase production, the role of cell culture was investigated in a fed batch process.

As indicated, fed-batch cultivation allowed enhanced biomass concentrations to be achieved (4 fold increase). To maximize amidase production, induction under these culture conditions required optimisation. Induction was carried out using three approaches (enbloc, continuous and intermittent addition of inducer), while selection pressure for the amidase-encoding plasmid was provided by the continuous or intermittent addition of ampicillin. Inducer concentration was increased to cater for the improved biomass concentration in the fed-batch system. The initial specific growth rate at the start of feeding was maintained at 0.2 h^{-1} to prevent acetate formation and also

support plasmid stability. There was a gradual reduction in the population of recombinant cells to less than 2% at the end of the fed batch process. This indicated reduced plasmid stability. While biomass and protein concentration were not affected, volumetric amidase activity reduced greatly when compared with values from batch studies. Hence, specific amidase activity was low across all fed-batch experiments, further indicating the influence of loss in recombinant organisms. Simple agar-plate well experiments showed that ampicillin had been degraded from the start of the experiment in the batch phase. Hence, selection pressure was not maintained for the recombinant strain for the duration of the experiment. The culture became vulnerable to out-competition by the wild type organism on lost of selection pressure and this was aggravated by the presence of additional nutrients to support growth in the fed-batch phase. The selection pressure could not be maintained by intermittent or continual addition of ampicillin through the fed-batch culture. It is postulated that β-lactamase, which itself is a by-product of the recombinant *E. coli* cultivation, was responsible for the degradation of the β-lactam antibiotic, ampicillin. Therefore, amidase expression could not be improved through fed batch studies. While β-lactam antibiotics have been widely used to maintain selection pressure for recombinant studies, these findings are supported by two previous reports that β-lactam antibiotics are less than ideal for maintaining selective pressure in *E. coli* cultivation. Therefore, a revised plasmid construct using an improved mechanism or approach to maintain selective pressure for maximum amidase production in the bioreactor is required.

6.1.2 EXPANDED BED ADSORPTION

The steps involved in downstream processing of amidase, an intracellularly secreted enzyme, could require up to 8 unit operations. Merging of unit operations to reduce the process train will lead to less exposure of the enzyme to contaminating biomolecules (especially nucleases), reduction in processing time, a higher yield and hence increased productivity. The use of expanded bed adsorption combines three steps in the downstream processing: centrifugation, microfiltration and initial capture of the desired protein. The aim of this study was to use the EBA process to recover amidase from crude homogenate in a single purification process step. The critical factors for the successful operation of this process were investigated initially in batch experiments using shake flask and subsequently column experiments. These factors include choice

of adsorbent, operating conditions that favour amidase adsorption over general protein and the influence of feedstock.

Initial studies were carried out in batch shake flask experiments to determine the choice of adsorbents and operating conditions of pH and ionic strength. Ion exchange purification method was used for amidase recovery. Thus, the anionic DEAE and cationic SP adsorbents were compared for maximum enzyme adsorption. The use of anionic DEAE adsorbent gave the best amidase separation from general protein, with maximum enzyme retention (approximately 80% adsorption) found at pH 7.7 using 50 mM phosphate buffer (conductivity of 15.60 mS). Subsequently, the use of clarified samples (characteristic of the feed used in packed bed) was compared to the use of crude homogenate as feedstock (typical of the EBA process). Hence, the extent of interference of biomass and cell debris on protein adsorption was estimated. Preliminary studies were carried out in the shake flask experiments and afterwards in the column. Similar adsorption performance for protein and amidase (less than 2% difference in percent adsorption) was observed in the shake flask suspension when clarified or crude homogenate were used. This suggests little interference of biomass at the shake flask level. However, pronounced interaction was observed in the column.

Three forms of crude homogenate were examined in the column: 1) whole *E. coli* cells in BSA solutions, 2) partially broken and 3) completely disrupted *E. coli* cells. Amidase was preferentially separated from other proteins irrespective of whether clarified or crude homogenate was used as feedstock. This confirms early studies in the shake flask experiment. This was evident in the late breakthrough exhibited by the enzyme. Biomass interaction with the DEAE adsorbent was minimal when whole cells were used, while cell debris from partially and completely disrupted feedstock interacted more with the adsorbent. This led to 27% decrease in maximum enzyme loading capacity, early amidase breakthrough and reduction in dynamic binding capacity. Cell debris from complete cell homogenisation was released from the column gradually compared with the release of whole cells. This suggests that the micronised debris interacted more strongly with the adsorbent than the cell fragments from partially homogenised solution. Electrostatic interaction between the cells and DEAE absorbent and increased viscosity are postulated as contributory factors to the reduction of protein dynamic binding capacity when crude samples were used. This study has shown that amidase can be

selectively captured from crude homogenate using EBA. However, further optimisation studies need to be carried out to improve amidase adsorption directly from unclarified feedstock.

6.2 RECOMMENDATIONS

Based on the findings of this current study, the following recommendations can be made:

- To improve specific amidase production, thorough studies need to be carried out on ways to improve selective pressure for the cloned, plasmid-borne gene. This could include choice of plasmid, antibiotics or manipulation of process conditions in the bioreactor.

- Although the use of IPTG is preferred to lactose as inducer due to catabolite repression, the former is toxic and expensive for use on a process scale. Hence, the use of IPTG may be unsafe and non-economical. Further studies need to be carried out on the use of lactose as an inducer to make this project executable on industrial scale.

- Beta-lactam antibiotic, ampicillin, is an amide and thus constitutes a substrate for excess amidase that may leak into the culture. Although the degradation of ampicillin was mostly due to the expression of beta-lactamase by the plasmid, it will be necessary to know if amidase acting on ampicillin contributes to the problem. This will assist in further process development.

- To enhance amidase capture from crude homogenate, further investigation is needed to reduce interference of biomass and cell debris. This will also prevent the harsh cleaning in place conditions that are needed to regenerate the adsorbents. Moreover, the recovery of the amidase needs to be investigated.

- In view of the fact that the DEAE is a non-specific adsorbent, it is recommended that the use of hydrophobic or more specific adsorbents be considered for amidase purification.

References

Agarkar, V. B., Kimani, S. W., Cowan, D. A., Sayed, M. F. R. and Sewell, A. T. (2006). The quatenary structure of the amidase from *Geobacillus pallidus* RAPc8 is revealed by its crystal packing. *Acta Crystallographica* **F62,** 1-5.

Amersham-Biosciences (1997). Expanded bed adsorption handbook; principles and methods.

Anand, H., Balasundaram, B., Pandit, A. B. and Harrison, S. T. L. (2007). The effect of chemical pretreatment combined with mechanical disruption on the extent of disruption and release of intracellular protein from *E.coli*. *Biochemical Engineering Journal,* **35,** 116-173.

Anderson, D. C. and Krummen, L. (2002). Recombinant protein expression for the therapeutic applications. *Current Opinion in Biotechnology* **13,** 117 - 123.

Anonymous (2006). www.bio.uu.nl. Accessed July, 2006.

Anspach, F. B., Curbelo, D., Hartmann, R., Garke, G. and Deckwer, W. (1999). Expanded bed chromatography in primary protein purification. *Journal of Chromatography A,* **865,** 129-144.

Asano, Y., Tachibana, M., Tani, Y. and Yamada, H. (1982). Purification and characterisation of amidase which participates in nitrile degradation. *Agricultural and Biological Chemistry,* **46,** 1175-1181.

Babaeipour, V., Shojaosadati, S. A., Robatjazi, S. M., Khalilzadeh, R. and Maghsoudi, N. (2007). Over-production of human interferon-γ by HCDC of recombinant *Escherichia coli*. *Process Biochemistry,* **42,** 112-117.

Baek, D. H., Kwon, S., Hong, S., Kwak, M., Lee, M., Song, J. J., Lee, S., Yoon, K. and Sung, M. (2003). Characterisation of a thermostable D-stereospecific alanine amidase from *Brevibacillus borstelensis* BCS-1. *Applied and Environmental Microbiology,* **69,** 980-986.

Bailey, J. E. and Ollis, D. F. (1986). *Biochemical Engineering Fundamentals,* Singapore, McGraw-Hill Inc.

Balasundaram, B. (2004). A detailed investigation of microbial cell disruption by hydrodynamic cavitation for selective product release. Ph.D Thesis, Department of Chemical Engineering University of Cape Town.

Balasundaram, B. and Harrison, S. T. L. (2006a). Disruption of brewers' yeast by hydrodynamic cavitation: Process variables ad their influence on selective release. *Biotechnology and Bioengineering,* **94,** 303-311.

Balasundaram, B. and Harrison, S. T. L. (2006b). Study of physical and biological factors involved in the disruption of *E.coli* by hydrodynamic cavitation. *Biotechnology progress,* **22,** 907-913.

Balasundaram, B. and Harrison, S. T. L. (2008a). Influence of the extent of disruption of baker's yeast on protein adsorption in expanded beds. *Journal of Biotechnology,* **133,** 360-369.

Balasundaram, B. and Harrison, S. T. L. (2008b). Influence of the micronisation of the *E. coli* cell debris during high pressure homogenisation on the adsorption of protein and β-galactosidase by EBA. *Journal of Biotechnology,* **In prep**.

Balasundaram, B. and Pandit, A. B. (2001a). Selective release of invertase by hydrodynamic cavitation. *Biochemical Engineering Journal,* **8,** 251-256.

Balasundaram, B. and Pandit, A. B. (2001b). Significance of location of enzymes on their release during microbial disruption. *Biotechnology and Bioengineering,* **75,** 607-614.

Banerjee, A., Sharma, R. and Banerjee, U. C. (2002). The nitrile degrading enzyme: current status and future prospects. *Applied Microbiology and Biotechnology,* **60,** 33-44.

Barnfield-Frej, A., Hjorth, R. and Hammarstrom, A. (1994a). Pilot scale recovery of recombinant annexin V from unclarified *Escherichia coli* homogenate using expanded bed adsorption. *Biotechnology and Bioengineering,* **44,** 922-929.

Barnfield-Frej, A. K., Hjorth, A. and Hammarstroem, A. (1994b). Cell/adsorbent interactions in expanded bed adsorptions of proteins. *Bioseparation,* **8,** 99-109.

Basaez, L. and Vanysek, P. (1999). Transport studies of β-lactam antibiotics and their degradation products across electrified water/oil surface. *Journal of Pharmaceutical and Biomedical Analysis,* **19,** 183-192.

Beilen, J. B. and Li, Z. (2002). Enzyme technology: an overview. *Current Opinion in Biotechnology,* **13,** 338-344.

Bentley, W. E., Davis, R. H. and Kompala, D. S. (1991). Dynamics of induced CAT expression in *E.coli*. *Biotechnology and Bioengineering,* **38,** 749-760.

Bierau, H., Zhang, Z. and Lyddiatt, A. (1999). Direct process integration of cell disruption and fluidised bed adsorption for the recovery of intracellular proteins. *Journal of Chemical Technology and Biotechnology,* **74,** 208-212.

Blanch, H. W. and Clarke, D. S. (1996). *Biochemical Engineering,* New York, M. Dekker.

Bommarus, A. S. and Polizzi, K. M. (2006). Novel biocatalysts: Recent developments. *Chemical Engineering Science,* **61,** 1004-1016.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry,* **72,** 248-254.

Brobjer, M. (1999). Development and scale up of a capture step (expanded bed chromatography) for a fusion protein expressed intracellularly in *Escherichia coli*. *Bioseparation,* **8,** 219-228.

Brown, T. A. (1989). *Genetics: a molecular approach,* London, Van Nostrand Reinhold (International) Co. Ltd.

Bruce, L. J., Ghose, S. and Chase, H. A. (1999). The effect of column verticality on seperation efficiency in expanded bed adsorption. *Bioseparation,* **8,** 69-75.

Burton, S. G., Cowan, D. A. and Woodley, J. M. (2002). The search for the ideal biocatalyst. *Nature Biotechnology,* **20,** 37-45.

Cabral, J. M. S. (2001). Biotransformations. In: Ratledge, C., and Kristiansen, B. (Ed.) *Basic Biotechnology.* Cambridge, Cambridge University Press.

Cameron, R. A., Sayed, M. and Cowan, D. A. (2005). Molecular analysis of the nitrile catabolism operon of the thermophile *Bacillus pallidus* RAPc8. *Biochimica et Biophysica Acta* **1725,** 35 – 46.

Chang, Y. K. and Chase, H. A. (1996a). Development of operating conditions for protein purification using expanded bed techniques: The effect of degree of bed expansion on adsorption performance. *Biotechnology and Bioengineering,* **49,** 512-526.

Chang, Y. K. and Chase, H. A. (1996b). Ion exchange purification of G6PDH from unclarified yeast cell homogenates using expanded bed adsorption. *Biotechnology and Bioengineering,* **49,** 204-216.

Chase, H. A. (1994). Purifiction of proteins by adsorption chromatography in expanded beds. *Trends in Biotechnology,* **12,** 296-303.

Chase, H. A. and Draeger, N. M. (1992). Affinity purification of proteins in an expanded bed. *Journal of Chromatography* **597,** 129-145.

Chen, S. J., Chang, M. C. and Cheng, C. Y. (1997). Effect of Induction Conditions on Production and Excretion of *Aeromonas hydrophila* Chitinase by Recombinant *Escherichia coli*. *Journal of Fermentation and Bioengineering,* **84,** 610-613.

Cheong, T. K. and Oriel, P. J. (2000). Cloning of a wide spectrum amidase from *Bacillus stearothermophilus* BR388 in *Escherichia coli* and marked enhancement of amidase expression using directed evolution. *Enzyme and Microbial Technology,* **26,** 152-158.

Chisti, Y. and Moo-Young, M. (1986). Disruption of microbial cells for intracellular products. *Enzyme and Microbial Technology,* **8,** 194-204.

Choi, J. H., Keum, K. C. and Lee, S. Y. (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli*. *Chemical Engineering Science,* **61,** 876-885.

Chou, C., Bennett, G. N. and San, K. (1996). Genetic manipulation of stationary-phase genes to enhance recombinant protein production in *Escherichia coli*. *Biotechnology and Bioengineering,* **50,** 636-642.

Cowan, D., Cramp, R., Pereira, R., Graham, D. and Almatawah, Q. (1998). Biochemistry and biotechnology of mesophilic and thermophilic nitrile metabolising enzymes. *Extremophiles,* **2,** 207-216.

Davis, W. W. and Stout, T. R. (1971). Disc plate method of microbiological antibiotic assay. *Applied Microbiology,* **22,** 659-665.

Dedhia, N. N., Hottiger, T. and Bailey, J. E. (1994). Overproduction of glycogen in *Escherichia coli* blocked in the aetate pathway improves cell growth. *Biotechnology and Bioengineering,* **44,** 132-139.

Domig, K. J., Mayrhofer, S., Zitz, U., Mair, C., Petersson, A., Amtmann, E., Mayer, H. K. and Kneifel, W. (2007). Antibiotic susceptibility testing of *Bifidobacterium thermophilum* and *Bifidobacterium pseudolongum* strains: Broth microdilution vs. agar disc diffusion assay. *International Journal of Food Microbiology,* **120,** 191-195.

Dong, X. and Sun, Y. (2001). Nd-Fe-B alloy-densified agarose gel for expanded bed adsorption of proteins. *Journal of Chromatography A,* **943,** 63-75.

Donovan, R. S., Robinson, C. W. and Glick, B. R. (1996). Review: optimising inducer and culture conditions for expression of foreign proteins under the control of the lac promoter. *Journal of Industrial Microbiology,* **16,** 145-154.

Doran, J. P., Duggan, P., Masterson, M., Turner, P. D. and O' Reilly, C. (2005). Expression and purification of a recombinant enantioselective amidase. *Protein Expression and Purification,* **40,** 190-196.

Doran, P. M. (1995). *Bioprocess Engineering Principles,* London, Academic Press Limited.

Dordick, J. S., Khmelnitsky, Y. L. and Sergeeva, M. V. (1998). The evolution of biotransformation technologies. *Current Opinion in Microbiology,* **1,** 311-318.

Duan, K. J., Lin, M. T., Hung, Y. C., L, C. T., Chen, C. W. and Sheu, D. C. (2000). Production of GST-SOD fusion protein by recombinant *E. coli* XL1 Blue. *Journal of Chemical Technology and Biotechnology,* **75,** 722-728.

Durany, O., Caminal, G., Mas, C. and Lopez-Santin, J. (2004). Studies on the expression of recombinant fuculose-1-phosphate aldolase in *E.coli*. *Process Biochemistry,* **39,** 1677-1684.

Egorova, K., Trauthwein, H., Verseck, S. and Antranikian, G. (2004). Purification and properties of an enantioslective and thermoactive amidase from the thermophilic actinomycete *Pseudonocardia thermophila*. *Applied Microbiology and Biotechnology,* **65,** 38-45.

Engler, C. R. (1985). Disruption of microbial cells. In: Moo-Young, M. (Ed.) *Comprehensive Biotechnology.* Oxford, Permagon Press.

Fahrner, R. L., Blank, G. S. and Zapata, G. A. (1999). Expanded bed protein A affinity chromatography of a recombinant humanized monoclonal antibody: process development, operation, and comparison with a packed bed method. *Journal of Biotechnology,* **75,** 273-280.

Fernandez-Lahore, H. M., Geilenkirchen, S., Boldt, K., Nagel, A., Kula, M. R. and Thommes, J. (2000). The influence of cell adsorbent interactions on protein adsorption in expanded beds. *Journal of Chromatography A,* **873,** 195-208.

Fernandez-Lahore, H. M., Kleef, R., Kula, M. R. and Thommes, J. (1999). The influence of complex biological feedstock on the fluidisation and bed stability in expanded bed adsorption. *Biotechnology and Bioengineering,* **64,** 484-496.

Feuser, J., Walter, J., Kula, M. and Thommes, J. (1999). Cell/adsorbent interactions in expanded bed adsorption of proteins. *Bioseparation,* **8,** 99-109.

Follows, M., Hetherington, P. J., Dunnill, P. and Lilly, M. D. (1971). Release of enzymes from baker's yeast by disruption in an industrial homogeniser. *Biotechnology and Bioengineering,* **13,** 549-560.

Fournand, D. and Arnand, A. (2001). Aliphatic and enantioselective amidases: from hydrolysis to acyl transfer activity. *Journal of Applied Microbiology,* **91,** 381-393

Fournand, D., Bigey, F. and Arnand, A. (1998). Acyl transfer activity of an amidase from rhodococcus sp. strain R312: Formation of a wide range of hydroxamic acids. *Applied and Environmental Microbiology,* **64,** 2844-2852.

Gavasane, M. R. and Gaikar, V. G. (2003). Aqueous two-phase parttioning of penicilin acylase from *E.coli* in presence of PEG-derivatives *Enzyme and Microbial Technology,* **32,** 665-675.

Glick, B. R. and Pasternak, J. J. (2003). Large scale production of proteins from micro-organisms. *Molecular Biotechnology.* ASM Inc.

Gondkar, S., Manudhane, K., Amritkar, N., Pai, A. and Lali, A. (2001). Effect of adsorbent porosity on performance of expanded bed chromatography of proteins. *Biotechnology Progress,* **17,** 522-529.

Grabherr, R., Nilsson, E., Striedner, G. and Bayer, K. (2002). Stabilising plasmid copy number to improve recombinant protein production. *Biotechnology and Bioengineering,* **77,** 142-147.

Gupta, R., Sharma, P. and Vyas, V. V. (1995). Effect of growth environment on the stability of a recombinant shuttle plasmid, pCPPS-31, in *Escherichia coli*. *Journal of Biotechnology,* **41,** 29-37.

Hahm, D. H., Kim, S. H., Pan, J. and Rhee, J. S. (1995). Maximum yield of foreign lipase in *Escherichia coli* HB101 limited by duration of protein expression. *Journal of Fermentation and Bioengineering,* **79,** 236-241.

Harper, D. (1977). Fungal degradation of aromatic nitriles: enzymology of C-N cleavage by *Fusarium solani*. *Biochemistry Journal,* **167,** 685-692.

Harrison, R. G., Todd, P., Rudge, S. R. and Petrides, D. P. (2003). *Bioseparation Science and Engineering,* New York, Oxford University Press.

Harrison, S. T. L. (1991). Bacterial cell disruption: A key operation in the recovery of intracellular products. *Biotechnology Advances,* **9,** 217-240.

Harrison, S. T. L., Dennis, J. S. and Chase, H. A. (1991). Combined chemical and mechanical processes for the disruption of bacteria. *Bioseparation,* **2,** 95-105.

Hasan, F., Shah, A. A. and Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology,* **39,** 235-251.

Hatti-Kaul, R. and Mattiasson, B. (2001). Downstream processing in biotechnology. In: Ratledge, C., and Kristiansen, B. (Ed.) *Basic Biotechnology.* 2nd ed. Cambridge, Cambridge University Press.

Held, M., Schmid, A., Van Beilen, J. B. and Witholt, B. (2000). Biocatalysis, biological systems for the production of chemicals. *Pure Applied Chemistry,* **72,** 1337-1343.

Hetherington, P. J., Follows, M., Dunnill, P. and Lilly, M. D. (1971). Release of protein from Bakers yeast (Saccharomyces cerevisiae) by disruption in an industrial homogeniser. *Trans. Instn. Chem. Engrs.,* **49,** 142-148.

Hirrlinger, B., Stolz, A. and Knackmuss, H. J. (1996). Purification and properties of an amidase from *Rhodococcus erythropolos* MP50 which enantioselectively hydrolyses 2-arylpropionamides. *Journal of Bacteriology,* **178,** 3501-3507.

Hoffmann, F., Heuvel, J., Zidek, N. and Rinas, U. (2004). Minimising inclusion body formation during recombinant protein production in *Escherichia coli* at bench and pilot plant scale. *Enzyme and Microbial Technology,* **34,** 235-241.

Hu, S. Y., Wu, J. L. and Huang, J. H. (2004). Production of tilapia insulin like growth factor-2 in high cell density cultures of recombinant *Escherichia coli*. *Journal of Biotechnology,* **107,** 161-171.

Huang, R. B., Andrews, B. A. and Asenjo, J. A. (1991). Differential product release (DPR) of proteins from yeast: A new technique for selective product recovery from microbial cells. *Biotechnology and Bioengineering,* **38,** 977-985.

Hubbuch, J. J., Brixius, P. J., Lin, D. Q., Mollerup, I. and Kula, M. R. (2006). The influence of homogenisation conditions in biomass-adsorbent interactions during ion-exchange expanded bed adsorption. *Biotechnology and Bioengineering,* **94,** 543-553.

Jeong, K. J., Choi, J. H., Yoo, W. M., Keum, K. C., Yoo, N. C., Lee, S. Y. and Sung, M. H. (2004). Constitutive production of human leptin by fed batch culture of recombinant rpsS *Escherichia coli*. *Protein Expression and Purification,* **36,** 150-156.

Kakimoto, T. and Funamizu, N. (2007). Factors affecting the degradation of amoxicillin in composting toilet. *Chemosphere,* **66,** 2219-2224.

Karau, A., Benken, C., Thommes, J. and Kula, M. R. (1997). The influence of particle size distribution and operating conditions on the adsorption performance in fluidised beds. *Biotechnology and Bioengineering,* **55,** 54-64.

Keshavarz, E., Hoare, M. and Dunnill, P. (1987). Biochemical engineering aspects of cell disruption. In: Verrall M.S. And Hudson, J. (Ed.) *Seperations for Biotechnology.* Ellis Horwood Ltd.

Khushoo, A., Pal, Y., Singh, B. N. and Makherjee, K. J. (2004). Extracellular expression and single step purification of recombinant *Escherichia coli* L–asparaginase II. *Protein Expression and Purification,* **38,** 29-36.

Kilikian, B. V., Suarez, I. D., Liria, C. W. and Gombert, A. K. (2000). Process strategies to improve heterologous protein production in *Escherichia coli* under lactose or IPTG induction. *Process Biochemistry,* **35,** 1019-1025.

Kim, M., Elvin, C., Brownlee, A. and Lyons, R. (2007). High yield expression of recombinant pro-resilin: Lactose-induced fermentation in *E.coli* and facile purification. *Protein Expression and Purification,* **52,** 230-236.

Kim, S. H. and Oriel, P. (2000). Cloning and expression of the nitrile hydratase and amidase genes from *Bacillus sp.* BR449 into *Escherichia coli*. *Enzyme and Microbial Technology,* **27,** 492-501.

Korpimaki, T., Kurittu, J. and Karp, M. (2003). Surprisingly fast disappearance of β-lactam selection pressure in cultivation as detected with novel biosensing approaches. *Journal of Microbiological Methods,* **53,** 37-42.

Korz, D. J., Rinas, U., Hellmuth, K., Sanders, E. A. and Deckwer, W. D. (1995). Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *Journal of Biotechnology,* **39,** 59-65.

Kotlova, E. K., Chestukhina, G. G., Astaurova, O. B., Leonova, T. E., Yanenko, A. S. and Debabov, V. G. (1999). Isolation and primary characteristion of an amidase from *Rhodococcus rhodochrous* M8. *Biochemistry,* **64,** 384-389.

Krijgsman, J. (1992). *Product recovery in bioprocess technology,* Oxford, Butterworth-Heinemann Ltd.

Kuboi, R., Umakoshi, H., Takagi, N. and Komasawa, I. (1995). Optimal disruption methods for selective recovery of β-galactosidase from *Escherichia coli*. *Journal of Fermentation and Bioengineering,* **79,** 335-341.

Kweon, D. H., Han, N. S., Park, K. M. and Seo, J. H. (2001). Overproduction of *Phytolacca insularis* protein in batch and fed batch culture of recombinant *Escherichia coli*. *Process Biochemistry,* **36,** 537-542.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature,* **227,** 680-685.

Laffend, L. and Shular, M. L. (1993). Structured model of genetic control via the *lac* promoter in *Escherichia coli*. *Biotechnology and Bioengineering,* **43,** 399-410.

Lau, J., Tran, C., Licari, P. and Galazzo, J. (2004). Development of a high cell density fed-batch bioprocess for the heterologous production of 6-deoxyerythronolide B in *Escherichia coli*. *Journal of Biotechnology,* **110,** 95-103.

Lee, C., Sun, W., Burgess, B. W., Junker, B. H., Reddy, J., Buckland, B. C. and Greasham, R. L. (1997). Process optimisation for large scale production of TGF-α-PE40 in recombinant *Escherichia coli*: effect of medium composition and induction timing on protein expression. *Journal of Industrial Microbiology and Biotechnology,* **18,** 260-266.

Lee, S. Y. (1996). High density culture of *Escherichia coli*. *Trends in Biotechnology,* **14,** 98-105.

Li, Y., Chen, J., Mao, Y., Lun, S. and Koo, Y. (1998). Effect of additives and fed batch culture strategies on the production of glutathione by recombinant *Escherichia coli*. *Process Biochemistry,* **33,** 709-714.

Lim, H. K., Jung, K. H., Park, D. H. and Chung, S. I. (2000). Production characteristics of interferon-α using L-arabinose promoter system in a high cell density culture. *Applied Microbiology and Biotechnology,* **53,** 201-208.

Lin, D. Q., Fernandez-Lahore, H. M., Kula, M. R. and Thommes, J. (2001a). Minimising biomass/adsorbent interactions in expanded bed adsorption processes: a methodological design approach. *Bioseparation,* **10,** 7-19.

Lin, D. Q., Kula, M. R., Liten, A. and Thommes, J. (2003). Stability of expanded beds during the application of crude feedstock. *Biotechnology and Bioengineering,* **81,** 21-26.

Lin, D. Q., Thommes, J., Kula, M. R. and Hubbuch, J. J. (2004). The influence of biomass on the hydrodynamic behaviour and stability of expanded beds. *Biotechnology and Bioengineering,* **87,** 337-346.

Lin, H. Y., Mathiszik, B., Xu, B., Enfors, S. O. and Neubauer, P. (2001b). Determination of the maximum specific uptake capacities for glucose and oxygen in glucose-limited fed-batch cultivations of *Escherichia coli*. *Biotechnology and Bioengineering,* **73,** 347-357.

Lowe, D. A. (2001). Production of enzymes. In: Ratledge, C. andKristiansen, B. (Eds.) *Basic Biotechnology.* Cambridge, Cambridge University Press.

Lutomski, D., Imam-Sghiouar, N., Blondeau, K., Caron, M. and Joubert-Caron, R. (2004). Purification of human galectin-1 produced in high density cultures of recombinant *Escherichia coli*: a comparison with classic shake flask cultivation. *Journal of Chromatography B,* **808,** 105-109.

Makhongela, H. S. (2006). Characterisation of a thermostable amidase and development of a bioreactor process for lactic acid production. M.Sc dissertation, Department of Chemical Engineering, University of Cape Town.

Makhongela, H. S., Glowacka, A. E., Agarkar, V. B., Sewell, B. T., Weber, B., Cameron, R. A., Cowan, D. A. and Burton, S. G. (2007). A novel thermostable nitrilase superfamily amidase from *Geobacillus pallidus* showing acyl transfer activity. *Applied Microbiology and Biotechnology,* **75,** 801-811.

Martins, S., Karmali, A. and Serralheiro, M. L. (2006). Kinetic properties of wid-type and altered recombinant amidases by the use of ion-selective electrode assay method. *Analytical Biochemistry,* **355,** 232-239.

Matsui, T., Shinzato, N., Yokota, H., Takahashi, J. and Sato, S. (2006). High cell density cultivation of recombinant *E.coli* with a pressurised culture. *Process Biochemistry,* **41,** 920-924.

Mattanovich, D., Kramer, W., Luttich, C., Weik, R., Bayer, K. and Katinger, H. (1997). Rational design of an improved induction scheme for recombinant *Escherichia coli*. *Biotechnology and Bioengineering,* **58,** 296-298.

Mayaux, J. F., Cerbelaud, E., Soubrier, F., Faucher, D. and Petre, D. (1990). Purification, cloning and primary structure of an enantiomer-selective amidase from *Brevibacterium sp.* strain R312: structural evidence for genetic coupling with nitrile hydratase. *Journal of Bacteriology,* **172,** 6764-6773.

Menzella, H. G., Ceccarelli, E. A. and Gramajo, H. C. (2003). Novel *Escherichia coli* strain allows efficient recombinant protein production using lactose as inducer. *Biotechnology and Bioengineering,* **82,** 809-817.

Miao, F. and Kompala, D. S. (1992). Overexpression of cloned genes using recombinant *Escherichia coli* regulated by a T7 promoter: Batch cultures and kinetic modelling. *Biotechnology and Bioengineering,* **40,** 787-796.

Middelberg, A. P. J. (1995). Process scale disruption of micro-organisms. *Biotechnology Advances,* **13,** 491-551.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* **31,** 426.

Moosa, S., Nemati, M. and Harrison, S. T. L. (2002). A kinetic study on the anaerobic reduction of sulphate, Part 1: Effect of sulphate concentration. *Chemical Engineering Science,* **57,** 2773-2780.

Muller, D. and Gabriel, J. (1999). Bacterial degradation of the herbicide bromoxylinil by *Agrobacterium radiobacter* in biofilm. *Folia Microbiol.,* **44,** 377-379.

Mylerova, V. and Martinkova, L. (2003). Synthetic applications of nitrile-converting enzymes. *Current Organic Chemistry,* **7,** 1-17.

Narciandi, R. E. (1996). High level production of p36 from HIV-2 in fed-batch culture of recombinant *Escherichia coli*. *Journal of Fermentation and Bioengineering,* **81,** 360-362.

Nawaz, M. S., Khan, A. A., Bhattacharayya, D., Siitonen, P. H. and Cerniglia, C. E. (1996). Physical, biochemical, and immunological characterisation of a thermostable amidase from *Klebsiella pneumoniae* NCTR 1. *Journal of Bacteriology,* **178,** 2397-2401.

Nawaz, S. M., Khan, A. A., Seng, J. E., Leakey, J. E., Siltonen, P. H. and Cemiglia, C. E. (1994). Purification and characterisation of an amidase from an acrylamide-degarading *Rhodococcus sp. Applied and Environmental Microbiology,* **60,** 3343-3348.

Nayak, D. P. and Vyas, V. V. (1999). Improved stability and expression of a recombinant shuttle plasmid in *Escherichia coli* during fedbatch cultivation. *World Journal of Microbiology and Biotechnology,* **15,** 65-71.

Neubauer, P., Lin, H. Y. and Mathiszik, B. (2003). Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli*. *Biotechnology and Bioengineering,* **83,** 153 - 164.

Ng, M. Y. T., Tan, W. S., Abdullah, N., Ling, T. C. and Tey, B. T. (2007). Direct purification of recombinant hepatitis B core antigen from two different pre-conditined unclarified *Escherichia coli* feedstocks via expanded bed adsorption chromatography. *Journal of Chromatography A,* **1172,** 47-56.

Puschmann, M., Grabherr, R., Striedner, G., Clementschitsch, F. and Bayer, K. (2002). Optimising recombinant microbial fermentation processes, an integrated approach. *Biopharm,* **July,** 1-9.

Rito-Palomares, M. and Lyddiatt, A. (2002). Process ntegration using aqeous two-phase partition for the recovery of intracellular proteins. *Chemical Engineering Journal,* **87,** 313-319.

Robas, N., Zouheiry, H., Branlant, G. and Branlant, C. (1992). Improved penicillin amidase production using a genetically engineered mutant of *Escherichia coli* ATCC 11105. *Biotechnology and Bioengineering,* **41,** 14-24.

Rothen, S. A., Sauer, M., Sonnleitner, B. and Witholt, B. (1998). Growth characteristics of *Escherichia coli*HB101[pGEc47] on defined medium. *Biotechnology and Bioengineering,* **58,** 92-100.

Sanden, A. M., Prytz, I., Tubulekas, I., Forberg, C., Le, H., Hektor, A., Neubauer, P., Pragai, Z., Harwood, C., Ward, A., Picon, A., Mattos, J. T., Postma, P., Farewell, A., Nystrom, T., Reeh, S., Pedersen, S. and Larsson, G. (2003). Limiting factors in *Escherichia coli* fed batch production of recombinant proteins. *Biotechnology and Bioengineering,* **81,** 158-166.

Schmidt, M., Babu, K. R., Khanna, N., Marten, S. and Rinas, U. (1999). Temperature induced production of recombinant human insulin in high density cultures of recombinant *Escherichia coli*. *Journal of Biotechnology,* **68,** 71-83.

Schutte, H. and Kula, M. R. (1990). Pilot and process scale techniques for cell disruption. *Biotechnol. Applied Biochem.,* **12,** 599-620.

Seader, J. D. and Henley, E. J. (1998). *Seperation Process Principles,* New York, John Wiley & Sons, Inc.

Seeger, A., Schneppe, B., Mccarthy, J. E. G., Deckwer, W. and Rinas, U. (1995). Comparison of temperature and isopropyl-β-D-thiogalacto-pyranoside-induced synthesis of basic fibroblast growth factor in high cell density cultures of recombinant *Escherichia coli*. *Enzyme and Microbial Technology,* **17,** 947-953.

Sheesan, D., Meade, G., Foley, V. and Dowd, C. A. (2001). Structure, function and evolution of glutathione transferases: Implications for classification of non-mammalian members of an ancient enzyme family. *Biochemistry Journal,* **360,** 1-6.

Shepard, S. R., Boyd, G. A. and Schrimsher, J. L. (2001). Routine manufacture of recombinant proteins using expanded bed adsorption chromatography. *Bioseparation,* **10,** 51-56.

Shiloach, J. and Fass, R. (2005). Growing *E.coli* to high density - A historical perspective on method development. *Biotechnology Advances,* **23,** 345-357.

Shuler, M. L. and Kargi, F. (2002). *Bioprocess Engineering, Basic Concepts,* New York, Prentice Hall PTR.

Sivakesava, S., Xu, Z. N., Chen, Y. H., Hackett, J., Huang, R. C., Lam, E., Lam, T. L., Siu, K. L., Wong, R. S. C. and Wong, W. K. R. (1999). Production of excreted human epidermal growth factor (hEGF) by an efficient recombinant *Escherichia coli* system. *Process Biochemistry,* **34,** 893-900.

Spiridonova, V. A., Lygina, A. S., Anohina, M. M. and Tupitsyn, N. N. (2007). Preparation of functionally active recombinant human interleukin-6. *Biochemistry (Moscow),* **72,** 424-429.

Srivastava, P. and Mukherjee, K. J. (2005). Kinetic studies of recombinant human interferon-alpha (rhIFN-α) expression in transient state continuous cultures. *Biochemical Engineering Journal,* **26,** 50-58.

Su, Z. G. and Feng, X. L. (1999). Process integration of cell disruption and aqueous two phase extraction. *Journal of Chemical Technology and Biotechnology,* **74,** 284-288.

Suarez, D. C. and Kilikian, B. V. (2000). Acetic acid accumulation in aerobic growth of recombinant *Escherichia coli*. *Process Biochemistry,* **35,** 1051-1055.

Swanson, P. E. (1999). Dehalogenases applied to industrial scale catalysis. *Current Opinion in Biocatalysis,* **10,** 365-369.

Tauber, M. M., Cavaco-Paulo, A., Robra, K. H. and Gubitz, G. M. (2000). Nitrile hydratase and amidase from *Rhodococcus rhodochrous* hydrolyse acrylic fibers and granular polyacrylonitriles. *Applied and Environmental Microbiology,* **66,** 1634-1638.

Teich, A., Lin, H. Y., Andersson, L., Meyer, S. and Meubauer, P. (1998). Amplification of ColE1 related palsmids in recombinant cultures of *Escherichia coli* after IPTG induction. *Journal of Biotechnology,* **64,** 197-210.

Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology,* **72,** 211-222.

Thommes, J., Weiher, M., Karau, A. and Kula, M. R. (1995). Hydrodynamics and performance in fluidised bed adsorption. *Biotechnology and Bioengineering,* **48,** 367-374.

Tong, X. D., Dong, X. Y. and Sun, Y. (2002). Lysozyme adsorption and purification by expanded bed adsorption chromatography with a small sized dense adsorbent. *Biochemical Engineering Journal,* **12,** 117-124.

Trott, S., Burger, S., Calaminus, C. and Stolz, A. (2002). Cloning and heterologous expression of an enantioselective amidase from *Rhodococcus erythropolis* strain MP50. *Applied and Environmental Microbiology,* **68,** 3279-3286.

Tsai, P. S., Hatzimanikatis, V. and Bailey, J. E. (1996). Effect of *Vitreoscilla* hemoglobin dosage on microaerobic *Escherichia coli* carbon and energy metabolism. *Biotechnology and Bioengineering,* **49,** 139-150.

Vennapusa, R., Hunegnaw, S. M., Cabrera, R. B. and Fernandez-Lahore, M. (2008). Assessing adsorbent-biomass interactions during expanded bed adsorption onto ion exchangers utilising surface energetics. *Journal of Chromatography A,* **1181,** 9-20.

Vergnault, H., Mercier-Bonin, M. and Willemot, R. (2004). Physicochemical parameters involved in the interaction of *Saccharomyces cerevisiae* cells with ion-exchange adsorbents in expanded bed chromatography. *Biotechnology Progress,* **20,** 1534-1542.

Vidal, L., Ferrer, P., Alvaro, G., Benaiges, M. D. and Caminal, G. (2005). Influence of induction and operation mode on recombinant rhamnulose 1-phosphate aldolase production by *Escherichia coli* using the T5 promoter. *Journal of Biotechnology,* **118,** 75-87.

Voute, N. and Boschetti, E. (1999). Highly dense beaded sorbents suitable for fluidised bed applications. *Bioseparation,* **8**, 115-120.

Walsh, G. (2002). *Protein biochemistry and biotechnology,* West Sussex, England.

Wang, Z., Le, G., Shi, Y. and Wegrzyn, G. (2001). Medium design for plasmid DNA production based on stoichiometric model. *Process Biochemistry,* **36,** 1085-1093.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry,* **39,** 971-974.

Weickert, M. J., Doherty, D. H., Best, E. A. and Olins, P. O. (1996). Optimisation of heterologous protein production in *Escherichia coli*. *Current Opinion in Biotechnology,* **7,** 494 - 499.

Weng, Y. P., Hsu, F. C., Yang, W. S. and Chen, H. P. (2006). Optimisation of the expression of glutamate mutase S component under the control of T7 system by using lactose and IPTG as the inducers. *Enzyme and Microbial Technology,* **38,** 465-469.

Wood, T. K. and Peretti, S. W. (1991). Effect of chemically induced, cloned gene expression on protein synthesis in *E.coli*. *Biotechnology and Bioengineering,* **38,** 397-412.

Wulfing, C. and Pluckthun, A. (1993). A versatile and highly repressible *Escherichia coli* expression system based on invertible promoters: expression of a gene encoding a toxic product. *Gene,* **136,** 199-203.

Xiu, P. L. and Rodrigues, A. E. (2005). Experimental and modelling study of protein adsorption in expanded bed. *Separations,* **51,** 2965-2977.

Xu, F. (2005). Applications of oxido-reductases. *Industrial biotechnology,* **Spring 2005,** 38-50.

Xu, Y., Rosenkranz, S., Weng, C., Scharer, J. M., Moo-Young, M. and Chou, C. P. (2006). Characterisation of the T7 promoter system for expressing penicillin acylase in *Escherichia coli*. *Applied Microbiology and Biotechnology,* **72,** 529-536.

Xu, Z., Jing, K., Liu, Y. and Cen, P. (2007). High-level expression of recombinant glucose dehydrognase and its application in NADPH regeneration. *Journal of Industrial Microbiology and Biotechnology,* **34,** 83-90.

Xue-Jun, C., Xng-Yan, W., Fonseca, L. J. P., Cabral, J. M. S. and Marcos, J. C. (2004). Production of 6-aminopenicillanic acid in aqueous two phase systems by recombinant *Escherichia coli* with intracellular penicillin acylase. *Biotechnology Letters,* **26,** 97-101.

Yao, Y. F., Weng, Y. M., Hu, H. Y., Ku, K. L. and Lin, L. L. (2006). Expression optimisation and biochemical characterization of a recombinant γ-Glutamyltranspeptidase from *Escherichia coli* Novablue. *The Protein Journal,* **25,** 431-441.

Yee, L. and Blanch, H. W. (1992). Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. *Bio/technology,* **10,** 1550-1556.

Yildir, C., Onsan, Z. I. and Kirdar, B. (1998). Optimisation of starting time and period of induction and inducer concentration in the production of the restriction enzyme *Eco*RI from recombinant *Escherichia coli* 294. *Turk. Journal of Chem.,* **22,** 221-226.

Yildirim, S., Konrad, D., Calvez, S., Drider, D., Prevost, H. and Lacroix, C. (2007). Production of recombinant bacteriocin diversin V41 by high cell density *Escherichia coli* batch and fed-batch cultures. *Biotechnological Products and Process Engineering* **77,** 525-531.

Zhao, H., Chockalingam, K. and Chen, Z. (2002). Directed evolution of enzymes and pahways for industrial biocatalysis. *Current Opinion in Biotechnology,* **13,** 104-110.

Zhao, J. B., Wei, D. Z. and Tong, W. Y. (2007). Identification of *Escherichia coli* host cell for high plasmid stability and improved production of antihuman ovarian carcinoma x antihuman CD3 single-chain bispecific antibody. *Applied Microbiology and Biotechnology,* **76,** 795-800.

Zheng, Z. Y., Yao, S. J. and Lin, D. Q. (2005). Using a kinetic model that considers cell segreagation to optimise hEGF expression in fed-batch cultures of recombinant *Escherichia coli*. *Bioprocess and Biosystems Engineering,* **27,** 143-152.

Zhou, Z., Schnake, P., Xiao, L. and Lal, A. A. (2004). Enhanced expression of a recombinant malaria candidate vaccine in *Escherichia coli* by codon optimisation. *Protein Expression and Purification,* **34,** 87-94.

Appendix A1

Determination of glucose concentration using the dinitrosalicylic acid assay

Reagents

DNS Reagent: Weigh 10 g of dinitrosalicylic acid, 0.5 g of sodium sulphate and 10 g of sodium hydroxide into 1 litre volumetric flask. Make up with distilled water up to the 1 litre mark.

Method

- Weigh 2 g of glucose in a 1 l volumetric flask
- Fill flask up with distilled water to the 1 l mark and mix using stirrer bar for 10 minutes
- Pipette each of 0.375, 0.750, 1.125 and 1.500 ml of sample into a 10 ml test tube
- Make the sample volume up to 3 ml using distilled water
- Add 3 ml of DNS reagent to each test tube
- Cap the test tubes and place in a 90ºC water bath for 10 minutes
- Add 1 ml of 40% potassium tartate solution (w/v) to each test tube after heating
- Cool to room temperature
- Read the absorbance at 575 nm against blank (distilled water) treated similar to sample

Standard curve

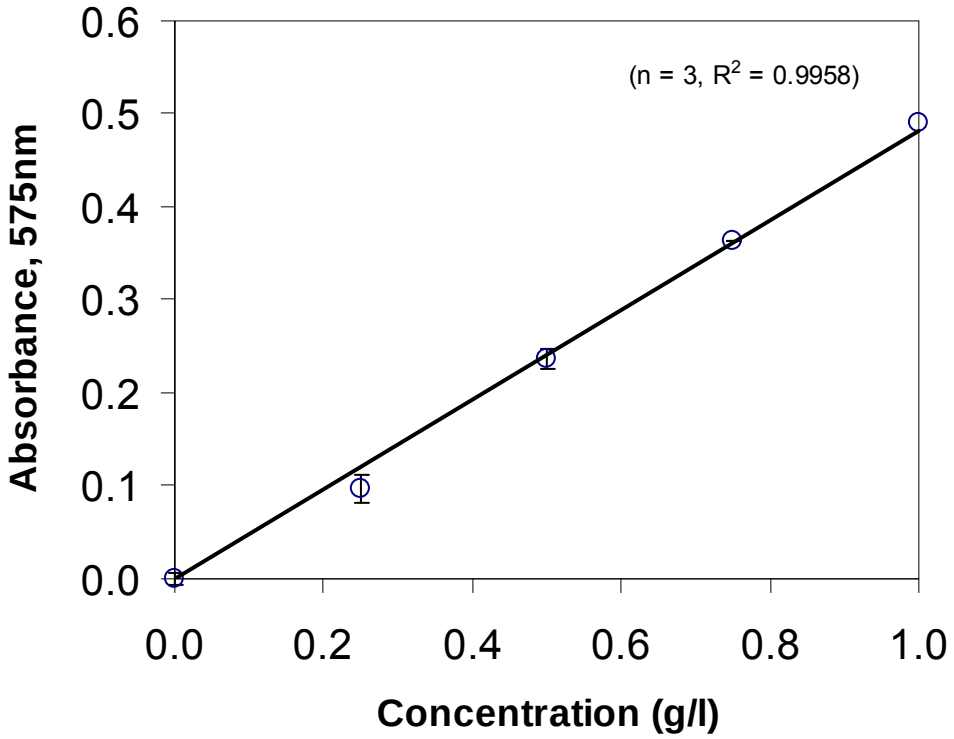


Figure A1: Glucose standard curve

Appendix A2

Bradford's method for determination of total soluble protein concentration

Reagents

Bradford Reagent: Dissolve 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol. Add 100 ml of 85% (w/v) phosphoric acid to this solution. Make the volume up to a final volume of 1 litre using distilled water

Bovine serum albumin standard (BSA): Prepare standard solutions of BSA in distilled water from 10 to 100 µg ml^{-1}.

Method

- Pipette 0.1 ml of BSA sample into a 2 ml plastic cuvette
- Add 1 ml of Bradford's reagent to the above and incubate at room temperature for 5 minutes
- Read the absorbance after 2 minutes (before 60 minutes) at 595 nm against a blank that was treated the same as the sample

Standard curve

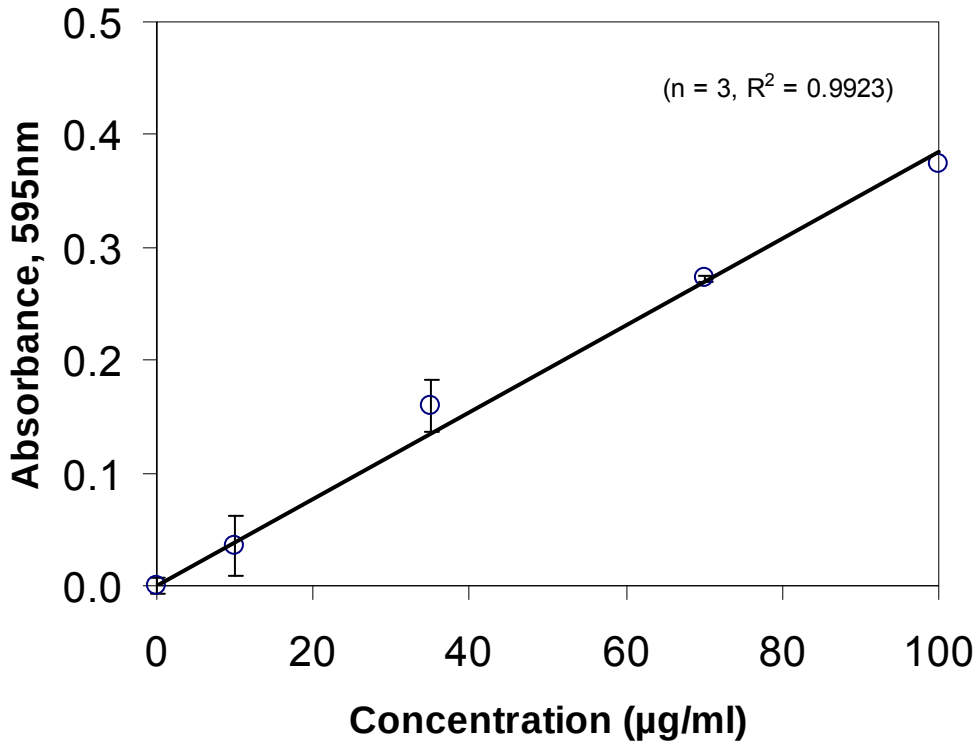


Figure A2: Protein standard curve using bovine serum albumin

Appendix A3

Ammonia standard

Reagents

Reagent A: 10 g l^{-1} phenol and 0.05 g l^{-1} sodium nitroprusside

Reagent B: 5 g l^{-1} sodium hydroxide and 0.0084 g l^{-1} sodium hypochlorite

Method

- Add 100 µl of the standard ammonium solution to 500 µl reagent A present in the 1.5 ml Eppendorf tube.
- Add 500 µl of reagent B to the reaction mixture above and vortex.
- Leave to incubate at room temperature for 30 minutes.
- Transfer contents of Eppendorf tube to 2 ml plastic cuvette and read absorbance at 625 nm

Standard curve

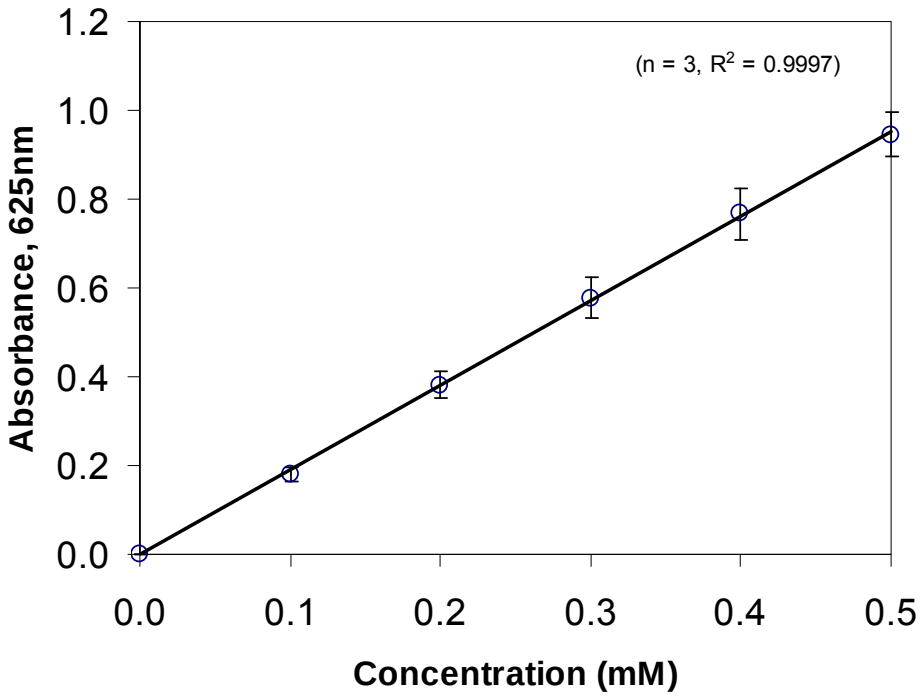


Figure A3: Ammonium chloride standard curve

Appendix **B**

Raw data of experiments presented in Chapter 4

Appendix B1

Table B1: Effect of IPTG concentration on biomass production in batch cultivation in the current study

Time (hrs)	Biomass concentration, DCW (g l^{-1}) at different IPTG concentrations					
	IPTG concentration (μM)					
	0	40	100	400	600	1000
1	0.03	0.07	0.07	0.05	0.07	0.08
3	0.14	0.59	0.36	0.45	0.30	0.28
5	0.54	1.61	1.06	1.23	0.91	0.77
6	0.93	2.20	1.58	1.77	1.66	1.29
7	1.48	2.94	2.13	2.42	2.20	1.71
8	2.05	3.66	2.64	3.10	2.96	2.18
9	2.67	4.33	3.24	3.49	3.27	2.66
10	3.12	4.79	3.79	4.07	3.84	3.20
11	3.62	5.50	4.44	4.54	4.59	3.66
12	3.90	5.63	5.00	5.01	5.24	4.19
13	4.20	5.77	5.36	5.33	5.64	4.72
14	4.40	6.58	5.75	5.49	5.74	4.90

Table B2: Protein concentration across various IPTG concentrations used in the batch process of the current study

Time (hrs)	Protein concentration (g l^{-1}) at different IPTG concentrations					
	IPTG concentration (μM)					
	0	40	100	400	600	1000
1	0.07	0.14	0.08	0.11	0.21	0.09
3	0.12	0.24	0.18	0.17	0.41	0.20
5	0.43	0.26	0.25	0.28	0.59	0.27
6	0.76	1.45	0.94	0.56	1.15	0.69
7	1.01	1.62	1.50	1.18	1.54	1.57
8	1.20	2.13	1.81	1.29	2.08	2.07
9	1.30	2.19	1.93	1.91	2.36	2.34
10	1.45	2.80	2.60	2.29	2.98	2.52
11	1.62	3.40	2.87	2.33	3.44	3.34
12	1.72	3.19	3.43	2.78	3.64	3.74
13	1.71	3.50	3.97	3.32	3.90	3.78
14	1.82	3.76	4.43	3.32	4.29	3.93

Table B3: Specific amidase expression at various IPTG concentrations investigated in batch cell cultivation

Time (hrs)	Specific amidase activity (U mg^{-1} protein) at different IPTG concentrations					
	IPTG concentration (μM)					
	0	40	100	400	600	1000
1	1.46	7.23	24.86	3.79	14.67	18.69
3	0.58	5.99	12.74	2.36	7.48	8.97
5	0.52	7.16	21.62	7.95	5.50	7.14
6	0.48	5.77	47.99	76.18	25.05	81.51
7	0.53	19.42	50.07	110.49	49.36	70.84
8	0.50	29.26	61.57	168.57	94.30	87.03
9	0.72	35.55	76.31	166.65	98.94	95.91
10	0.74	31.15	65.31	174.33	122.37	110.32
11	1.05	27.10	65.36	185.25	128.25	93.56
12	1.50	30.14	60.32	167.18	128.99	92.43
13	2.74	23.02	53.71	156.29	129.89	91.32
14	2.79	0.00	48.96	128.73	95.16	87.70

Table B4: Specific amidase production across the IPTG concentrations used in the batch process of the current study

Time (hrs)	Specific amidase activity (U mg^{-1} biomass) at different IPTG concentrations					
	IPTG concentration (μM)					
	0	40	100	400	600	1000
1	2.65	9.53	19.45	5.78	29.79	13.29
3	0.35	1.59	4.28	0.61	6.94	4.25
5	0.27	0.77	3.46	1.20	2.37	1.65
6	0.26	2.54	19.01	16.02	11.58	29.11
7	0.24	7.14	23.51	36.01	23.06	43.51
8	0.19	11.35	28.15	46.80	44.26	54.98
9	0.23	11.97	30.30	60.91	47.57	56.29
10	0.23	12.13	29.84	65.50	63.34	57.80
11	0.31	11.15	28.15	63.21	64.05	56.91
12	0.44	11.38	27.54	61.82	59.75	55.14
13	0.75	9.31	26.51	64.92	59.84	48.76
14	0.77		25.15	51.85	47.40	46.91

Table B5: Cell growth and amidase expression in fed batch studies, inducing at the start of feeding (10^{th} hour) with a final IPTG concentration of 400 µM

Time (hr)	Absorbance (A_{660nm})	Biomass, DCW (g/l)	Glucose (g/l)	$Y_{x/s}$ (g g^{-1})	Specific activity/ biomass (U mg^{-1} cell)	Specific activity/ protein (U mg^{-1} protein)
0	0.00	0.50	29.68			
10	17.03	9.09	0.16	0.28	0.59	1.76
11	20.65	11.27	0.23	0.30	2.72	9.51
12	23.43	13.00	0.40	0.30	5.61	19.70
14	33.03	17.67	0.59	0.32		22.25
15	35.11	20.69	0.55	0.33	6.64	23.88
16	41.50	22.98	0.64	0.33	5.64	24.59
17	49.20	24.20	0.81	0.31	5.31	24.63
18	60.25	27.02	0.76	0.31	5.03	23.98
19	65.75	30.20	1.06	0.30	4.49	23.34
20	80.25	32.64	1.23	0.29	4.43	24.20
21	95.75	34.00	1.19	0.27	4.13	24.33
22	70.35	37.36	1.76	0.27	4.22	24.59
23	78.85	40.80	2.27	0.26	4.41	27.41
24	92.95	40.67	1.64	0.23	4.15	27.87

Table B6: Cell growth and amidase expression in fed batch studies, inducing at the 3rd hour of batch cultivation and with continuous induction at the start of feeding (10th hour) with a final IPTG concentration of 400 µM

Time (hr)	Absorbance (A_{660nm})	Biomass DCW (g l^{-1})	Glucose (g l^{-1})	$Y_{x/s}$ (g g^{-1})	Acetate (g l^{-1})	Plasmid stability (%)	Specific activity/ biomass (U mg^{-1} cell)	Specific activity/ protein (U mg^{-1} protein)
0	0.00	0.47	23.18			95.54		
3	0.78	0.60	19.66	0.04	0.00	98.71	5.88	45.11
10	9.64	4.60	13.12	0.41	0.00	57.62	48.75	123.9
11	12.42	6.57	14.14	0.39	4.40		35.71	114.1
12	15.52	8.83	15.05	0.38	4.40		12.91	38.81
13	19.92	12.47	13.44	0.40	0.00	55.47	11.52	34.96
14	25.84	14.17	9.07	0.33	7.20		8.92	28.59
15	40.79	19.50	0.32	0.32	8.80		2.54	8.32
16	46.74	21.77	0.21	0.32	12.40	49.09	1.78	5.86
17	59.79	25.30	0.47	0.34	8.40		1.68	5.85
18	65.79	27.53	0.40	0.33	0.00	4.79	1.23	4.75
19	73.19	30.07	0.40	0.32	0.00		0.82	3.06
20	79.49	32.37	0.59	0.31	0.00		0.73	3.06
21	76.19	35.07	0.51	0.30	0.00	2.94	0.46	2.06
22	69.79	37.03	0.62	0.28	0.00		0.58	2.40
23	67.19	32.60	0.68	0.22	0.00		0.26	1.00
24	64.09	33.87	0.68	0.21	0.00	0.61	0.56	2.35

Table B7: Cell growth and amidase expression in fed batch studies, inducing at the 14th hour of cultivation and with intermittent addition of IPTG up to a final concentration of 1.6 mM

Time (hr)	Absorbance (A_{660nm})	Biomass, DCW (g l^{-1})	Glucose (g l^{-1})	$Y_{x/s}$ (g g^{-1})	Acetate (g l^{-1})	Plasmid stability (%)	Specific activity/ biomass (U mg^{-1} cell)	Specific activity/ protein (U mg^{-1} protein)
0	0.00	0.37	31.71		0.76	75.00		
10	17.01	9.33	0.37	0.29	2.16	20.47	0.47	1.19
11	21.56	11.50	0.31	0.31	0.97		2.76	5.94
12	24.69	13.33	0.35	0.32	0.00	13.85	4.03	9.03
13	30.81	14.03	0.50	0.29	0.00		5.75	12.55
14	34.73	16.77	0.47	0.31	0.00	6.43	5.52	12.17
15	40.09	19.00	0.66	0.31	0.00		6.91	16.15
16	46.99	20.93	0.77	0.31	0.00	5.78	6.86	15.72
17	78.99	23.33	0.94	0.30	0.00		5.42	13.41
18	80.79	29.07	1.17	0.34	0.00	1.67	6.03	15.47
19	92.09	28.63	1.09	0.30	0.00		5.65	14.14
20	89.69	30.17	1.27	0.28	0.00	0.68	6.69	17.76
21	76.69	31.97	1.47	0.26	0.00		4.37	12.24
22	78.09	35.37	1.66	0.26	0.00	0.38	4.11	12.13
23	103.9	37.03	1.73	0.25	0.00		3.96	11.96
24	95.59	38.40	1.73	0.24	0.00	0.00	4.94	15.70

Appendix B2

Statistical analyses of the final biomass accumulation in batch cultivation

The one tailed t-Test was applied as follows:

1. Test the null hypothesis that the mean final biomass concentration using various IPTG concentrations is at least 4.4 g l^{-1} at the 5% significance level
2. H_o: μ equal or larger than 4.4 g l^{-1}
3. H_1: μ smaller than 4.4 g l^{-1}
4. α = 0.05
5. The test statistic is: $t_o = \frac{d - \mu}{S_d / \sqrt{n}}$
6. Do not reject H_o if $t_o > t_{0.05,2} = -2.9199$
7. Computation: The sample average (d) and standard deviation (S_d) of the differences are d = 5.692 g l^{-1} and S_d = 0.6046 g l^{-1},

   The test $t_o = \frac{1.292}{0.6046/\sqrt{5}} = 4.7783$

Conclusion: Since t_o = 4.7783 > -2.9199, it was concluded that the final biomass concentration obtained without induction was not significantly different from the final biomass accumulation obtained using IPTG up to 1000 μM (with induction at the 5th hour of cell cultivation). Hence, addition of IPTG was not detrimental to biomass formation.

Appendix **C**

Raw data of experiments presented in Chapter 5

Table C1: The breakthrough data for total soluble protein (C/Co) and biomass (A/Ao) using BSA as standard protein

Volume of eluent (ml)	BSA concentration in feed (mg ml^{-1})								
	0.24			1.74			5.12		
	BSA in eluent (mg m^{-1})	C/Co	A/Ao	BSA in eluent (mg m^{-1})	C/Co	A/Ao	BSA in eluent (mg m^{-1})	C/Co	A/Ao
16.90	0.01	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24.70	0.00	0.02	0.00	0.00	0.00	0.17	0.00	0.00	0.01
28.60	0.01	0.04	0.00	0.18	0.10	0.42	0.00	0.00	0.22
32.50	0.01	0.04	0.02	0.50	0.28	0.55	0.00	0.00	0.43
36.40	0.02	0.09	0.42	0.72	0.41	0.64	2.66	0.52	0.54
40.30	0.03	0.12	0.60	0.84	0.48	0.68	3.18	0.62	0.62
44.20	0.04	0.17	0.67	0.93	0.53	0.72	3.77	0.74	0.68
48.10	0.07	0.31	0.72	1.02	0.59	0.77	4.22	0.82	0.71
52.00	0.11	0.46	0.77	0.81	0.47	0.80	4.26	0.83	0.75
55.90	0.14	0.59	0.79	1.31	0.75	0.82	4.71	0.92	0.78
59.80	0.14	0.60	0.82	1.34	0.77	0.87	4.66	0.91	0.81
63.70	0.15	0.62	0.82	1.30	0.75	0.88	4.39	0.86	0.82
67.60	0.17	0.70	0.85	1.40	0.80	0.89	4.79	0.93	0.85
71.50	0.17	0.73	0.87	1.06		0.91	4.85	0.95	0.86
75.40	0.16	0.69	0.88	1.12		0.92	4.86	0.95	0.89
79.30	0.18	0.76	0.89	1.43	0.82	0.93	4.90	0.96	0.90
83.20	0.18	0.74	0.90	1.46	0.84	0.94	4.66	0.91	0.90
87.10	0.18	0.77	0.91	1.52	0.87	0.96	4.90	0.96	0.92
91.00	0.19	0.78	0.92	1.54	0.89	0.98	5.03	0.98	0.93
94.90	0.19	0.81	0.93	1.56	0.89	0.98	4.73	0.92	0.93
98.80	0.18	0.76	0.92	1.60	0.92	0.98	4.90	0.96	0.95
102.7	0.19	0.79	0.94	1.63	0.94	0.99	4.74	0.92	0.95
106.6	0.19	0.78	0.94	1.56	0.89	0.99	4.93	0.96	0.95
110.5	0.20	0.85	0.95	1.67	0.96	0.98	4.91	0.96	0.96
114.4	0.20	0.83	0.92	1.65	0.95	0.98	4.94	0.96	0.95
118.3	0.21	0.86	0.95	1.56	0.89	0.99	4.71	0.92	0.97
122.2	0.19	0.80	0.95	1.57	0.90	1.00	4.99	0.97	0.96
126.1	0.19	0.80	0.95	1.66	0.95	0.98	4.88	0.95	0.97
130.0	0.20	0.82	0.96	1.67	0.96	1.01	4.74	0.93	0.98

Table C2: The breakthrough data for protein, amidase and biomass using unclarified feedstock

Volume of eluent (ml)	Complete disruption					Partial disruption				
	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co	A/Ao	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co	A/Ao
18.20	0.01	0.02			0.00	0.00	0.01	0.12	0.01	0.00
22.40	0.02	0.06	0.37	0.03	0.07	0.01	0.03	0.18	0.02	0.13
26.60	0.04	0.08			0.14	0.02	0.06	0.33	0.04	0.22
30.80	0.04	0.10	0.43	0.04	0.18	0.03	0.08	0.43	0.05	0.28
35.00	0.06	0.13			0.22	0.04	0.12	0.64	0.07	0.34
39.20	0.05	0.12	0.62	0.06	0.29	0.04	0.12	0.60	0.07	0.41
43.40	0.08	0.18			0.31	0.05	0.15	0.74	0.08	0.45
47.60	0.07	0.17	1.11	0.11	0.34	0.07	0.18	0.76	0.09	0.50
51.80	0.12	0.27			0.39	0.07	0.19	0.76	0.09	0.54
56.00	0.08	0.18	1.37	0.13	0.39	0.06	0.18	0.89	0.10	0.57
60.20	0.11	0.26			0.42	0.06	0.17	0.88	0.10	0.58
64.40	0.12	0.27	1.51	0.14	0.46	0.09	0.26	0.99	0.11	0.68
68.60	0.15	0.34			0.50	0.11	0.31	1.15	0.13	0.73
72.80	0.15	0.35	1.64	0.15	0.51	0.10	0.29	1.02	0.11	0.76
77.00	0.16	0.38			0.51	0.11	0.31	1.23	0.14	0.80
81.20	0.16	0.36	1.71	0.16	0.52	0.13	0.35	1.27	0.14	0.83
85.40	0.17	0.40			0.55	0.14	0.38	1.22	0.14	0.84
89.60	0.17	0.40	1.95	0.18	0.55	0.14	0.40	1.25	0.14	0.86
93.80	0.16	0.38			0.59	0.15	0.41	1.35	0.15	0.88
98.00	0.13		1.87	0.18	0.63	0.13	0.35	1.36	0.15	0.90
102.2	0.14				0.59	0.15	0.42	1.34	0.15	0.92
106.4	0.14		1.84	0.17	0.62	0.13	0.36	1.38	0.15	0.93
110.6	0.14				0.69	0.15	0.43	1.40	0.16	0.93
114.8	0.14		1.86	0.18	0.65	0.15	0.42	1.29	0.14	0.95
119.0	0.14				0.66	0.15	0.41	1.40	0.16	0.96
123.2	0.15		1.67	0.16	0.66	0.17	0.47	1.76	0.20	0.96
127.4	0.19	0.44			0.72	0.16	0.45	1.77	0.20	0.96

Table C2 (contd.): The breakthrough data for protein, amidase and biomass using unclarified feedstock

Volume of eluent (ml)	Complete disruption					Partial disruption				
	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co	A/Ao	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co	A/Ao
131.6	0.22	0.52	1.72	0.16	0.68	0.17	0.46	1.78	0.20	1.00
135.8	0.23	0.52			0.70	0.14	0.39	1.76	0.20	0.97
140.0	0.21	0.48	1.75	0.17	0.81	0.17	0.47	1.81	0.20	1.00
144.2	0.23	0.53			0.85	0.15	0.42	1.71	0.19	0.97
148.4	0.22	0.52	2.05	0.19	0.73	0.16	0.45	2.07	0.23	0.96
152.6	0.22	0.51			0.73	0.17	0.47	1.82	0.20	0.96
156.8	0.23	0.53	1.66	0.16	0.80	0.17	0.47	1.71	0.19	0.96
161.0	0.23	0.53			0.77	0.18	0.49	1.75	0.20	0.99
165.2	0.23	0.53	1.66	0.16	0.90	0.17	0.46	1.54	0.17	0.97
169.4	0.23	0.55			0.85	0.18	0.49	1.71	0.19	0.98
173.6	0.24	0.56	1.28	0.12	0.75	0.17	0.47	1.73	0.19	0.97
177.8	0.25	0.59			0.77	0.16	0.45	1.66	0.19	0.98
182.0	0.23	0.54	1.22	0.12	0.91	0.17	0.48	1.61	0.18	0.98
186.2	0.23	0.54			0.90	0.18	0.48	1.66	0.19	0.98
190.4	0.24	0.55	1.23	0.12	0.80	0.17	0.46	1.58	0.18	0.98
194.6	0.24	0.57			0.79	0.17	0.46	1.56	0.18	0.99
198.8	0.24	0.55	1.25	0.12	0.81	0.18	0.50	1.63	0.18	0.99
203.0	0.24	0.56			0.98	0.19	0.52	1.51	0.17	0.99
207.2	0.23	0.54	1.25	0.12	0.86	0.19	0.52	1.50	0.17	0.98
211.4	0.24	0.55			0.82	0.18	0.50	1.50	0.17	0.98
215.6	0.25	0.57	1.27	0.12	0.81	0.19	0.51	1.54	0.17	0.99
219.8	0.23	0.53				0.19	0.53	1.53	0.17	0.99
224.0	0.26	0.60	1.23	0.12	0.88	0.19	0.54	1.37	0.15	0.99
Co	**0.43**		**10.58**			**0.36**		**8.91**		

Table C3: The breakthrough data for protein (C/Co), amidase and biomass (A/Ao) using clarified feedstock

Volume of eluent (ml)	Complete disruption				Partial disruption			
	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co
18.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22.40	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00
26.60	0.01	0.03	0.06	0.00	0.01	0.02	0.04	0.00
30.80	0.02	0.06	0.14	0.01	0.02	0.08	0.19	0.02
35.00	0.03	0.07	0.18	0.01	0.03	0.08	0.27	0.02
39.20	0.04	0.09	0.23	0.02	0.03	0.11	0.32	0.03
43.40	0.04	0.09	0.31	0.03	0.04	0.13	0.39	0.04
47.60	0.04	0.10	0.79	0.07	0.05	0.15	0.35	0.03
51.80	0.06	0.13	0.52	0.04	0.05	0.17	0.51	0.05
56.00	0.07	0.18	0.59	0.05	0.07	0.21	0.65	0.06
60.20	0.07	0.17	0.63	0.05	0.07	0.23	0.75	0.07
64.40	0.08	0.20	0.73	0.06	0.08	0.25	0.65	0.06
68.60	0.09	0.21	0.74	0.06	0.07	0.22	0.69	0.06
72.80	0.09	0.21	0.77	0.07	0.08	0.26	0.99	0.09
77.00	0.11	0.27	0.86	0.07	0.09	0.29	1.00	0.09
81.20	0.11	0.27	0.91	0.08	0.11	0.33	1.17	0.11
85.40	0.13	0.32	1.12	0.09	0.11	0.34	0.86	0.08
89.60	0.13	0.31	1.05	0.09	0.15	0.47	1.15	0.10
93.80	0.13	0.32	1.09	0.09	0.11	0.34	1.29	0.12
98.00	0.15	0.35	1.20	0.10	0.13	0.41	1.25	0.11
102.2	0.15	0.35	1.17	0.10	0.12	0.37	1.10	0.10
106.4	0.17	0.40	1.15	0.10	0.13	0.41	1.26	0.11
110.6	0.17	0.40	1.18	0.10	0.11	0.34	1.30	0.12
114.8	0.17	0.40	1.25	0.11	0.11	0.35	1.24	0.11

Table C3 (contd.): The breakthrough data for protein (C/Co), amidase and biomass (A/Ao) using clarified feedstock

Volume of eluent (ml)	Complete disruption				Partial disruption			
	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co	Protein concentration (mg/ml)	C/Co	Amidase activity (U ml^{-1})	C/Co
119.0	0.18	0.41	1.21	0.10	0.13	0.41	1.15	0.10
123.2	0.19	0.44	1.09	0.09	0.13	0.41	1.55	0.14
127.4	0.18	0.43	1.20	0.10	0.12	0.38	1.45	0.13
131.6	0.19	0.45	1.25	0.11	0.13	0.40	1.36	0.12
135.8	0.18	0.42	1.23	0.10	0.13	0.41	1.46	0.13
140.0	0.20	0.48	1.34	0.11	0.14	0.44	1.47	0.13
144.2	0.21	0.49	1.30	0.11	0.14	0.44	1.55	0.14
148.4	0.21	0.49	1.23	0.10	0.14	0.45	1.32	0.12
152.6	0.19	0.45	1.30	0.11	0.13	0.42	1.44	0.13
156.8	0.19	0.45	1.30	0.11	0.13	0.42	1.56	0.14
161.0	0.21	0.48	1.09	0.09	0.13	0.42	1.52	0.14
165.2	0.19	0.46	1.19	0.10	0.13	0.42	1.50	0.14
169.4	0.21	0.49	1.18	0.10	0.15	0.47	1.43	0.13
173.6	0.20	0.48	1.17	0.10	0.15	0.47	1.53	0.14
177.8	0.21	0.50	1.25	0.11	0.14	0.45	1.45	0.13
182.0	0.22	0.53	1.25	0.11	0.15	0.48	1.45	0.13
186.2	0.23	0.54	1.22	0.10	0.14	0.44	1.48	0.13
190.4	0.25	0.58	1.21	0.10	0.14	0.44	1.47	0.13
194.6	0.23	0.55	1.23	0.10	0.15	0.46	1.46	0.13
198.8	0.23	0.55	1.23	0.10	0.15	0.48	1.45	0.13
203.0	0.21	0.50	1.13	0.10	0.15	0.48	1.50	0.14
207.2	0.24	0.57	1.08	0.09	0.15	0.49	1.48	0.13
211.4	0.22	0.53	1.15	0.10	0.15	0.46	1.48	0.13
215.6	0.24	0.57	1.15	0.10	0.15	0.49	1.47	0.13
219.8	0.24	0.57	1.14	0.10	0.13	0.41	1.49	0.13
224.0	0.25	0.58	1.12	0.09	0.14	0.46	1.44	0.13
Co	**0.42**		**11.80**		**0.32**		**11.08**	