



# LUND UNIVERSITY

## **Bench-Scale Production of Heterologous Proteins from Extremophiles- Escherichia coli and Pichia pastoris based expression systems**

Ramchuran, Santosh

2005

[Link to publication](#)

*Citation for published version (APA):*

Ramchuran, S. (2005). *Bench-Scale Production of Heterologous Proteins from Extremophiles- Escherichia coli and Pichia pastoris based expression systems*. [Doctoral Thesis (compilation), Biotechnology]. Santosh O. Ramchuran, Biotechnology (LTH), Lund University.

*Total number of authors:*

1

### **General rights**

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00

Santosh O. Ramchuran

Bench-Scale Production of Heterologous Proteins from Extremophiles

Lund 2005

# Bench-Scale Production of Heterologous Proteins from Extremophiles

*Escherichia coli* and *Pichia pastoris*  
based expression systems

Santosh O. Ramchuran

---

Department of Biotechnology  
Lund University  
2005



LUND UNIVERSITY

ISBN 91-89627-29-6  
ISRN LUTKDH/TKBT--05/1082--SE



# Bench-Scale Production of Heterologous Proteins from Extremophiles

*Escherichia coli* and *Pichia pastoris* based expression systems



**LUND**  
UNIVERSITY

Santosh Omrajah Ramchuran  
Department of Biotechnology  
Doctoral Thesis  
April 2005

Akademisk avhandling som för avläggande av teknologie doktorsexamen vid tekniska fakulteten vid Lunds Universitet kommer att offentlig försvaras torsdagen den 7 April 2005, kl 10:30 i hörsal A, på Kemientrum, Getingevägen 60, Lund.

Academic thesis which, by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Thursday 7 April 2005, at 10:30 in lecture hall A, at the Center for Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Professor Sven-Olof Enfors, Department of Biotechnology, Royal Institute of Technology, Stockholm, Sweden.

Doctoral Thesis 2005  
Lund University  
Department of Biotechnology  
Center for Chemistry and Chemical Engineering  
Box 124  
SE-22100 Lund  
Sweden

© 2005 Santosh Omrajah Ramchuran  
ISBN: 91-89627-29-6  
ISRN LUTKDH/TKBT--05/1082--SE  
Printed by Media-Tryck (Lund, 2005)

<b>Organization</b> Lund University Department of Biotechnology	<b>Document name</b> DOCTORAL DISSERTATION	
	<b>Date of issue</b> April 2005	
	<b>CODEN</b> ISRN LUTKDH/TKBT--05/1082--SE	
<b>Author(s)</b> Santosh Omrajah Ramchuran	<b>Sponsoring organization</b>	
<b>Title and subtitle</b> Bench-scale production of heterologous proteins from extremophiles <i>Escherichia coli</i> and <i>Pichia pastoris</i> based expression systems		
<b>Abstract</b> Over the past few years considerable research attention has been assigned to extremophiles as sources of extremozymes due to their applicability in industrial processes, and the development of eco-friendly technologies. The establishment of efficient production strategies for heterologous proteins is an empirical process requiring broad background knowledge on available expression systems together with their major advantages and shortcomings. The studies conducted during the course of this thesis has included four enzymes originating from thermophiles namely, thermostable glycoside hydrolases, xylanase and cellulase from <i>Rhodothermus marinus</i> , cyclomaltodextrinase from <i>Anoxybacillus flavithermus</i> and a phospholipase from alkaliphilic <i>Bacillus halodurans</i> . Batch cultivation of <i>R. marinus</i> in the presence of xylan allowed low production of the native xylanase in sufficient amounts to probe cell-attachment studies by enzymatic and immunological techniques. Higher levels of the target proteins were achieved by intracellular and extracellular heterologous production using an <i>Escherichia coli</i> and <i>Pichia pastoris</i> based expression system respectively. The production of a functional enzyme is intimately related to the host's cellular machinery furthermore, as a prerequisite, the establishment of efficient bioprocess strategies is crucial for attaining optimum enzyme production yields. The results presented include bench-scale production strategies employing high cell density fed-batch cultivations with <i>E. coli</i> as a host. In addition, extracellular production of thermostable xylanase and alkaliphilic phospholipase using the methylotrophic yeast <i>P. pastoris</i> as a host is reported.		
<b>Key words</b> Heterologous protein, Glycoside hydrolase, High cell density cultivation, Expression systems, Fed-batch		
<b>Classification system and/or index terms (if any)</b>		
<b>Supplementary bibliographical information</b>		<b>Language</b> English
<b>ISSN and key title</b>		<b>ISBN</b> 91-89627-29-6
<b>Recipient's notes</b>	<b>Number of pages</b>	<b>Price</b>
	<b>Security classification</b>	

Distribution by (name and address)

Santosh Ramchuran, Lund University, Department of Biotechnology, Box 124, SE-22100 Lund, Sweden

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature: 

Date: 2005-04-07

## Abbreviations

HCD	High cell density
PO <sub>2</sub>	Dissolved oxygen concentration
CAZy	Carbohydrate active enzyme database
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyl transferase
GHF	Glycoside hydrolase family
CBM	Carbohydrate binding module
SCP	Single cell protein
AOX	Alcohol oxidase
HCDC	High cell density cultivation
$q_g$	Specific glucose uptake rate
$q_g^{crit}$	Critical glucose uptake rate
$q_o$	Specific oxygen uptake
SLFB	Substrate limited fed-batch
TLFB	Temperature limited fed-batch
<i>R. marinus</i>	<i>Rhodothermus marinus</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>P. pastoris</i>	<i>Pichia pastoris</i>
GRAS	Generally Regarded As Safe
ppGpp	Guanosine tetraphosphate
OM	Outer membrane
TCA	Tricarboxylic Acid
DSP	Down Stream Processing

## Table of Contents

<b>Abstract</b>	iii
<b>Abbreviations</b>	iv
<b>1. Introduction</b>	<b>1</b>
1.1. Scope of the Thesis	4
1.2. List of Papers	6
<b>2. Impact of Gene Technology on Enzyme Production</b>	<b>7</b>
2.1. Enzyme Requirements for Industrial Application	9
2.2. Designing an Enzyme Production Process	11
<b>3. Biocatalysts from Extremophiles</b>	<b>14</b>
3.1. Extremophiles	14
3.2. Extremozymes and Industry	14
3.3. Glycoside Hydrolases from Thermophiles	16
3.3.1. Xylanase	18
3.3.2. Cellulase	20
3.3.3. Cyclomaltodextrinase	21
3.4. Phospholipase from an Alkaliphile	23
<b>4. Prokaryotic and Eukaryotic Expression Systems</b>	<b>26</b>
4.1. Optimising Heterologous Gene Expression in <i>E. coli</i>	27
4.1.1. Transcriptional Regulation of Expression	27
4.1.2. Factors Affecting Production Yields	28
4.2. Heterologous Gene expression in <i>Pichia pastoris</i>	31
4.2.1. Methanol Metabolism and the AOX1 Promoter	32

4.2.2. Host Strains and Vector Design	34
4.2.3. Post-translational Modifications	35
<b>5. Bioprocess Technology</b>	<b>36</b>
5.1. Fermentation Technology employing Extremophiles	36
5.2. Fed-batch Process employing <i>E. coli</i> for Enzyme Production	37
5.2.1. Cellular Responses during Cultivation	38
5.2.2. Cellular Response during Protein Over-expression	40
5.2.3. Process Control	41
5.3. Fermentation Technology employing <i>P. pastoris</i>	43
<b>6. Summary of the Present Investigation</b>	<b>45</b>
<b>7. Acknowledgements</b>	<b>48</b>
<b>8. References</b>	<b>50</b>
<b>Papers I-VI</b>	



## **1. Introduction**

Biotechnology represents a number of versatile and environmentally friendly technologies for delivering industrially clean products and processes. Recently assembled evidence also indicates that biotechnology is competitive in terms of cost and risks and is not limited by scale of operation [1] thus having a major impact in the establishment of industrially “green” processes. A central pursuit of the modern biotechnology industry during this paradigm shift towards clean technology has been to increase efficiency in the area of enzyme production. This thesis focuses on the expression of genes encoding industrially important enzymes as well as the ability to produce these enzymes employing bench scale fermentation processes.

Enzymes are remarkable catalysts and have a long established role in mankind’s attempts to utilise biological systems for a variety of purposes. Enzymes are routinely utilised at present for diverse applications ranging from the manufacture of various industrial commodities, to diagnostics and therapeutics. The primary sources of these enzymes today are from mesophilic microorganisms and the applications of these enzymes are hence restricted due to their limited stability under adverse conditions, e.g. extreme pH and temperature. Extremophiles are a source of extremozymes (i.e. enzymes functional under more extreme conditions) which offers new opportunities for biocatalysis and biotransformations as a result of their extreme stability [2]. Extremophilic microorganisms are adapted to survive in ecological niches such as high temperatures, extremes of pH, high salt concentrations and pressure thus producing unique biocatalysts that function under extreme conditions in many cases comparable to those prevailing in various industrial processes [3].

The advent of recombinant DNA technology has further boosted the industrial production of useful enzymes and many enzymes are now being produced by recombinant microorganisms, animals and plant cells [4]. Moreover, developments in genetic and protein engineering have improved the stability, economy, specificity and overall application potential of industrial enzymes. Thus, it is not surprising that together with the increasing number of biotechnological endeavours the industrial market continues to grow rapidly and has an ever increasing demand for additional biocatalysts.

Protein expression technology plays a major role in harnessing novel proteins and developing high-production expression systems among which bacterial and yeast expression systems are the most commonly used. In the context of this thesis, *Escherichia coli* and *Pichia pastoris* based expression systems were investigated. Regarding the former, *E. coli* still remains one of the most commonly used hosts for the production of heterologous proteins due to it being a well characterised system [5]. However, in spite of the vast array of knowledge that exists on the genetics and molecular biology of *E. coli*, not every gene can be efficiently expressed in this microorganism. The major drawbacks of *E. coli* based expression systems include the inability to perform many post-translational modifications, the lack of a secretion mechanism, limited ability to facilitate extensive disulfide bond formation and the formation of insoluble aggregates (inclusion bodies) [6]. Due to intracellular accumulation of heterologous protein in *E. coli*-based expression systems productivity is proportional to the final cell density. Thus, the primary goal in process technology has focused on high cell density cultivations (HCDC) to maximise product yields [7] exploiting the fed-batch mode of cultivation [8-9]. Although, the developments in HCDC-techniques for *E. coli* has led to successful production of various

heterologous proteins, these techniques have several drawbacks such as substrate inhibition, limited oxygen transfer capacity, the formation of inhibitory by-products, and limited heat dissipation[10-17].

Process optimisation in *E. coli* based expression systems is often extended further to include the genetic-based solutions, traditionally employing strong expression systems to gain maximum heterologous protein yields [18]. However, such systems often override host cell metabolism [19] and an efficient or optimal process requires the synchronisation of gene expression rates and host cell's metabolic capabilities. Therefore, establishing equilibrium between heterologous and cellular protein biosynthesis is the key for ensuring stable and prolonged heterologous protein production levels due to the host cell being responsible for providing the protein synthesis machinery, building blocks and energy for the production of heterologous proteins. Attempts made in this thesis to investigate and establish the equilibrium between heterologous and cellular protein biosynthesis has ensured the successful production of heterologous proteins at higher titers than previously reported.

As an alternative to *E. coli* based expression systems, the methylotrophic yeast *Pichia pastoris* has developed into a successful system for the production of a variety of heterologous proteins [20]. Exploiting the *Pichia* based expression system offers the opportunity of extracellular protein accumulation thus directly facilitating easy down-stream processing. Furthermore, the capability of performing complex post-translational modifications and the commercial availability of *Pichia pastoris* based expression systems has significantly increased its popularity for the production of heterologous proteins [21]. Similarly, in this work a lipolytic enzyme was efficiently produced exploiting the *P. pastoris* based expression

system due to previous attempts using an *E. coli* based expression system being unsuccessful.

Finally, it should be noted that choosing an expression system for high level heterologous protein production depends on several factors and no universal expression system exists. Fortunately, some empirical “rules” are in place to guide and design expression systems for heterologous protein production. In addition, the selection of an appropriate expression system requires evaluating its merits in terms of process, design and economics (especially for large scale) during high level heterologous protein production.

## **1.1 Scope of the Thesis**

The research work presented in this thesis involves enzymes from extremophiles (**Papers I-VI**). These so-called “extremozymes” have received considerable attentions due to their applicability in industrial processes. However, only a limited number of enzymes are produced at industrial scale and before any scale-up can be considered, expression of the gene encoding the target protein using a relevant expression system must be made. One of the major bottlenecks is attaining optimum heterologous expression levels of the desired protein using available expression systems. The choice of an expression system for the high-level production of heterologous proteins depends on many factors including commercial availability, process design, and scale.

In this thesis the focus has been on two alternative expression hosts: *E. coli* and *P. pastoris* for which commercial expression vectors are available. Efforts have been made to optimise enzyme production, using the selected systems at “bench-scale” (2-5 L). *E. coli* based expression systems are commonly used for intracellular expression and an integrated approach has

been applied which includes aspects of host metabolism and process engineering, to ensure maximum heterologous protein yields. The key to maximum cellular capability has been on determining the highest ratio between cellular and heterologous gene expression (**Papers II, III, IV**). The other approach taken was expressing the enzymes extracellularly using the *Pichia pastoris* based expression system. This not only has a significant impact on DSP costs but also allowed successful production of enzymes that previously could not be expressed in active form using *E. coli* based expression systems. (**Papers V, VI**).

## 1.2 List of Papers

- I. Nordberg Karlsson E, Abou Hachem M, **Ramchuran S O**, Costa H, Holst O, Svenningsen Å F and Hreggvidsson G O. (2004) The modular xylanase Xyn10A. from *Rhodothermus marinus* is cell-attached, and its C-terminal domain have several putative homologues among cell-attached proteins within the phylum Bacteroidetes. FEMS Microbiol. Lett. 241:233-242.
- II. **Ramchuran S O**, Nordberg Karlsson E, Velut S, de Maré L, Hagander P and Holst O (2002) Production of heterologous thermostable glycoside hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of *Escherichia coli* BL21(DE3). Appl. Microbiol. Biotechnol. 60:408-416.
- III. **Ramchuran S O**, Holst O and Nordberg Karlsson E. (2005) Effect of post-induction nutrient feed composition and use of lactose as inducer during production of thermostable xylanase in *Escherichia coli* glucose limited fed-batch cultivations. J. Biosci. Bioeng. (*In press*)
- IV. Nordberg Karlsson E, **Ramchuran S O**, Turner P, de Maré L, Cimander C, Velut S, Ekman Å, Wennerberg C, Hagander P and Holst O. Production of two glycoside hydrolases of thermophilic origin in *E. coli* and analysis of released endotoxins using substrate limited and temperature limited fed-batch cultivation strategies. Journal of Biotechnology. (*Submitted*)
- V. **Ramchuran S O**, Mateus B, Holst O and Nordberg Karlsson E. (2005) The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase from the bacterium *Rhodothermus marinus*. FEMS Yeast Research. (*In press*)
- VI. **Ramchuran S O**, Vargas V, Hatti-Kaul R and Nordberg Karlsson E. The methylotrophic yeast *Pichia pastoris* as a host for the expression of a lipolytic enzyme from *Bacillus halodurans* LBB2. (Manuscript)

## **2. Impact of Gene Technology on Enzyme Production**

Protein over-expression refers to the directed synthesis of large amounts of desired proteins [22]. The process requires the consideration of 2 key issues. Firstly, the introduction of foreign DNA into the host cell with the major focus on selection and construction of the vector carrying the foreign gene. Secondly, expression control and factors affecting the expression of the protein in the chosen expression system. With regards to the former there is an array of vectors available to initiate DNA transfer in and out of the cell (plasmids, lambda phage, cosmids, phagemids, artificial chromosomes from yeasts and bacteria) and can either be integrated into the host's genome or remain independent. Genetic elements for expression control are in most cases included in these vectors, while many other factors need investigation during the optimisation of cultivation parameters.

The rapid expansion in the area of gene technology has had a direct impact on the increased number of recombinant enzymes available today. Also, advances in gene cloning have led to rapid re-examination of known potential enzymes which were previously looked upon as economically unfavourable to produce. A classic example is cyclodextrin glycosyl transferase (CGTase) produced by *Thermoanaerobacter*, which proved difficult to cultivate at large scale at 95°C and also showed a low enzyme yield, but where heterologous production using *Bacillus macerans* enabled successful expression of CGTase in yields suitable for large scale production [23]. Likewise, very low xylanase yields were obtained during the cultivation of the thermophilic bacterium *Rhodothermus marinus* (**Paper I**) however, heterologous production of xylanase using both *E. coli* (**Papers II, II, IV**) and *P. pastoris* (**Paper V**) based expression systems enabled significant improvements in enzyme production yields. In general, the

benefits of genetic engineering for the production of industrial enzymes can be summarised as follows: (1) naturally occurring enzymes present in minute concentrations and in microorganisms often difficult to cultivate may be produced by genetically modified host microorganisms cultivated on cheap raw material substrates, (2) efficient gene construction and selection allows increased productivity, (3) improvements in enzyme stability, activity and specificity can be obtained by genetic engineering.

**Table 1.** Some applicable host-vector systems according to Australian regulation ([www.ogtr.gov.au](http://www.ogtr.gov.au))

Microorganism	Host	Vector
<b>Bacteria</b>	<i>Escherichia coli</i> K12 /B	1. Non-conjugative plasmids 2. Bacteriophage
	<i>Bacillus subtilis</i>	Host range does not include <i>B. cereus</i> , <i>B. anthracis</i> pKT 262, 263, 264 Certified plasmids: SCP2, SLP1,2 PIJ101 and derivatives
	<i>B. licheniformis</i>	
	<i>Pseudomonas putida</i> KT 2440	
	<i>Streptomyces coelicolor</i>	
	<i>Streptomyces lividans</i>	
	<i>Streptomyces parvulus</i>	
<i>Streptomyces griseus</i>		
<b>Yeast and Fungi</b>	<i>Pichia pastoris</i>	All vectors
	<i>Saccharomyces cerevisiae</i>	All vectors
	<i>Schizosaccharomyces pombe</i>	All vectors
	<i>Kluyveromyces lactis</i>	All vectors
	<i>Trichoderma reesei</i>	All vectors

However, many guidelines and restrictions surround the expression and production of heterologous proteins. Specifically, with regards to host vector systems, regulations govern not only the host but also the vector itself. A list of some acceptable bacterial and yeast host vector systems are presented in Table 1, however, it should be noted that the list is subject to



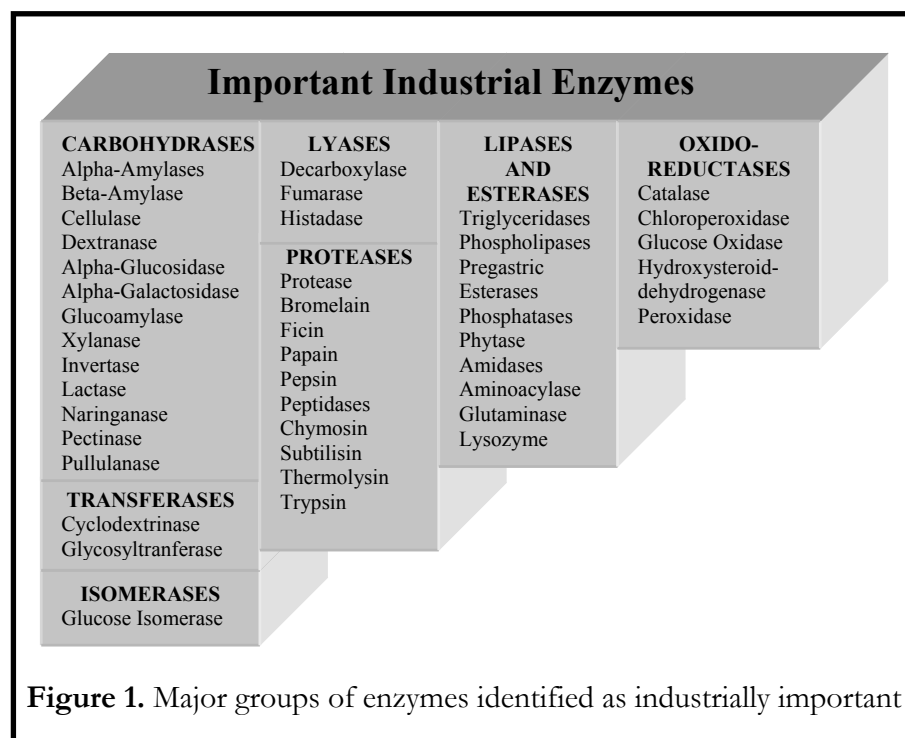
change depending on the regulations concerning the use of genetically modified microorganism in a specific country.

## **2.1 Enzyme Requirements for Industrial Application**

Presently, only a limited number of all known enzymes are commercially available. With regards to the use of industrial enzymes the major markets currently are detergent enzymes and food and feed processing [24], but there is also a rapidly growing market demand for industrial enzymes in the textile, pulp, paper and leather industries. A summary of the enzymes predominantly focused on for large-scale production and application is shown in Figure 1, with carbohydrases and proteases dominating the world-wide enzyme sale markets due to their widespread use in many industries.

Several factors influence the selection of an enzyme destined for industrial production such as specificity, reaction rate, pH, temperature optima, stability and substrate affinity. The safety of consumers using enzyme-based products and the safety of personnel during the production process are also factors that need to be considered. Efficient use of an enzyme in a process can be looked at simply as adding the enzyme to the reactant stream to obtain the desired catalytic reaction/s, after which the enzyme is gradually inactivated by the process. Enzymes, however, are susceptible to harsh denaturing conditions that are typically found in the chemical processing industry which impair their catalytic rates and functioning [24]. Two key parameters ensuring catalytic versatility of enzymes in industrial applications are hence pH and thermostability. Thus, there is an on-going search for improved biocatalysts which extends to the discovery of natural enzymes from extreme environments, the so-called extremozymes. A broad pH range provides an increased operating margin

in a process while on the other hand a narrow pH range (but maybe at an extreme pH) is useful in specific reactions. Enzymes from alkaliphiles (**Paper VI**), offers the possibility of functioning at high pH under alkaline conditions. Thermostable extremozyme variants are favoured in industrial processes requiring an elevated operating temperature and in this thesis, enzymes from the glycoside hydrolase family are investigated (**Papers I, II, III, IV, V**).



**Figure 1.** Major groups of enzymes identified as industrially important

## **2.2 Designing an Enzyme Production Process**

The selection of an enzyme and the development of the enzyme production process is complicated because each phase of the process needs to be defined in sequential order. Procedures and guidelines put in place early in the sequence ultimately influence later steps such as DSP, which ultimately influence the reproducibility of the process and quality of the enzyme (Figure 2). In order to utilise and develop biocatalysts for applied purposes rational approaches need to be considered. Primarily the starting point is a product which can be produced by one or more biocatalytic reactions during which the conversion of suitable substrates to the desired product occurs. Therefore, the goals are centred on finding and producing the best biocatalyst for the given task.

The enzyme production process can be broken down into several phases (Figure 2). The process generally begins with screening and identification of the target enzyme in the native microorganism. Early in the development stages, limiting aspects/properties of the desired biocatalyst may be engineered, gradually leading to an economically feasible industrial process. Improvements in key technologies such as protein engineering and molecular evolution [25-26] have directly influenced and led to rapid growth in biocatalysis for applied purposes. A good example is directed evolution where notable improvements in enzyme properties may be obtained when several generations of random mutagenesis, recombination and screening have been deployed [27-28]. Another approach is the use of site-directed mutagenesis to obtain enzymes with altered properties [29-30] which may render the enzyme capable of more or new reactions thus increasing its scope of application. Also, entirely new processes may be developed by exploiting the extreme characteristics that these enzymes possess.

Several factors need to be considered when choosing a suitable production host. One of these is the scale of production. Ideally for large scale, extracellular production is favoured as it simplifies the recovery and purification process as compared to intracellularly produced enzymes. The production host should ideally also possess a GRAS-status (**Generally Regarded As Safe**) especially if the intended enzyme is to be used in food and pharmaceutical processes. In addition, the production host should be adequately capable of producing large amounts of desired enzyme.

After the selected production host has been genetically modified to overproduce the enzyme of interest, an economically suitable fermentation process needs to be developed, optimised and scaled-up. Most large-volume industrial enzymes are produced in 50-500 m<sup>3</sup> fermenters. The final scale of the production and processing an enzyme is subjected to is largely dependent on its intended application. The present work however focuses on the “bench-scale production” of enzymes and in the following chapters the choice of the biocatalyst (**Chapter 3**), host-vector systems (**Chapter 4**), and process technologies (**Chapter 5**) for enzyme production are discussed.

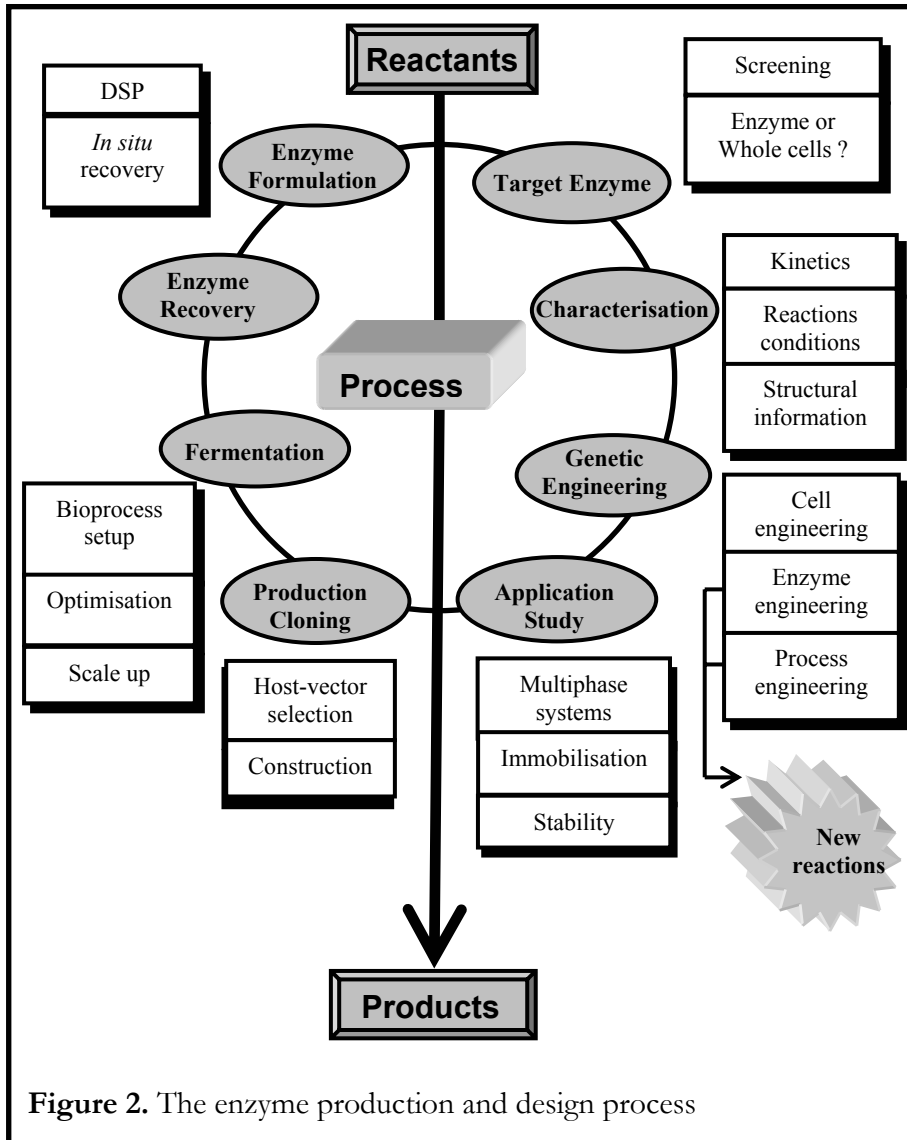


Figure 2. The enzyme production and design process

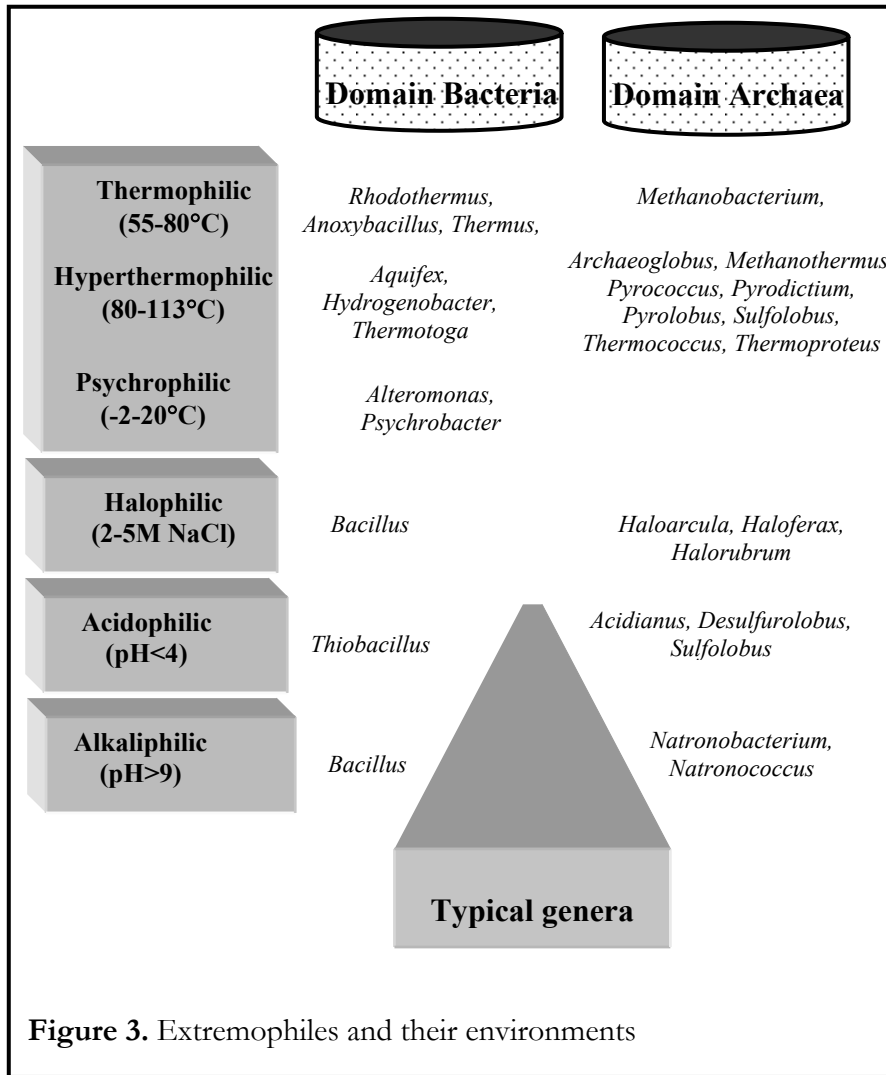
### **3. Biocatalysts from Extremophiles**

#### **3.1 Extremophiles**

Extremophilic microorganisms have adapted to survive in a range of hostile natural habitats. The discovery of extremophiles (particularly those that inhabit high temperature environments) stimulated attempts to define the most extreme conditions that remain compatible with the existence of life [31]. Currently, microorganisms with the highest growth temperatures (103-110 °C) are members of the genera *Pyrobaculum* [3]. Extremophiles have been detected in a wide range of environments as can be seen in Figure 3. Although, the majority of extremophiles are members of the domain Archaea [32], large numbers of extremophilic bacteria have also been identified. Many of these species are classified to genera located close to the root of the universal phylogenetic tree (e.g. *Thermotoga*, *Thermus*) [2], while a few (e.g. *Rhodothermus*) may have acquired these properties later in evolution.

#### **3.2 Extremozymes and Industry**

There is an increasing interest in extremophiles (Thermophiles, Psychrophiles, Alkaliphiles and Acidophiles) both as whole cells and as a source of enzymes (extremozymes). Microorganisms from these habitats can have several interesting properties. Acidophiles for example, typically share other extremophilic habitat properties such as thermophilicity, halophilicity or heavy-metal resistance, and are hence considered for the bioprocessing of minerals [33]. The enzymes from extremophiles also have properties adapted for function in their natural habitat which can significantly increase the range of conditions where biocatalysis is an option. A few of these extremozymes and their potential applications are presented in Table 2.



Polymer degrading enzymes such as amylases, pullulanases, xylanases and cellulases have potential roles in the food, chemical and pharmaceutical, feed, paper, pulp and waste-treatment industries. Proteases, lipases and cellulases from alkaliphiles has had a huge impact on detergent

formulation, also low molecular weight metabolites from these microorganisms can be of industrial interest e.g. cyclodextrins, compatible solutes and industrial lipids [34-35].

**Table 2.** Industrial applications of extremozymes

<b>Extremophile</b>	<b>Extremozymes</b>	<b>Application and products</b>	<b>Reference</b>
<b>Thermophiles</b>	Amylase	Food Industry	
	Xylanase	Paper bleaching	[36-38]
	Lipase	Waste water treatment, detergent	[39]
	Protease	Baking, brewing, detergents	[2, 40-41]
	DNA polymerase	Genetic engineering	[40-42]
<b>Psychrophiles</b>	Amylase, protease, lipase Dehydrogenase	Polymer degradation in detergents Biosensors	[2, 43]
<b>Alkaliphiles</b>	Cellulase, protease	Detergents	[2, 34]
	Amylase, lipase	Food additives	[34, 44]
<b>Acidophiles</b>	Sulphur oxidation	Desulphurisation of coal	[45]

### 3.3 Glycoside Hydrolases from Thermophiles

The majority of carbohydrate materials predominant in nature are in the form of polysaccharides (sugar residues linked by glycosidic bonds) of varying lengths and of complex composition. Furthermore, they can display diverse conformation due to branching in different ways. Similarly, enzymes responsible for the degradation of polysaccharides display diverse modes of hydrolysis [46]. Glycoside hydrolases are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety



[47-48]. A classification system [49] based on sequence similarity, has led to the establishment of 97 different families (GHF) [CAZy web site (CArbohydrate-Active enZymes)].

The interest in glycoside hydrolases is largely due to their application potential in various industrial processes and as a result of their potential use in the conversion of natural polysaccharides. Due to the low solubility of polymeric substrates such as starch, cellulose and hemicellulose, processes are carried out at elevated temperatures. The elevated temperature has a significant influence on bioavailability and solubility as well as an accompanied decrease in viscosity of the substrates. Thermostable enzymes, which have been isolated from thermophilic microorganisms from different exotic ecological zones of the earth, display an inherent catalytic stability at elevated temperatures.

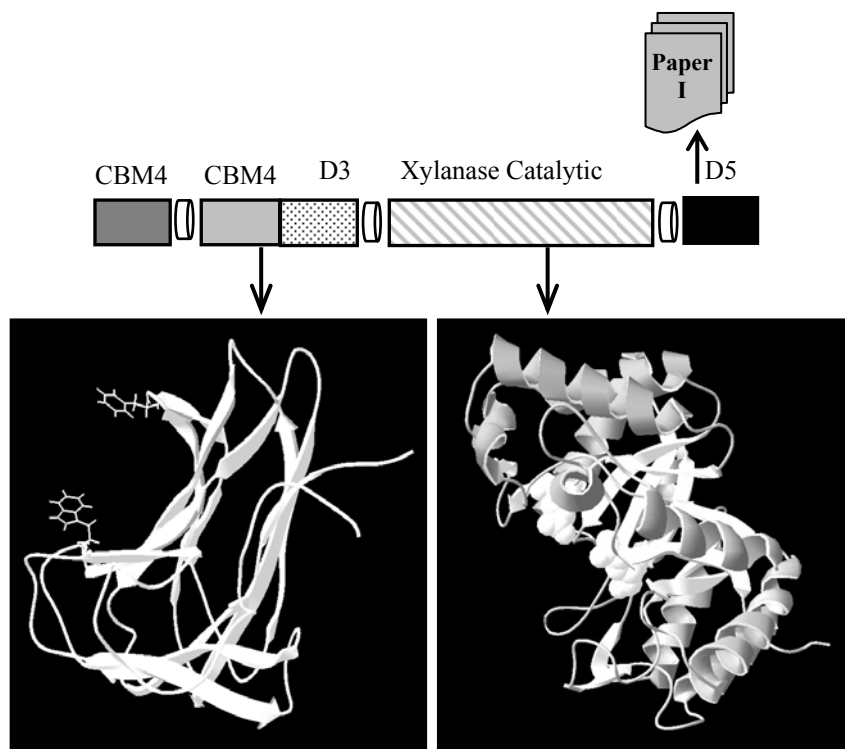
The thermophilic microorganism *Rhodothermus marinus* secretes several hydrolytic enzymes including a xylanase (**Paper I**) and a cellulase [50]. The microorganism which was first isolated from a shallow water marine hot-spring off the Icelandic coast is an aerobic bacterium, slightly halophilic that displays an optimum growth temperature of 65 °C in a neutral to slightly alkaline pH [51]. The moderately thermophilic bacterium *Anoxybacillus flavithermus* was isolated from geothermal sites growing optimally at 60 °C [52-53] and encodes a multi-domained cyclomaltodextrinase ( $\alpha$ -amylase family) from glycoside hydrolase family 13 [54]. These thermostable glycoside hydrolases were produced in this investigation i.e. xylanase (**Papers I, II, III, IV, V**), cellulase (**Paper II**) and cyclomaltodextrinase (**Paper IV**); using different induction and process strategies to evaluate their production potential in the selected host-vector systems.

### 3.3.1 Xylanase

Xylans are major constituents of plant cell walls and the most abundant hemicellulose displaying great variability depending on the biological role and cytological localisation [55]. Xylanase enzyme systems are responsible for the degradation of xylan and can be divided into main-chain degrading enzymes and accessory enzymes responsible for the hydrolysis of side-chain constituents. The most studied are main-chain degrading endo-1,4- $\beta$ -xylanases (EC 3.2.1.8), which are commonly referred to as xylanases and 1,4- $\beta$ -xylosidases (EC 3.2.1.37) [56-57] with the former being the largest, containing 178 members classified into the two families, 10 and 11. Although both families (GHF 10 and GHF 11) hydrolyse similar substrates the GHF 10 xylanases exhibit substantial differences from GHF 11. Early characterisation suggested that the former were typically acidic with low isoelectric point (pI) values with higher molecular mass catalytic modules ( $\sim$ 40kDa), in contrast to the GHF 11 members having basic pI values and low molecular masses ( $<$ 30kDa) [57]. Furthermore, approximately 40% of the GHF 10 members display modular architecture [58].

The thermostable xylanase (GHF 10) originating from *R. marinus* is modular consisting of 5 modules/domains [59] individually connected by a recognisable linker sequence (Figure 4). The linker regions are often rich in proline or hydroxy-amino acids with varying lengths and they are susceptible to proteolytic cleavage [60]. The catalytic module has a relatively high molecular weight ( $\sim$ 40kDa) [61-62] (**Papers II, III, V**) and typically displays a TIM-barrel fold (cylindrical 8-fold  $\alpha/\beta$ -barrel structure (Figure 4). The two N-terminally repeated carbohydrate-binding modules (CBM4-1 and CBM4-2) encoded by the *xyn10A* gene have affinity for both insoluble

xylan and amorphous cellulose and although they show an 88% sequence identity they differ in their substrate affinities and stabilities [63-64].



**Figure 4.** Schematic representation of the modular *Xyn10* gene from *R. marinus*. **(Left)** Ribbon model depicting type B Xyn10 CBM4-2. The two aromatic residues using the xylan binding cleft are indicated. **(Right)** Side view of a homologue to the Xyn10A catalytic module. The ribbon model shows the two side-chains of the catalytic residues in space-fill.

Furthermore, the structure of the CBM4-2 (Figure 4) has been resolved which has a  $\beta$ -jelly roll structure formed by 11 strands, and contains a prominent cleft [65]. The third (D3) and fifth domain (D5) encoded by the *xyn10A* gene from *R. marinus* has previously been reported as being with

unknown function. However, with regards to the latter this domain represents a novel type of module that mediates cell attachment in proteins originating from members of the phylum Bacteroidetes (**Paper I**).

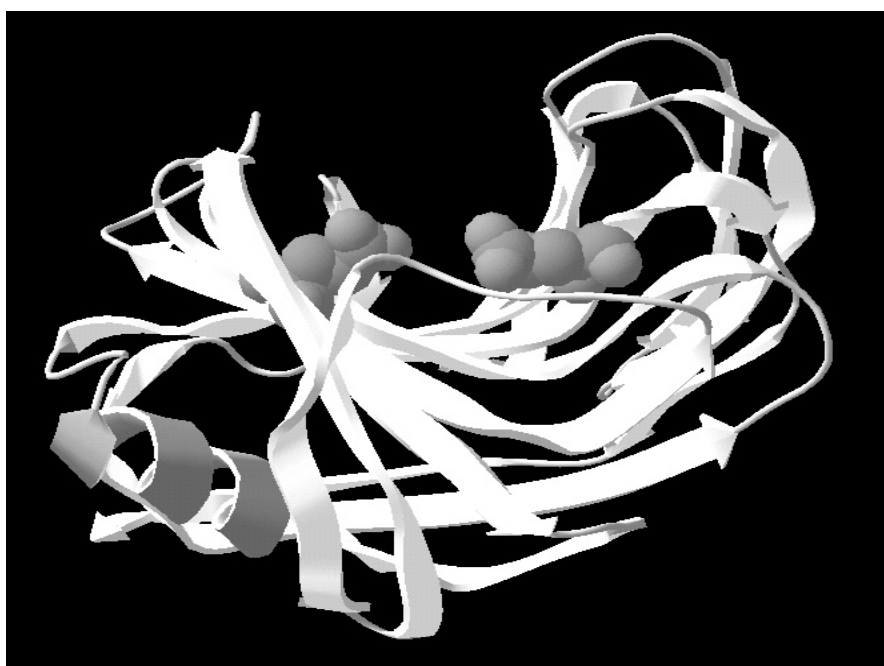
Xylanases exhibit a number of potential industrial applications. Currently, the most effective use of xylanase is in the prebleaching of kraft pulp to minimise the use of harsh chemicals in the subsequent treatment stages of kraft pulp [66-68]. However, the applications of xylanase further extends to the food, feed and agricultural industries [57, 69-71].

### **3.3.2 Cellulase**

Cellulose mainly exists as a structural component in cell walls of higher plants making up around 30% of dry mass in grasses and cereals and between 40-50% of wood [72]. Efficient depolymerisation of the polymer is brought about by cellulases with the ability to hydrolyse  $\beta$ -1,4 glucosidic bonds. It should be noted that cellulases and xylanases sometimes display overlapping specificities and modes of action [73]. Cellulases fall into three major groups depending on their mode of attack: (1) endoglucanases (EC 3.2.1.4) which carry out random cleavage of internal bonds in the amorphous or less ordered region of cellulose as well as cleavage in a variety of other soluble glucan polymers containing  $\beta$ -1,4 linked D-glucose; (2) exoglucanases (EC 3.2.1.91) which function in a processive manner on the reducing or non-reducing ends of the cellulose chain releasing cellobiose as their main product; (3)  $\beta$ -glucosidases (EC 3.2.1.21) which are involved in the hydrolysis of cellobiose to glucose [74].

The extremely thermostable endoglucanase, Cel12A from *R. marinus* [75] belong to the family 12 glycoside hydrolases. Generally members of the GHF 12 display a high catalytic activity on mixed linkage glucans such

as  $\beta$ -glucan and lichenan, while their activities on both amorphous and crystalline cellulose are exceedingly low [76]. The three-dimensional structure of the catalytic module displays a  $\beta$ -jellyroll fold (Figure 5) very similar to that of family 11 xylanase [77]. Applications of cellulases extend from use in drainage, deinking and fiber modification in the pulp and paper industry to food, feed and agricultural industries [78].



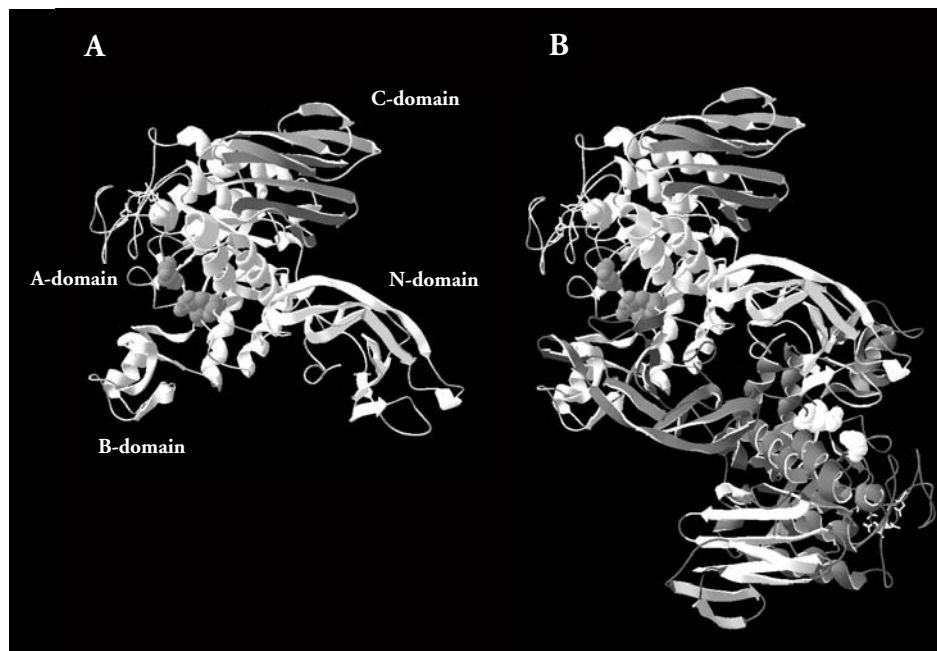
**Figure 5.** Ribbon model of the *R. marinus* Cel 12A from a side-view with the side-chains of the two catalytic residues shown in space-fill.

### 3.3.3 Cyclomaltodextrinase

The  $\alpha$ -amylase family (GHF 13) is one (and the largest) of five structural families of starch-modifying enzymes, specifically acting on the  $\alpha$ -1,4- and  $\alpha$ -1,6-*O*-glycosidic linkages of starch and starch like polymers [79]. Substrate specificity revealed that the family 13 also includes

cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133), and neopullulanases (NPases; EC 3.2.1.135) which readily hydrolyse cyclodextrins, pullulan and starch [80]. These enzymes are distinguished from typical  $\alpha$ -amylases by containing a novel N-terminal domain and exhibiting preferential substrate specificities for cyclomaltodextrins (CDs) over starch. CDases hydrolyse CDs much faster than starch [81-82] and do not produce CDs from starch in contrast with cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) that forms CDs from starch and has hydrolytic activity towards CDs [83].

Since the first reported CDase from *Bacillus macerans* [84] there has been an increasing number of reports on CDases [85-89]. Recently a multi-domain CDase from the thermophilic bacterium *Anoxybacillus flavithermus* has been cloned and expressed in *E. coli* [54]. Although the  $\alpha$ -amylase family display large variation in domain number and structure [90-91], the CDases are generally composed of four domains termed N, A, B and C (Figure 6). In **paper IV** a temperature-limited fed-batch (TLFB) was performed in order to improve production yields as the  $\alpha$ -amylase family enzymes has proven to be difficult to express in an active form.



**Figure 6.** Ribbon models of *Anoxybacillus flavithermus* CDase13 in its monomeric (**A**) and its native dimeric (**B**) state.

### 3.4 Phospholipase from an Alkaliphile

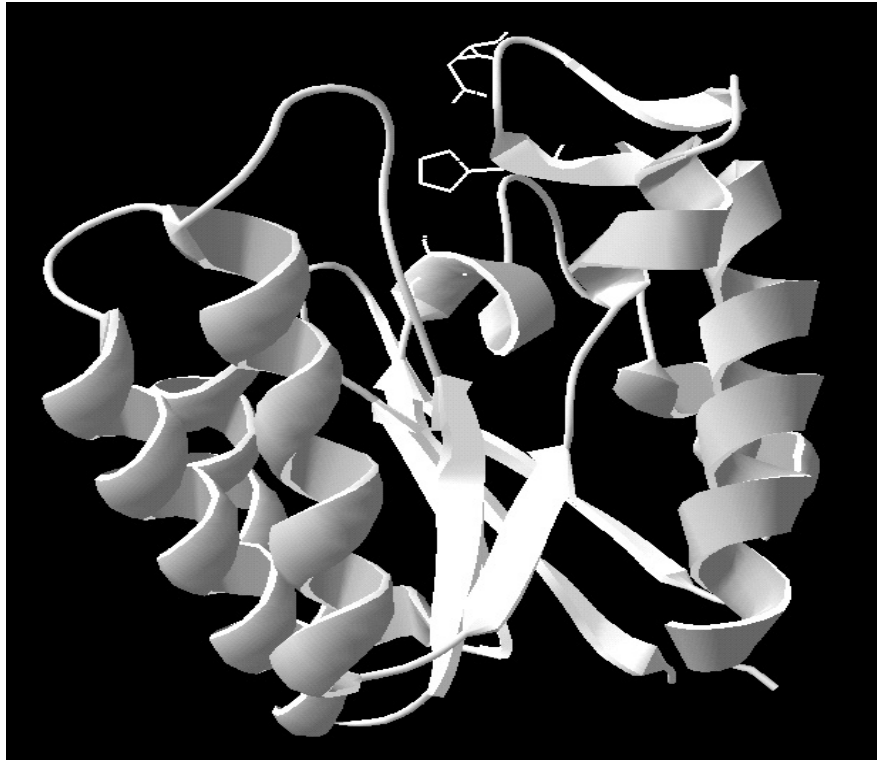
Alkaliphiles are defined as microorganism displaying optimum growth at least two pH units above neutrality. Soda lakes and soda deserts are the most naturally occurring alkaline habitats where large amounts of carbonate minerals results in the establishment of pH values around 11.5 and above as a result of geological, geographical and climate conditions [92-93]. These environments allow the dense populations of aerobic organotrophic and alkaliphilic bacteria, which are potential sources of alkali-stable enzymes, to proliferate [94-95]. Among the alkaliphilic bacteria the genus *Bacillus* produces extracellular, alkaline active enzymes which has had

considerable impact on industrial processes requiring alkaline conditions. A number of alkaline active enzymes such as amylases, xylanases, proteases, galactosidases, pectinases and pullulanases have been reported from *B. halodurans* [44, 96-98]. Furthermore, *B. halodurans* C-125 has been biochemically and genetically characterised [99].

Recently, lipolytic enzymes have been reported in *B. halodurans* from samples originating from a Kenyan alkaline soda lake [100]. Microbial lipase displays a wide range of industrial applications and is also an interesting catalyst in organic media [101]. Although many new bacterial lipolytic enzymes have been studied only a few reports have attempted to organise information such as biochemical properties, for comparative purposes [102-105]. Usually, lipolytic enzymes are characterised by their ability to catalyse a broad range of reactions. However, the wide diversity of methods used for lipase assays (such as the hydrolysis of *p*-nitrophenyl esters, the pH-stat method and the monolayer technique) prevents a direct comparison of results on substrate specificity [103, 106].

In the present investigation (**Paper VI**), a gene encoding lipase identical to BH3288, deposited in the NCBI database [<http://www.ncbi.nlm.nih.gov>] has been amplified from the strain of *B. halodurans* isolated in the Kenyan alkaline soda lake mentioned above [100] and was successfully cloned and expressed using a *Pichia pastoris* host-vector system. This enzyme was found to display phospholipase activity which is an essential property in applications for baking, degumming of vegetable oils and as emulsifiers in mayonnaise industry. Although lipases belong to many different sub-families with low sequence similarities, they have the same overall architecture, the  $\alpha/\beta$ -hydrolase fold (Figure 7) and the true lipases display a conserved active site signature, the GX SXG-motif [107], which was also found in the *B. halodurans* sequence.





**Figure 7.** A representation of the  $\alpha/\beta$ -hydrolase fold conserved among lipases. The side-chains shown are the conserved catalytic triad [S, H, D], with the serine being part of the GXSXG-motif.

#### 4. Prokaryotic and Eukaryotic Expression Systems

Prokaryotic and eukaryotic systems are the two general categories of expression systems. Prokaryotic systems are generally easier to handle and are satisfactory for most expression purposes. However, there are serious limitations in using prokaryotic cells for the production of eukaryotic proteins which undergo a variety of post-translational modifications e.g. glycosylation, and phosphorylation. All expression systems pose advantages and disadvantages that one should be aware of and selection involves evaluating the possibilities to obtain good yields, proper glycosylation and folding, prior to considering scale up. The production trials and optimisation work reported in this thesis focuses on two hosts: *E. coli* (**Papers II, III, IV**) and *P. pastoris* (**Papers V, VI**) and as a starting point Table 3 outlines some important characteristics of these host's which influences their selection as an appropriate host vector system.

**Table 3.** Comparison of host characteristics

	<i>E. coli</i>	<i>P. pastoris</i>
Complexity of growth medium	Minimum	Minimum
Cost of growth medium	Low	Low
Expression level	High	Low - high
Extracellular expression	secretion to periplasm	secretion to medium
<i>Post-translational modifications</i>		
Protein folding	usually required	may be required
N-linked glycosylation	No	Yes
O-linked glycosylation	No	Yes
Phosphorylation	No	Yes
Acetylation	No	Yes
Acylation	No	Yes

Among prokaryotic host expression systems, *E. coli* is a widely employed host due to the vast body of knowledge that exists about its genetics and physiology, including its complete genome sequence and a number of available vectors which greatly facilitates gene cloning [108-109]. As alternative hosts, yeasts (mainly the genus *Saccharomyces* and *Pichia* both being classified as GRAS) are favoured for the expression of proteins destined for use in medical, food and pharmaceutical industries [110-111].

#### **4.1 Optimising Heterologous Gene Expression in *E. coli***

As previously mentioned the gram-negative bacterium *E. coli* remains one of the most attractive host for the production of heterologous proteins. A wide range of advantages possessed by *E. coli* ensures that it remains a valuable microorganism for high-level protein production [6, 108, 112-113]. Although there are no guarantees that the desired heterologous protein will accumulate to high levels in a fully biological active form in *E. coli*, a considerable amount of effort has been directed towards improving the performance and versatility of this host for heterologous gene expression. Below, a few key features of *E. coli*-based expression systems are examined and strategies employed to overcome limitations are also addressed.

##### **4.1.1 Transcriptional Regulation of Expression**

The suitability of promoters for high-level gene expression is governed by several criteria [114]. Firstly, the promoter must be strong, capable of protein production in excess (at least 10-30% of the total cellular protein). Secondly, the promoter should exhibit a minimal level of basal transcription. Thirdly, promoters should be capable of induction in a simple and cost-effective manner [115]. For many years the *E. coli lac*

operon has served as one of the paradigms of prokaryotic regulations. Thus, many promoters used to control transcription of heterologous proteins have been constructed from *lac*-derived regulatory elements [116-117].

Over the years, the pET vectors (Novogen, Madison, WI) have gained increasing popularity. In this system, target genes are positioned downstream of the bacteriophage T7 promoter or a T7/*lac*-promotor fusion on medium copy number plasmids. The highly processive T7 RNA polymerase is encoded in the production host via insertion of a prophage ( $\lambda$ DE3) encoding the enzyme under control of the IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside)-inducible *lacUV5* promoter [118]. Using this system up to 40-50% of the total cellular protein could be heterologous and transcription is tightly controlled. It is often argued that the cost and toxicity of IPTG limits the usefulness of these promoters. However, these drawbacks may be overcome by cultivation techniques that allow use of lactose as an inducer instead of IPTG (**Paper III**). Other interesting reports have demonstrated useful protein expression systems for different applications *via* the *araE*, *rhaBAD*, and *nar* promoters, which uses arabinose, rhamnose and microaerobic conditions, respectively to induce heterologous gene expression [119-121].

#### **4.1.2 Factors Affecting Production Yields**

There has been considerable research effort over the years to overcome the limitations in protein expression in *E. coli* (Table 4). Although, there is the flexibility of selecting the target destination (periplasm, cytoplasm) for the accumulation of the desired protein there are a number of advantages and disadvantages that need to be taken into account. The periplasm provides a more oxidised environment for protein

folding than the cytoplasm but incomplete *in vivo* signal peptide cleavage may pose a problem. In the cytoplasm, proteolysis and the formation of inclusion bodies are seen as major bottle-necks affecting the production of heterologous proteins and are discussed below.

**Table 4.** Potential strategies to resolve protein production problems

Problems	Strategy for resolution	Reference
Inclusion bodies, Proteolysis, [protein insolubility, refolding to gain activity]	Low growth temperature	[122]
	Amino acid substitution	[123-125]
	Coexpression of molecular chaperons	[123, 126-127]
	Fusion partners	[128-130]
	Rich growth media	[131]
	Sucrose/raffinose in growth media	[132]
	Mutagenesis of cleavage sites	[133-135]
	Fermentation conditions	[118, 133, 136-137]
Signal peptide does not always facilitate transport, signal peptide cleavage	Altered pH	[138]
	Protease deficient strains	[139]
	Coexpression-signal peptidase I	[140]
Reduced folding	Coexpression of <i>sec</i> genes	[126]
	Fusion proteins	[141]
	Amino acid substitutions	[142]
	Coexpression of disulfide isomerase	[143-144]

### *Proteolytic degradation*

Proteolysis is a selective, highly regulated process that plays an important role in cellular physiology [145-146] such as removal of abnormal and incorrectly folded proteins. A large number of protease are present in *E. coli* and are localised in the cytoplasm, periplasm and inner and outer membranes (Table 5). Strategies for minimising proteolysis of

heterologous proteins in *E. coli* include among others, targeting proteins to the periplasm, using protease deficient strains, cultivation at low temperature, construction of N- and/or C-terminal fusion proteins and replacing protease cleavage sites [6, 125]. Also, in the present study it was shown that the presence of protease is dependent on the induction time in relation to the cell density obtained as well as nutrient composition during cultivation and type of inducer employed during heterologous protein production (**Papers II & III**).

**Table 5.** Examples of Proteases present in *E. coli*

Protease	Location	Comments	Reference
<b>Cytoplasm</b>			
Lon (La)		Degrades abnormal proteins	[147-151]
Do		Serine protease	[152]
Protease II		Serine active site	[152-155]
Ci		Metalloprotease	[156]
Fa		Endoprotease	[157]
Rec A		Recombinant functions	[158-161]
So		Serine protease	[162-163]
Clp		Degrades abnormal proteins	[164]
<b>Periplasm</b>			
Deg P			
Pi		Necessary for high temperature growth	[165-167]
Mi			
Peri 7			
Peri8		Cleavage between tyr-leu and phe-tyr	
<b>Cell membrane</b>			
Protease IV		Prefers hydrophobic amino acids	[168-169]
OmpT		Cuts at paired residues	[170-172]
Protease VI		Membrane associated	[173]

### ***Formation of Inclusion bodies***

Overproduction of heterologous proteins intracellularly in *E. coli* is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies. A number of environmental conditions such as pH and temperature are reported to influence the formation of inclusion bodies [17-175]. Formation of inclusion bodies may be advantageous in rendering protection of heterologous proteins against proteases and simplifying protein purification. However, there are no guarantees that *in vitro* refolding will yield large amounts of active heterologous protein.

Thus, greater effort has been focused on attaining high yields of soluble active protein through fermentation engineering (**Paper IV**). The data obtained showed that the generally accepted decrease of temperature is not always successful but may also require modification of the process-strategy to yield improvements. Other approaches include co-expression of molecular chaperons [176] and the fusion of the desired protein to highly soluble proteins [54] such as the *E. coli* Nus A protein [177]. Fusion proteins offer other advantages, such as improving folding characteristics, increased solubility or specific affinity sites thus limiting proteolysis and providing generic protein purification schemes respectively [178-179]. However, the main disadvantages of protein fusion technologies are that liberation of the target or passenger protein requires additional steps which may include expensive proteases (e.g. Factor Xa or enterokinase) and a reduction in yield due to incomplete cleavage.

## **4.2 Heterologous Gene Expression in *Pichia pastoris***

Initially the ability of *Pichia pastoris* to metabolise methanol as a sole carbon source prompted immediate attention in its use as a potential

source of single-cell proteins (SCP). Over the years researchers have developed the methylotrophic yeast *Pichia pastoris* into a highly successful system for the production of a variety of heterologous proteins [20]. Several factors have attributed to the increasing popularity of this expression system such as: (1) molecular and genetic manipulation of *P. pastoris* (similar to *Saccharomyces cerevisiae*), which makes it a well-characterised system; (2) high yields of heterologous protein attainable either intracellularly or extracellularly; and (3) its capability of performing post-translational modifications such as glycosylation, disulphide bond formation and proteolytic processing.

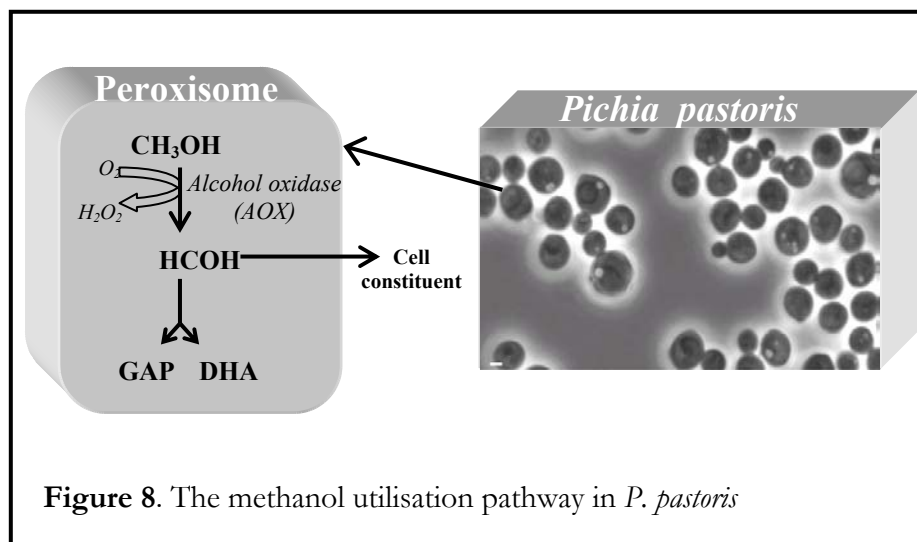
#### **4.2.1 Methanol Metabolism and the AOX1 Promoter**

Only a limited number of species belonging to the genera *Hansenula*, *Candida*, *Torulopsis* and *Pichia* are capable of growth on methanol as a sole carbon source [180-182]. In the *P. pastoris* expression system the conceptual basis stems from the observation that some enzymes required for methanol metabolism are present in substantial amounts only when cells are grown on methanol [183-184]. Methanol utilisation requires a unique metabolic pathway involving several unique enzymes [185] with the enzyme alcohol oxidase (AOX) catalysing the first step: the oxidation of methanol to formaldehyde and hydrogen peroxide. The hydrogen peroxide is degraded to oxygen and water by catalase which, together with AOX is sequestered in peroxisomes. A portion of the formaldehyde leaves the peroxisomes and is further oxidised to provide a source of energy for the cells growing on methanol (Figure 8).

Alcohol oxidases are present at high levels in cells grown on methanol as a sole carbon source but not when most other carbon sources (e.g. glucose or glycerol) are present. *P. pastoris* cells grown in fermentation



cultures where methanol is fed as a growth limiting substrate have shown an accumulation of AOX >30% of the total soluble protein [186-187].



**Figure 8.** The methanol utilisation pathway in *P. pastoris*

Although two genes encode alcohol oxidase in *P. pastoris*: *AOX1* and *AOX2*, the *AOX1* is responsible for the vast majority of the alcohol oxidase activity and hence expression systems have been developed utilising the promoter of the *AOX1* gene. [188]. Regulation of the *AOX1* gene is a mechanism resembling that of the *GAL1* gene of *Saccharomyces cerevisiae* involving an induction/derepression as well as induction mechanism. However, unlike *GAL1* regulation, where the absence of a carbon source such as glucose in the medium results in transcription, the lack of a carbon source does not result in substantial *AOX1* transcription. The presence of methanol is hence essential to induce high level transcription of alcohol oxidase via this promoter [188].

#### 4.2.2 Host Strains and Vector Design

The most commonly utilised promoter for heterologous protein production in *P. pastoris* is the *AOX1* promoter described above which is commercially available (Invitrogen, Carlsbad, CA). A number of heterologous proteins from bacterial origin have been produced using this promoter and with the results gained in this thesis using the *AOX1* promoter the list of enzymes (Table 6) can now be extended with representatives of extremophilic origin; including extracellularly produced thermostable family 10 bacterial xylanase (**Paper V**) and a phospholipase originating from an alkaliphilic strain of *B. halodurans* (**Paper VI**). Other alternate promoters to the *AOX1* promoter are *P. pastoris GAP* (glyceraldehyde 3-phosphate gene), *FLD1* (glutathione-dependent formaldehyde gene), *PEX8* (genes for peroxisomal matrix proteins) and *YPT1* (gene for GTPase involved in secretion) promoters.

Most *P. pastoris* host strains grow on methanol at the wild-type rate (Mut<sup>+</sup>, methanol utilisation plus phenotype). However, other mutant host strains are available which vary with regards to methanol metabolism due to deletion of one or both *AOX* genes and some reports have shown better production of heterologous proteins in these modified strains [194-196]. Also, several protease deficient strains have been shown to reduce degradation of heterologous proteins [197-198].

Vectors for generating heterologous methylotrophy are capable of integration into the genome of the host either by random or homologous recombination and expression-vectors have been designed as *E. coli/P. pastoris* shuttle vectors. In addition, to facilitate secretion of the desired heterologous protein, vectors are available where, in-frame fusion of the foreign protein and a secretion signal of *P. pastoris* such as acid phosphatase (*PHO1*) or *S. cerevisiae*  $\alpha$ -factor can be generated. The latter was used in the

present investigation (**Papers V, VI**), which yielded efficient secretion of the target proteins in the protease deficient *P. pastoris* strain (SMD 1168H) that was selected as the host.

**Table 6.** Bacterial proteins produced in *P. pastoris*

Bacterial Source	Protein	Comment	Reference
<i>B. licheniformis</i>	$\alpha$ -amylase	E (2.5 g/L)	[189]
<i>B. stearothermophilus</i>	Carboxypeptidase	E (100 mg/L)	[190]
<i>Escherichia coli</i>	Acid phytases	E (29 U/mg)	[191]
<i>Escherichia coli</i>	$\beta$ -galactosidase	I ( $2 \times 10^3$ U/mg)	[20]
<i>Staphylococcus aureus</i>	Staphylokinase	E (50 mg/L)	[192]
<i>Streptococcus equisimilis</i>	Streptokinase	I (77 mg/L)	[193]
<i>R. marinus</i>	Xylanase	E (~2 g/L)	<b>Paper V</b>
<i>B. halodurans</i>	Phospholipase	E (~1 g/L)	<b>Paper VI</b>

E (Extracellular) I (Intracellular)

#### 4.2.3 Post-translational Modifications

*P. pastoris* is capable of adding both *N*- and *O*-linked carbohydrates to secreted proteins. However, the capability is limited to the addition of mannose only to the *N*-glycosylation core and in the case of *O*-glycosylation; the oligosaccharides are composed solely of mannose (Man) residues [199-200]. *P. pastoris* appears to exhibit an advantage over *S. cerevisiae* in that the tendency towards overglycosylation of heterologous proteins is less pronounced when using *P. pastoris* as an expression host.

In **paper V**, a 200-fold increase in heterologous xylanase activity was observed after partial unfolding and refolding of the protein and it should be mentioned that the reasons behind the reactivation need of the enzyme could be due to *O*-glycosylation, which is more difficult to monitor than *N*-glycosylation. Although glycosylation has not been reported for *R. marinus*, *O*-glycosylated *P. pastoris* produced heterologous proteins have been reported in some cases [201-202].

## 5. Bioprocess Technology

### 5.1 Fermentation Technology employing Extremophiles

The primary objective of fermentation technology in research and industry is attaining optimum productivity ( $\text{g L}^{-1} \text{h}^{-1}$ ), i.e. obtaining the highest amount of product in a volume within a certain time. High-cell-density (HCD) is thus a prerequisite for high productivity when product accumulation is proportional to cell mass concentration. There are several reported high-cell-density-cultivation (HCDC) strategies for bacteria, archaea and yeast (Table 7) however; microorganisms with modified metabolism and specialised additional biosynthetic capacities provide new challenges to bioengineers. The main problems associated with HCDC are substrate solubility, limitation and/or inhibition of substrates with respect to growth, instability and volatility of substrates and products, product or by-product accumulation to a growth-inhibitory level, degradation of products, high evolution rates of  $\text{CO}_2$  and heat, high oxygen demand as well as the increasing viscosity of the medium as the cultivation progresses [203].

Additional problems arise with HCDC of extremophiles due to their need for extreme conditions for optimal growth. These problems are mainly related to different physicochemical aspects of their fermentation and may require adaptations/design of the bioreactor; nevertheless considerable effort has gone into the development of fermentation strategies [203-204]. In the context of this thesis *R. marinus* (**Paper I**) was successfully cultivated in batch fermentation resulting in a final cell dry weight of approximately 4 g/L with the goal not focused on high productivity but in order to obtain samples for enzyme characterisation.

**Table 7.** Various microorganisms grown to cell densities higher than 100 grams cell dry weight per litre of culture volume.

Microorganism	Characteristics	CDW (g L <sup>-1</sup> )	Pr (g L <sup>-1</sup> h <sup>-1</sup> )	Reference
<b>Bacteria</b>				
<i>E. coli</i>	Mesophile	190	1.04	[205]
		180	1.08	[205]
		148	3.36	[206]
		145	4.53	[207]
<i>B. subtilis</i>		184	6.57	[208]
<i>S. laurentii</i>		157	0.71	[209]
<i>P. putida</i>		100	3.33	[210]
<i>M. extorquens</i>		233	1.37	[211]
<b>Archaea</b>				
<i>Marinococcus</i> M52	Halophile	132	N.A	[203]
<i>Sulfolobus shibatae</i>	Thermoacidophile	114	0.04	[203]
<b>Eukarya</b>				
<i>C. brassicae</i>	Mesophile	268	9.57	[212]
<i>S. cerevisiae</i>	Mesophile	235	1.07	[213]
<i>P. pastoris</i>	Mesophile	100	0.78	[199]

CDW = maximum cell dry weight    Pr = overall biomass productivity

## 5.2 Fed-batch Process employing *E. coli* for Enzyme Production

As mentioned previously *E. coli* has emerged as the pioneer microorganism with respect to molecular strategies for the production of heterologous proteins. Due to product accumulation occurring intracellularly considerable effort has been placed in conjunction with fermentation technology to develop HCD fed-batch strategies ensuring efficient heterologous protein titres. The fed-batch cultivation process is performed without any outflow of the culture medium but with a continuous inflow of a growth-limiting substrate. The process typically starts as a pure batch culture prior to the growth limited fed-batch phase.

Since high amounts of substrates are needed to reach a high concentration of biomass, these substrates must be fed in a controlled manner in order to overcome metabolic and engineering limitations during the establishment of high cell densities. Exploiting substrate control ensured the successful establishment of HCD in the present investigation (**Papers II, III, IV**).

A fed-batch process requiring an induction step to initiate heterologous protein synthesis is typically divided into pre- and post-induction phases. During the pre-induction phase the focus is centred on directing the host cell metabolism solely towards cell mass production and limiting by-product formation while in the post-induction phase the goal is to attain maximum product accumulation without degradation of the desired protein. However, as stated above the attainment of high cell densities is a prerequisite in order to obtain maximum product concentrations and both the pre- and post-induction phase needs consideration with respect to host cell-metabolism.

### **5.2.1 Cellular Responses during Cultivation**

A major limiting factor during the cultivation of *E. coli* is the production of acidic by-products such as acetate which reduces growth rate, biomass yields, and maximum attainable cell densities [14, 214-215]. More importantly, acetate accumulation has been reported to have a detrimental effect on recombinant cells concomitantly reducing heterologous protein production levels [216-218]. Acetate is produced when the carbon flux into the central metabolic pathway exceeds the biosynthetic demands and the capacity for energy generation within the cell [219-221], adding to saturation of the tricarboxylic acid (TCA) cycle and/or the electron transport chain [14, 219].

Acetate production usually occurs when *E. coli* is grown under anaerobic or oxygen limiting conditions; however, acetate is also produced under aerobic conditions in the presence of excess glucose and is termed overflow metabolism [17, 222]. This occurs when the specific glucose uptake rate ( $q_g$ ) exceeds a critical value ( $q_g^{crit}$ ) [14]. Also, the onset of acetate formation has been observed when the specific oxygen uptake rate ( $q_o$ ), which normally increases with  $q_g$ , reaches an apparent maximum ( $q_o^{max}$ ) [223-226].

A number of strategies have been developed to limit acetate accumulation [5]. Alterations in media composition such as the use of glycerol as a carbon source reduced acetate formation [206, 220]. Also, by the addition of certain amino acids the harmful effects of acetate can be alleviated thus resulting in an increase in growth rate and heterologous protein production [13]. Manipulating process variables such as cultivation at lower temperature to lower nutrient uptake and growth [12, 227-229] or increasing the dissolved oxygen concentration ( $pO_2$ ) by pure oxygen sparging [175, 230] has been beneficial in lowering acetate levels. Furthermore, acetate production is highly strain dependent but by exploiting the tools of metabolic engineering to manipulate/redirect metabolic pathways it is possible to acquire *E. coli* strains with lower acetate production capabilities. Some examples include a reduction in glucose uptake [231], improving oxygen uptake [232], and blocking acetate excretion [233].

However, it should be noted that one of the most routinely used method to avoid overflow metabolism is by manipulation of the glucose feed rate during fed-batch cultivation (**Papers II, III, IV**) and this is discussed in more detail under the process control section.

### 5.2.2 Cellular Response during Protein Over-expression

The determining factor in the yield of heterologous proteins is the mutual interplay between the strength of the genetic system and the host-cell's metabolic capacity. Therefore, efficiency of bioprocesses for heterologous protein production relies upon understanding the relationship between expression of heterologous-protein encoding genes and host-cell metabolism. Often the use of too strong expression systems leads to rapid loss in host-cell metabolic activity due to the redirection of cellular metabolism [234] towards heterologous-protein production, thus over utilising the shared protein synthesis machinery, precursors and energy resources during the post induction phase.

Determination of the metabolic load imposed during heterologous protein synthesis is hence a key issue for process optimisation. The development of a “metabolic load sensor” based on the stringent-response network appears as one possibility to monitor this load [235]. In the stringent response, the synthesis of ribosomal and transfer RNAs is inhibited when protein synthesis is blocked by amino acid starvation. During amino acid starvation a regulatory nucleotide, guanosine tetraphosphate (ppGpp), accumulates and inhibits rRNA and tRNA synthesis [236]. However, this triggers host-cell proteases to degrade abnormal proteins in order to replenish amino acid resources and redirect free amino acids for incorporation into essential growth associated proteins [237].

Also, optimisation of the post induction phase by incorporation of certain key metabolites and precursors, during this phase of the cultivation has rendered significant improvements in target protein yields as well as lowering cell associated factors such as *in vivo* proteolysis (**Papers II, III**). Furthermore, heterologous protein production rates may be adapted for



optimal exploitation of the host-cell's biosynthetic capacity. Reducing the promoter strength by feeding limiting quantities of the inducer or using alternative inducers eventually with lower affinity for the specific repressor molecule is an effective way of tuning the expression rate (**Paper III**).

### 5.2.3 Process Control

The mode of feeding influences *E. coli* fed-batch cultivations by defining the growth rate and the effectiveness of the carbon cycle for product formation and minimisation of acetate formation. By controlling the glucose feed rate it is possible to avoid overflow metabolism and oxygen limiting conditions. There are a number of feeding strategies available to reduce or avoid acetate accumulation. The nutrient feed mode can be defined based on an open-loop, if an exact mathematical model is at disposal or a feedback control example pH (pH-stat) or dissolved oxygen (DO-stat) [5, 238-239].

In the present investigation (**Papers II, III, IV**) glucose limited *E. coli* fed-batch cultivations were performed based on on-line detection of acetate formation using dissolved oxygen responses [240]. Under glucose-limited conditions pulses superimposed to the glucose feed rate gave rise to changes in the glucose uptake that can be seen in the dissolved oxygen ( $pO_2$ ) measurements [241], which in turn was used as a feedback control to adjust the nutrient feed rate [242]. By exploiting this nutrient feed strategy, overflow metabolism was avoided and very low acetate levels were obtained.

It has been shown that performing *E. coli* fed batch cultivations under glucose limitation is successful for attaining high cell densities and reduced acetate levels however; the effect on heterologous protein production levels also needs evaluation. In the current investigation (**Paper II**) heterologous

cellulase production levels proceeded normally irrespective of the cell mass concentration at the time of induction. On the other hand, heterologous xylanase production levels were dependent on the cell mass concentration at the point of induction as well as the nutrient feed composition (**Papers II and III**). Thus, in some instances glucose-limitation may not be beneficial during the post induction phase and the nutrient feed composition should be optimised to avoid cellular stress responses such as degradation of the heterologous protein. This substrate-limited fed-batch (SLFB) strategy still supplies glucose at limiting levels but also provides other essential metabolites or precursors during the post-induction phase. Furthermore, cultivation under glucose limitation allows lactose to be immediately metabolised when it is supplied in excess as an inducer (**Paper III**).

In some reports [243] however, performing *E. coli* substrate limited fed-batch cultivation resulted in the release of higher amounts of lipopolysaccharide (LPS) outer membrane (OM) components called endotoxins. The levels of endotoxin was reduced by employing a temperature-limited fed-batch (TLFB) technique in which the dissolved oxygen concentration was regulated by temperature while all substrate components were fed uncontrolled as glucose limitation was the cause of excessive endotoxin release. In this thesis evaluation of heterologous protein production levels between two glycoside hydrolases using the SLFB and TLFB techniques were performed. Using the TLFB strategy seems to be beneficial to heterologous protein production during a longer post-induction phase but with the parameters used here, endotoxin release was not significantly affected (**Paper IV**).

### 5.3 Fermentation Technology employing *P. pastoris*

Although *E. coli* has been the "factory" of choice for the expression and production of many proteins the inherent problems of intracellular accumulation, endotoxin production and the fact that some proteins are produced in a non-functional, unfinished form due to the lack of certain post-translational modifications has resulted in the development of alternate expression systems. *Pichia pastoris* has been developed to be an outstanding host for the production of foreign proteins since its alcohol oxidase promoter was isolated and cloned [197, 244].

*Pichia pastoris* offers the advantage of good growth and cell mass accumulation in a shake flask. However, cultivation in shake flasks can pose an array of problems, including pH control, oxygen limitation, nutrient limitation and temperature fluctuation. Moreover, researchers found that cultivation in a fermenter has resulted in an increase in heterologous protein production levels of over 140% [245]. A substrate limited fed-batch mode of operation has been utilised by several researches in order to achieve maximum heterologous protein production levels [246-249]. Although, the produced protein can be obtained extracellularly, high cell density cultivations are often performed in the pre-induction phase using glycerol as a carbon source. This is due to high levels of methanol, which is used in the post-induction phase, being toxic to the cells. Therefore, the transition from using glycerol as a growth limiting substrate to methanol as well as the residual concentration of methanol in the fermenter requires subtle feeding control. Furthermore, growth on methanol is slow and results in extended fermentation time thus ultimately relating to total productivity.

In this thesis an alternate screening and production strategy termed "Batch Induced Cultivation" is presented (**Papers V, VI**). This involves

performing batch cultivations with complex growth media and the inclusion of methanol as the sole carbon source. As a prerequisite *AOX1* transcription is initiated during the inoculum stage prior to transfer into the fermenter. This strategy enabled production of heterologous xylanase (**Paper V**) and phospholipase (**Paper VI**) to proceed approximately 4-10 hours after fermentation start thus reducing production time to 60 hrs as opposed to extended fermentations using the fed-batch strategy [244, 250-251].

## 6. Summary of the Present Investigation

The studies conducted during the course of this thesis has included four enzymes namely, xylanase and cellulase from *R. marinus*, cyclomaltodextrinase from *Anoxybacillus flavithermus* and a phospholipase from *Bacillus halodurans*. In figure 9, the general overview as well as the breakdown into the individual papers in which the relevant enzymes were investigated is mapped. The first part of the work probes the modular architecture of the family 10 xylanase (*RmXyn10A*). **Paper I** deals with elucidating/assigning characteristics to the previously undefined domain (D5) of the full-length xylanase which was produced by batch cultivation of the thermophile. The results suggest that this domain represents a novel type of module that mediates cell attachment in proteins originating from members of the phylum Bacteroidetes.

In **Paper II** the production of the individual catalytic modules of the cellulase and xylanase, both originating from *R. marinus* were evaluated in an *E. coli* based expression system. Induction with IPTG using the strong T7/*lac* promoter showed significant difference in the production patterns of both these enzymes. While the specific cellulase activity was predominately lower compared to the xylanase there was an incremental increase in heterologous cellulase yield in contrast to the xylanase which showed a decreasing production level 2 hours after induction at a high cell mass concentrations. Using the cellulase production trend as a model, the aim was focused on optimisation of the post induction phase for xylanase production. Results indicated that heterologous xylanase production at a high cell mass concentration was largely dependent on the post-induction nutrient feed composition. In **Paper III**, a further investigation into the metabolic stress imposed during heterologous xylanase production was carried out. The effect of some selected nutrient feed additives [TCA-

intermediates] as a strategy to relieve the metabolic burden imposed during heterologous xylanase production showed promising results. The presence of either succinic acid or malic acid as feed-additives resulted in an increase in production of approximately 40% of the heterologous thermostable xylanase. Furthermore, use of lactose as an alternative inducer of the T7/lac-promoter, was also proven to be a suitable strategy that significantly prolonged the heterologous protein production phase as compared to induction with IPTG. These results gave new complementary information regarding the protein-synthesising machinery of the cell, during heterologous protein-production.

In **Paper IV** processes control strategies are exploited for *E. coli* based expression systems in order to attain maximum heterologous protein yields. The enzymes selected were two glycoside hydrolases: cyclomaltodextrinase and the full-length modular thermostable xylanase. A comparison and evaluation of both substrate and temperature limited fed-batch strategies were carried out and this paper describes the production profiles of the respective enzymes and the amount of released endotoxins using the described feeding strategies.

Extracellular heterologous protein production was also evaluated using a *Pichia pastoris* based expression system. In **Paper V** the catalytic module of the family 10 xylanase originating from *Rhodothermus marinus* was cloned and expressed in the methylotrophic yeast *Pichia pastoris*. This is the first reported thermostable gram-negative bacterial xylanase efficiently secreted by *P. pastoris*. Furthermore, this host-vector system seems promising for the development of high cell density fed-batch processes for the production of thermostable xylanase of bacterial origin for applied purposes. Also, in **Paper VI**, the *Pichia* system was used for the expression of phospholipase from alkaliphilic *Bacillus halodurans*. Previous attempts to produce this

enzyme using the *E. coli* based expression system resulted in the formation insoluble aggregates (inclusion bodies).

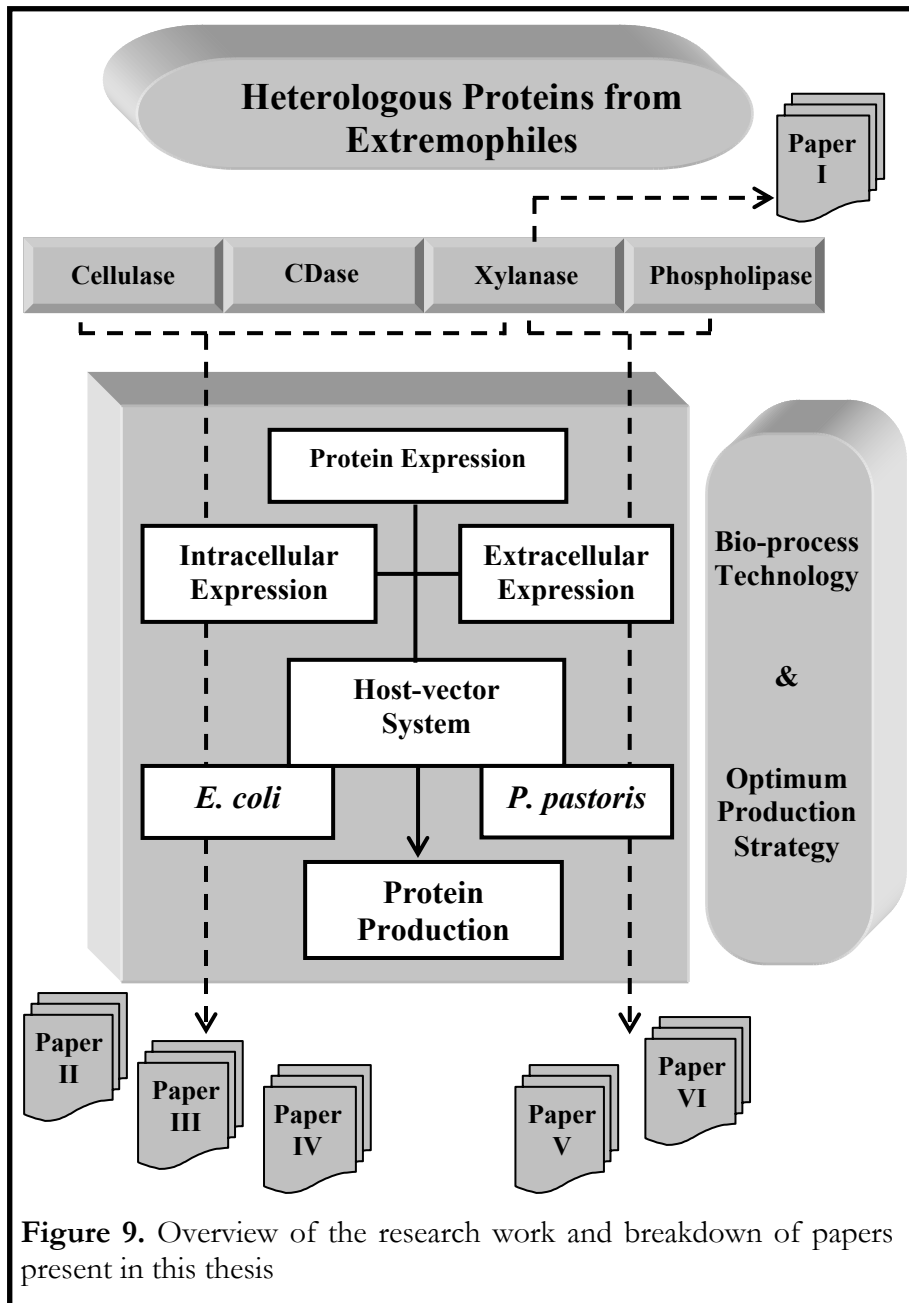


Figure 9. Overview of the research work and breakdown of papers present in this thesis

## **7. Acknowledgements**

Firstly I would like to express my gratitude to Lund University and Sweden for giving me the opportunity to reside and study so far away from home.

Seeing that it has taken 4 and half years to complete this degree I can safely say I have too many people to mention so in order to save time and space, All past and present members of the Biotechnology department are acknowledged.

Professor Bo Mattiasson is thanked for giving me the opportunity to carry out the research at the department of Biotechnology.

Professor Olle Holst; for giving me the opportunity to pursue a PhD in his research group, encouraging me to attend conferences and constantly reminding me of the economic status of my project and urging me to write lots of applications.

Department of Automatic Control; Professor Per Hagander, Stéphane, Lena, Mats as well as other research collaborators and co-authors are thanked for their assistance and input.

The secretaries Kersti and Siv, thanks for all the administrative help over the years.

Dr. Eva Nordberg Karlsson; for her excellent supervision, support and for being a good friend. It was truly an honor to work with you and I look forward to hopefully starting some new collaborative projects in the future. Keep up the good work and all the best to you and your family.

Pernilla for the many laughs we shared in the lab. Good luck in your research. As well as past members of the “Fermentation Group” Olof, Katarina, Maher and Christina.

Frans Peder, thanks for your help with the computers and for keeping me informed on current stock prices as well as giving me regular Swedish News updates.

Vicky, thanks for your advice over the years and for being a friend. I wish you all the best in life and, in that four-letter word that you told me exists.

To my adopted sister Maru och pojkvän Mario, you guys are the best and coolest couple I have met. Going to miss you'll lots. “Roots Rock Reggae” will never die. Awaiting your arrival in South Africa.



Raul for teaching me that the Spanish Omelet (*Tortilla de Patatas*) is essential for survival during ones PhD studies. All the best to you mate and good luck with your thesis.

Camilla, Betti, Bruno, Marco, Ricardo, Luis, Giulia, Ezio and Alessia, in the short time we spent together we got lots of fun memories to keep. Take care my friends and remember our deal to meet in PRAG 2008 (I will be in club Da.....)

AnaSantos, thank you for your friendship and for being YOU. I wish you all the best in the years to come.

Boaz, thanks for everything mate, we had some cool party-times. Good luck in your research.

Marika and her brother Tapio for showing me what Xmas should really be like and for introducing me to the wonderful world of sauna, ice-fishing, skiing and Lapin Kulta.

Finally, to me parents and the rest of my extended family and friends in South Africa, thank you for all the love, encouragement and support you have given me over the years. Hopefully I will come back home someday.

This work was supported financially by:

STINT- The Swedish Foundation for International Co-operation in Research and Higher Education

Vinnova – (2001-02098, P10432-2)

The European Project “Datagenom” (LSHB-CT-2003-503017)

*Cheers*

**Santosh**

## 8. References

- [1] Bull, A.T., Bunch, A.W. and Robinson, G.K. (1999) Biocatalysts for clean industrial products and processes. *Current Opinion in Microbiology*. **2**, 246-251.
- [2] Hough, D.W. and Danson, M.J. (1999) Extremozymes. *Current Opinion in Chemical Biology*. **3**, 39-46.
- [3] Niehaus, F., Bertoldo, C., Kahler, M. and Antranikian, G. (1999) Extremophiles as a source of novel enzymes for industrial application. *Applied Microbiology and Biotechnology*. **51**, 711-729.
- [4] Headon, D.R. and Walsh, G. (1994) The Industrial-Production of Enzymes. *Biotechnology Advances*. **12**, 635-646.
- [5] Lee, S.Y. (1996) High cell-density culture of *Escherichia coli*. *Trends in Biotechnology*. **14**, 98-105.
- [6] Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiological Reviews*. **60**, 512-538.
- [7] Datar, R.V., Cartwright, T. and Rosen, C.G. (1993) Process Economics of Animal Cell and Bacterial Fermentations: A case-study analysis of tissue plasminogen-activator. *Bio-Technology*. **11**, 349-357.
- [8] 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.
- [9] Riesenber, D. (1991) High cell density cultivation of *Escherichia coli*. *Current Opinion in Biotechnology*. **2**, 380-384.
- [10] Lin, H.Y. and Neubauer, P. (2000) Influence of controlled glucose oscillations on a fed-batch process of recombinant *Escherichia coli*. *Journal of Biotechnology*. **79**, 27-37.
- [11] Neubauer, P., Haggstrom, L. and Enfors, S.O. (1995) Influence of substrate oscillations on acetate formation and growth-yield in *Escherichia coli* glucose limited fed-batch cultivations. *Biotechnology and Bioengineering*. **47**, 139-146.
- [12] Hellmuth, K., Korz, D.J., Sanders, E.A. and Deckwer, W.D. (1994) Effect of growth rate on stability and gene expression of recombinant plasmids during continuous and high cell density cultivation of *Escherichia coli* TG1. *Journal of Biotechnology*. **32**, 289-298.
- [13] Han, K., Hong, J. and Lim, H.C. (1993) Relieving effects of glycine and methionine from acetic acid inhibition in *Escherichia coli* fermentation. *Biotechnology and Bioengineering*. **41**, 316-324.
- [14] Han, K., Lim, H.C. and Hong, J. (1992) Acetic acid formation in *Escherichia coli* fermentation. *Biotechnology and Bioengineering*. **39**, 663-671.
- [15] Oconnor, G.M., Sanchezriera, F. and Cooney, C.L. (1992) Design and evaluation of control strategies for high cell density fermentations. *Biotechnology and Bioengineering*. **39**, 293-304.
- [16] Konstantinov, K., Kishimoto, M., Seki, T. and Yoshida, T. (1990) A balanced DO-stat and its application to the control of acetic acid excretion by recombinant *Escherichia coli*. *Biotechnology and Bioengineering*. **36**, 750-758.
- [17] Luli, G.W. and Strohl, W.R. (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Applied and Environmental Microbiology*. **56**, 1004-1011.
- [18] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology*. **185**, 60-89.

- [19] Liang, S.T., Xu, Y.C., Dennis, P. and Bremer, H. (2000) mRNA composition and control of bacterial gene expression. *Journal of Bacteriology*. **182**, 3037-3044.
- [20] Cregg, J.M., Cereghino, J.L., Shi, J.Y. and Higgins, D.R. (2000) Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology*. **16**, 23-52.
- [21] Cereghino, J.L. and Cregg, J.M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews*. **24**, 45-66.
- [22] Rai, M. and Padh, H. (2001) Expression systems for production of heterologous proteins. *Current Science*. **80**, 1121-1128.
- [23] Falch, E.A. (1991) Industrial Enzymes: Developments in production and application. *Biotechnology Advances*. **9**, 643-658.
- [24] Bull, A.T. (2001) Biotechnology for industrial sustainability. *Korean Journal of Chemical Engineering*. **18**, 137-148.
- [25] Arnold, F.H. (1993) Engineering enzymes for nonnatural environments - Improved biocatalysts for the chemical and biotechnology industries. *Protein Engineering*. **6**, 59-59.
- [26] Arnold, F.H. (1993) Engineering proteins for nonnatural environments. *FASEB Journal*. **7**, 744-749.
- [27] Giver, L., Gershenson, A., Freskgard, P.O. and Arnold, F.H. (1998) Directed evolution of a thermostable esterase. *Proceedings of the National Academy of Sciences of the United States of America*. **95**, 12809-12813.
- [28] MacBeath, G., Kast, P. and Hilvert, D. (1998) Redesigning enzyme topology by directed evolution. *Science*. **279**, 1958-1961.
- [29] Dufour, E., Tam, W., Nagler, D.K., Storer, A.C. and Menard, R. (1998) Synthesis of amidrazones using an engineered papain nitrile hydratase. *FEBS Letters*. **433**, 78-82.
- [30] Holloway, P., Knoke, K.L., Trevors, J.T. and Lee, H. (1998) Alteration of the substrate range of haloalkane dehalogenase by site-directed mutagenesis. *Biotechnology and Bioengineering*. **59**, 520-523.
- [31] Demirjian, D.C., Moris-Varas, F. and Cassidy, C.S. (2001) Enzymes from extremophiles. *Current Opinion in Chemical Biology*. **5**, 144-151.
- [32] Adams, M.W.W. and Kelly, R.M. (1998) Finding and using hyperthermophilic enzymes. *Trends in Biotechnology*. **16**, 329-332.
- [33] Norris, P.R., Burton, N.P. and Foulis, N.A.M. (2000) Acidophiles in bioreactor mineral processing. *Extremophiles*. **4**, 71-76.
- [34] Horikoshi, K. (1999) Alkaliphiles: Some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews*. **63**, 735-741.
- [35] Schiraldi, C. and De Rosa, M. (2002) The production of biocatalysts and biomolecules from extremophiles. *Trends in Biotechnology*. **20**, 515-521.
- [36] Di Lernia, I., Morana, A., Ottombrino, A., Fusco, S., Rossi, M. and De Rosa, M. (1998) Enzymes from *Sulfolobus shibatae* for the production of trehalose and glucose from starch. *Extremophiles*. **2**, 409-416.
- [37] Buchert, J., Tenkanen, M., Kantelinen, A. and Viikari, L. (1994) Application of xylanases in the pulp and paper industry. *Bioresource Technology*. **50**, 65-72.
- [38] Damaso, M.C.T., de Castro, A.M., Castro, R.M., Andrade, C. and Pereira, N. (2004) Application of xylanase from *Thermomyces lanuginosus* IOC-4145 for enzymatic hydrolysis of corncob and sugarcane bagasse. *Applied Biochemistry and Biotechnology*. **113-116**, 1003-1012.
- [39] Becker, P., AbuReesh, I., Markossian, S., Antranikian, G. and Markl, H. (1997) Determination of the kinetic parameters during continuous cultivation of the lipase

- producing thermophile *Bacillus* sp. IHI-91 on olive oil. *Applied Microbiology and Biotechnology*. **48**, 184-190.
- [40] Madigan, M.T. and Mairs, B.L. (1997) Extremophiles (Scientific American). **276**, pg 82.
- [41] Pennisi, E. (1997) Biotechnology - in industry, extremophiles begin to make their mark. *Science*. **276**, 705-706.
- [42] Herbert, R.A. (1992) A Perspective on the biotechnological potential of extremophiles. *Trends in Biotechnology*. **10**, 395-402.
- [43] Russell, N.J. (2000) Toward a molecular understanding of cold activity of enzymes from psychrophiles. *Extremophiles*. **4**, 83-90.
- [44] Boyer, E.W. and Ingle, M.B. (1972) Extracellular alkaline amylase from a *Bacillus* sp. *Journal of Bacteriology*. **110**, 992-&.996.
- [45] Olsson, G., Larsson, L., Holst, O. and Karlsson, H.T. (1989) Microorganisms for desulfurization of coal: The influence of leaching compounds on their growth. *Fuel*. **68**, 1270-1274.
- [46] Sinnott, M.L. (1990) Catalytic mechanisms of enzymatic glycosyl transfer. *Chemical Reviews*. **90**, 1171-1202.
- [47] Henrissat, B. (1998) Glycosidase families. *Biochemical Society Transactions*. **26**, 153-156.
- [48] McCleary, B.V. and Matheson, N.K. (1986) Enzymatic analysis of polysaccharide structure. *Advances in Carbohydrate Chemistry and Biochemistry*. **44**, 147-276.
- [49] Henrissat, B. and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases. *Current Opinion in Structural Biology*. **7**, 637-644.
- [50] Hreggvidsson, G.O., Kaiste, E., Holst, O., Eggertsson, G., Palsdottir, A. and Kristjansson, J.K. (1996) An extremely thermostable cellulase from the thermophilic eubacterium *Rhodothermus marinus*. *Applied and Environmental Microbiology*. **62**, 3047-3049.
- [51] Alfredsson, G.A., Kristjansson, J.K., Hjorleifsdottir, S. and Stetter, K.O. (1988) *Rhodothermus marinus*, gen-nov, sp-nov, a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *Journal of General Microbiology*. **134**, 299-306.
- [52] Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., Nikitin, D., Osipov, G. and Laurinavichius, K. (2000) *Anoxybacillus pushchinensis* gen-nov, sp-nov, a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*. **50**, 2109-2117.
- [53] D'Souza, D.R., Morgan, R.D., Parashar, V., Capalash, N. and Sharma, P. (2004) Characterization of B $\beta$ /I - a thermostable, Co<sup>++</sup>-requiring isoschizomer of BsYI from *Anoxybacillus flavithermus*. *World Journal of Microbiology & Biotechnology*. **20**, 593-598.
- [54] Turner, P., Holst, O. and Karlsson, E.N. (2005) Optimized expression of soluble cyclomaltodextrinase of thermophilic origin in *Escherichia coli* by using a soluble fusion-tag and by tuning of inducer concentration. *Protein Expression and Purification*. **39**, 54-60.
- [55] Joseleau, J.P. and Chambat, G. (1984) Structure of the primary-cell walls of suspension-cultured *rosa-glauca* cells 2. Multiple forms of xyloglucans. *Plant Physiology*. **74**, 694-700.
- [56] Biely, P., Vrsanska, M., Tenkanen, M. and Kluepfel, D. (1997) Endo-beta-1,4-xylanase families: differences in catalytic properties. *Journal of Biotechnology*. **57**, 151-166.

- [57] Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. (1988) Multiplicity of Beta-1,4-xylanase in microorganisms - Functions and Applications. *Microbiological Reviews*. **52**, 305-317.
- [58] Davies, G. and Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases. *Structure*. **3**, 853-859.
- [59] Karlsson, E.N., Bartonek-Roxa, E. and Holst, O. (1997) Cloning and sequence of a thermostable multidomain xylanase from the bacterium *Rhodothermus marinus*. *Biochimica et Biophysica Acta-Gene Structure and Expression*. **1353**, 118-124.
- [60] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. (1991) Domains in microbial beta-1,4-glycanases - Sequence conservation, Function, and Enzyme families. *Microbiological Reviews*. **55**, 303-315.
- [61] Karlsson, E.N., Dahlberg, L., Torto, N., Gorton, L. and Holst, O. (1998) Enzymatic specificity and hydrolysis pattern of the catalytic domain of the xylanase *Xyn1* from *Rhodothermus marinus*. *Journal of Biotechnology*. **60**, 23-35.
- [62] Karlsson, E.N., Holst, O. and Tocaj, A. (1999) Efficient production of truncated thermostable xylanases from *Rhodothermus marinus* in *Escherichia coli* fed-batch cultures. *Journal of Bioscience and Bioengineering*. **87**, 598-606.
- [63] Abou Hachem, M., Karlsson, E.N., Bartonek-Roxa, E., Raghothama, S., Simpson, P.J., Gilbert, H.J., Williamson, M.P. and Holst, O. (2000) Carbohydrate-binding modules from a thermostable *Rhodothermus marinus* xylanase: cloning, expression and binding studies. *Biochemical Journal*. **345**, 53-60.
- [64] Abou-Hachem, M., Olsson, F. and Karlsson, E.N. (2003) Probing the stability of the modular family 10 xylanase from *Rhodothermus marinus*. *Extremophiles*. **7**, 483-491.
- [65] Simpson, P.J., Jamieson, S.J., Abou-Hachem, M., Karlsson, E.N., Gilbert, H.J., Holst, O. and Williamson, M.P. (2002) The solution structure of the CBM4-2 carbohydrate binding module from a thermostable *Rhodothermus marinus* xylanase. *Biochemistry*. **41**, 5712-5719.
- [66] Pfabigan, N., Karlsson, E.N., Ditzelmueller, G. and Holst, O. (2002) Prebleaching of kraft pulp with full-length and truncated forms of a thermostable modular xylanase from *Rhodothermus marinus*. *Biotechnology Letters*. **24**, 1191-1197.
- [67] Bajpai, P. (1999) Application of enzymes in the pulp and paper industry. *Biotechnology Progress*. **15**, 147-157.
- [68] Nagarathnamma, R. and Bajpai, P. (1999) Decolorization and detoxification of extraction-stage effluent from chlorine bleaching of kraft pulp by *Rhizopus oryzae*. *Applied and Environmental Microbiology*. **65**, 1078-1082.
- [69] Beg, Q.K., Kapoor, M., Mahajan, L. and Hoondal, G.S. (2001) Microbial xylanases and their industrial applications. *Applied Microbiology and Biotechnology*. **56**, 326-338.
- [70] Puchart, V., Katapodis, P., Biely, P., Kremnický, L., Christakopoulos, P., Vrsanska, M., Kekos, D., Macris, B.J. and Bhat, M.K. (1999) Production of xylanases, mannanases, and pectinases by the thermophilic fungus *Thermomyces lanuginosus*. *Enzyme and Microbial Technology*. **24**, 355-361.
- [71] Kuhad, R.C. and Singh, A. (1993) Lignocellulose biotechnology - Current and future prospects. *Critical Reviews in Biotechnology*. **13**, 151-172.
- [72] Marchessault, R. H., Coulombe, S., Morikawa, H. and Robert, D. (1982) Characterization of Aspen exploded wood lignin. *Canadian Journal of Chemistry*. **60**, 2372-2382.
- [73] Shen, H. et al. (1995) *Cellulomonas fimi* cellobiohydrolases in: Enzymatic Degradation of Insoluble Carbohydrates **Vol. 618**, pp. 174-196.
- [74] Gilbert, H.J. and Hazlewood, G.P. (1993) Bacterial cellulases and xylanases. *Journal of General Microbiology*. **139**, 187-194.

- [75] Halldorsdottir, S., Thorolfsdottir, E.T., Spilliaert, R., Johansson, M., Thorbjarnardottir, S.H., Palsdottir, A., Hreggvidsson, G.O., Kristjansson, J.K., Holst, O. and Eggertsson, G. (1998) Cloning, sequencing and overexpression of a *Rhodothermus marinus* gene encoding a thermostable cellulase of glycosyl hydrolase family 12. *Applied Microbiology and Biotechnology*. **49**, 277-284.
- [76] Wicher, K.B., Abou-Hachem, M., Halldorsdottir, S., Thorbjarnardottir, S.H., Eggertsson, G., Hreggvidsson, G.O., Karlsson, E.N. and Holst, O. (2001) Deletion of a cytotoxic, N-terminal putative signal peptide results in a significant increase in production yields in *Escherichia coli* and improved specific activity of Cel12A from *Rhodothermus marinus*. *Applied Microbiology and Biotechnology*. **55**, 578-584.
- [77] Crennell, S.J., Hreggvidsson, G.O. and Karlsson, E.N. (2002) The structure of *Rhodothermus marinus* Cel12A, a highly thermostable family 12 endoglucanase, at 1.8 angstrom resolution. *Journal of Molecular Biology*. **320**, 883-897.
- [78] Allen, W.G. (1976) Potential applications for cellulase enzymes. *Biotechnology and Bioengineering*. 303-305.
- [79] Park, K.-H., Kim, T.-J., Cheong, T.-K., Kim, J.-W., Oh, B.-H. and Svensson, B. (2000) Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the  $\alpha$ -amylase family. *Biochimica et Biophysica Acta- Protein Structure and Molecular Enzymology*. **1478**, 165-185.
- [80] Lee, H.S., Kim, M.S., Cho, H.S., Kim, J.I., Kim, T.J., Choi, J.H., Park, C., Oh, B.H. and Park, K.H. (2002) Cyclomaltodextrinase, Neopullulanase, and Maltogenic Amylase are nearly indistinguishable from each other. *Journal of Biological Chemistry*. **277**, 21891-21897.
- [81] Bender, H. (1985) Studies of the inhibition by malto-oligosaccharides of the cyclization reaction catalyzed by the cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* M5 with glycogen. *Carbohydrate Research*. **135**, 291-302.
- [82] Bender, H., Siebert, R. and Stadlerszoke, A. (1982) Can cyclodextrin glycosyltransferase be useful for the investigation of the fine-structure of amylopectins, questionable characterization of highly branched clusters isolated from digests with potato and maize starches. *Carbohydrate Research*. **110**, 245-259.
- [83] Mori, S., Goto, M., Mase, T., Matsuura, A., Oya, T. and Kitahata, S. (1995) Reaction conditions for the production of gamma-cyclodextrin by cyclodextrin glucanotransferase from *Brevibacterium* sp no. 9605. *Bioscience Biotechnology and Biochemistry*. **59**, 1012-1015.
- [84] Depinto, J.A. and Campbell, L.L. (1968) Purification and properties of cyclodextrinase of *Bacillus macerans*. *Biochemistry*. **7**, 121-126.
- [85] Kitahata, S., Taniguchi, M., Beltran, S.D., Sugimoto, T. and Okada, S. (1983) Purification and some properties of cyclodextrinase from *Bacillus coagulans*. *Agricultural and Biological Chemistry*. **47**, 1441-1447.
- [86] Oguma, T., Kikuchi, M. and Mizusawa, K. (1991) Some culture conditions for the production of cyclodextrin-hydrolyzing enzymes from *Bacillus sphaericus*. *Agricultural and Biological Chemistry*. **55**, 1661-1662.
- [87] Oguma, T., Matsuyama, A., Kikuchi, M. and Nakano, E. (1993) Cloning and sequence analysis of the cyclomaltodextrinase gene from *Bacillus sphaericus* and expression in *Escherichia coli* cells. *Applied Microbiology and Biotechnology*. **39**, 197-203.
- [88] Kim, T.J. et al. (1998) Analysis of the gene encoding cyclomaltodextrinase from alkalophilic *Bacillus* sp. I5 and characterization of enzymatic properties. *Archives of Biochemistry and Biophysics*. **353**, 221-227.

- [89] Galvin, N.M., Kelly, C.T. and Fogarty, W.M. (1994) Purification and properties of the cyclodextrinase of *Bacillus sphaericus* ATCC 7055. *Applied Microbiology and Biotechnology*. **42**, 46-50.
- [90] Svensson, B. (1994) Protein engineering in the alpha-amylase family - Catalytic mechanism, Substrate-specificity, and Stability. *Plant Molecular Biology*. **25**, 141-157.
- [91] Henrissat, B. (1991) A classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochemical Journal*. **280**, 309-316.
- [92] Grant, W.D. (1987) The enigma of the Alkaliphile. *Microbiological Sciences*. **4**, 251-255.
- [93] Jones, B.E., Grant, W.D., Duckworth, A.W. and Owenson, G.G. (1998) Microbial diversity of soda lakes. *Extremophiles*. **2**, 191-200.
- [94] Martins, R.F., Davids, W., Abu Al-Soud, W., Levander, F., Radstrom, P. and Hatti-Kaul, R. (2001) Starch-hydrolyzing bacteria from Ethiopian soda lakes. *Extremophiles*. **5**, 135-144.
- [95] Gessesse, A. and Gashe, B.A. (1997) Production of alkaline protease by an alkaliphilic bacteria isolated from an alkaline soda lake. *Biotechnology Letters*. **19**, 479-481.
- [96] Kurono, Y. and Horikosh.K (1973) Production of enzymes by alkalophilic microorganisms. Alkaline catalase produced by *Bacillus* No KU-1. *Agricultural and Biological Chemistry*. **37**, 2565-2570.
- [97] Ikura, Y. and Horikoshi, K. (1979) Isolation and some properties of beta-galactosidase producing bacteria. *Agricultural and Biological Chemistry*. **43**, 85-88.
- [98] Hashim, S.O., Delgado, O., Hatti-Kaul, R., Mulaa, F.J. and Mattiasson, B. (2004) Starch hydrolysing *Bacillus halodurans* isolates from a Kenyan soda lake. *Biotechnology Letters*. **26**, 823-828.
- [99] Takami, H. and Horikoshi, K. (2000) Analysis of the genome of an alkaliphilic *Bacillus* strain from an industrial point of view. *Extremophiles*. **4**, 99-108.
- [100] Vargas, V.A., Delgado, O.D., Hatti-Kaul, R. and Mattiasson, B. (2004) Lipase-producing microorganisms from a Kenyan alkaline soda lake. *Biotechnology Letters*. **26**, 81-86.
- [101] Jaeger, K.E. and Reetz, M.T. (1998) Microbial lipases form versatile tools for biotechnology. *Trends in Biotechnology*. **16**, 396-403.
- [102] Gilbert, E.J. (1993) *Pseudomonas* Lipases: Biochemical properties and Molecular cloning. *Enzyme and Microbial Technology*. **15**, 634-645.
- [103] Svendsen, A., Borch, K., Barfoed, M., Nielsen, T.B., Gormsen, E. and Patkar, S.A. (1995) Biochemical properties of cloned lipases from the *Pseudomonas* family. *Biochimica et Biophysica Acta-Lipids and Lipid Metabolism*. **1259**, 9-17.
- [104] Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., Vanheuver, M. and Misset, O. (1994) Bacterial lipases. *Fems Microbiology Reviews*. **15**, 29-63.
- [105] Gotz, F., Verheij, H.M. and Rosenstein, R. (1998) *Staphylococcal* lipases: molecular characterisation, secretion, and processing. *Chemistry and Physics of Lipids*. **93**, 15-25.
- [106] Rogalska, E., Cudrey, C., Ferrato, F. and Verger, R. (1993) Stereoselective hydrolysis of triglycerides by animal and microbial lipases. *Chirality*. **5**, 24-30.
- [107] Chapus, C., Rovey, M., Sarda, L. and Verger, R. (1988) Minireview on pancreatic lipase and colipase. *Biochimie*. **70**, 1223-1234.
- [108] Shatzman, A.R. and Rosenberg, M. (1987) Expression, Identification, and Characterization of recombinant gene products in *Escherichia coli*. *Methods in Enzymology*. **152**, 661-673.

- [109] Casadaban, M.J., Martinezarias, A., Shapira, S.K. and Chou, J. (1983) Beta-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and Yeast. *Methods in Enzymology*. **100**, 293-308.
- [110] Hitzeman, R.A., Hagie, F.E., Levine, H.L., Goeddel, D.V., Ammerer, G. and Hall, B.D. (1981) Expression of a human gene for interferon in Yeast. *Nature*. **293**, 717-722.
- [111] Andersen, D.C. and Krummen, L. (2002) Recombinant protein expression for therapeutic applications. *Current Opinion in Biotechnology*. **13**, 117-123.
- [112] Georgiou, G. and Valax, P. (1996) Expression of correctly folded proteins in *Escherichia coli*. *Current Opinion in Biotechnology*. **7**, 190-197.
- [113] Hodgson, J. (1993) Expression Systems: A users guide. *Bio-Technology*. **11**, 887-893.
- [114] Yansura, D.G. and Henner, D.J. (1990) Use of *Escherichia coli* Trp-promoter for direct expression of proteins. *Methods in Enzymology*. **185**, 54-60.
- [115] Hannig, G. and Makrides, S.C. (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends in Biotechnology*. **16**, 54-60.
- [116] Backman, K., Ptashne, M. and Gilbert, W. (1976) Construction of plasmids carrying *Ci* gene of bacteriophage lambda. *Proceedings of the National Academy of Sciences of the United States of America*. **73**, 4174-4178.
- [117] Yanischperron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and Puc19 vectors. *Gene*. **33**, 103-119.
- [118] Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology*. **10**, 411-421.
- [119] Wilms, B., Hauck, A., Reuss, M., Syltatk, C., Mattes, R., Siemann, M. and Altenbuchner, J. (2001) High cell density fermentation for production of L-N-carbamoylase using an expression system based on the *Escherichia coli* rhaBAD promoter. *Biotechnology and Bioengineering*. **73**, 95-103.
- [120] Han, S.J., Chang, H.N. and Lee, J. (2001) Characterization of an oxygen-dependent inducible promoter, the *nar* promoter of *Escherichia coli*, to utilize in metabolic engineering. *Biotechnology and Bioengineering*. **72**, 573-576.
- [121] Khlebnikov, A., Risa, O., Skaug, T., Carrier, T. and Keasling, J.D. (2000) Regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture. *Journal of Bacteriology*. **182**, 7029-7034.
- [122] Schein, C.H. (1989) Production of soluble recombinant proteins in bacteria. *Bio-Technology*. **7**, 1141-1147.
- [123] Dale, G.E., Broger, C., Langen, H., Darcy, A. and Stuber, D. (1994) Improving protein solubility through rationally designed amino-acid replacements-Solubilization of the trimethoprim resistant Type S1 dihydrofolate reductase. *Protein Engineering*. **7**, 933-939.
- [124] Izard, J., Parker, M.W., Chartier, M., Duche, D. and Baty, D. (1994) A single amino-acid substitution can restore the solubility of aggregated Colicin-A mutants in *Escherichia coli*. *Protein Engineering*. **7**, 1495-1500.
- [125] Murby, M., Samuelsson, E., Nguyen, T.N., Mignard, L., Power, U., Binz, H., Uhlen, M. and Stahl, S. (1995) Hydrophobicity engineering to increase solubility and stability of a recombinant protein from respiratory syncytial virus. *European Journal of Biochemistry*. **230**, 38-44.
- [126] Wall, J.G. and Pluckthun, A. (1995) Effects of Overexpressing folding modulators on the in vivo folding of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology*. **6**, 507-516.



- [127] Yasukawa, T., Kaneiishi, C., Maekawa, T., Fujimoto, J., Yamamoto, T. and Ishii, S. (1995) Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. *Journal of Biological Chemistry*. **270**, 25328-25331.
- [128] Lavallie, E.R., Diblasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F. and McCoy, J.M. (1993) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *Escherichia coli* cytoplasm. *Bio-Technology*. **11**, 187-193.
- [129] Nygren, P.A., Stahl, S. and Uhlen, M. (1994) Engineering proteins to facilitate bioprocessing. *Trends in Biotechnology*. **12**, 184-188.
- [130] Wilkinson, D.L., Ma, N.T., Haught, C. and Harrison, R.G. (1995) Purification by immobilized metal affinity chromatography of human atrial natriuretic peptide expressed in a novel thioredoxin fusion protein. *Biotechnology Progress*. **11**, 265-269.
- [131] Moore, J.T., Uppal, A., Maley, F. and Maley, G.F. (1993) Overcoming inclusion body formation in a high level expression system. *Protein Expression and Purification*. **4**, 160-163.
- [132] Bowden, G.A. and Georgiou, G. (1988) The effect of sugars on beta-lactamase aggregation in *Escherichia coli*. *Biotechnology Progress*. **4**, 97-101.
- [133] Baneyx, F. and Georgiou, G. (1992) Degradation of secreted proteins in *Escherichia coli*. *Annals of the New York Academy of Sciences*. **665**, 301-308.
- [134] Hellebust, H., Murby, M., Abrahmsen, L., Uhlen, M. and Enfors, S.O. (1989) Different approaches to stabilize a recombinant fusion protein. *Bio-Technology*. **7**, 165-168.
- [135] Murby, M., Uhlen, M. and Stahl, S. (1996) Upstream strategies to minimize proteolytic degradation upon recombinant production in *Escherichia coli*. *Protein Expression and Purification*. **7**, 129-136.
- [136] Chesshyre, J.A. and Hipkiss, A.R. (1989) Low-temperatures stabilize interferon-alpha-2 against proteolysis in *Methylophilus methylotrophus* and *Escherichia coli*. *Applied Microbiology and Biotechnology*. **31**, 158-162.
- [137] Lee, S.C. and Olins, P.O. (1992) Effect of overproduction of heat-shock chaperones GroESL and DnaK on human procollagenase production in *Escherichia coli*. *Journal of Biological Chemistry*. **267**, 2849-2852.
- [138] Sugimoto, S., Yokoo, Y., Hatakeyama, N., Yotsuji, A., Teshiba, S. and Hagino, H. (1991) Higher culture Ph is preferable for inclusion body formation of recombinant salmon growth-hormone in *Escherichia coli*. *Biotechnology Letters*. **13**, 385-388.
- [139] Gottesman, S. (1990) Minimizing proteolysis in *Escherichia coli* - Genetic Solutions. *Methods in Enzymology*. **185**, 119-129.
- [140] Vandijl, J.M., Dejong, A., Smith, H., Bron, S. and Venema, G. (1991) Signal peptidase-I overproduction results in increased efficiencies of export and maturation of hybrid secretory proteins in *Escherichia coli*. *Molecular & General Genetics*. **227**, 40-48.
- [141] Grisshammer, R., Duckworth, R. and Henderson, R. (1993) Expression of a rat neurotensin receptor in *Escherichia coli*. *Biochemical Journal*. **295**, 571-576.
- [142] Knappik, A. and Pluckthun, A. (1995) Engineered turns of a recombinant antibody improve its *In vivo* folding. *Protein Engineering*. **8**, 81-89.
- [143] Humphreys, D.P., Weir, N., Mountain, A. and Lund, P.A. (1995) Human protein disulfide-isomerase functionally complements a dsbA mutation and enhances the yield of pectate-lyase-C in *Escherichia coli*. *Journal of Biological Chemistry*. **270**, 28210-28215.

- [144] Ostermeier, M., DeSutter, K. and Georgiou, G. (1996) Eukaryotic protein disulfide isomerase complements *Escherichia coli* dsbA mutants and increases the yield of a heterologous secreted protein with disulfide bonds. *Journal of Biological Chemistry*. **271**, 10616-10622.
- [145] Goldberg, A.L., Howell, E.M., Li, J.B., Martel, S.B. and Prouty, W.F. (1974) Physiological significance of protein degradation in animal and bacterial cells. *Federation Proceedings*. **33**, 1112-1120.
- [146] Goldberg, A.L. and Dice, J.F. (1974) Intracellular protein degradation in mammalian and bacterial cells. *Annual Review of Biochemistry*. **43**, 835-869.
- [147] Goff, S.A., Casson, L.P. and Goldberg, A.L. (1984) Heat-shock regulatory gene *HtpR* influences rates of protein degradation and expression of the ion gene in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*. **81**, 6647-6651.
- [148] Goff, S.A. and Goldberg, A.L. (1985) Production of abnormal proteins in *Escherichia coli* stimulates transcription of ion and other heat-shock genes. *Cell*. **41**, 587-595.
- [149] Gottesman, S., Gottesman, M., Shaw, J.E. and Pearson, M.L. (1981) Protein degradation in *Escherichia coli* - the ion mutation and bacteriophage-N lambda-N and Cii protein stability. *Cell*. **24**, 225-233.
- [150] Stout, V., Torrescabassa, A., Maurizi, M.R., Gutnick, D. and Gottesman, S. (1991) RcsA, an unstable positive regulator of capsular polysaccharide synthesis. *Journal of Bacteriology*. **173**, 1738-1747.
- [151] Torrescabassa, A.S. and Gottesman, S. (1987) Capsule synthesis in *Escherichia coli* K 12 is regulated by proteolysis. *Journal of Bacteriology*. **169**, 981-989.
- [152] Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annual Review of Genetics*. **30**, 465-506.
- [153] Kanatani, A., Masuda, T., Shimoda, T., Misoka, F., Lin, X.S., Yoshimoto, T. and Tsuru, D. (1991) Protease Ii from *Escherichia coli* - sequencing and expression of the enzyme gene and characterization of the expressed enzyme. *Journal of Biochemistry*. **110**, 315-320.
- [154] Kanatani, A., Yoshimoto, T., Nagai, H., Ito, K. and Tsuru, D. (1992) Location of the Protease Ii gene on the physical map of the *Escherichia coli* chromosome. *Journal of Bacteriology*. **174**, 7881-7881.
- [155] Pacaud, M. and Richaud, C. (1975) Protease Ii from *Escherichia coli* - Purification and characterization. *Journal of Biological Chemistry*. **250**, 7771-7779.
- [156] Kims, K.I., Baek, S.H., Hong, Y.M., Kang, M.S., Ha, D.B., Goldberg, A.L. and Chung, C.H. (1995) Purification and characterization of protease-Ci, a cytoplasmic metalloendoprotease in *Escherichia coli*. *Journal of Biological Chemistry*. **270**, 29799-29805.
- [157] Goldberg, A.L., Voellmy, R., Chung, C.H., Larimore, F.S. and Swamy, K.H.S. (1980) Regulation and mechanisms of protein degradation in *Escherichia coli*. *Federation Proceedings*. **39**, R7-R7.
- [158] Little, J.W., Edmiston, S.H., Pacelli, L.Z. and Mount, D.W. (1980) Cleavage of the *Escherichia coli* LexA protein by the RecA protease. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*. **77**, 3225-3229.
- [159] Roberts, J.W. and Roberts, C.W. (1975) Proteolytic cleavage of bacteriophage lambda repressor in induction. *Proceedings of the National Academy of Sciences of the United States of America*. **72**, 147-151.
- [160] Roberts, J.W., Roberts, C.W. and Mount, D.W. (1977) Inactivation and proteolytic cleavage of phage lambda repressor invitro in an ATP-dependent reaction.

- Proceedings of the National Academy of Sciences of the United States of America.* **74**, 2283-2287.
- [161] Roberts, J.W., Roberts, C.W. and Craig, N.L. (1978) *Escherichia coli* RecA gene product inactivates phage lambda-repressor. *Proceedings of the National Academy of Sciences of the United States of America.* **75**, 4714-4718.
- [162] Chung, C.H. and Goldberg, A.L. (1983) Purification and characterization of protease So, a cytoplasmic serine protease in *Escherichia coli*. *Journal of Bacteriology.* **154**, 231-238.
- [163] Lee, Y.S., Park, S.C., Goldberg, A.L. and Chung, C.H. (1988) Protease So from *Escherichia coli* preferentially degrades oxidatively damaged glutamine-synthetase. *Journal of Biological Chemistry.* **263**, 6643-6646.
- [164] Maurizi, M.R., Clark, W.P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. and Gottesman, S. (1990) Sequence and structure of Clp P, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli*. *Journal of Biological Chemistry.* **265**, 12536-12545.
- [165] Erickson, J.W. and Gross, C.A. (1989) Identification of the sigma E subunit of *Escherichia coli* RNA-polymerase - a 2nd alternate sigma-factor involved in high temperature gene expression. *Genes & Development.* **3**, 1462-1471.
- [166] Lipinska, B., Fayet, O., Baird, L. and Georgopoulos, C. (1989) Identification, characterization, and mapping of the *Escherichia coli* *htrA* gene, whose product is essential for bacterial growth only at elevated temperatures. *Journal of Bacteriology.* **171**, 1574-1584.
- [167] Strauch, K.L. and Beckwith, J. (1988) An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. *Proceedings of the National Academy of Sciences of the United States of America.* **85**, 1576-1580.
- [168] Ichihara, S., Suzuki, T., Suzuki, M. and Mizushima, S. (1986) Molecular cloning and sequencing of the *Sppa* gene and characterization of the encoded protease-IV, a signal peptide peptidase, of *Escherichia coli*. *Journal of Biological Chemistry.* **261**, 9405-9411.
- [169] Suzuki, T., Itoh, A., Ichihara, S. and Mizushima, S. (1987) Characterization of the *Sppa* gene coding for protease-IV, a signal peptide peptidase of *Escherichia coli*. *Journal of Bacteriology.* **169**, 2523-2528.
- [170] Henderson, T.A., Dombrosky, P.M. and Young, K.D. (1994) Artifactual processing of penicillin binding proteins 7 and 1B by the Ompt protease of *Escherichia coli*. *Journal of Bacteriology.* **176**, 256-259.
- [171] Manning, P.A. and Reeves, P. (1976) Properties of protein III- A major outer membrane protein in *Escherichia coli* K-12. *Proceedings of the Australian Biochemical Society.* **9**, 62-62.
- [172] Nakata, N., Tobe, T., Fukuda, I., Suzuki, T., Komatsu, K., Yoshikawa, M. and Sasakawa, C. (1993) The Absence of a surface protease, Ompt, determines the intercellular spreading ability of *Shigella* - The Relationship between the Ompt and Kcpa loci. *Molecular Microbiology.* **9**, 459-468.
- [173] Palmer, S.M. and St. John, A.C. (1987) Characterization of a membrane associated serine protease in *Escherichia coli*. *Journal of Bacteriology.* **169**, 1474-1479.
- [174] Strandberg, L. and Enfors, S.O. (1991) Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Applied and Environmental Microbiology.* **57**, 1669-1674.
- [175] Strandberg, L. and Enfors, S.O. (1991) Batch and fed-batch cultivations for the temperature induced production of a recombinant protein in *Escherichia coli*. *Biotechnology Letters.* **13**, 609-614.

- [176] Thomas, J.G. and Baneyx, F. (1996) Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells overexpressing heat-shock proteins. *Journal of Biological Chemistry*. **271**, 11141-11147.
- [177] Davis, G.D., Elisee, C., Newham, D.M. and Harrison, R.G. (1999) New fusion protein systems designed to give soluble expression in *Escherichia coli*. *Biotechnology and Bioengineering*. **65**, 382-388.
- [178] Abrahmsen, L., Moks, T., Nilsson, B. and Uhlen, M. (1986) Secretion of heterologous gene products to the culture medium of *Escherichia coli*. *Nucleic Acids Research*. **14**, 7487-7500.
- [179] Bardwell, J.C.A. (1994) Building bridges - Disulfide bond formation in the Cell. *Molecular Microbiology*. **14**, 199-205.
- [180] Hazeu, W., Bos, P. and Debruyne, J.C. (1972) Methanol assimilation by Yeasts. *Archiv Fur Mikrobiologie*. **87**, 185-188.
- [181] Tani, Y., Kato, N., Izumi, Y., Shimizu, S., Ogata, K. and Yamada, H. (1978) Microbial utilization of methanol. *Journal of the Society of Fermentation Technology*. **56**, 527-537.
- [182] Barnett, J.A. (1987) The Yeasts. *Nature*. **329**, 680-680.
- [183] Veenhuis, M., Vandijken, J.P. and Harder, W. (1983) The significance of peroxisomes in the metabolism of one-carbon compounds in Yeasts. *Advances in Microbial Physiology*. **24**, 1-82.
- [184] Egli, T., Vandijken, J.P., Veenhuis, M., Harder, W. and Fiechter, A. (1980) Methanol metabolism in Yeasts - Regulation of the synthesis of catabolic enzymes. *Archives of Microbiology*. **124**, 115-121.
- [185] Cregg, J.M., Vedvick, T.S. and Raschke, W.C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. *Bio-Technology*. **11**, 905-910.
- [186] Couderc, R. and Baratti, J. (1980) Oxidation of methanol by the Yeast, *Pichia pastoris* - Purification and properties of the alcohol oxidase. *Agricultural and Biological Chemistry*. **44**, 2279-2289.
- [187] Roggenkamp, R., Janowicz, Z., Stanikowski, B. and Hollenberg, C.P. (1984) Biosynthesis and regulation of the peroxisomal methanol oxidase from the methylotrophic Yeast *Hansenula polymorpha*. *Molecular & General Genetics*. **194**, 489-493.
- [188] Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A. and Gingeras, T.R. (1987) Expression of the *LacZ* gene from 2 methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Research*. **15**, 3859-3876.
- [189] Montesino, R., Garcia, R., Quintero, O. and Cremata, J.A. (1998) Variation in N-linked oligosaccharide structures on heterologous proteins secreted by the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification*. **14**, 197-207.
- [190] Despreaux, C.W. and Manning, R.F. (1993) The *Daca* gene of *Bacillus stearothermophilus* coding for D-alanine carboxypeptidase - Cloning, structure and expression in *Escherichia coli* and *Pichia pastoris*. *Gene*. **131**, 35-41.
- [191] Rodriguez, E., Han, Y.M. and Lei, X.G. (1999) Cloning, sequencing, and expression of an *Escherichia coli* acid phosphatase/phytase gene (*appA2*) isolated from pig colon. *Biochemical and Biophysical Research Communications*. **257**, 117-123.
- [192] Miele, R.G., Prorok, M., Costa, V.A. and Castellino, F.J. (1999) Glycosylation of asparagine-28 of recombinant staphylokinase with high-mannose-type oligosaccharides results in a protein with highly attenuated plasminogen activator activity. *Journal of Biological Chemistry*. **274**, 7769-7776.

- [193] Hagenson, M.J., Holden, K.A., Parker, K.A., Wood, P.J., Cruze, J.A., Fuke, M., Hopkins, T.R. and Stroman, D.W. (1989) Expression of streptokinase in *Pichia pastoris* yeast. *Enzyme and Microbial Technology*. **11**, 650-656.
- [194] Tschopp, J.F., Sverlow, G., Kosson, R., Craig, W. and Grinna, L. (1987) High level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. *Bio-Technology*. **5**, 1305-1308.
- [195] Chiruvolu, V., Cregg, J.M. and Meagher, M.M. (1997) Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fed-batch fermentations. *Enzyme and Microbial Technology*. **21**, 277-283.
- [196] Cereghino, G.P.L., Cereghino, J.L., Ilgen, C. and Cregg, J.M. (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Current Opinion in Biotechnology*. **13**, 329-332.
- [197] Brierley, R.A., Bussineau, C., Kosson, R., Melton, A. and Siegel, R.S. (1990) Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene - bovine lysozyme. *Annals of the New York Academy of Sciences*. **589**, 350-362.
- [198] White, C.E., Hunter, M.J., Meininger, D.P., White, L.R. and Komives, E.A. (1995) Large-scale expression, purification and characterization of small fragments of thrombomodulin: The roles of the sixth domain and of methionine 388. *Protein Engineering*. **8**, 1177-1187.
- [199] Gellissen, G. (2000) Heterologous protein production in methylotrophic yeasts. *Applied Microbiology and Biotechnology*. **54**, 741-750.
- [200] Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B. and Rasmussen, J.R. (1991) The oligosaccharides of glycoproteins - Bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Bio-Technology*. **9**, 1347-1355.
- [201] Heimo, H., Palmu, K. and Suominen, I. (1997) Expression in *Pichia pastoris* and purification of *Aspergillus awamori* glucoamylase catalytic domain. *Protein Expression and Purification*. **10**, 70-79.
- [202] Juge, N., Andersen, J.S., Tull, D., Roepstorff, P. and Svensson, B. (1996) Overexpression, purification, and characterization of recombinant barley alpha-amylases 1 and 2 secreted by the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification*. **8**, 204-214.
- [203] Krahe, M., Antranikian, G. and Markl, H. (1996) Fermentation of extremophilic microorganisms. *FEMS Microbiology Reviews*. **18**, 271-285.
- [204] Holst, O., Manelius, A., Krahe, M., Markl, H., Raven, N. and Sharp, R. (1997) Thermophiles and fermentation technology. *Comparative Biochemistry and Physiology*. **118A**, 415-422.
- [205] Nakano, K., Rischke, M., Sato, S. and Markl, H. (1997) Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor. *Applied Microbiology and Biotechnology*. **48**, 597-601.
- [206] 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.
- [207] Horn, U., Strittmatter, W., Krebber, A., Knupfer, U., Kujau, M., Wenderoth, R., Muller, K., Matzku, S., Pluckthun, A. and Riesenberg, D. (1996) High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high cell density fermentation under non-limited growth conditions. *Applied Microbiology and Biotechnology*. **46**, 524-532.

- [208] Park, Y.S., Kai, K., Iijima, S.J. and Kobayashi, T. (1992) Enhanced beta-galactosidase production by high cell density culture of recombinant *Bacillus subtilis* with glucose-concentration control. *Biotechnology and Bioengineering*. **40**, 686-696.
- [209] Suzuki, T., Yamane, T. and Shimizu, S. (1987) Mass production of thioestrepton by fed-batch culture of *Streptomyces laurentii* with Ph-stat modal feeding of multi-substrate. *Applied Microbiology and Biotechnology*. **25**, 526-531.
- [210] Kim, G.J., Lee, I.Y., Choi, D.K., Yoon, S.C. and Park, Y.H. (1996) High cell density cultivation of *Pseudomonas putida* BM01 using glucose. *Journal of Microbiology and Biotechnology*. **6**, 221-224.
- [211] Suzuki, T., Yamane, T. and Shimizu, S. (1986) Mass production of poly-beta-hydroxybutyric acid by fed-batch culture with controlled carbon nitrogen feeding. *Applied Microbiology and Biotechnology*. **24**, 370-374.
- [212] Yano, T., Kobayashi, T. and Shimizu, S. (1985) High concentration cultivation of *Candida brassicae* in a fed-batch system. *Journal of Fermentation Technology*. **63**, 415-418.
- [213] Suzuki, T., Kamoshita, Y. and Ohashi, R. (1997) A dense cell culture system for microorganisms using a shake flask incorporating a porous ceramic filter. *Journal of Fermentation and Bioengineering*. **84**, 133-137.
- [214] Pan, J.G., Rhee, J.S. and Lebeault, J.M. (1987) Physiological constraints in increasing biomass concentration of *Escherichia coli* B in fed-batch culture. *Biotechnology Letters*. **9**, 89-94.
- [215] Jensen, E.B. and Carlsen, S. (1990) Production of recombinant human growth-hormone in *Escherichia coli* - Expression of different precursors and physiological effects of glucose, acetate, and salts. *Biotechnology and Bioengineering*. **36**, 1-11.
- [216] Koh, B.T., Nakashimada, U., Pfeiffer, M. and Yap, M.G.S. (1992) Comparison of acetate inhibition on growth of host and recombinant *Escherichia coli* K12 strains. *Biotechnology Letters*. **14**, 1115-1118.
- [217] Shimizu, M., Iijima, S. and Kobayashi, T. (1992) Production of insecticidal protein of *Bacillus thuringiensis* by cultivation of recombinant *Escherichia coli*. *Journal of Fermentation and Bioengineering*. **74**, 163-168.
- [218] Macdonald, H.L. and Neway, J.O. (1990) Effects of medium quality on the expression of human interleukin-2 at high cell density in fermenter cultures of *Escherichia coli* K-12. *Applied and Environmental Microbiology*. **56**, 640-645.
- [219] Elmansi, E.M.T. and Holms, W.H. (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *Journal of General Microbiology*. **135**, 2875-2883.
- [220] Holms, W.H. (1986) Evolution of the glyoxylate bypass in *Escherichia coli* - An hypothesis which suggests an alternative to the Krebs Cycle. *FEMS Microbiology Letters*. **34**, 123-127.
- [221] Varma, A. and Palsson, B.O. (1994) Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type *Escherichia coli* W3110. *Applied and Environmental Microbiology*. **60**, 3724-3731.
- [222] Kleman, G.L. and Strohl, W.R. (1994) Acetate metabolism by *Escherichia coli* in high cell density fermentation. *Applied and Environmental Microbiology*. **60**, 3952-3958.
- [223] Paalme, T., Elken, R., Kahru, A., Vanatalu, K. and Vilu, R. (1997) The growth rate control in *Escherichia coli* at near to maximum growth rates: The A-stat approach. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*. **71**, 217-230.
- [224] Ko, Y.F., Bentley, W.E. and Weigand, W.A. (1993) An integrated metabolic modeling approach to describe the energy efficiency of *Escherichia coli* fermentations

- under oxygen-limited conditions - Cellular energetics, Carbon flux, and Acetate production. *Biotechnology and Bioengineering*. **42**, 843-853.
- [225] Varma, A., Boesch, B.W. and Palsson, B.O. (1993) Stoichiometric interpretation of *Escherichia coli* glucose catabolism under various oxygenation rates. *Applied and Environmental Microbiology*. **59**, 2465-2473.
- [226] Reiling, H.E., Laurila, H. and Fiechter, A. (1985) Mass culture of *Escherichia coli* - medium development for low and high density cultivation of *Escherichia coli* B/R in minimal and complex media. *Journal of Biotechnology*. **2**, 191-206.
- [227] Lee, J.H., Choi, Y.H., Kang, S.K., Park, H.H. and Kwon, I.B. (1989) Production of human-leukocyte interferon in *Escherichia coli* by control of growth rate in fed-batch fermentation. *Biotechnology Letters*. **11**, 695-698.
- [228] Lee, Y.L. and Chang, H.N. (1990) High cell density culture of a recombinant *Escherichia coli* producing penicillin acylase in a membrane cell recycle fermenter. *Biotechnology and Bioengineering*. **36**, 330-337.
- [229] Ohta, K., Shibui, T., Morimoto, Y., Iijima, S. and Kobayashi, T. (1993) High level production of human Proapo A-I by fed-batch culture of recombinant *Escherichia coli*. *Journal of Fermentation and Bioengineering*. **75**, 155-157.
- [230] Matsui, T., Yokota, H., Sato, S., Mukataka, S. and Takahashi, J. (1989) Pressurized culture of *Escherichia coli* for a high concentration. *Agricultural and Biological Chemistry*. **53**, 2115-2120.
- [231] Chou, C.H., Bennett, G.N. and San, K.Y. (1994) Effect of modified glucose-uptake using genetic engineering techniques on high level recombinant protein production in *Escherichia coli* dense cultures. *Biotechnology and Bioengineering*. **44**, 952-960.
- [232] Khosla, C., Curtis, J.E., Demodena, J., Rinas, U. and Bailey, J.E. (1990) Expression of intracellular hemoglobin improves protein synthesis in oxygen-limited *Escherichia coli*. *Bio-Technology*. **8**, 849-853.
- [233] Bauer, K.A., Benbassat, A., Dawson, M., Delapuepte, V.T. and Neway, J.O. (1990) Improved expression of human interleukin-2 in high cell density fermenter cultures of *Escherichia coli* K-12 by a phosphotransacetylase mutant. *Applied and Environmental Microbiology*. **56**, 1296-1302.
- [234] Ramirez, D.M. and Bentley, W.E. (1995) Fed-batch feeding and induction policies that improve foreign protein-synthesis and stability by avoiding stress responses. *Biotechnology and Bioengineering*. **47**, 596-608.
- [235] Cserjan-Puschmann, M., Kramer, W., Duerrschmid, E., Striedner, G. and Bayer, K. (1999) Metabolic approaches for the optimisation of recombinant fermentation processes. *Applied Microbiology and Biotechnology*. **53**, 43-50.
- [236] Nystrom, T. (2004) Growth versus maintenance: a trade-off dictated by RNA polymerase availability and sigma factor competition. *Molecular Microbiology*. **54**, 855-862.
- [237] Harcum, S.W. and Bentley, W.E. (1993) Response Dynamics of 26-Kda, 34-Kda, 39-Kda, 54-Kda, and 80-Kda proteases in induced cultures of recombinant *Escherichia coli*. *Biotechnology and Bioengineering*. **42**, 675-685.
- [238] Meszaros, A. and Bales, V. (1992) A Contribution to optimal control of fed-batch biochemical processes. *Bioprocess Engineering*. **7**, 363-367.
- [239] Riesenber, D. and Guthke, R. (1999) High cell density cultivation of microorganisms. *Applied Microbiology and Biotechnology*. **51**, 422-430.
- [240] Akesson, M., Karlsson, E.N., Hagander, P., Axelsson, J.P. and Tocaj, A. (1999) On-line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. *Biotechnology and Bioengineering*. **64**, 590-598.

- [241] Akesson, M., Hagander, P. and Axelsson, J.P. (2001) Probing control of fed-batch cultivations: analysis and tuning. *Control Engineering Practice*. **9**, 709-723.
- [242] Akesson, M., Hagander, P. and Axelsson, J.P. (2001) Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. *Biotechnology and Bioengineering*. **73**, 223-230.
- [243] Svensson, M., Han, L., Silfversparre, G., Häggström, L., and Enfors, S-O. (2005) Control of endotoxin release in *E. coli* fed-batch cultures. *Bioproc Biosyst Eng.* **(in press)**.
- [244] Chen, Y.L., Cino, J., Hart, G., Freedman, D., White, C. and Komives, E.A. (1997) High protein expression in fermentation of recombinant *Pichia pastoris* by a fed-batch process. *Process Biochemistry*. **32**, 107-111.
- [245] Chen, Y.L., Krol, J., Cino, J., Freedman, D., White, C. and Komives, E. (1996) Continuous production of thrombomodulin from a *Pichia pastoris* is fermentation. *Journal of Chemical Technology and Biotechnology*. **67**, 143-148.
- [246] Schneider, G., Strehaiano, P. and Taillandier, P. (2001) Improvement of a fed-batch process for high level xylanase production by a *Bacillus* strain. *Journal of Chemical Technology and Biotechnology*. **76**, 456-460.
- [247] Ohman, L., Ljunggren, J. and Haggstrom, L. (1995) Induction of a metabolic switch in insect cells by substrate limited fed-batch cultures. *Applied Microbiology and Biotechnology*. **43**, 1006-1013.
- [248] Ljunggren, J. and Haggstrom, L. (1995) Specific growth rate as a parameter for tracing growth limiting substances in animal cell cultures. *Journal of Biotechnology*. **42**, 163-175.
- [249] Ljunggren, J. and Haggstrom, L. (1994) Catabolic control of hybridoma cells by glucose and glutamine limited fed-batch cultures. *Biotechnology and Bioengineering*. **44**, 808-818.
- [250] Hong, F., Meinander, N.Q. and Jonsson, L.J. (2002) Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnology and Bioengineering*. **79**, 438-449.
- [251] Thorpe, E.D., d'Anjou, M.C. and Daugulis, A.J. (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnology Letters*. **21**, 669-672.