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Ramchuran, Santosh

2005

LUND UNIVERSITY

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

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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 offentligen försvaras torsdagen den 7 April 2005, kl 10:30 i hörsal A, på Kemicentrum, 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

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

Organization Lund University Department of Biotechnology	**Document name** DOCTORAL DISSERTATION
	Date of issue April 2005
	CODEN ISRN LUTKDH/TKBT--05/1082--SE
Author(s) Santosh Omrajah Ramchuran	**Sponsoring organization**

Title and subtitle

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Abstract

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 *Rhodothermus marinus*, cyclomaltodextrinase from *Anoxybacillus flavithermus* and a phospholipase from alkaliphilic *Bacillus halodurans*. Batch cultivation of *R. marinus* 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 *Escherichia coli* and *Pichia pastoris* 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 *E. coli* as a host. In addition, extracellular production of thermostable xylanase and alkaliphilic phospholipase using the methylotrophic yeast *P. pastoris* as a host is reported.

Key words

Heterologous protein, Glycoside hydrolase, High cell density cultivation, Expression systems, Fed-batch

Classification system and/or index terms (if any)

Supplementary bibliographcal information		**Language** English
ISSN and key title		**ISBN** 91-89627-29-6
Recipient´s notes	**Number of pages**	**Price**
	Security classification	

Distribution by (name and address)

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

Abbreviations

HCD	High cell density
PO_2	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
R. marinus	*Rhodothermus marinus*
E. coli	*Escherichia coli*
P. pastoris	*Pichia pastoris*
GRAS	Generally Regarded As Safe
ppGpp	Guanosine tetraphosphate
OM	Outer membrane
TCA	Tricarboxylic Acid
DSP	Down Stream Processing

Table of Contents

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)

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

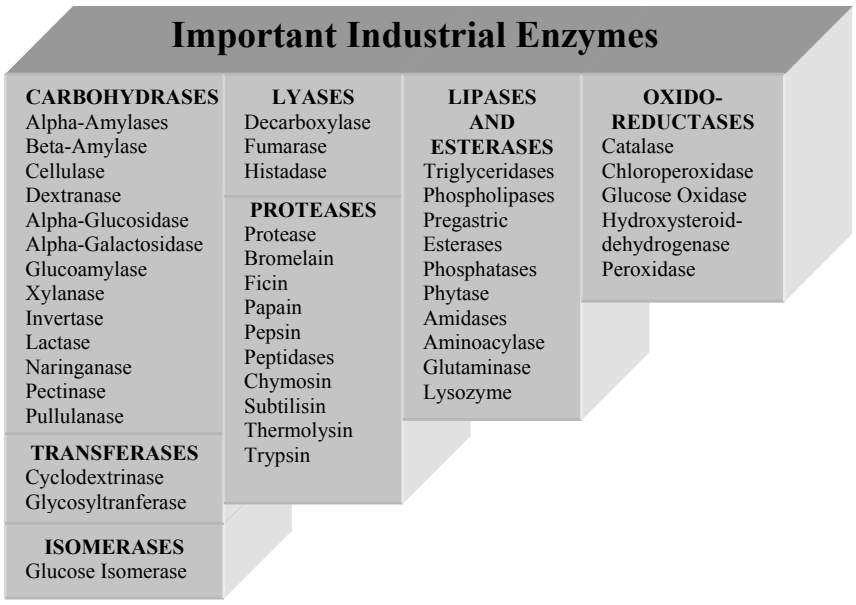

Figure 1. Major groups of enzymes identified as industrially important

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 were 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 posses.

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 posses a GRAS-status (**G**enerally **R**egarded **A**s **S**afe) 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^3 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.

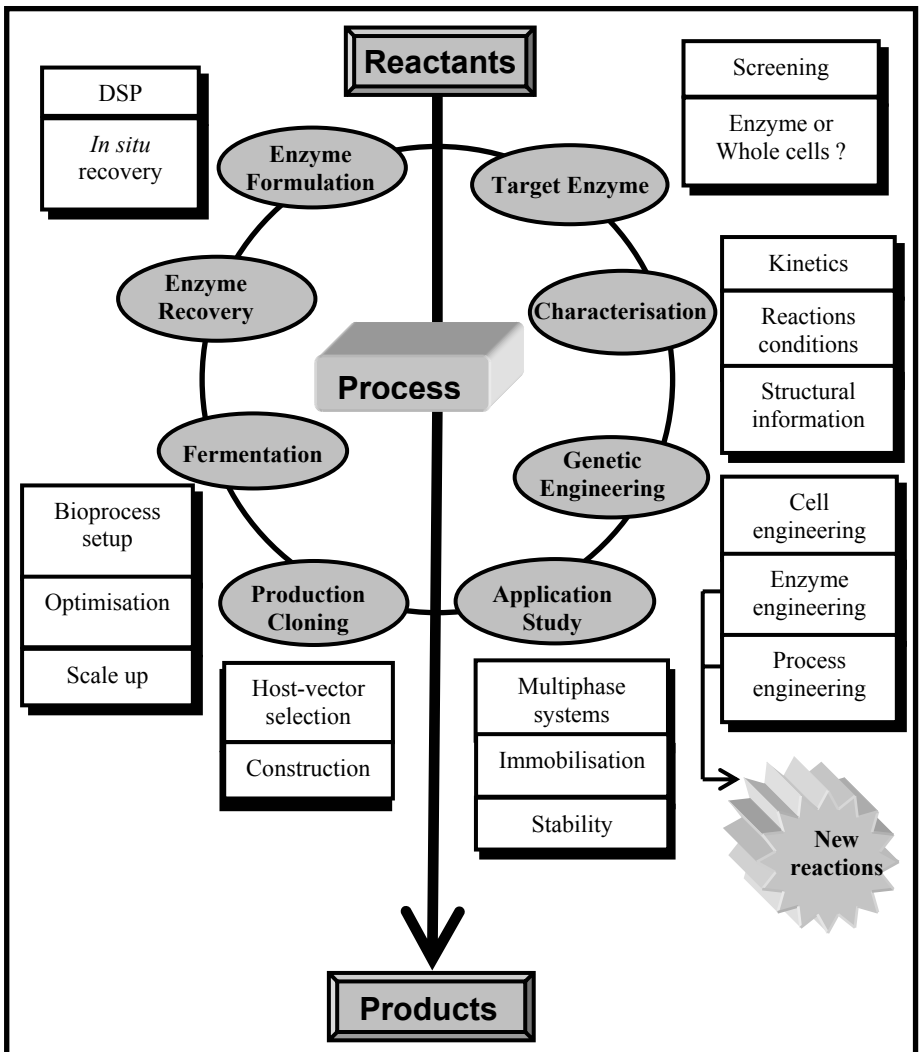

Figure 2. The enzyme production and design process

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 theses 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 increased the range of conditions where biocatalysis is an option. A few of these extremozymes and their potential applications are presented in Table 2.

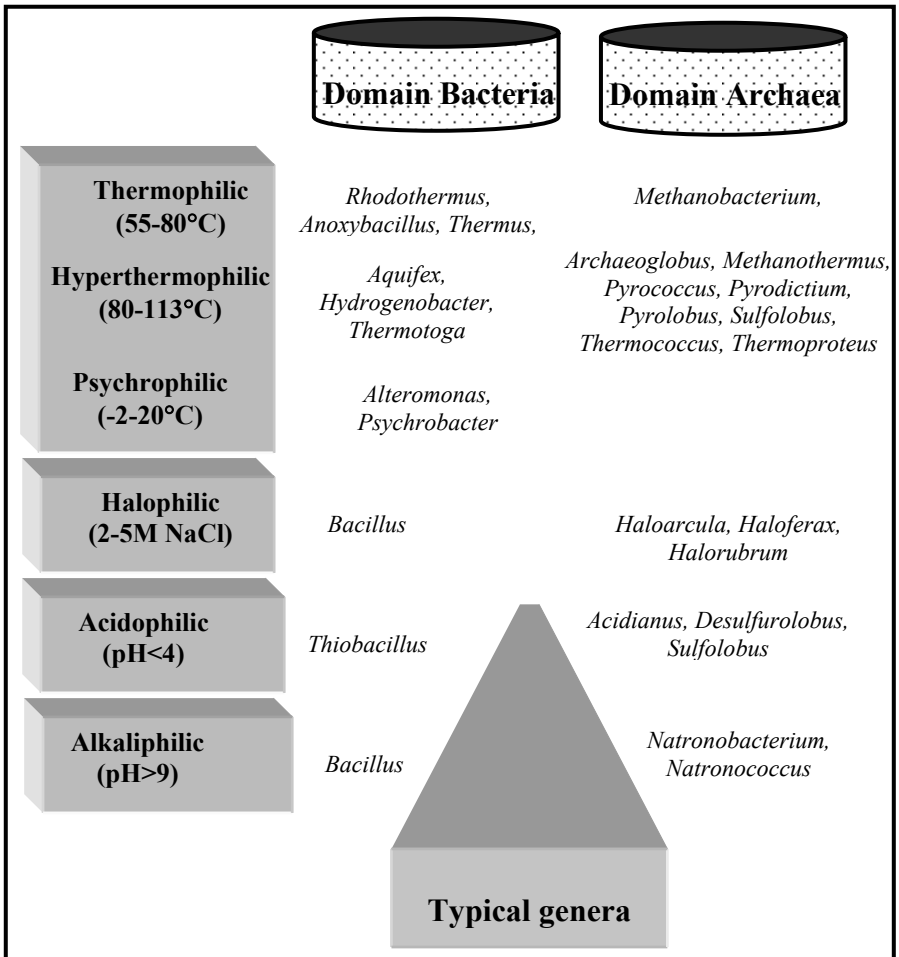

Figure 3. Extremophiles and their environments

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

Extremophile	Extremozymes	Application and products	Reference
Thermophiles	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]
Psychrophiles	Amylase, protease, lipase	Polymer degradation in detergents	[2, 43]
	Dehydrogenase	Biosensors	
Alkaliphiles	Cellulase, protease	Detergents	[2, 34]
	Amylase, lipase	Food additives	[34, 44]
Acidophiles	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 (**CA**rbohydrate-**A**ctive en**Z**ymes)].

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 (α-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-β-xylanases (EC 3.2.1.8), which are commonly referred to as xylanases and 1,4-β-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 (~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 (~40kDa) [61-62] (**Papers II, III, V**) and typically displays a TIM-barrel fold (cylindrical 8-fold α/β-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].

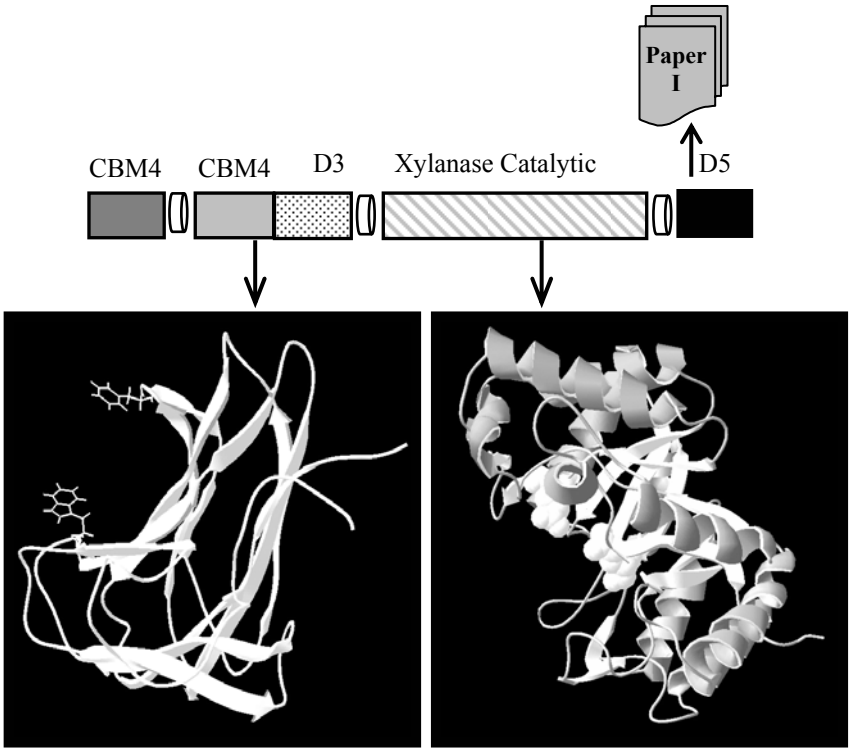

Figure 4. Schematic representation of the modular *Xyn10* gene from *R. marinus*. **(Left)** Ribbon model depicting type B Xyn10 CBM4-2. The two aromatic residues using the xylan binding cleft are indicated. **(Right)** Side view of a homologue to the Xyn10A catalytic module. The ribbon model shows the 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 β-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 β-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 β-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) β-glucosidases (EC 3.2.1.21) which are involved in the hydrolysis of cellobiose to glucose [74].

The extremely thermostable endoglucanase, Cel12A form *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 β-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 β-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].

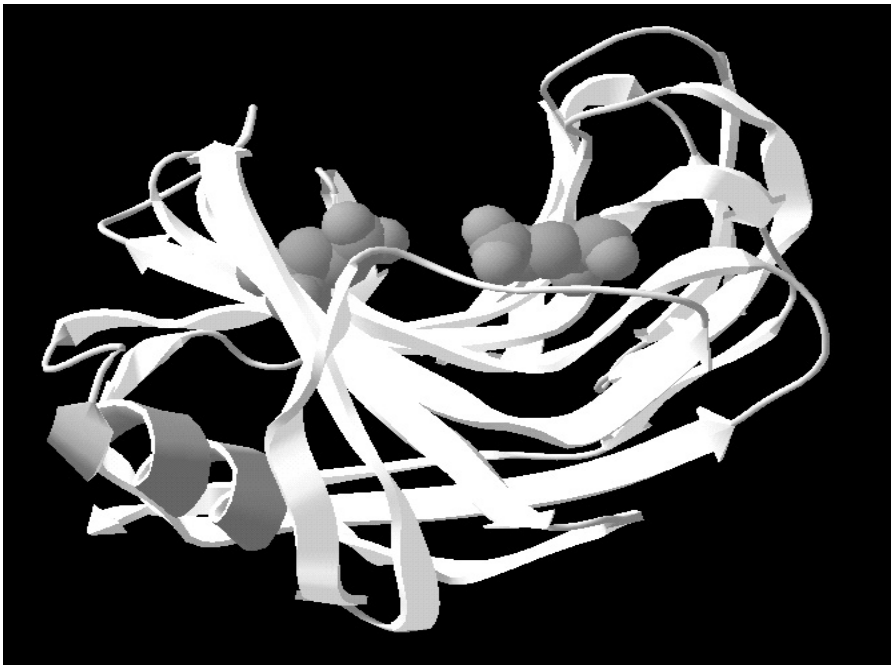

Figure 5. Ribbon model of the *R. marinus* Cel 12A from a side-view with the side-chains of the two catalytic residues shown in space-fill.

3.3.3 Cyclomaltodextrinase

The α-amylase family (GHF 13) is one (and the largest) of five structural families of starch-modifying enzymes, specifically acting on the α-1,4- and α-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 α-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 glucanosyltransferase (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 α-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 α-amylase family enzymes has proven to be difficult to express in an active form.

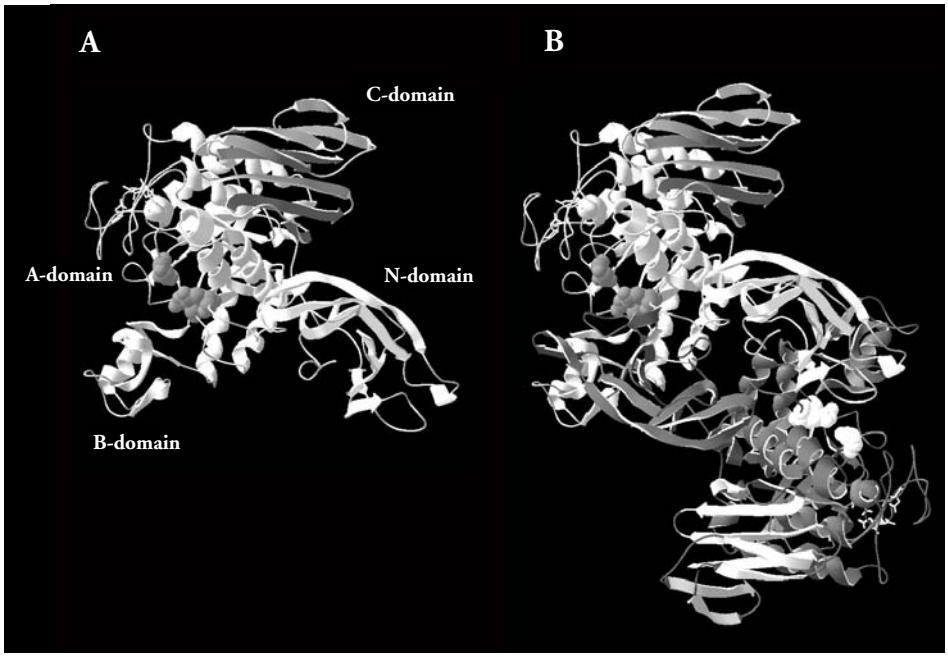

Figure 6. Ribbon models of *Anoxybacillus flavithermus* CDase13 in its monomeric **(A)** and its native dimeric **(B)** state.

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 α/β-hydrolase fold (Figure 7) and the true lipases display a conserved active site signature, the GXSXG-motif [107], which was also found in the *B. halodurans* sequence.

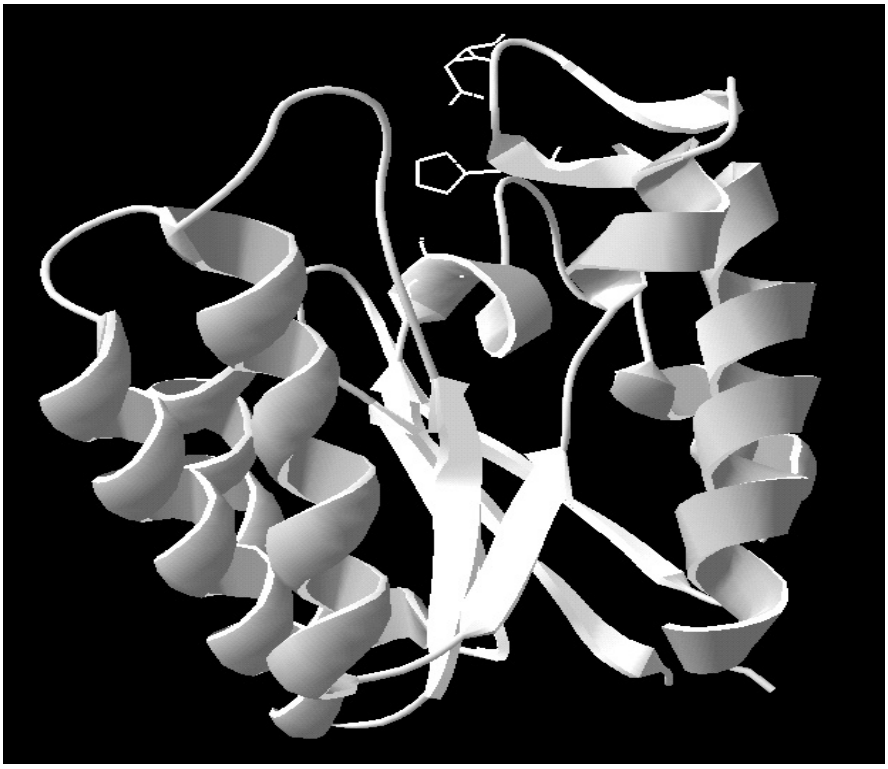

Figure 7. A representation of the α/β-hydrolase fold conserved among lipases. The side-chains shown are the conserved catalytic triad [S, H, D], with the serine being part of the GXSXG-motif.

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

	E. coli	***P. pastoris***
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
Post-translational modifications		
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 (λDE3) encoding the enzyme under control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lac*UV5 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]
	Altered pH	[138]
	Protease deficient strains	[139]
Signal peptide does not always facilitate transport, signal peptide cleavage	Coexpression-signal peptidase I	[140]
	Coexpression of *sec* genes	[126]
	Fusion proteins	[141]
Reduced folding	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
	Cytoplasm		
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]
	Periplasm		
Deg P			
Pi		Necessary for high temperature	
Mi		growth	[165-167]
Peri 7			
Peri8		Cleavage between tyr-leu and phe-tyr	
	Cell membrane		
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 were methanol is fed as a growth limiting substrate have shown an accumulation of AOX >30% of the total soluble protein [186-187].

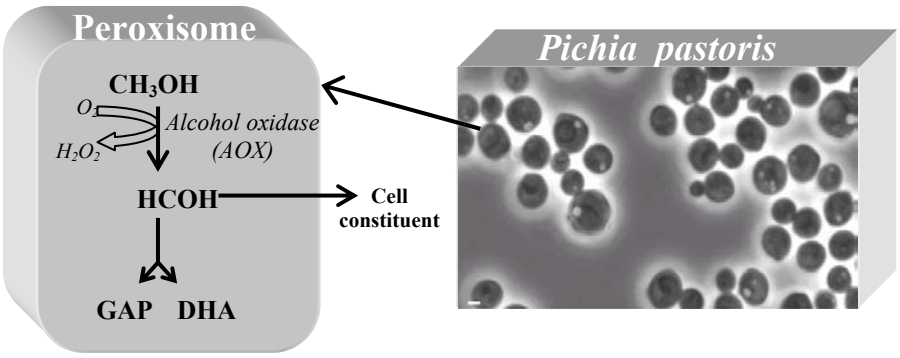

Figure 8. The methanol utilisation pathway in *P. pastoris*

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-phasphate 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^+, 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* α-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
B. licheniformis	α-amylase	E (2.5 g/L)	[189]
B. stearothermophilus	Carboxypeptidase	E (100 mg/L)	[190]
Escherichia coli	Acid phytases	E (29 U/mg)	[191]
Escherichia coli	β-galactosidase	I (2×10^3 U/mg)	[20]
Staphylococcus aureus	Staphylokinase	E (50 mg/L)	[192]
Streptococcus equisimilis	Streptokinase	I (77 mg/L)	[193]
R. marinus	Xylanase	E (~2 g/L)	**Paper V**
B. halodurans	Phospholipase	E (~1 g/L)	**Paper VI**

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 (g L^{-1} 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 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 ($g\ L^{-1}$)	Pr ($g\ L^{-1}\ h^{-1}$)	Reference
		Bacteria		
E. coli	Mesophile	190	1.04	[205]
		180	1.08	[205]
		148	3.36	[206]
		145	4.53	[207]
B. subtilis		184	6.57	[208]
S. laurentii		157	0.71	[209]
P. putida		100	3.33	[210]
M. extorquens		233	1.37	[211]
		Archaea		
Marinococcus M52	Halophile	132	N.A	[203]
Sulfolobus shibatae	Thermoacidophile	114	0.04	[203]
		Eukarya		
C. brassicae	Mesophile	268	9.57	[212]
S. cerevisiae	Mesophile	235	1.07	[213]
P. pastoris	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 (*Rm*Xyn10A). **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 theses 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).

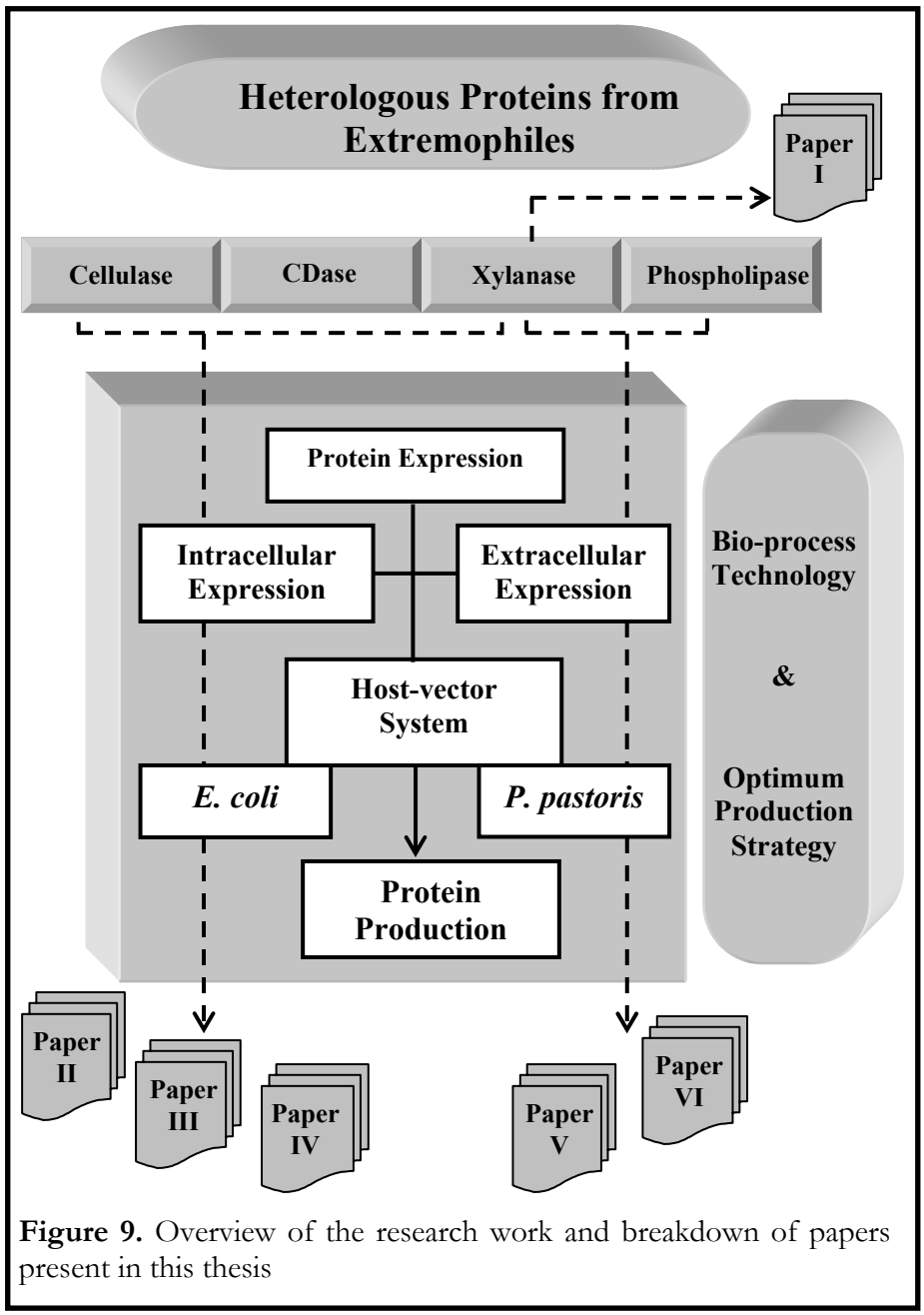

Figure 9. Overview of the research work and breakdown of papers present in this thesis

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

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