RECOMBINANT *Pyrococcus furiosus* EXTRACELLULAR ALPHA-AMYLASE EXPRESSION in *Pichia pastoris*

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ABSTRACT

RECOMBINANT *Pyrococcus furiosus* EXTRACELLULAR α-AMYLASE EXPRESSION in *Pichia pastoris*

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Pyrococcus furiosus extracellular α-amylase is a hyperthermostable glucosyl hydrolyzing enzyme which shows unique biochemical properties that may have impact on improving starch hydrolysis process; however, it is insignificantly expressed in its native archaeal host. In this study, it was aimed to express the *P. furiosus* extracellular alpha-amylase (PFA) in *Pichia pastoris*, which is a well-recognized overexpression host used in production of heterologous proteins. In this context, first, *P. furiosus* was grown under anaerobic conditions in capped bottles for t= 12 h at T=90°C and then its genomic DNA was isolated. PFA coding cDNA frame was amplified using two specifically designed oligonucleotides and cloned into pPICZαA expression vector. Then wild type *P. pastoris* X-33 cells were transfected with pPICZαA::PFA construct. In shake flask production medium, existence of recombinant PFA activity was tested and biochemical characterization of the recombinant product was done. This was the first time PFA is expressed in an eukaryotic host. Optimum working temperature and pH of the rPFA were found to be 95 °C and within the range of 4.5-6.5, respectively.

rPFA is independent to metal ions and inhibition by production medium of *P. pastoris* was observed, in presence of divalent metal ions. Although *Saccharomyces cerevisiae* α-factor secretion signal was fused to the N terminal of rPFA, minute amount of extracellular secretion was detected but the majority of the enzymatic activity remained in the intracellular medium. The best producer strain was selected by measuring α-amylase activity in cell extracts by DNS method. Effects of pH on cell growth and recombinant protein production were determined by shake flask experiments and maximum of 4800 U/l rPFA was detected with 7.30 g/l wet cell density in pH=6 buffered medium. In order to achieve higher rPFA production, two bioreactor experiments were designed at two different pH operation conditions, namely pH=4 and pH=5, in a working volume of 1 L. The dissolved oxygen tension was kept over 20% and predetermined exponential methanol feeding strategy was employed in order to fix specific cell growth rate, μ, at 0.03 h^{-1}. At pH=4 operation, maximum of 73,400 U/l α-amylase activity was detected at the t=27 h of production phase when the wet cell density was 209 g/l.

Keywords: Recombinant, *Pyrococcus furiosus* alpha-amylase, *Pichia pastoris*

ÖZ

Pyrococcus furiosus HÜCRE DIŞI ALFA-AMİLAZ ENZİMİNİN REKOMBİNANT *Pichia pastoris* İLE EKSPRESYONU

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Pyrococcus furiosus hücre dışı α-amilaz'ı (PFA), glukozil bağlarını parçalayan, yüksek sıcaklığa dayanıklı, ve sıra dışı özellikleri ile nişasta hidrolizi işlemini geliştirebilecek potansiyele sahip bir tür enzim olup, doğal arkeal konağında çok az miktarda üretilmektedir. Bu çalışmada *P. furiosus* hücre dışı alfa-amilaz enziminin, bugüne kadar yüzlerce proteinin rekombinant olarak üretiminin arttırılmasında başarı ile kullanılan *P. pastoris* ile üretilmesi amaçlanmıştır. Bu kapsamda, *P. furiosus* küçük ölçekli biyoreaktörlerde oksijensiz ortamda 12 saat süresince 90°C 'de çoğaltıldıktan sonra genomik DNA izole edilmiştir. PFA enzimini kodlayan gen, tasarlanan ileri ve geri primerle çoğaltıldıktan sonra pPICZαA plasmidine klonlanmıştır. *P. pastoris* X-33 suşu, pPICZαA:PFA plasmidi ile transfekte edildikten sonra erlenmeyer üretim ortamında rPFA enzim aktivetisinin varlığı saptanmış ve rekombinant enzimin biyokimyasal karakterizasyonu yapılmıştır. Bu aynı zamanda PFA enziminin ökaryotik bir hücrede üretildiği ilk çalışmadır. rPFA'nın optimum 95 °C sıcaklık ve pH 4.5-6.5 aralığında metal iyonlarına ihtiyaç duymadan çalıştığı saptanmıştır. Ancak,

divalent metal iyonları varlığında enzim inhibe olmuştur. *Saccharomyces cerevisiae* α-faktör salgı sinyalinin enzimin amino ucuna eklenmesine rağmen hücre-dışı enzim aktivitesi düşük hücre-iç enzim aktivitesi yüksek bulunmuştur. En iyi rPFA üreticisi olan suş, hücre-içi enzim aktivitelerinin DNS metodu ile belirlenmesiyle saptanmıştır. Enzim üretimi ve hücre çoğalması üzerine pH etkisi, erlenmeyer deneyleri ile incelenmiş, pH=6 tampon çözeltisinde 4800 U/l α-amilaz aktivitesi ve 7.30 g/l yaş hücre derişimi tesbit edilmiştir. Daha yüksek miktarda rPFA üretmek amacıyla pH=4 ve pH=5 pH kontrollü işletim koşullarında, 1 litre çalışma hacminde iki farklı biyoreaktör deneyi tasarlanmıştır. Her iki deneyde de ortamdaki çözünmüş oksijen miktarı 20% nin üzerinde tutulurken, spesifik hücre çoğalma hızını $0.03\ h^{-1}$ de tutacak şekilde önceden belirlenen üstel metanol besleme stratejisi kullanılmıştır. En yüksek α-amilaz aktivitesi pH=4 koşulunda, üretim fazının t=27 st'inde, yaş hücre derişimi 209 g/l iken 73,400 U/l olarak belirlenmiştir.

Anahtar kelimeler: Rekombinant, *Pyrococcus furiosus* alfa-amilaz, *Pichia pastoris*

To my family

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NOMENCLATURE

DO	Dissolved oxygen	%
N	Agitation rate	min^{-1}
OUR	Oxygen uptake rate	mol m^{-3} sec^{-1}
OTR	Oxygen transfer rate	mol m^{-3} sec^{-1}
OD_{600}	Optical density measured at 600 nm.	
Q	Feed inlet rate	l h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
r	Reaction rate	g $l^{-1}h^{-1}$
t	Time	H
T	Bioreaction medium temperature,	°C
U	One unit of an enzyme	
V	Volume	l
WCW	Wet cell weight	g
Y	Yield (overall)	g g^{-1}

Greek Letters

μ_0	Desired specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
μ_t	Total specific growth rate	h^{-1}

Subscripts

$_0$	Refers to "initial condition"
AOX	Refers to "alcohol oxidase"
Gly	Refers to "glycerol"
MeOH	Refers to "methanol"
O	Refers to "oxygen"
p	Refers to "protein" or "product"
rPFA	Refers to "recombinant *Pyrococcus furiosus* amylase"
S	Refers to "substrate"
X	Refers to "cell"

Abbreviations

AOX	Alcohol oxidase
BLA	*Bacillus licheniformis* amylase
BSM	Basal salt medium
DNA	Deoxyribonucleic acid
GB	Glycerol batch
GFB	Glycerol fed-batch
HPLC	High pressure liquid chromatography
MFB	Methanol fed-batch
MT	Methanol transition
PFA	*Pyrococcus furiosus* amylase
PTM1	Trace salt solution

CHAPTER 1

INTRODUCTION

α-Amylases (E.C.C. number 3.13.1.1) belong to the glycosyl hydrolyses group of enzymes. This group comprises the enzymes that have the capability to hydrolyze the 1-4 glycosidic bonds in starch granules.

Alpha amylases are used in variety of industries such as textile, detergent, food, brewing, pulp and paper. However the major use of α-amylase is in starch industry, in which starch is converted to crystalline dextrose, dextrose syrup and high fructose corn syrup (HFCS) (de Souza and e Magalhaes 2010). Native starch molecules are quite insoluble in cold water; however, with the increasing temperature starch granules start to swell irreversibly. The temperature at which starch molecules start to swell is called gelatinization temperature (van Oort 2010). In order to assure the removal of any residual amylose-lipid complex, generally gelatinization temperatures over 100 °C are preferred. Above gelatinization temperature, starch is first hydrolyzed randomly by α-amylase. This step is called starch liquefaction as the viscosity of the starch slurry is decreased due to fragmentation of starch into shorter length dextrins. Up to date amylases from various *Bacillus* species have been used for starch liquefaction. Van der Maarel et al. (2002) states that the drawbacks of the current α-amylases are their relatively high optimum pH, which makes pH adjustment of the starch slurry necessary, and Ca^{2+} requirement as a cofactor, that subsequently plugs the

process lines and has the potential to inhibit the downstream enzymatic processes (van der Maarel, et al. 2002). In an economical point of view these increase the chemical costs and make the ion-exchange refining of final product necessary for salt removal (Crabb and Mitchison 1997). An ideal starch processing enzyme should be independent of metal ions as cofactors, have low optimum working pH, preferentially as close as possible to the natural pH of the starch solution, pH=4. In addition to high optimum working temperature it has to be stable at that temperature. Amylases of archaeal sources offer good alternatives with their exceptional thermostability and durability under harsh industrial conditions.

P. furiosus is an hyperthermophilic archaea which was first isolated from the geothermally heated marine sediments of Vulcano Island, Italy (Fiala and Stetter 1986). It is an absolute anaerob which grows optimally at 98 °C. *P. furiosus* utilizes starch as a carbon source by its extracelluar α-amylase (PFA). PFA works optimally at pH range of 5.5-6 and at a temperature of 98 °C. PFA has a half life of 13.2 h in its optimum working conditions (Laderman, et al. 1993). Jorgensen et al. (1997) addressed the *P. furiosus* extracellular α-amylase (PFA) as an promising agent for starch liquefaction processes due to its high thermostability, independence on metal ions as cofactors, unique product pattern and substrate specifity. (Jorgensen, Vorgias and Antranikian 1997). However *P. furiousus* cultivation is not well suited for industrial practice as it grows at T=100°C and secretes its α-amylase in very low amounts, 1000 units/liter (Jorgensen, Vorgias and Antranikian 1997).

In case expression of an protein product in its native host is not efficient or the host is not well suited for industrial applications, the cDNA sequence of the protein product can be isolated and cloned into a foreign host and be expressed in it. These type of protein products are said to be heterologous or recombinant.

The success of the heterologous protein expression is mostly depended on the selection of the correct host and vector combination. Discrepancies in codon usage bias, redox state, post translational modifications between the recombinant host and the native host may lead to poor expression figures or misfolded protein product. Also solubility of the product in the host is an important parameter which determines whether the product forms insoluble protein aggregates or not.

Pichia pastoris is an methylotrophic yeast which has been widely used as an heterologous protein expression host since 1980s. Over 600 proteins has been produced intracellularly and extracellularly at satisfactory levels by *P. pastoris* since 1980s. Ease of its genetic manipulation and existence of some eukaryotic post translational mechanisms makes it a popular expression host (Cregg, Cereghino, et al. 2000).

Objective of this research is to produce *P. furiosus* extracellular α-amylase in *P. pastoris* expression system under the control of AOX promoter induced by methanol. For this purpose *P. furiosus* DSM 3638 strain was grown, then its genomic DNA has been isolated and cloned on pPICZαA expression vector. Using *E. coli* XL-1 as a shuttle, pPICZαA::PFA construct was then integrated on to *P. pastoris* X-33 genome. With shake flask experminents localization of the recombinant enyzme and its biochemical characteristics were determined. After determining the effect of pH on rPFA production, in order to increase rPFA yield, two different sets of bioreactor experiments at pH=4 and pH=5 operation conditions were carried out in 1 l of working volume with predetermined exponential methanol feeding strategy specific to μ=0.03 h^{-1}.

CHAPTER 2

LITERATURE SURVEY

2.1 Recombinant DNA Technology

Recombinant DNA technology refers to production of new DNA strands via joining two nucleic acid sequences which normally doesn't occur in the same organism. Possibility of this technology was first foreseen by a graduate student of A.Dale Kaiser, Peter Lobban following the discovery of the DNA cleaving enzymes, restriction endonucleases, by Werner Arber, Daniel Nathans, and Hamilton Smith in 1970, for which they have been awarded to the Nobel Prize in Physiology or Medicine in 1978. Lobban's work has never been published, but referred in ensuing studies. Techniques of creating a transgenic bacteria, isolation and amplification of DNA fragments and their insertion to the host was described in detail by Jackson et al. in 1972. In 1973 Stanley Cohen from Stanford University and Herbert Boyer from University of California isolated a gene from a toad using restriction enzymes, expressed the gene in *E. coli*. Since then recombinant DNA technology became a power tool for production of the therapeutically important proteins in foreign hosts.

In industry genetic recombination practices decrease the costs by both increasing the product yield and making purification step easier (Lui 2010). Designing of the

heterologous protein production process starts with the selection of the expression host and expression plasmid for expression of the desired gene.

2.1.1 Host cell selection

Host cell's biochemical enviorenment must be compatible with the native host's in terms of protein solubility, redox state, translational and post translational machinery in order to achieve a successful production of the functional product (Greene 2004). In case of a discrepancy, several solutions are proposed.

2.1.1.1 Translational compatibility

There's a strong correlation between the level of gene expression and the degree of codon bias. There needs to be significant overlap between the bias of the codons present in the host's genome and those in the native host's. Otherwise tRNA pool of the host can easily face to a bottleneck in translation machinery if less preferred tRNAs are demanded by the recombinant mRNA transcripts. Possible solutions include substitution of rare codons in the heterologous gene with prevalent codons via site directed mutagenesis or use of host cells in which rare tRNA genes are transformed.

Codon Adaptation Index (CAI) is used to determine translational compatibility of a gene script transcribed in a heterologous host. In order to calculate CAI, first of all Relative Synonymous Codon Usage (RSCU) value for each codon is calculated, which is simply calculated by actual frequency of a codon divided by hypothetical frequency of it in case utilized equally with its synonyms. Relative adaptiveness of a codon, w, is derived for any codon by dividing its RSCU value to the RSCU of the optimal codon coding for same amino acid.

		E.coli		Yeast	
		RSCU	w	RSCU	w
Ser	UCU	2.571	1.000	3.359	1.000
	UCC	1.912	0.744	2.327	0.693
	UCA	0.198	0.077	0.122	0.036
	UCG	0.044	0.017	0.017	0.005
Pro	CCU	0.231	0.070	0.179	0.047
	CCC	0.038	0.012	0.036	0.009
	CCA	0.442	0.135	3.776	1.000
	CCG	3.288	1.000	0.009	0.002

Fig 2.1 Sample RSCU and w table for *E. coli* and Yeast for selected amino acids (Sharp and Li 1987).

The Codon Adaptation Index (CAI) specific for a gene is calculated by the geometric mean of the RSCU values corresponding to each of the codons in that gene divided by the maximum possible CAI for a gene of the same amino acid composition. CAI scores greater than 0.70 are generally associated with high level of expression both in yeast and *E. coli* (Sharp and Li 1987).

2.1.1.2 Redox state and protein folding compatibility

Chaperons & foldases are oxidoreductase group of enzymes that ensures proper folding of a protein. Each oxidoreductase has its own optimal redox state to execute proper folding. 20 mV potential differences between native and recombinant host may easily lead to a misfolding of a protein by itself or by a chaperone. This problem may be solved by fusing a soluble oxidoreductase enzyme, such as thioredoxin, to the protein product itself (LaVallie, et al. 2003) or by using thioredoxin reductase and glutathione reductase deficient strains of

bacterial host (Lobel, Pollak and Lustbader, et al. 2002, Lobel, Pollak and Klein, et al. 2001). In both cases cytoplasmically produced recombinant protein is correctly folded by promotion of the disulfide bond formation.

2.1.1.3 Protein solubility compatibility

Either misfolding or overexpression of the target protein can result in a insoluble product. Wilkinson and Harrison developed a model which predicts the solubility of the proteins in *E. coli* primarily depending on the hydrophilicity (fraction of the polar and nonpolar amino acids), average charge, turn-forming residue fraction, and total number of amino acid residues present in the protein. In order to increase the solubility of any protein, a native soluble protein may be fused to the heterologous one even though the fusion protein might be substantially larger than the target protein alone (Davis, et al. 2000).

2.1.1.4 Post translational modifications and host compatibility

If an heterogous protein from a higher-order eukaryote to be produced, some post translational modifications such as proteolytic cleavage, glycolisation, phosphorylation and some amino acid modifications (acetylation, amidation, sulfation, isoprenylation and fatty acid addition) may be required before the fully functional protein is obtained. Not all of the expression hosts can accommodate these modification machineries. In table 2.1 post translational facilities of the several expression hosts are explained.

Table 2.1 Capabilities of various host cells as for gene expression (Greene 2004)

Host Cells	Proteolytic processing	Phosphorylation	Glycolisation	Amino acid modification	
Bacteria	Non-existent	Possible	No	No	
Yeast \ Fungi	Some	Some	Yes	Some	
Insect	Some	Some	Moderate	Some	
Plants	Moderate	Some	Moderate	Some	—
Plant cells	Moderate	Some	Moderate	Some	
Animals	High	High	High	Moderate	
Animal cells	High	High	High	Full	
Mammalian	Yes	Full	Full	Full	

2.1.2 Challenges and solutions in production of recombinant proteins

In a heterologous expression system loss of expression, abnormalities in post translational mechanism, transport and localization problems may occur if the expression system is not optimized.

2.1.2.1 Loss of expression

No matter where the recombinant gene is located, it can be located on a plasmid, on host's chromosome or delivered by a virus, the gene may disappear from host cells or it can be disrupted by structural changes which lead to a decreased production rates.

2.1.2.1.1 Loss of expression in plasmid based systems

Plasmids impose a metabolic load on cells since cellular resources must be utilized for their replication as well as it is used for cells' itself. Increasing insert size, temperature, expression level and recombinant protein yield and toxicity to the host cell increases the load. Plasmid bearing cells replicate more slowly.

These results in increasing percentage of wild type cells in the culture, and eventually plasmid-free cells overtake the culture.

During cell replication, plasmids are not always distrubuted at equal numbers to daughter cells. Cells with low copy number plasmids are quite prone to yield plasmid-free daughter cells (Summers 1998). This is called segregational instability. It can be prevented by applying a selective pressure on wild type cells. An essential gene can be deleted from the bacterial chromosome and included in the plasmid or growth repressor might be introduced in the chromosome and its antidote might be placed in the plasmid. These require genetic manipulation of the host chromosome. A simpler method can be the insertion of a antibiotic resistance gene in the plasmid and inclusion of the the antibiotic in the growth medium, however this technique is undesirable in both food and pharmaceutical industry besides bein costly (Palomares, Estrada-Mondaca and Ramirez, Production of Recombinant Proteins 2004).

2.1.2.1.2 Loss of expression in case of choromosomally integrated recombinant gene

Although chromosomal integration of a foreign DNA is labor intensive and yields less expression due to low copy number, it is especially preffered in mamalian expression systems where production phase lasts for months and extended stability of the expression host is required. The major problem with chromosomal integration is the probability that the expression casette might be integrated into a inactive region of the chromatin. Locus control regions can be utilized to check transcriptional regulation of the transgene (Palomares, Estrada-Mondaca and Ramirez 2004).

2.1.2.1.3 Loss of expression in viral vector systems

Viral expression systems are generally preffered due to their simplicity. Viruses deliver their genetic material to the host in an efficient and non-destructive way. There a direct relation between amount of virus attached to the cells and and protein production (Petricevich, et al. 2001). Viral vector systems work quite the same with the plasmid based systems. The time at which virus is introduced in to the production medium (TOI, Time For Infection) is analogous to the induction stage of the plasmid based systems an the multiplicities of infection (MOI) is to the plasmid copy number. Is TOI is too early, then viruses will evolve by time and deferective interfering particles (DIPs) will form. DIPs will not promote any protein expression and expose a metabolic load on the cells, which leads to a decreased recombinant yield in turn. If MOI is less than 0.1, then DIPs will attenuate as DIPs require a intact virus in the same organism for its survival . Late TOI will also lead to decreased protein production due to in-sufficient utilization of the metabolites in the medium for protein expression.

2.1.2.2 Problems in post translational processing

Overexpression of heterelogous proteins often results in formation of misfolded protein aggregates known as "inclusion bodies". Nonphysiological concentrations of product formation (Carrio and Villaverde 2002), lack of formation of disulfide bond formation due to reducing environment of the cytosol (Baneyx 1999) and saturation of the cellular folding machinery (Schlieker, Bukau and Mogk 2002) could be the reasons for misfolded proteins . It is impossible to predict whether a protein will remain soluble or not in its new expression system. Fusing a soluble protein to the recombinant protein or changing the hydrophobic amino acid residues with hydrophilic ones by protein engineering methods are the possible solutions against inclusion body formation.

Although it seems like a problem at first glance, if an efficient method to refold the protein is found, this can be an advantage as inclusion bodies are resistant to proteolysis. Purification of the inclusion bodies can be easily performed by a simple centrifugation step. Moreover, toxic effects of some heterologous proteins are hindered by expressing them in the form of inclusion body (Swartz 2001).

In *E. coli* expression systems, methionine is automatically added to N-terminal of the protein only if the second amino acid is alanine, glycine, proline, serine, threonine or valine. N-terminal methionine is removed by methionine aminopeptidase (MAP) enzyme. In case native MAP enzyme activity is not enough, it can be co-expressed with the recombinant protein in expense of reduced product yield as production of two heterelogous proteins at the same time imposes an extra metabolic load (Hwang, et al. 1999).

Some proteins require hydrocarbons attached to their specific amino acid groups, for their correct folding, localization, solubility and stability. This process is called glycolisation and requires several enzymes working in conjuction with each other. For therapeutic proteins, authentic glycolisation is essential, because non-authentic glycolisation may trigger the immune response of the patients. Non-authentic glycolisation may be the result of depletion of the lipid and sugar pools. Sugar pool depletion is associated with prolonged starvation on glucose or glutamine and can be avoided by adding sugar nucleotide precursors in the medium. Culture conditions also affect the degree of glycolisation. Toxic byproducts decrease the degree of sialylation and extend of glycolisation as a result. Although reduced growth rate may result in a reduced protein production rate, it positively affects the degree of glycolisation.

2.1.2.3 Transport and localization

Recombinant proteins can be directed to different cellular compartments by signal peptides or fusion proteins depending on the purpose of the process. In phage display applications proteins are targeted to the virus surface through fusion proteins, while some proteins are targeted to the periplasm of *E. coli* for correct folding (Wingfield 2002). In recombinant protein production processes extracellular secretion allows easier purification, however secreted protein will be highly diluted thus should be concentrated by ultrafiltration first. In case a bottleneck in the secretion pathway reduces the protein yield, intracellular production of the recombinant protein should be preferred to maximize the protein yield.

If the heterologous protein is a secreted one, cleavage of the fused signal peptide is essential, if not, it stacks in the endoplasmic reticulum. In case of an inefficient removal of the signal peptide, peptidase enzymes can be co-expressed (Van Dijl, et al. 1991). Similarly co-expression of endoproteases for correct processing of some proteins such as proteases and insulin are essential.

2.1.2.4 Yeast and Fungi specific problems and their solution

Yeast species are known by human since Neolithic age and the oldest biotechnological processes are carried out by them such as cheese and wine making. *S. cerevisiae* is the first yeast utilized as an expression host in heterelogous protein production. It has the advantage of having secretion and post translational mechanisms and being GRAS. On the other hand expression of some therapeutic proteins in *S. cerevisiae* results in hyperglycolisated products which causes immunogenic responses in human. For the production of glycolisated proteins, facultative methylotrophic yeasts such as *Pichia pastoris*, *P. methanolica*, *Candida boidinii* and *Pichia angusta* are preffered as they have similar

glycolisation pathways with mammalian cells. *P. pastoris* can grow up to very high cell densities and secretes protein products at gram per liter levels in the extracellular media.

2.1.3 Bioengineering approaches for the solution of the common problems associated with the heterologous gene expression

Most of the obstacles in heterologous protein expression pathway may be solved without molecular level manipulations, by modifying the process parameters.

2.1.3.1 Induction strategies

The type of promoter utilized in the expression system will determine whether the expression system will be constitutive or inductive. Constitutive promoters will always remain active throughout the growth phase till the death phase in expense or increased plasmid instability and potential inhibition effect of the heterologous protein expressed. In many cases optimum condition for recombinant protein expression is different than that for growth. In this type situation inducible systems are preferred. pH and temperature shifts, anearobiosis, antibiotic addition can be used as inducers as well as starvation or addition of a specific nutrient. Dynamics of the bioreactor should be considered while selecting the method of induction. Out of mass heat and momentum transfer problems in large scale bioreactors, heat transfer seems to be the most difficult issue to be solved, thus utilization of the heat shock promoters should be avoided if production to be carried out in large scale.

Time of induction is an important parameter to be tuned and should be set as early as possible if the plasmid copy number is low. However if the cell growth is inhibited by heterelogous protein expression then accumulation of biomass

should be awaited. In case of extreme biomass accumulation prior to induction, loss of expression may occur due to insufficient nutrient availability. This is called cell density effect and can be partially avoided by addition of concentrated nutrient following induction. In some cases this solution might not work as well due to accumulation of toxic metabolites and depletion of trace elements (Palomares, Estrada-Mondaca and Ramirez, Principles and applications of the insect cell-baculovirus expression vector system 2005).

The state of the cells at the time of induction also effects the expression levels. Eriksen and his co-workers reported higher expression levels at early exponential phase due to greater solubility of the recombinant protein compared to those in late exponential and lag phases (Eriksen, et al. 2001). Temperature is also known to affect the protein solubility and cold-shock proteins' promoters could be used in conjuction with decreased temperature at induction phase.

The extend of the induction is directly related with time and magnitude of the inducer. In case inducer is an expensive chemical, concentrations slightly higher than the stimulation threshold offer, the most economical solution to the problem (Ramirez, et al. 1994).

2.1.3.2 Growth control

Growth rate of the cell culture can be manipulated through pre-determined substrate feeding, controlled dissolved oxygen in the medium and temperature. Recombinant protein yield is directly related to the growth rate as maintenance coefficient, metabolic fluxes, RNA polymerase activities, ribosome number, plasmid number and stability are all dependent to the specific growth rate as well as the distribution of the cells in the cell-cycle phases. Decreasing the temperature is an efficient way to apply in animal cell culture s as it traps the

cells in G1 phase of the cell cycle (Kaufmann, et al. 2001). In many cases it is very hard explain the correlation between growth and specific heterelogous protein production (Palomares, Estrada-Mondaca and Ramirez 2004).

2.1.3.3 Bioreactor and operation strategies

The set of environmental parameters inside a bioreactor vessel is called "envirome". These parameters vast and include temperature, pH, dissolved gas and nutrient concentrations or inlet rates, agitation rate, redox potential, pressure, fluid dynamics, cell concentration and growth rate. Bioreactors have potential of increasing the recombinant product by manipulating envirome which has a impact on transcriptional, translational and post translational mechanisms, cell growth rate and cellular metabolic states.

The control of dissolved oxygen tension (DOT) is one of the basic control parameter in bioreactor. Oxygen dissolves in water at very low amount, and bioreactors are designed to maximize oxygen transfer rate (OTR) as much as possible. In general DOT higher than 20% will not limit the growth while in immobilized or aggregated cultures this should be no less than 50% (Yegneswaran, Thompson and Gray 1991). Increasing the agitation speed or aeration rate can damage the fragile animal and filamentous fungi cells. In this case shear protective additives can be used to decrease cell membrane fluidity or hemoglobin can be co-expressed in the same host (Bollinger, Bailey and Kallio 2001). On the contrary excessive amount of DO may decrease the recombinant protein yield by imposing oxidative stress on cells and damaging the oxygen sensitive proteins such as cylohexanone and monooxygenase which should be produced under absolutely anaerobic conditions (Konz, King and Cooney 1998).

In some cases excessive amounts of a substrate may result in formation of toxic byproducts through activation of some metabolic pathways such as in case of "Carbtree effect" which can be explained as the alcoholic or acid fermentation in aerobic conditions due to high concentrations of glucose. Switching to fed-batch mode of substrate addition might be a solution as long as substrate starvation doesn't trigger the plasmid instability.

2.1.4 Clone building

A common way to produce a genetically modified organism starts with isolating the DNA fragment responsible for the expression of the gene, joining it to an expression vector, and goes on with introduction of it into host cell and selection of the positive transformants. Several different methods are available for implementation of those steps and a possible pathway for cloning a gene is described by Primrose et al. (2001).

In our research DNA fragment is amplified via PCR method, restriction sites were created subsequently in this step via specifically designed primer oligonucleotides, each having corresponding restriction sites to those of cloning vector. Following ligation, vector is transformed first into *E. coli* XL-1 blue strain, then to the expression host *P. pastoris*. Positive transformants were selected at the *E. coli* stage via PCR and agarose gel analysis.

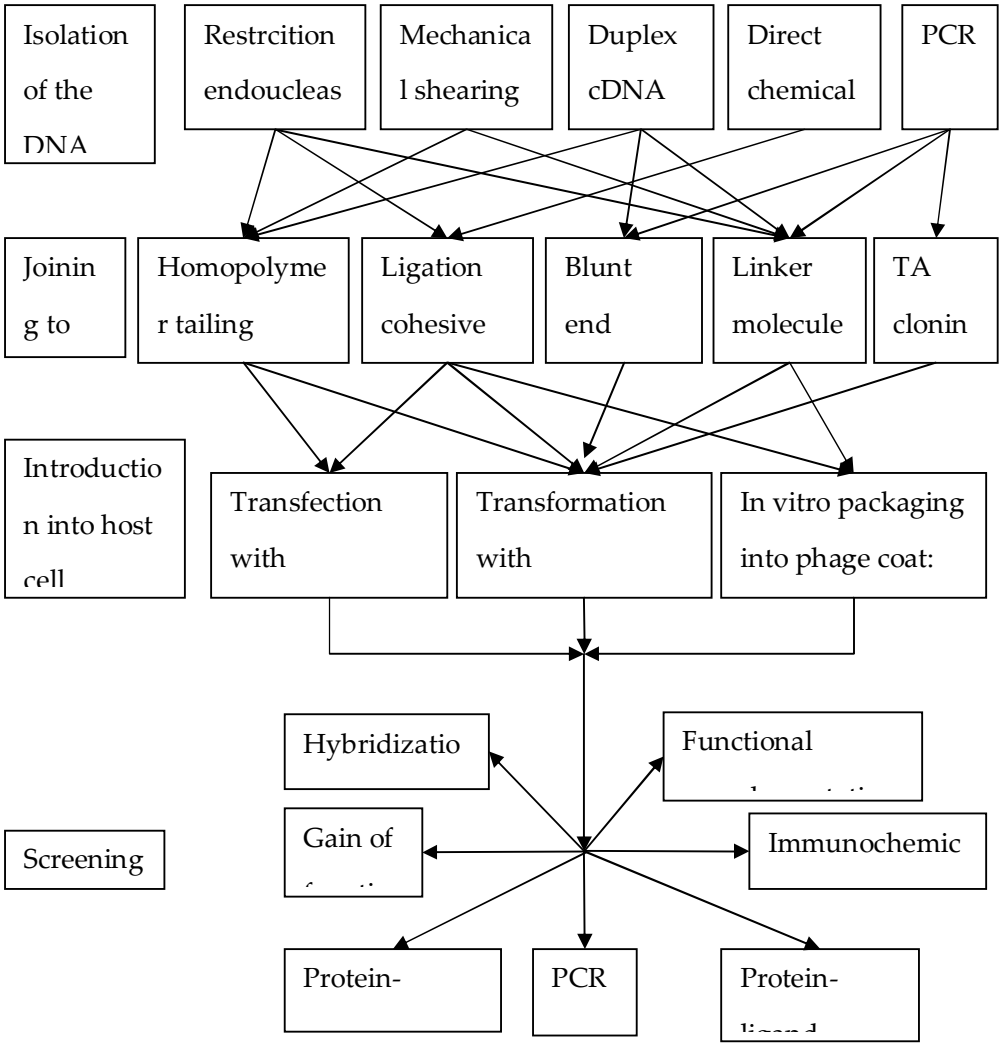

Fig. 2.2 Illustration of the possible pathways for clone building (Primrose, Twyman and Old 2001).

2.1.4.1 Cloning by PCR

Cloning by PCR is relatively efficient method compared with the traditional shotgun cloning, where genomic DNA is digested with restriction enzymes and gene of interest is searched for by screening transformants. In PCR, once the C & N terminal sequences of the gene of interest are known, complementary oligonucleotides are synthesized as primers which initiate the transcription of the gene of interest in vitro. It also enables site specific mutations and restriction site additions to the N & C terminals of the ORF (Lui 2010).

2.1.4.1.1 Basic steps of the PCR

PCR is an iterative process which consists of 3 main steps; Denaturation, Annealing and Extension.

Denaturation step: This is the step where double stranded template DNA and primers are unzipped at elevated temperature. 94-95°C is enough to unzip dsDNA. In repetitive cycles it should be held at 94 °C for 45 s., however in the initial denaturation step it can be as long as 5 minutes in order to assure complete denaturation of long DNA templates. Higher temperatures may be required if the G+C content of the template or DNA target sequence is greater than 55%.

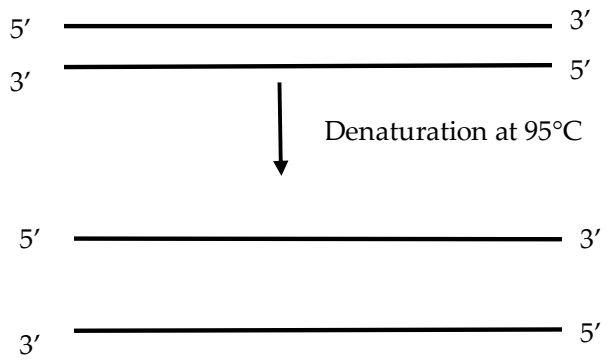

Fig 2.3 Denaturation of the double stranded DNA

Annealing of primers to Template DNA: Reaction mixture is cooled down to 5 °C below melting temperature of the primers, primers will anneal to their complementary regions on the template DNA. If this temperature is kept too high primers will bind poorly, and if kept too low non-specific bindings will occur, thus selection of this temperature is critical.

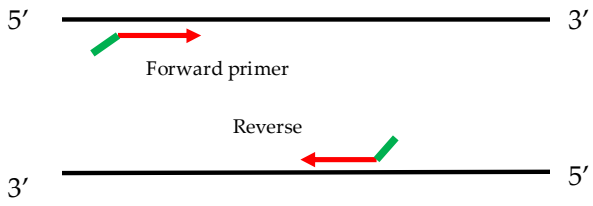

Fig 2.4 Annealing of the specially designed oligonucleotides to the unzipped DNA

Extension of oligonucleotide primers: In this step in vitro DNA transcription takes place with presence of the polymerase enzymes. Extension temperature is generally set at 72°C, however duration of this step differs depending on the type of the polymerase enzyme used. For each 1000 bp to be synthesized 1 minute is sufficient in case Taq Polymerase is used, time doubles if the Pfu Polymerase enzyme is chosen.

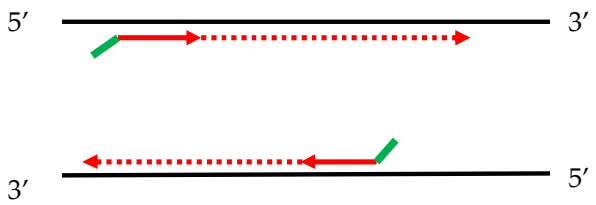

Fig 2.5 Elongation of the newly synthesized complementary DNA strands

Generally 30 steps are enough to yield sufficient amount of DNA products, with higher cycle numbers concentration of the non specific products will arise. Starting from the 3rd step of the PCR double stranded DNA molecule which comprises precisely the target region will arise and its number increases geometrically. At the 22th cycle one template molecule will be amplified about one million fold in ideal case (Primrose, Twyman and Old 2001, Sambrook and Russel 2001).

Essential components of the PCR are, thermostable DNA polymerase enzyme, pair of synthetic oligonucleotides to prime DNA synthesis, dNTP bases, divalent cations in order to activate polymerase enzyme, pH buffer, monovalent cations

and template DNA. Out of these components design of the synthetic oligonucleotides requires special attention, since any of the design parameters listed below affects the outcome of the PCR reaction.

2.1.4.1.2 Primer Design basics

Base composition: There should be a homogeneous distribution of the bases, polypurine or polypyrimidine repeats should be avoided. In ideal case G+C content should be somewhere between 40-60%. For primers with low GC content length of the primer should be kept long enough in order to prevent low melting temperature.

Length: Complementary region of the primer to the template DNA of 18-25 bp proven to be optimum. Difference in length of the primer couples in the same reaction shouldn't exceed 3 bp.

Self complementary sequences: Oligonucleotides having inverted repeat sequences or complementary regions tend to form hairpin structure, this type of self annealing will decrease the PCR efficiency.

Complementarity between members of a primer pair: 3' site of the primers will tend to bind other primers if they detect complementary regions present in them. As a rule of thumb maximum of 3 consecutive nucleotides on one primer should be complementary to the other.

Melting temperatures: Melting temperatures of the primer pairs shouldn't differ more than 5 °C. To ensure complete denaturation, melting temperature of the

amplified region should be within the 10 °C range of the malting temperature of the primers

3′ termini of the primers: G or C base should be preferred at the 3′ termini if possible, however NNGC or NNCG variations should be avoided since they promote the hairpin formation.

Restriction site at the 5′ termini: Normally there won't exist a desired restriction site at the 5′ of the oligonucleotide, they're added by the researcher at the 5′ site. Restriction site selection is unlimited, however it shouldn't present in insert DNA and in plasmid DNA more than once. Normally while designing the 5′ ends, some extra bases will be added in order to maximize cleavage efficiency. Number of bases to be added varies depending on the restriction site (Dallas-Yang, Guoqiang and Sladek 1998).

Commercially important protein molecules have been known since over half a decade before and they were used to be produced employing traditional techniques before the discovery of methods to alter cell metabolism by genetic mutations. Using traditional methods, 5 mg of somatostatin was first isolated from half a million sheep brains and a smaller amount of epidermal growth factor from 160 metric cubes of human urine. With the introduction of Recombinant DNA technologies it became possible to produce same amount of material from a few liters of bacterial culture (R.W. Old & S.B.Primrose 1994, 5).

Human insulin was the first recombinant protein to be produced commercially in 1982. Then it was followed by human growth hormone, hGH, under the brand name of "Humatrope". Since then dozens of proteins has been produced commercially by employing techniques of genetic recombination.

2.1.4.2 The central role of *E. coli* in Transformations

E. coli has always been a popular model system for molecular genetic engineering studies as there exist numerous well-characterized mutants, wide selection of plasmids together with a good understanding of the gene regulation in it. So, cloning in *E. coli* is relatively easier than any other organism, as a result, occasionally, insert DNA is first propagated in *E. coli* then it is cloned into its actual host. Without the ability to clone and manipulate DNA in E.coli, the application of recombinant DNA technology to other organisms would be greatly hindered (Primrose, Twyman and Old 2001).

2.2 α-Amylase

α-Amylases (or-1,4-glucan-4- glucanohydrolase; E.C.3.2.1.1) hydrolyse internal a-1,4-glycosidic linkages in starch, essentially at random fashion, to give shorter maltodextrins.

α-Amylases have two unique features that distinguish them from all other amylotic enzymes. They form α configuration at the reducing end anomeric carbon of the product, and they have endo-mechanism of attacking the starch polymer. Since fragmentation of the starch molecule with endo-acting mechanism decreases the viscosity of the starch slurry, α-amylases are sometimes called as liquefying amylases (Robyt 2009).

α-Amylases are used in variety of industrial fields. It is used in baking industry to improve the flour, as additive in detergents, to produce modified starches in paper industry, as desizing agents in textile industry, for partial replacement of the expensive malt ingredient in brewing and to hydrolyze the substrate in starch processing (Nielsen and Borchert 2000). However the major use of α-amylase is

in the starch industry in which it is used for production of starch hydrolysates (Gupta, et al. 2003).

2.2.1 Starch Processing and α-Amylase

Initiation of the starch industry dates back to 1811 when German scientist Kirchoff discovered that the dilute acid treatment of the starch-water suspension results sweet tasting syrup, but it took a century before a large scale starch-hydrolyzing industry was developed till Newkirk described a commercial process in 1921. In this process, starch is solubilized at elevated temperatures and then acid hydrolyzed.

Recently, the acid hydrolysis method has been replaced by the enzymatic hydrolysis process with emerge of the thermostable amylotic enzymes in 1950s (van der Maarel, et al. 2002). For complete conversion of the starch slurry into high glucose syrup, gelatinized starch slurry at 95-105 °C is liquefied into water soluble shorter-chain-length dextrins by α-amylase. Thermostability and thermophilicity of the enzyme is the key in this step since it takes about 90 minutes to yield satisfactory liquefaction. Temperatures above 100 °C are preferred in order to assure the complete removal of the starch-lipid complexes (Gupta, et al. 2003).

2.2.1.1 Conventional starch liquefying α-amylases

Initially *Bacillus amyloliqufaciens* is used in starch liquefaction process. It is stable between pH 5-8 and at temperature range of 50-60°C. It is not obligate to metal ions but thermostability and activity are enhanced in presence of them. Relative

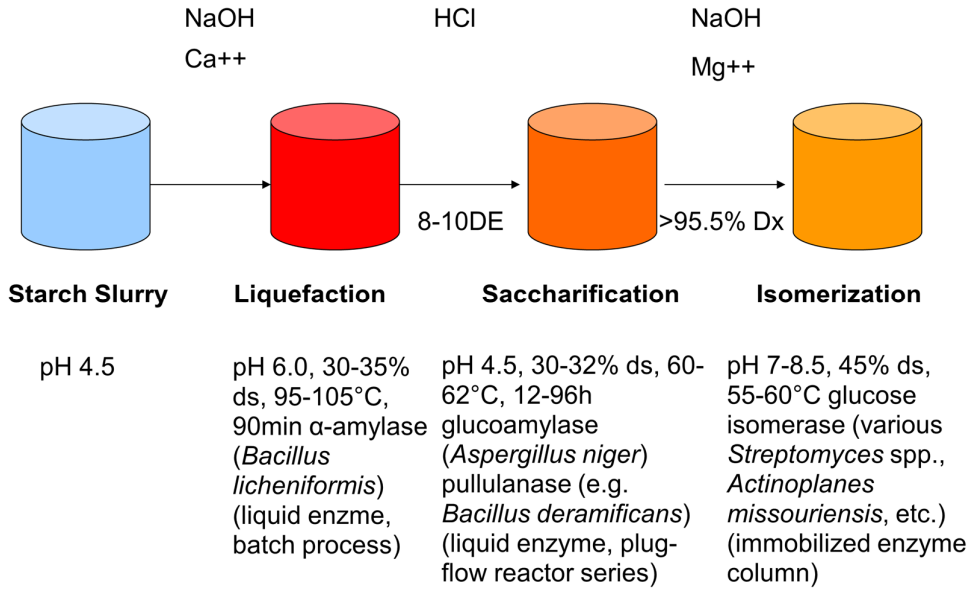

Fig. 2.6 Enzymatic hydrolysis of starch

activity sharply decreases beyond 70 °C. With its raw starch digestion capability and thermal denaturation it was like more suitable for baking industry than it is for starch processing (Ghangadharan et al., 2009). By discovery of the thermostable amylases it was replaced *Bacillus stearothermophilus* (BSA) and *Bacillus licheniformis* α-amylases (BLA). (van der Maarel et al., 2002).

B. stearothermophilus α-amylase is quite thermostable. While BLA loses 19% of its activity after 1h traetment at 90 °C, BSA amylase maintains 92% of its activity after treatment at 100 °C for 1. However has optimum working temperature of 50 °C and optimum pH of 7, which is far away from the optimum conditions of starch liquefaction (Chackraborty et al., 2000).

Half life of the wild type BLA amylase is about 9.8h at 90 °C at ph 6.5 and it requires 5 mM Ca^{2+} ions in these conditions. In absence of Ca^{2+} ions, half life of BLA is less than 1h (G. Dong, C. Vieille, et al. 1997, Wang, et al. 2007, van der Maarel, et al. 2002).

Although these enzymes are able to operate at temperatures as high as 105 °C, the process won't run much below pH 5.9 since the thermostability of aforesaid amylases decrease by decreasing pH. To overcome this problem, the pH of the starch slurry is adjusted from its natural pH of 4.5 to pH 5.8-6.5, and Ca^{2+} is added to improve enzyme stability. The next process step (saccharitication) requires pH adjustment back down to pH 4.2-4.5. These adjustments increase the chemical costs and require additional ion-exchange refining of the final product for salt removal. Moreover, Ca^{2+} itself is a potent inhibitor for the xylose isomerase which can be used in downstream processes, it also forms calcium oxalate which plugs the process lines (van der Maarel, et al. 2002). An AA able to operate at lower pH would reduce these costs, simplify the process and reduce high-pH byproduct (e.g. maltulose) formation in the liquefact (Crabb and Mitchison 1997).

Most of the enzymes are not well suited for the harsh conditions adopted in industry because they're rapidly denaturated at the operational conditions. Extremophilic industrial enzymes (extremozymes) provide interesting alternatives for working at extreme process paramaters such as temperature and pH but none of them have been produced at commercially viable levels (Cobucci-Ponzano, et al. 2007). *P. furiosus* has an α-amylase enzyme which shows promising characteristics for enhancement of liquefaction step of starch processing (Jorgensen, Vorgias and Antranikian 1997).

2.2.1.2 *Pyrococcus furiosus* α-amylase

P. furiosus is an obligately anaerobic heterotroph hyperthermophilic archea, first characterized by German scientists Karl O. Stetter and Gerhard Fiala in 1986 (Fiala and Stetter 1986). It grows in the geothermal hot marine sediments of Vulcano Island, Italy. It thrives elevated temperatures for its survival, grows between 70-103 °C and optimally at 100 °C.

In 1990, Brown S.H. and his co-workers reported the existence of the amylotic enzyme activities in *P. furiosus*. They detected the α-amylase, α-glucosidase and pullulanase activities and observed that both of those enzymes shows high degrees of thermostability accompanied with the optimum working temperatures over 100°C (Brown, Costantino and Kelly 1990).

The α-amylase from *P. furiosus* was first purified to homogeneity and characterized by Kenneth A. Laderman and his co-workers in 1993, this was the intracellular α-Amylase of *P. furiosus* with molecular mass of 66 kDa. Depending on its heat denaturation thermodynamic data, cold denaturation has been foreseen at -3 °C (Laderman, et al. 1993).

In 1997 Steen Jorgensen and his co-workers identified a new α-amylase of *P. furiosus*, cloned, sequenced and expressed it in *E. coli* and *B. subtilis*. It was stated that this extracellular α-amylase showed no sequence homology to the previously discovered sub-cellular α-amylase of the same organism. The ORF of the gene encoding extracellular α-amylase was 1383 base pairs long, coding for 461 amino acids. The 25 amino acids of signal sequence were as identified by comparing the results of the protein sequencing and DNA sequence of the open

reading frame. The enzyme showed its highest activity at pH 4.5 and optimum temperature was said to be close to 100 °C (Jorgensen S. 1997).

In the same year Dong and his co-workers cloned the same gene encoding extracellular α-amylase from *P. furiosus* by activity screening in *E. coli*. However they determined the signal sequence to be 1 amino acid longer than that determined by Jorgensen. They reported that gene encoded 460 amino acid sequence 26 of which was the signal sequence. The enzyme showed its highest activity between pH 5.5-6 and at 98 °C with the 3900 U/mg of specific activity. The enzyme didn't require any of the metal ions for its activity. No loss of enzyme activity was observed after treatment of the *E. coli* homogenate at 100 °C for 20 minutes. It was emphasized that PFA was significantly more thermostable than the commercially available *B. licheniformis* α-amylase (Taka-Therm™). On gel filtration PFA enzyme showed MW to be 100 kDa, which is twice of the calculated 52 kDa. This shows that he enzyme is homo-dimer. rPFA monomers stay partially folded even after the extended period of denaturation steps with 2% SDS and 0.64 mercaptoethanol. On SDS page, apparent molecular weight of the rPFA is 66000 kDa if it is denaturated below 60 °C, and 44000 kDa when denaturated above 90 °C. (G. Dong, C. Vieille, et al. 1997). This is a well known behavior of the *P. furiosus* enzymes (G. Dong, C. Vieille, et al. 1997, Rudiger A. 1995).

Dong and his co-workers did also cloned rPFA gene on pET213 vector excluding its secretion signal sequence. Under the control of T7 promoter 38,000 U/l α-amylase activity was detected in *E. coli* culture broth. Maximum of 109,000 U/l α-amylase activity was observed in culture broth if rPFA is co-expressed with thioredoxin. Overexpression studies have been done by Dong and his co-workers and maximum of 28 mg/liter production achieved under the control of T7 promoter in *E. coli* with pET213 plasmid (G. Dong, C. Vieille, et al. 1997).

Jorgensen et al. (1997) addressed the *P. furiosus* α-amylase as an promising agent for starch liquefaction processes due to its high thermostability, independence on metal ions as cofactors and unique product pattern and substrate specifity, mentioned the importance of application of genetic and fermentation techniques for the production of these type of enzymes (Jorgensen, Vorgias and Antranikian 1997).

2.3 Pichia pastoris

Existence of the first methanol utilizing yeast, *P. pastoris*, was reported by Ogata et al. (1969) where MeOH was used as the sole carbon source. They reported that newly discovered strain assimilates glycerol and EtOH much better than other carbohydrates, can utilize amino acids, organic acids, yields formaldehyde as metabolite and grows between pH 2.5-9.0 and 5-45 °C. It was also observed that newly isolated strain grows much faster with the increase in MeOH concentration. Till that time MeOH utilization by microorganisms has been known only in bacteria such as *Pseudomanas sp. PRLW-4, Pseudomanas methanica, Methanomanas methanooxidans,* etc. (Ogata K. 1969). During 1970s, till the beginning of the oil crisis, interest in *P. pastoris* has emerged rapidly, and Phillips Petroleum company has made many researches on this methylotrophic yeast, and was able to grow it up to 130 g/l dry cell weight (Cereghino and Cregg 2000).

2.3.1 Methanol utilization system of *P. pastoris*

P. pastoris assimilates MeOH in a similar way with the other methylotrophic yeasts, the only difference is the existence of the S-formylglutathione hydrolase activity.

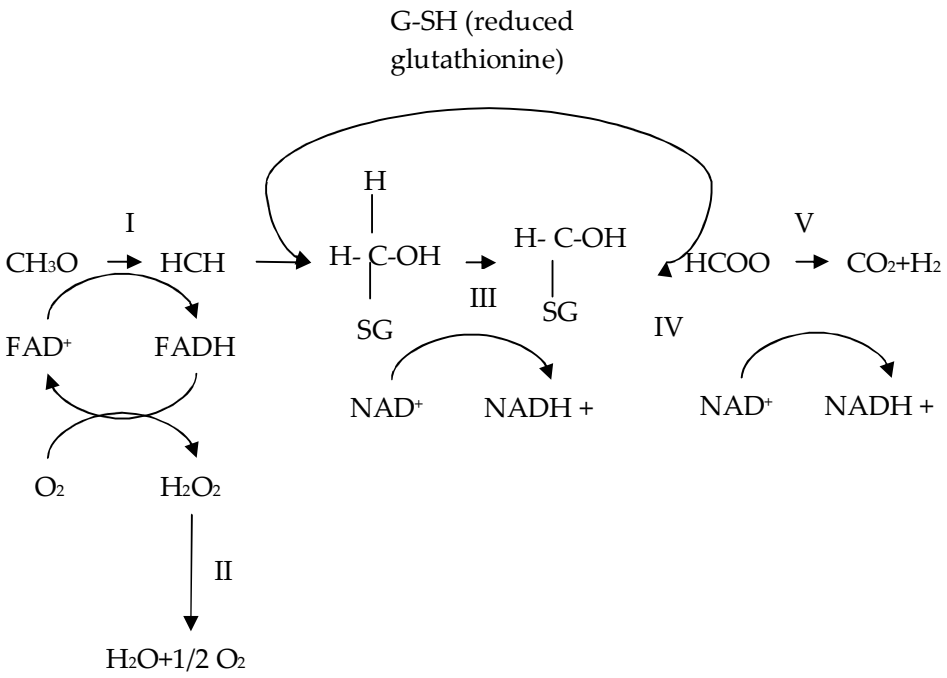

Fig 2.7 Oxidation of methanol by the yeast *Pichia pastoris*. I)Alcohol oxidase, II) catalase, III) formaldehyde dehydrogenase, IV) S-FG hydrolase, V) formate dehydrogenase

The first step is the oxidation of MeOH with the molecular oxygen via alcohol oxidase enzyme. These series of reactions yields formaldehyde and hydrogen peroxide which are toxic to any living cell. In order to sequester these toxic products away from the rest of the cell, this process takes place in a special organelle called peroxisome (van der Klei, et al. 2006). Formaldehyde is converted to dihydroxyacetone by DHA synthase. DHA is converted to GAP in the cytosol through a series of reactions.

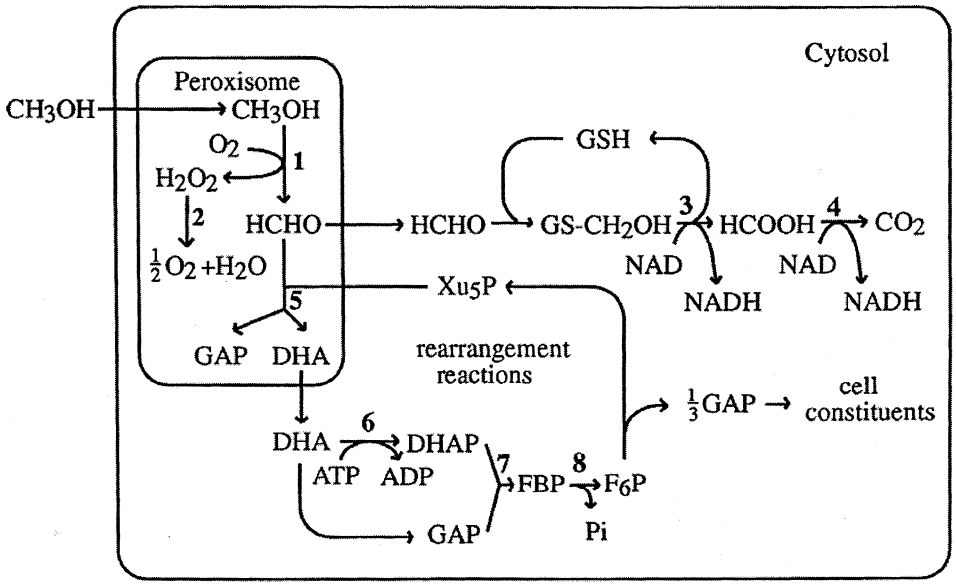

Figure 2.8 Methanol metabolism in *P. pastoris* (Cereghino and Cregg 2000).

2.3.1.1 Methanol utilization phenotypes of *P. pastoris*

The first step of the MeOH oxidation process is the oxidation of the MeOH to formaldehyde via alcohol oxidase enzyme. Alcohol oxidase has low affinity to oxygen and in order to compensate the poor catalytic activity, cells will express this enzyme in large quantities. In some cases alcohol oxidase may constitute 33% of the total proteins expressed in *P. pastoris* (Couderc and Barratti 1980). Alcohol oxidase is transcribed from 2 different locuses (AOX1 & AOX2) on the *Pichia* genome (Ellis, et al. 1985, Cregg, Madden, et al. 1989). Although AOX1 and AOX2 shows 92% similarity in their ORF and 97% homology in deduced amino acid sequence, majority of the alcohol oxidase activity within the cell is regulated by AOX1 (Koutz, et al. 1989, Cregg, Madden, et al. 1989).Depending on the ability or disability of *P. pastoris* strains to express these enzymes, 3 different

phenotypes are characterized, Mut^+, Mut^s, Mut^-. The wild type *P. pastoris* and expresses both alcohol oxidases, which is named as Mut^+ while Mut^s (acronym for Methanol Utilization Slow) strain has disruption in AOX1 locus thus metabolizes MeOH more slowly. Mut^- is unable to grow on MeOH as a sole carbon source since it has both AOX genes deleted (Macauley-Patrick, et al. 2005).

2.3.2 *P. pastoris* as a heterologous protein production host

The budding yeast *S. cerevisiae* was the first yeast to be used for eukaryotic protein production, as it was best characterized. However it has some limitations due to its primitive glycolisation and amino acid modification systems, it started to be replaced by methylotrophic budding yeasts *H. polymorpha* & *P. pastoris* together with fission yeast *Schizosaccharomyces pombe*. After Cregg et Al. published the transformation guidelines for *P. pastoris* in 1985, *P. pastoris* has been a used for the expression of many heterologous proteins (Cregg, Barringer, et al. 1985) .Together with the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA) Philips Petroleum studied the *P. pastoris* as a system for heterelogous protein production. By 2007, At least 600 genes has been cloned and expressed in *P. pastoris* (Zhang, et al. 2009).

Pichia pastoris has many advantages over other species which makes it a possible candidate for heterelogous protein production. First of all, it facilitates a very strong and tightly regulated AOX1 promoter which is used to drive the high level expression of the recombinant protein both intracellularly and extracellularly (Cregg, Vedvick and Raschke, Recent advances in the expression of foreign genes in Pichia pastoris 1993, Cregg, Tschopp, et al. 1987).

Table 2.2 Some of the heterologous proteins produced by *P. pastoris* expression system and production figures of those.

Protein	**Expression Level**	**Phenotype / secretion**	**Reference**
Invertase	2.3 g/l	Mut^+ / extracellular	(Tschopp, et al. 1987)
B. licheniformis α-Amylase	2.5 g/l	Mut^- / extracellular	(Paifer, et al. 1994)
Phytase	4.2 g/l	extracellular	(Luo, et al. 2007)
Hepatitis B surface antigen	0.4 g/l	Mut^s / intracellular	(Cregg, Tschopp, et al. 1987)
Tumor necrosis factor (TNF)	10 g/l	Mut^s / intracellular	(Sreekrishna, et al. 1989)
Pertussis antigen P69	3 g/l	Mut^s / intracellular	(Romanos, et al. 1991)
Tetanus toxin fragment C	12 g/l	Mut^s & Mut^+ / extracellular	(Clare, Rayment and Ballentine, et al. 1991)
Tick anticoagulant protein	1.7 g/l	Mut^s / extracellular	(Laroche, et al. 1994)

P. pastoris prefers respiratory mode of growth. As opposed to fermentative cultures it doesn't secrete toxic products such as ethanol and formaldehyde at excessive amounts (Cereghino and Cregg 2000).

P. pastoris is able to grow at high cell densities in a simple defined medium (Zhang et al., 2007). Its concentration can reach up to 130g dry cell/liter medium (G. Wegner 1990). Because of the positive correlation with the biomass production and the recombinant protein production, ability to grow at high cell

densities is a major advantage (J. M. Cregg, Distinctions Between Pichia pastoris and Other Expression Systems 2007)

Eukaryotic protein synthesis pathway which makes disulfide bond formation available, thus correct folding of the eukaryotic proteins is possible, as opposed to *S. cerevisiae*, it doesn't hyper-glycolisate the secreted proteins (Cereghino and Cregg 2000).

Integrated vectors ensure the genetic stability of the recombinant DNA elements even in case of extended runs of continuous and batch runs in large-scale (M. Romanos 1995).

In this research *P. pastoris* has been chosen as the expression system due to its advantages as an expression system.

2.3.3 Promoters used in *Pichia pastoris* systems

Several promoters are available for heterologous protein expression in *P. pastoris.* However the most widely used ones are AOX1 FLD1 and GAP promoters.

2.3.3.1 pAOX1

AOX1 expression is contolled by both induction and catabolite repression/derepression mechanisms and tightly regulated at the transcriptional level (Tschopp, et al. 1987). AOX1 promoter is repressed by glucose and by most of the carbon sources, de-repressed in response to carbon source limitation and induced in presence of MeOH in the medium (Koutz, et al. 1989).

AOX1 is one of the strongest, most regulated promoters known, thus utilized as a promoter on expression vectors for production of heterelogous proteins in *P. pastoris* (Cereghino and Cregg, Applications of yeast in biotechnology: protein production and 1999). The protein expression in these yeast strains are 10-100 times higher than the that of *S. cerevisiae* with the utilization of the AOX1 promoter by expression vectors (Gre0410). Invitrogen designs and sells variety of *P. pastoris* expression vectors. In our research pPICZαA expression vector is used.

2.3.3.2 pGAP

In 1997 Waterham and his co-workers isolated the glyceraldehyde-3-phosphate dehydrogenase gene and identified its promoter. Using pGAP expression vectors encoding *E. coli* β-lactamase, *P. pastoris* GAPDH and AOX enzymes has been construct. In each of these three cases, constructs with GAP promoter yielded better results compared to the ones utilizing AOX1 promoter (Waterham, et al. 1997). pGAP is as stong as AOX1 promoter and can be an alternative in processes where presence of MeOH is undesirable. Compared with AOX1 promoter, pGAP utilization simplifies the production processes by removing the need for switching between different carbon sources.

2.3.3.3 pFLD1

Glutathione-dependent formaldehyde dehydrogenase catalyzes the assimilation of formaldehyde and involved in metabolism of certain alkylated amines. It is expressed independently in response to MeOH or methylamine presence in the medium. The strength of FDL1 promoter in response to both inducers are found to be similar to that of pAOX1 (Shen, et al. 1998).

2.3.4 Inducible promoters vs. constitutive promoters

Depletion of rare tRNAs during translation of foreign mRNA is the principal reason for the reduction in growth of the host cells that can be named as an overall toxic response. This can have significant results in protein yield if the exogenous gene is expressed using a constitutive promoter. Transcription of the gene before the culture has reached optimal density may cause depletion of the host' rare tRNAs, resulting in growth reduction and even complete cessation of cell growth. Under these conditions cell densities are always low, with a commensurate reduction in protein yield. This problem can be largely circumvented through the use of an inducible expression vector, whereby promoter is normally inactive unless induced. When using an inducible expression system, the culture of transformed host cells are allowed to reach maximal density, then the cells are induced. The rationale here is that once the culture is saturated, the cells' translational resources presumably do not have to be used for cell proliferation, so they can be dedicated to expression of the foreign gene product. As a general rule, inducible expression vectors offer the greatest flexibility and the best chance of achieving maximal expression (Gre044).

2.3.5 Use of various secretion signals for secretion of the recombinant proteins

In 1994 Paifer and his research group cloned BLA coding DNA sequence with native and *S. cerevisiae* SUC2 signal sequences in *P. pastoris* MP 36 strain with His- phenotype. Mutants having bacterial signal sequences yielded 0.9 g/L rBLA while the one with yeast signal sequence yielded 2.5 g/L. Besides 95% of the rBLA production was found to be secreted into extracellular medium with SUC2 signal while that was restricted to 75% with bacterial one (Paifer, et al. 1994).

In some cases it is evident that better results might be yield with the native signal sequences just as the case in Korona's work (Korona et.al, 2006). Two different xylanase genes *xyn6* and *xynB* were isolated from *Aspergillus niger* and cloned into *P. pastoris* genome with native and *S. cerevisiae* α-factor secretion signal. The result was slightly better product yields with native signal sequences. *xynB* production decreased to 140 mg/L from 150 mg/L to in presence of heterologous secretion signal while decay in *xyn6* was more significant, to 180 mg/L from 220 g/L (Korona, Korona and Bielecki 2006).

2.3.6 pPICZαA expression vector

pPICZαA vector is used to express and secrete recombinant proteins in *P. pastoris*. It is also designed to be used as a shuttle vector, for its maintenance and screening in *E. coli*. It can be used with any *Pichia* strain and allows high level, methanol inducible expression of gene of interest. Gene map of the pPICZαA expression vector is shown in Fig 2.7

pPICZα vectors differ only in their multiple cloning sites and share the following elements in common;

- AOX1 promoter at the 5′ end for tightly regulated and strictly controlled MeOH induction of the expression of the insert gene
- α-factor secretion signal from *S. cerevisiae* for secretion of the recombinant protein from *Pichia pastoris*.

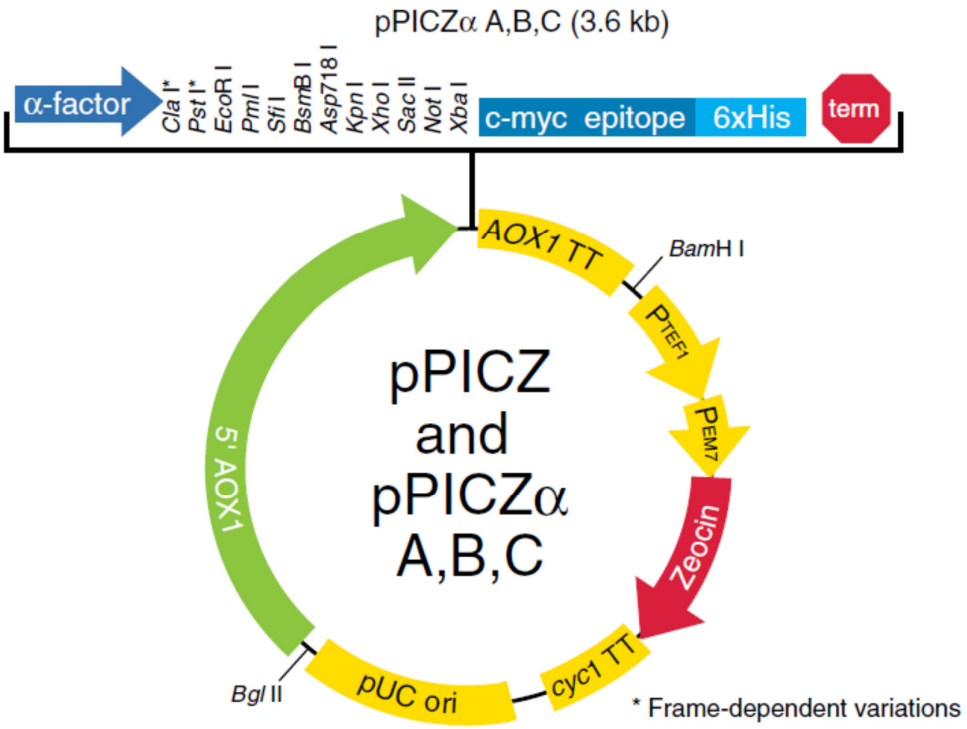

Figure 2.8 Map of the pPICZα vector (Pichia Pastoris Expression System product literature 2011)

- C terminal polypeptide containing C-myc epitope and polyhistidine (6Xhis) tag for easy purification of the recombinant protein in case needed.

- Zeocin ™ antibiotic resistance gene for selection both in *E. coli* and *P. pastoris* strains

Multiple cloning site of the pPICZαA plasmid is given in detail in Appendix D.

2.4 Cell Growth Kinetics

Derivations of the equations used in specific rate and yield calculations are presented below;

2.4.1 Kinetics of batch culture

Microbial growth is defined as increase in both cell size due to chemical reactions occur within the cell and cell count as a result of replication.

Mass balance in a batch reactor can be written as;

$$r_x V = \frac{d(C_x V)}{dt} \tag{2.1}$$

Specific growth rate is defined as the rate of cell formation in a definite time

$$\mu = \frac{dC_x}{dt} \tag{2.2}$$

Biomass formation rate is the product of specific growth rate and the cell concentration;

$$r_x = \mu C_x \tag{2.3}$$

In fed-batch operation volume is not constant due to intermittent nutrient additions, combining equations yields;

$$\mu C_x V = \frac{d(C_x V)}{dt} \tag{2.4}$$

$$\mu C_x V = V\frac{dC_x}{dt} + C_x\frac{dV}{dt} \tag{2.5}$$

Inlet flow due to nutrient addition is denoted by Q;

$$Q = \frac{dV}{dt} \tag{2.6}$$

Rearranging eqn. (2.5) yields

$$\mu C_x V = V\frac{dC_x}{dt} + QC_x \tag{2.7}$$

$$\mu = \frac{1}{C_x}\frac{dC_x}{dt} + \frac{Q}{V} \tag{2.8}$$

In our research, glycerol is given batchwise at glycerol batch phase, thus material balance for glycerol can be written as;

$$r_{GLY}V = \frac{d(C_{GLY}V)}{dt} \tag{2.9}$$

$$r_{GLY}V = V\frac{dC_{GLY}}{dt} + C_{GLY}\frac{dV}{dt} \tag{2.10}$$

Since specific glycerol consumption rate is defined as the glycerol consumed per unit mass of cell;

$$r_{GLY=}q_{GLY}C_x \tag{2.11}$$

Rearranging eqn. 2.10 with 2.11 and 2.6 gives;

$$q_{GLY} C_x V = V \frac{dC_{GLY}}{dt} + C_{GLY} Q \tag{2.12}$$

$$q_{GLY} = \frac{1}{C_x} \frac{dC_{GLY}}{dt} + \frac{C_{GLY} Q}{C_x V} = \frac{1}{C_x} \left(\frac{dC_{GLY}}{dt} + \frac{C_{GLY} Q}{V} \right) \tag{2.13}$$

Mass balance for the fed-batch phase is written for MeOH as;

Rate of change of MeOH = MeOH in – MeOH out + accumulation or consumption

$$\frac{d(C_M V)}{dt} = Q C_{Min} - 0 + r_M V \tag{2.14}$$

$$r_M = -q_M C_x \tag{2.15}$$

Inserting eqn. 2.15 into 2.14 and writing the derivative function explicitly yields;

$$V \frac{dC_M}{dt} + C_M \frac{dV}{dt} = Q C_{Min} - q_M C_x V \tag{2.16}$$

Assuming quasi steady state, in which nutrient fed is instantly consumed by microorganism and the concentration of the nutrient is close to "zero", thus;

$$C_M \frac{dV}{dt} \cong C_M Q \tag{2.17}$$

Rearranging eqn. 2.16,

$$V \frac{dC_M}{dt} + C_M \frac{dV}{dt} = V Q C_{Min} - q_M C_x V \tag{2.18}$$

Specific MeOH consumption rate can be calculated by rearranging the eqn. 2.18

$$q_M = -\frac{1}{C_X}\left\{\frac{dC_M}{dt} + \frac{C_M}{V}\frac{dV}{dt} - QC_{Min}\right\} \quad (2.19)$$

Where dC_M/dt is generally neglected in quasi state assumption

Running a similar mass balance for recombinant protein without inlet & outlet term, the mass balance ;

$$q_{rp} = \frac{1}{C_x}\left(\frac{dC_{rp}}{dt} + \frac{C_{rp}Q}{V}\right) \quad (2.20)$$

"CQ/V" term is occasionally referred as "extracellular dilution term" in literature. Omitting it will result in batchwise operation assumption.

To sum up, specific rates μ, q_{GLY}, q_M, q_{rp} can be calculated using equations 2.8, 2.13, 2.19 and 2.20 respectively.

2.4.2 Yield coefficients

In general, yield is defined as the unit product pruced per substrate in definite time. If the time is defined as the as the whole process, overall yield obtained;

$$Y_{P/S} = -\frac{\Delta P}{\Delta S}$$

Frequently used yield coefficients are summarized in table 2.3;

Table 2.3 Frequently used yield coefficients

Symbol	Definition	Unit
$Y_{X/S}$	Mass of cell produced per unit mass of substrate consumed	kg cell / kg substrate
$Y_{X/O}$	Mass of cells produced per unit mass of oxygen consumed	kg cell / kg oxygen
$Y_{S/O}$	Mass of substrate consumed per unit mass of oxygen consumed	kg cell / kg oxygen
$Y_{P/X}$	Mass of product formed per unit mass of cell	kg product / kg cell
$Y_{P/S}$	Mass of product formed per unit mass of substrate consumed	kg product / kg substrate

If the time window is scaled down to a specific period in the process, then instantaneous yield is obtained,

$$Y^{\iota}_{P/S} = -\frac{dP}{dS} = -\frac{dP/dt}{dS/dt}$$

2.5 Effect of bioreactor operation parameters on biomass and recombinant protein production in *P. pastoris*

Product yield of the process is mainly dependent on pH, temperature, aeration rate, and nutrient feeding strategies employed during fermentation.

2.5.1 Temperature

P. pastoris grows optimally at 30 °C, however optimum growth temperature and the temperature at which recombinant product yield is at its maximum is not necessarily need to be the same. Jin et al. (2010) reported that it is possible to increase porcine interferon-α production more than 100 fold by decreasing the temperature to 20 °C from 30 °C (Jin et al., 2010). At 30 °C, ratio of death cells to total cell count is nearly 5 fold higher than that at 22 °C (Wang, et al. 2009). This causes higher amount of proteases released from death cells, and higher rate of recombinant protein degradation subsequently.

2.5.2 Dissolved Oxygen

DO levels are very critical for cultivations of *P. pastoris*. In the first step of MeOH utilization, while MeOH is converted to formaldehyde, H_2O_2 is formed. This step is catalyzed by AOX in presence of O_2. If excessive amount of O_2 is supplied in MeOH unlimited conditions, cell inhibition may occur due to accumulation of H_2O_2 and formaldehyde in cells.

2.5.3 pH

P. pastoris grows between pH 3-7 and cell growth is not significantly affected within the range (E. H. Wegner 1983). However varying pH condition significantly affect the final recombinant protein concentration since stability of any protein extensively depends on pH and the activities of native proteases of *P. pastoris* are dependent on pH. Thus there is no common pH for maximum recombinant protein yield for every product, it must be examined for every single product independently. For example, while the optimum pH for production of mouse epidermal factor and Human serum albumin is reported to be 6.0, it is 3.00 for production of insulin-like growth factor_I and cytokine growth-blocking peptide (Koganesawa, et al. 2002; Brierley 1994; Kobayashi, Kuwae, et al. 2000; Clare, Rayment and Ballentine, et al. 1991).

2.6 Feeding Strategies

Various carbon feeding strategies have been developed for *P. pastoris* in order to use it as an expression system. Glycerol fed batch and methanol fed batch stages are proven to be crucial for the expression of the recombinant protein.

2.6.1 Glycerol fed-batch stage strategies

In glycerol fed-batch stage most of the strategies are focused on eliminating the repressing effect of glycerol at the end of the stage and getting the cells ready for the induction stage by derepressing the AOX1 promoter.

Decision of when to cease the glycerol fed-batch stage must be carefully made. Keeping glycerol fed-batch stage too long will increase the final biomass yield but adversely affect the specific product yield. 160-180 g wet cell per liter is

found to be good end point, and the exponential feeding of the glycerol at a rate of 0.16-0.18 h^{-1} is recommended (Wei, Zhou and Zhang 2008). Bahrami et al. reported that feeding glycerol to support specific growth rate of 0.21 in the beginning of the process and subsequently reducing it to 0.15 till the end of the stage will increase the final product yield almost 50% (Bahrami, et al. 2008)

2.6.2 Methanol fed-batch stage strategies

As *P. pastoris* cells are sensitive to the MeOH concentration in the medium, MeOH feeding strategy has an impact on recombinant protein production. Most of the research has been done on optimization of the MeOH fed-batch stage and several MeOH feeding strategies has been established. Which method to prefer generally depends on specific product of interest, strain and process conditions.

2.6.2.1 DO Stat Control

In DO stat control, MeOH feeding rate to the culture broth is determined by the dissolved oxygen (DO) measurements at constant aeration conditions. As the DO tends to decrease MeOH feed rate decreased and vice versa. Jimenez et al. (1997) reported that using DO stat control yields 5.135 g/l recombinant dextranase in MP36 Mut^s strain of the *P. pastoris* at 1 vvm aeration rate. Which was almost two fold higher than that was achieved by predetermined MeOH feeding in the same process conditions.

2.6.2.2 Constant methanol concentration

MeOH concentration during bioreactor operations is very curicial. Wu and his co-workers (2000) made a series of researches in order to find out the optimal MeOH concentration for r-Lipase production in different Mut phenotypes of *P.*

pastoris GS-115. 1g/L for the Mut^s phenotype and 7.5 g/L MeOH concentration for Mut$^+$ phenotype are found to be optimum for the maximal product yield per liter of medium (Wu, Chu, et al., Methanol Induction Optimization for Recombinant Human Consensus Interferon Mutant Production in Pichia pastoris 2010, Wu, Yu, et al. 2011). Katakura and his co-workers reported that increasing the MeOH concentration in the medium increases the specific recombinant protein production in expense of decreasing specific growth rates. At 31 g/l, DNA replication and membrane synthesis is inhibited severely and specific growth rate decreases to 0.0009 h^{-1} (Katakura, et al. 1998).

In 2004, Damasceno et al. also used GS115 Mut^s strain as the host and carried out several experiments in fed-batch MeOH feeding strategy in order to find optimum pH and MeOH concentration in the medium for maximum scFV production. They found that optimum MeOH concentration to be 5 g/l although they carried out their optimum pH experiments in presence of 0.1% MeOH and obtained 4.88 g/l scFV production. It was observed that when MeOH concentration is 0.01%, cell growth significantly decreases and scFV production becomes 0.26 g/l in this case (Damasceno, et al. 2004).

2.6.2.3 Predetermined linear methanol feeding

In 2002, Li et al. compared 3 different constant MeOH feeding rates in terms of their effect on endostatin production in GS115 Mut$^+$ strain. While 15 g/l.h MeOH feeding at induction stage yielded higher final cell density, 11g/l.h MeOH feed gave the best result in terms of final and specific endostatin production (Li, et al. 2002).

This result is very close to the MeOH feeding rate recommended by Invitrogen. Several published fermentation strategies are based on this linear feeding profile.

Although this procedure yields satisfactory results, it is not aimed for improving production efficiency with respect to time, yield or substrate composition. Feed forward control mechanism based on specific growth rate, μ, which results in a exponential feeding profile, is preferred recently in many researches (Dietzsch, Spadiut and Herwig 2011).

2.6.2.4 Predetermined exponential methanol feeding

In 2002, Trinh and his co-workers compared the efficiency of MeOH control with online gas sensor, DO signal control and predetermined feed rate on recombinant protein yield using *P. pastoris* GS115 Mut^+ phenotype as a host. They observed that DO signal control and online gas sensor control yielded twice as much biomass compared with predetermined feed rate control. Final endostatin concentration in the medium was about 50% higher in case MeOH is fed with a predetermined rate to control the growth at 0.02 h^{-1}. (92 mg/l endostatin was produced) (Trinh, Phue and Shiloach 2003).

Most of the complication of the exponential feeding arises when the cells achieve maturation and death phase subsequently. After that MeOH feeding should be decreased as opposed to what exponential strategy offers. In 2000, Kobayashi et al. suggested a model which takes morphological changes of the cell culture into account. They utilized *P. pastoris* GS115 strain under the control of a mutant AOX2 promoter. Starting with μ=0.025 h^{-1}, specific growth rate was gradually decreased to 0.002 h^{-1}, at the same time, MeOH feed rate was determined by a discrete equation in which maintenance coefficient, growth yield on MeOH, total volume, specific growth rate and total biomass were taken into account. At the end of 350 hours of induction, almost 6.5 g/l rHSA was obtained in the culture medium (Kobayashi, Kuwae, Ohya, Ohda, Ohyama, & Tomomitsu, High Level Secretion of Recombinant Human Serum Albumin by Fed-Batch Fermentation of

the Methylotrophic Yeast, *Pichia pastoris*, Based on Optimal MeOH Feeding Strategy, 2000).

In the end it seems like independent of the control mechanism used, keeping the MeOH concentration between 1-5 g/l usually yields best results for recombinant protein production.

2.6.3 Mixed feeding strategies

There has also several studies been done about co-feeding of a secondary carbon source together with methanol in order to both supplement growth and inhibit the proteases.

2.6.3.1 Methanol – Glycerol

For the first time in 1990 Brierley and his colleagues suggested that co-feeding of MeOH would cover the cons of Mut- cultivation by increasing the specific growth rate of the cells. Similar results to Mut^+ were obtained with Mut^s cells fed with 1.8 g/l.h glycerol in addition to unlimted MeOH. Speicifc product yield was increased 4.5 fold compared to Mut^s cells fed with MeOH as a sole C source in the induction phase. Bierley et al. stated that lower expression levels at higher glycerol feeding rates are most probably because of this EtOH inhibition effect EtOH is the byproduct of glycerol metabolism and a strong inhibitor of AOX1 promoter. 50 mg/l EtOH is enough for repression of the promoter. (Brierley, Bussineau, et al. 1990).

McGrew et al. (1997) employed the methanol-glycerol feeding scheme to Mut^+ strains at different ratios of the substrates. 2:1 MeOH:Glycerol ratio yielded the

highest specific product yield. Due to better cell growth at 1:1 ratio final product yield was higher in this case (McGrew et al. 1997).

Katakura and his coworkers observed that at constant MeOH concentration of 5.5%, feeding 5g glycerol/h.l medium increases the specific production 2.3 fold, and specific growth rate 20% (Katakura, et al. 1998).

2.6.3.2 Methanol – Sorbitol

In 1999, Thrope et al. compared glycerol and sorbitol mixed feed strategies and concluded product yield of both strategies quite similar, and sorbitol-methanol mixed feed strategy should be preferred because of the repressing effect of the glycerol in case limiting conditions for the co-substrate cannot be met (Thrope, d'Anjou and Daugulis 1999).

Çelik et al. (2009) stated that batchwise addition of the sorbitol at the beginning of the MeOH induction phase will increase the total rHuEpo production in Mut^+ strains 1.8 fold, and decreases the total production time. Maximum cell density was reached at the 24^{th} hour of the induction phase, duration of the lag phase is eliminated and oxygen demand of the cells diminishes, which may be a major design problem in large scale fermentations (Çelik, Çalık and Oliver 2009).

2.6.3.3 Methanol - Casamino acids

In 1998, Chauan et al. supplemented MeOH feed with the 0.1% casamino acids at every 24 hours of induction. While it doesn't have a significant effect on final cell density, they observed a two fold increase in total rHBsAg production (Chauhan, Arora and Khanna 1999). Addition of casamino acids inhibits the proteases by occupying their active sites.

2.6.4 Continuous Cultures

In continuous cultures, it is possible to achieve higher amounts of recombinant protein than that is possible with fed batch.

In 2007, Yamawaki et al. assessed the efficiency of the fed-batch and continuous systems on single-chain variable fragment antibody (scFv) production. They reported that in continuous cultures scFv concentration increases with decreasing dilution rate and maximum of 810 mg/l scFV produced at a dilution rate of 0.0094 h^{-1}, that production figure is almost 4 fold higher than what is reported for fed-batch feeding (Yamawaki, et al. 2007). However, the use of different Mut phenotype for the same protein may favor the fed-batch feeding instead of continuous just as the case in Damasceno's (2004) research.

2.7 Recombinant amylotic enzymes produced by *P. pastoris*

Fierobe and his coworkers (1997) has cloned the *Aspergillus niger* glucoamylase coding DNA sequence on to a pHIL-D2 expression vector with the *A. awamori* signal sequence, creating a Mut- phenotype making use of *P. pastoris* GS 115 strain. Centrifugation, ultrafiltration and column separation methods were used in order to purify the recombinant enzyme respectively, and the total concentration of it was calculated using the results of the glucose oxidation enzymatic assay and the known specific activity of the enzyme. They obtained 0.4 g/l recombinant *A.niger* glucoamylase at a single batch with 48 h of incubation and 0.75% initial MeOH concentration at BMMY medium. They also observed decreasing the volume of the production medium to 1ml increases the recombinant protein production to 1g/l, which may be as a result of better oxygen transfer to the cells (Fierobe, et al. 1997).

In 2000, Muslin and his co-workers cloned the Hordeum Vulgare α-Glucosidase to *P. pastoris* GS 115 strain via pPIC9 expression vector. Using α-factor signal peptide 2 g/l recombinant protein was yielded extracellularly in BMM medium under controlled production parameters with the use of a bioreactor (Muslin E.H. 2000).

In another research carried out by Chen (2009) and his co-workers, 3 g/l mannanase was obtained at the 96th hour of the 10 L bioreactor operation. *P. pastoris* x33 strain and pPICZαA vector were used to obtain a Mut^{+} phenotype at the end of transfection. After the transfection, the best producer strain has been selected by determining the one shows highest activity in enzymatic assay. Bioreactor was operated at 28 °C and between pH 5-5.5 (Chen X. 2009).

Also there has been some extremophilic enzymes has been made produced in *P. pastoris* extracellularly. In 2009 Luo and his coworkers transfected the *P. pastoris* GS 115 strain with the pPIC9 vector carrying the β-mannase coding DNA sequence of the acidophilic *Bispora* sp. MEY-1 fungus fused with the α-factor secretion signal of the *S. cerevisiae*. Cells were grown and induced in the media and conditions set by Invitrogen's *P. pastoris* expression manual, sampled at every 12 h. DNS method was used as the assay of the β-mannase activity and at the 120th hour of the bioreactor operation maximum yield on recombinant protein has been reported . Samples were purified for β-mannase by centrifugation, ammonium sulphate precipitation, dialysis in Tris-Cl buffer, FPLC, ultrafiltration and by gel elution respectively. SDS and Bradford assay were made used together for the concentration analysis of the recombinant protein. the Maximum β-mannase concentration was reported as 1.56 g/l at the 120th of the operation (Luo H. 2009).

CHAPTER 3

MATERIAL & METHODS

3.1 Chemicals

Unless otherwise stated analytical grade chemicals an solutions of Dico Laboratories, Fluka Ltd., Roche and Merck Ltd were used.

3.2 Buffers and stock solutions

Formulation for the all buffers and solutions were given in Appendix A. They have all been prepared using distilled water, sterilized either by autoclaving at 121 °C for 20 minutes or passing through 0.20 and 0.45 μm filters (Sartorius AG, Gottingen, Germany) depending on type of solutions and stored at +4 °C.

3.3 The Microorganisms

E.coli TOP10 and *P. pastoris* x-33 strains were obtained from Invitrogen. *P. furiosus* strain was obtained from DSMZ under the stain number of DSM 3638. Expression vector is pPICZαA and this has also been supplied from Invitrogen.

3.4 Growth of *P. furiosus*

Growth medium is slightly modified version of the *Pyrodictium* medium, defined by the DSMZ (DSMZ 2007). Composition of the *Pyrodictium* medium is given in Appendix B. pH is adjusted to 5.5 with 10N sulfuric acid, medium is de-oxygenated via sparging nitrogen gas prior to inoculation, since the organism is obsolete anaerobic. *P. furiosus* has been grown overnight in capped bottles immersed in water bath at 98 °C.

3.5 Genomic DNA isolation of *P. furiosus*

Cells are harvested from the 50ml of medium by centrifuging at 4000 rcf for 15 minutes, and suspended sulphur in the medium is seperated from the cell pellet in the same time. Cells are resuspended in 450 µl of TE buffer, transferred to 1.5 ml eppendorf tube and after the addition of 50 µl of 10% SDS buffer. Tube contents are mixed via inverting the tube for 5-6 times. 5 µl of RNAse A is added, incubated for 15 minutes at room temperature. 25 µl proteinase K is added and incubated for 1 hour at 55 °C. 0.5 ml of PCI is added and tube contents are mixed by inverting tubes for 15 minutes gently. Following centrifugation at 16000 rcf for 15 minutes, aqueous phase is transferred to a new 1.5 ml eppendorf tube. PCI extraction step is repeated until a clear aqueous phase with no interphase is obtained. 0.5 ml of chloroform is added and centrifuged for 15 min at 16000 rcf. Aqueous phase is transferred to an eppendorf tube and mixed with 400 µl isopropanol and the contents are centrifuged at 16000 rcf for 5 minutes. Pellet is washed with 70% EtOH and dried under vacuum at room temperature. Genomic DNA can be is dissolved in 25 µl of TE buffer (Koning 2011).

3.6 Gene amplification by PCR method

Extracellular α-amylase gene of *P. furiosus* has been deposit in gene bank by Jorgensen and his co-workers under the accession number of U96622. The sequence of this gene together with its protein binding and signal sequences is given in appendix C. Following forward and reverse primers specific to PFA coding region of the *P. furiosus* genome was used. Forward primer was designed so that it excludes the native secretion signal of PFA.

Forward primer:
GC GAATTC GCAAAAT ACTTGGAGCTTGAAGAG

Reverse primer:
GAG CCGCGG CTCACCCAACACCACAATAAC

EcoRI and SacII specific regions were added to the forward and reverse primers respectively together with the additional 2-3 bases to increase the cleavage efficiency.
Melting temperatures of the primers were calculated by the online Tm calculator application of the Applied Biosystems on the web (Applied Biosystems 2011). Annealing temperature of the PCR reactions are set 5°C less than the melting temperatures of the primers in the reaction medium.

PCR reaction mixture consists of;

5 ul 10X PCR buffer
4 µl 25mM $MgSO_4$
10 µl 2mM dNTP mix

2 μl Forward primer

2 μl Reverse primer

2 ng Template DNA

1.25 units Pfu DNA polymerase

26 μl dH_2O

PCR cycles were programmed as follows;

Initial denaturation step : 3 minutes at 96 °C

Denaturation step : 40 seconds each at 96 °C

Primer annealing step: 40 seconds at 52 °C

Extending step: 5 minutes at 72 °C

Final extending step: 5 minutes at 72 °C

Between initial denaturation and final extending steps; denaturation, annealing and extending steps are cycled 30 times.

3.7 Propagation of pPICZαA vector

pPICZαA vector is stored in *E. coli* XL-1 strains in 13% glycerol solution at -80 °C. For propagation 5ml of glycerol medium is spread over LB agar, at the end of 12h. incubation at 30 °C single colony is picked and inoculated in 50 ml of LB broth, in the exponential growth phase cells are harvested.

3.8 Plasmid isolation with Alkaline Lysis method

Plasmid DNA to be used in digestion reactions and electrophoresis analysis can be isolated using alkaline lysis method. Following steps should be followed for isolation of the plasmid DNA;

Preperation of the cells

1. Inoculate the 1 ml LB broth with the single colony of transformed bacteria. Incubate at 37 °C with vigorous shaking.
2. Pellet the cells with centrifugation at 2000 g for 10 minutes at 4 °C.
3. Remove the medium by gentle aspiration , leave the tubes as dry as possible.

Lysis of the cells

4. Add 200 µl of Alkaline lysis I solution, resuspend the cells with vigorous vortexing. Make sure that the pellet is dissolved completely.
5. 400 µl of freshly prepared Alkaline lysis II solution is added. Contents are mixed by inverting the tubes for 5 times. Tubes can be stored on ice.
6. 300 µl of ice-cold Alkaline lysis is added to the tube contents are mixed by inverting several times. Tubes are stored on ice for 3-5 minutes.
7. Tube contents are centrifuged in a microfuge at 10000g for 5 minutes at 4 °C. 600 µl of supernatant of the each tube is transferred to a new microfuge tube.

8. Phenol:chloroform is added at equal amount to supernatant and mixed by vortexing. After centrifugation at maximum speed for 2 minutes at 4 °C aqueous upper layer is transferred to a fresh tube.

Recovery of Plasmid DNA

9. Add 600 µl of isopropanol at room temperature, mix by vortexing and allow tubes to stand at room temperature for 2 minutes.
10. Centrifuge the tubes at room temperature at maximum speed for 5 minutes.
11. Drain the aqueous phase, allow the tubes to stand on a piece of towel at inverted position to allow any drops of liquid to drain away. You can also pipette the liquid drops adhering on the tube walls.
12. Wash the pellet with 1 ml of 70% ethanol, recover the DNA with centrifugation at maximum speed for 2 minutes at room temperature.
13. Drain EtOH and remove any drops adhering to the tube walls by pipetting, evaporate all the EtOH remaining (2-5 minutes).
14. Nucleic acids can be dissolved in 100 µl of TE buffer of distilled water. Distilled water is preffered if subsequent enzymatic reactions exist.

3.9 Digestion of the DNA fragments

In order to create sticky ends, cloning vector and the amplified gene fragments are digested with the SacII and EcoRI digestion enzymes at the following reaction conditions.

3.9.1 EcoRI/SacII & PmeI digestions

Single digests to create sticky ends at the EcoRI and SacII restricition sites of the DNA insert and to linearize the vector construct prior to the transformation into *Pichia* X-33. Reaction mixture consists of following ingredients;

20 μg DNA,	volume varies according to concentration
10xdigestion buffer,	2 μl
Digestion enzyme (10u/μl)	0.5 μl
dH_2O,	fill up to 20 μl

Contents are mixed by swirling and incubated for 4 hours at 37°C in a water bath.

3.9.2 Double digestion with EcoRI & SacII

Double digestion of the pPICZαA expression vector is carried out before ligation with the single digested DNA insert in following reaction mixture

20 μg pPICZαA plasmid DNA,	volume varies according to stock concentration
10x buffer B,	2 μl
EcoRI (10u/μl)	0.5 μl
SacII (10u/μl)	0.5 μl
dH_2O,	fill up to 20 μl

Contents are mixed by swirling and incubated for 4 hours at 37°C in a water bath.

3.9.3 Thermal deactivation of the digestion products

Digestion reaction mixtures are held at 65 °C for 20 minutes for the complete thermal deactivation of the restriction enzymes.

3.10 Agarose gel electrophoresis

For quantification of the PCR products and purification of specific DNA fragments gel electrophoresis method is used. Two different types o gels can be employed; agarose and polyacrylamide. Polyacrylamide gels are used for small DNA fragments up to few hundred bases, while the agarose gel is more suitable for DNA bands ranging from few hundred to 10Mb. In our experiments we used agarose gel for all runs. Agarose gel is prepared by dissolving 400 mg of agarose in 50ml of TE buffer. This mixture is heated up until it boils, then while cooling down 3 µl of ethidium bromide is added for staining of the DNA fragments under UV light. This mixture is poured into the gel cast with combs attached for the formation of the wells in which the DNA fragments are loaded. When it cools down to the room temperature gel solidifies and the combs are removed. It is then placed into the vessel full of 0.5x TBE buffer. As the electric field is applied between the two ends of the vessel, DNA loaded in the gel starts to move from positive to the negative end, through the holes of the gel matrix. The ones with higher molecular weight moves slower in the gel, and the DNA fragments will line up depending on their molecular weights. One expection here will be the case if circular DNA fragments to be run, in this case due to their compact structure they will move faster than their linear equiavalents. For our experiments 0.8% agarose gel is prepared by mixing 0.4 grams of agarose with 50 ml of TBE buffer. Mixture is heated up till it boils, then cooled to 60°C. Before it freezes 3µl of ethidium bromide is added than the gel is poured in cast and left

for freezing. Solidified gel is immersed in 1X TBE buffer in Biorad's Powerpac basic system's bath and charged with electric field at 90 volts for 90 minutes while loading 15 samples and 45 minutes while loading 30 samples.

3.11 Ligation reactions

First of all, single digested insert is ligated to the double digested linear plasmid on the EcoRI restriction site, and digested with SacII enzyme in the same buffer with T4 ligase. Linear vector construct which is estimated to have 4863 base pairs is purified from the agarose gel, then circularized by self circularization reaction. DNA insert ligation and self circularization protocols are as described below;

3.11.1 DNA insert ligation into vector DNA

In order to insert an expression cassette into a double digested plasmid, following steps are followed;

1. In a microcentrifuge tube 1 μg of digested vector DNA and 3 μg of digested insert DNA is mixed
2. Following reagents are added to the same tube;

10X ligation buffer,	2 μl
T4 DNA ligase,	2 units
dH2O,	to 20 μl

3. Contents are mixed by vortexing for 5 seconds.

4. Mixture is incubated at 22 °C for 1 hour.
5. T4 DNA ligase is deactivated by heating the mixture at 65 °C for 10 minutes.

3.11.2 Self circularization of linear DNA

In order to self circularize the linear DNA following procedure is followed;

1. Solution of 30ng linear DNA is preapared in a microcentrifuge tube
2. Following reagents are added in the same tube;

10x ligation buffer,	5 μl
T4 DNA ligase,	5 units
dH_2O,	to 50 μl

3. Mixture is incubated at 22 °C for 1 hour.
4. T4 DNA ligase activity is inhibited by heating the reaction medium to 65 °C for 10 minutes.
5. Resulting mixture can be used for transformation directly.

3.12 Plasmid transformation into *E. coli* via $CaCl_2$ Method

In order to perform $CaCl_2$ transformation first of all E. coli strains must be made passively permeable to DNA by exposing it to high concentrations of divalent cations. Competent *E. coli* XL1-Blue strain is prepared as follows;

1. *E. coli* XL1-Blue strain is grown on agar plates and single bacterial colony is picked from agar plates those have been incubated for 16-20 hours at 37 °C. These colonies are transferred to the 2 x 50 ml LB broths in 250 ml baffled Erlenmeyer flasks.

2. Harvest the bacterial cells to 50 ml polypropylene tubes that has been sterilized and cooled in advance once the OD reaches to 0.35. Keep the tubes on ice-water mix for 10 minutes to stop cellular activity.

3. Pellet the cells by centrifugation at 2700 g for 10 minutes at 4 °C.

4. Decant the cell supernatant, leave the tubes as dry as possible by inverting them on a piece of towel and leaving at this position for 1 minutes maximum. You can wipe out the last drops of the medium from the tube walls with a piece of towel.

5. Resuspend the pellet in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution by gentle vortexing and swirling (80mM $MgCl_2$, 20 mM $CaCl_2$)

6. Pellet the cells by centrifugation at 2700 g for 10 minutes at 4 °C.

7. Resuspend the each 50 ml cell culture cell pellets in 2 ml of ice-cold 0.1 M $CaCl_2$.

8. At this point, cells are named as competent; they can be either used for transformation directly or can be stored in aliquots at -70 °C with 12% glycerol added.

Whether the fresh or frozen competent cells are to be used for transformation, following protocol is followed;

1. 200 µl of competent cells are transferred into sterile and chilled 17x100mm propylene tubes using a chilled micropipette tip. Ligation mixture that contains maximum 50 ng DNA with maximum volume of 10 µl is added to the tube and contents are mixed with swirling. Tubes are stored on ice for 30 minutes.

2. Tubes are transferred to the water bath at 42 °C and kept at this temperature for 90 seconds

3. Tubes are transferred to ice bath and kept for 1-2 minutes.

4. 800 µl of LB medium without antibiotics is added to the each tube and incubated for 45 minutes at 37 °C

5. 50-200 µl of the medium is transferred to the LB agar plates. Wait until all the liquid is absorbed by the agar.

6. LB agar plates are inverted incubated at 37 °C. After 16 hours colonies carrying the plasmid should appear

3.13 LiCl transformation to *Pichia pastoris*

Selection of the strain is crucial in *Pichia* transformation as it determines the Mut phenotype of the mutants. Wild type *P. pastoris* X-33 strain is ideal for transformations, it is useful for large scale fermentation and selection on Zeocin, can grow in YPD and in minimal media. It yields Mut^+ phenotypes at the end of transformation by integrating at 5′ AOX1 region by single crossover (insertion). Schematic drawing of how single crossover integration event happens is shown in figure 3.2

Before starting transformation, linearization of the vector construct within the 5′ AOX1 region is recommended in order to promote integration (Invitrogen 2002).

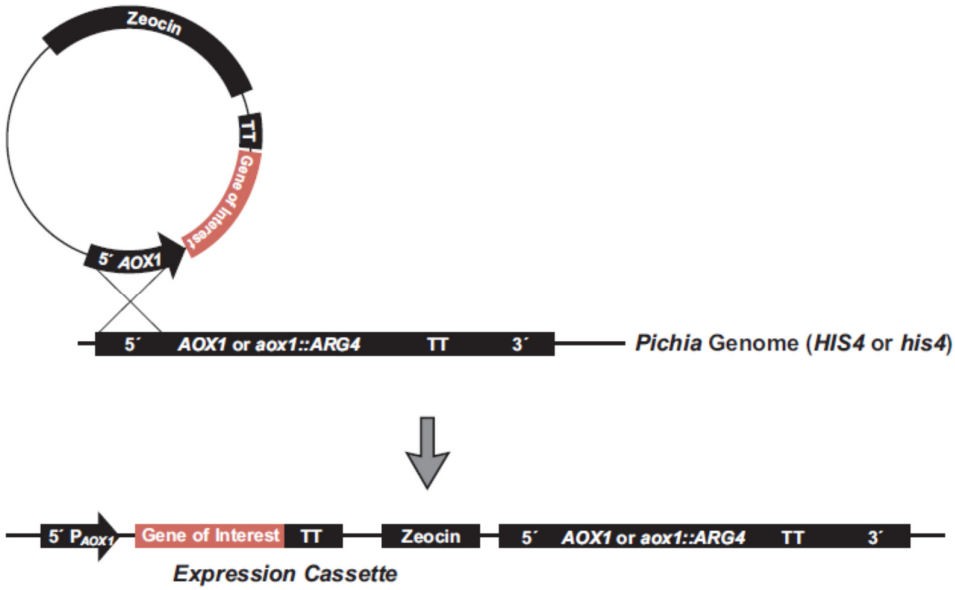

Fig 3.1 Single crossover event in *Pichia* genome

Care must be taken to select the restriction site so that it doesn't ruin the integrity of the expression cassette. pPICZαA:PFA vector construct is linearized at the PmeI resctriction site located the 414th bp of the construct. Small aliquot of reaction mixture is checked for complete linearization after following standard PmeI digestion protocol summarized in this chapter previously. If the vector is completely linearized, restriction enzyme is heat deactivated at 65 °C for 20 minutes, DNA is phenol/chloroform extracted once and precipitated with addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of pure EtOH. DNA is pelleted by centrifugation at 4 °C and 16000 g for 2 minutes, washed with

80% EtOH once, air dried, and resuspended in 10 µl of dH2O. Only after vector construct is linearized, LiCl transformation protocol is initiated by preparation of the competent *P. pastoris* X-33 cells as described below;

3.13.1 Preperation of the cells

Competent *P. pastoris* cells are prepared as follows;

1. *Pichia Pastoris* X-33 strain is incubated on YPD agar with 400µg/ml zeocin concentration for 60 hours at 30 °C . YPD agar preparation is described in Appendix B. Zeocin concentration of the agar plates are 400 µg/ml.
2. Single colony is picked from YPD agar and inoculated in YPD liquid medium. Cells are harvested once the OD_{600} reaches to 0.8-1.
3. Each 50 ml of medium is centrifuged at 1500 x g and 4 °C for 4 minutes and pellet is washed with 25 ml of sterile distilled water. Then again centrifuged at 1500 x g for 6 minutes.
4. Pellet is resuspended with 1 ml of 100mM LiCl solution and transferred to a 1.5 ml microcentrifuge tube.
5. Pellet the cells by centrifugation at maximum speed for 15 seconds on a tabletop centrifuge, remove the LiCl with a pipet.
6. Resuspend the cells in 400 µl of 100 mM LiCl.
7. Prepare 50 µl aliquots of the cell suspension for each transformation in eppendorf tubes. Aliquots should be used immediately.

3.13.2 Transformation

Transformation is carried out immediately after competent cells are prepared according to transformation guidelines described below:

1. Boil 50 µl of the salmon sperm DNA for 5 minutes and quickly chill on ice.
2. Pellet the cells in step 7 above with centrifugation at 4000 x g for 2 minutes. Remove the LiCl by pipetting.
3. Add the reagents to the cells in the following order;

240 µl 50% PEG
36 µl 1 M LiCl
25 µl 2 mg/ml salmon sperm DNA
Linear plasmid DNA (5-10 µg) in 50 µl sterile water

4. Mix the contents by vigorous vortexing until the cell pellet is completely dissolved
5. Incubate the tube for 30 minutes at 30 °C without shaking
6. Immerse the tubes in water bath at 42 °C for 20-25 minutes.
7. Centrifuge at 4000 x g, pellet the cells, and decant the supernatant.
8. Resuspend the cells in 1 ml of YPD free of antibiotics, incubate at 30 °C without shaking
9. At the end of 1st and 4th hour of the incubation plate 25 and 100 µl of the medium on YPD plate with 400µg/ml zeocin concentration.

3.14 Storage of *P. pastoris* strains

Single colonies are picked up from YPD agar and grew overnight in YPD liquid medium. Cells are harvested and resuspended in YPD medium containing 15% w/v glycerol to a final OD of 50-100. Cell banks are frozen in liquid nitrogen immediately and stored at -80 °C.

3.15 Selection of the best producer strains out of positive transformants

Because transformation may occur in many different ways in *Pichia*, and because the site of the transformation will affect the recombinant protein yield, it is essential to make a productivity test on at least 6 colonies (Invitrogen 2011).

Positive *Pichia* transformants were grown in BMGY medium, harvested when they reach 2-6 OD and inoculated into buffered BSM medium supplemented with 2% MeOH, to give initial OD of 1. 2 ml of MeOH per liter of BSM is added in the 24^{th} hour of production and cells are harvested at the 48^{th} hour. Crude enzyme mixture prepared from cell lysate is assayed for α-amylase activity by DNS method, the one that shows highest α-amylase activity per gram of wet cell is selected as the master cell bank for the remainder of the research.

3.16 Recombinant *P. furiousus* α-amylase production

Protein production has been carried out in baffled shake flasks and in a pilot scale bioreactor. Both of these methods have pre-cultivation of *P. pastoris* in common

3.16.1 Precultivation of *P. pastoris*

YPD agar plates with 200 μg zeocin™ /ml is inoculated with frozen *P. pastoris*:pPICZαA::PFA cells from our working cell bank. Composition of the YPD agar plates is given in Appendix B. Cells are grown on YPD agar at 30 °C for 60 h.

20 ml of YPD medium in 100 ml baffled shake flask is inoculated by a single colony from the YPD plate. Cells are harvested in their exponential growth phase by centrifugation at 4 °C and 4000 rcf for 5 min. At exponential phase, OD_{600} of the pre-cultivation medium is between 2 and 6.

3.16.2 rPFA production in shake flasks

Cells from pre-cultivation medium are harvested and inoculated into V=250 ml baffled and air filtered shake flasks containing V=50 ml buffered or un-buffered production medium of *P. pastoris*. The composition of the production medium is given in Appendix B. Inoculum volume is determined so that the initial OD_{600} of the production medium is equal to 1. At t=0 h. and t=24 h. flasks are supplemented with 2% methanol. Flasks are shaken for 48 hours at 225 rpm at 30°C.

3.16.3 *P. pastoris* Fermentation in Bioreactor

Pilot scale bioreactor experiments have been carried out in Biostat CT2-2 bioreactor system with maximum working volume of 3 liters. Vessel has 4 baffles, 2 sensor ports, 3 addition ports, sparger at the bottom, gas condenser and exhaust filter at the top. This unit has base, nutrient and antifoam addition peristaltic pumps which are controlled by the temperature, pH, dissolved oxygen, foam level sensors. Temperature is controlled by injecting steam or

chilled water into the vessel jacket. Both of them are controlled automatically by the control unit. Air sparger is controlled by a cascade mechanism between gas flow controller and the DO sensor probe to set the DO constant at 20% in the medium. pH of the medium is controlled by base addition pump cascaded to the pH probe. Agitation rate is kept constant at 900 rpm in all experiments with 2 separate 4 bladed Rushton blades. One of the blades are positioned at the bottom for stirring the medium inside and the other is positioned at top of the working volume, used for foam breaking. Schematic of the bioreactor system is given in fig. 3.1 below.

A typical fermentation experiment starts with the calibration of the pH probe. pH probe is calibrated using pH 7.00 and pH 4.01 buffers according to the users' manual of the Biostat CT2-2 system. After pH calibration, fermentation vessel is heat sterilized at 121.1 °C for 20 minutes with 900 ml BSM in it. Composition of the BSM is given in appendix B. Once sterilization is finished, vessel is cooled down to 30°C, pH is adjusted to predetermined value by addition of 2N NaOH solution. After temperature and pH is set to process conditions, calibration of the DO probe can be carried out. DO probe is calibrated by sparging N_2 and air at a rate of 10 liters/min while agitating speed is at its maximum, 900 rpm. When the medium is saturated with N_2 , DO reading is set to is set as 0%, and the condition at which medium is saturated with air is set as 100% dissolved oxygen condition. Peristaltic pumps are calibrated with the feeding hoses to be used in the experiment to determine the volumetric flow rates.

Once the sterilization and calibration of the sensors are finished, pre-culture seed is inoculated into the fermentation vessel containing 1 l BSM medium. The amount of inoculums seed is adjusted so that the initial OD_{600} of the culture medium is becomes 1. pH of the medium is adjusted with 2N NaOH solution. Cells are grown batch-wise at N=900 rpm, T=30°C, pH=5 and DO tension ≥ 20%.

When the wet cell density reaches 80 g/L all the glycerol in the medium is almost consumed. From this point on 50% w/v glycerol solution containing 12 ml/L PTM1 is fed at a predetermined exponential rate which is limiting. Glycerol feeding rate is determined by equation 3.1 ;

$$F_{Gly} = \nu_{Gly}.X_{OG}.V_{OG}e^{\mu_{Gly}.t}/\ (0.5\ \text{g ml}^{-1}) \quad (3.1)$$

Where F_{Gly} is the methanol feeding rate in ml.h^{-1}, X_{OG} and V_{OG} are the initial wet cell density (g/liter) and the volume (liter) of the production medium at the beginning of glycerol-fed batch phase respectively, μ_{Gly} is the predetermined specific growth rate (h^{-1}) at glycerol fed-batch phase which is 0.18 in all cases, t is the time in hours and ν_{Gly} is the specific consumption rate of glycerol (g.g^{-1}.h^{-1}). Relationship between ν_{Gly} and μ_{Gly} is determined by Wei and his colleagues as in eqn. 3.2 (Wei, Zhou and Zhang 2008);

$$\nu_{Gly} = 0.503\ g.g^{-1} \times \mu_{Gly} + 0.0065\ g.g^{-1}.h^{-1} \quad (3.2)$$

In the end of glycerol fed batch phase, pH of the medium is slowly adjusted to the set value of the production phase. When the wet cell density reaches 180 g/L, glycerol feed is ceased and %100 MeOH containing 12 ml/L PTM1 is started to be fed at a constant rate of 3.6 ml L^{-1}.h^{-1}. Methanol transition phase takes almost 3.5 hours. At the end of this phase predetermined exponential feeding of MeOH starts. MeOH feeding rate is determined by the equation 3.3;

$$F_{MeOH} = \nu_{MeOH}.X_{OM}.V_{OM}e^{\mu_{MeOH}.t}/\ (0.792\text{g.ml}^{-1}) \quad (3.3)$$

Where F_{MeOH} is the methanol feeding rate in ml.h^{-1}, X_{OM} and V_{OM} are the initial wet cell density (g/liter) and the volume (liter) of the production medium respectively, μ_{MeOH} is the predetermined growth rate (h^{-1}) at methanol fed-batch

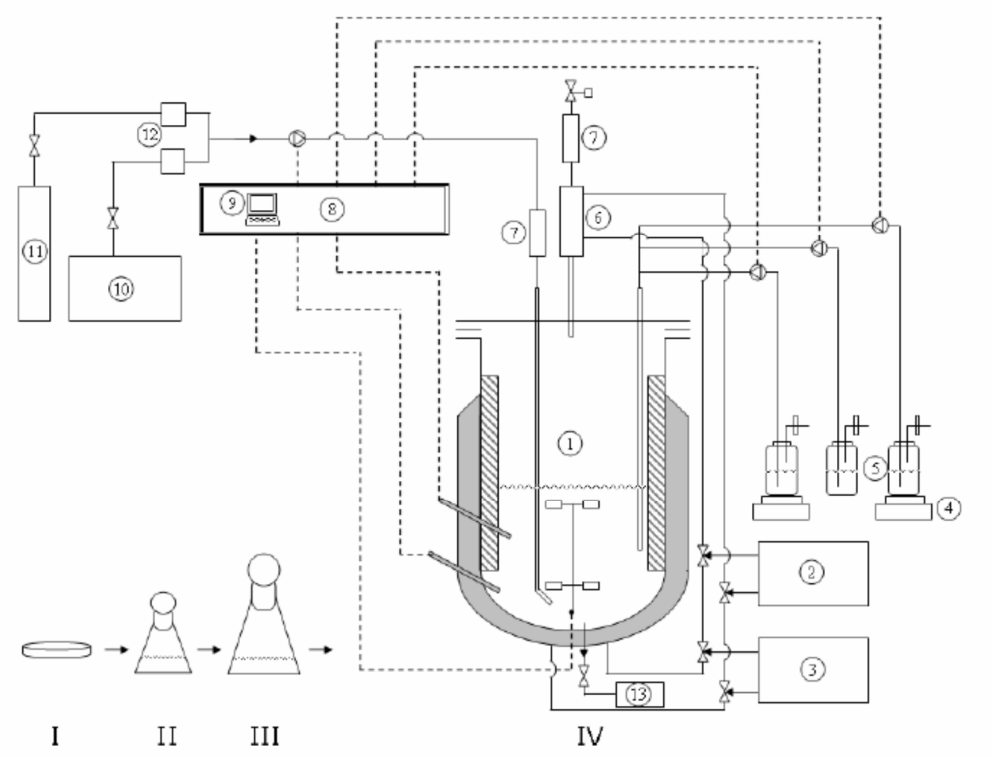

Figure 3.2 Scheme of the Biostat CT2-2 fermenter system (1) Bioreaction vessel, Biostat CT2-2 (2) Cooling circulator (3) Steam generator (4) Balances (5) Feed, base and antifoam bottles (6) Exhaust cooler (7) Gas filters (8) Controller (9) Biostat CT Software (10) Air compressor (11) Pure O_2 tank (12) Digital mass flow controllers (13) Sample valve (Çelik, 2008)

Phase which is equal to 0.03 h^{-1}, t is the time in hours and ν_{MeOH} is the specific consumption rate of methanol (g.g^{-1}.h^{-1}). Relationship between ν_{MeOH} and μ_{MeOH} is determined by Wei and his colleagues as in eqn. 3.4 (Wei, Zhou and Zhang 2008);

$$v_{MeOH} = 0.6\ g.g^{-1} \times \mu_{MeOH} + 0.0094\ g.g^{-1}.h^{-1} \quad (3.4)$$

Once wet cell weight and oxygen demand starts decreasing production phase is ended, cells are harvested by centrifuging at 4000 rcf and 4°C. Cell supernatant and pellet are stored separately for further analysis.

3.17 Crude enzyme preparation

Crude enzyme is prepared according to following guidelines;

1. 0.6 ml of production medium is pipetted into 1.5 ml eppendorf tube and centrifuged in tabletop centrifuge at 4000 rcf and 4°C for 5 minutes.
2. Supernatant is discarded and equal volume of dH_2O is added instead
3. Cell pellet is resuspended by vigorous vortexing for 1 minute.
4. 0.3 grams of glass beads is added
5. The tube is closed tightly and the contents are mixed at a frequency of 12 s^{-1} in a orbital shaker
6. The bottom of the microfuge tubes are drilled by a needle, an empty microfuge tube is placed underneath and cell lysate is recovered by centrifuging at 100 rcf.
7. This cell lysate can be used as the crude enzyme preparation

3.18 Amylase activity assay, DNS method

α-Amylase activity is detected by modified version of the Bernfeld's method. This method is based on the colorimetric detection of the free reducing groups. Dinitrosalicyclic acid reagent reacts with reducing ends of the starch hydrolysate to form 3-amino-5-nitrosalysilic acid which absorbs the light at 540 nm

wavelength. Alkali pH is required for redox reaction is maintained by NaOH and sodium potassium tartarate (Rochelle salt) protects the reagent from dissolved oxygen. 1 unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmole of reducing end in 1 minute in assay conditions. DNS reagents are prepared as described in appendix A. Assay procedure is as follows;

1. Add 360 μl of 2% starch solution to blank and test tubes.
2. Add 40 μl of crude enzyme preparation to the test tube, vortex vigorously for 30 seconds and then incubate in a water bath at 90°C for 5 minutes then cool on ice.
3. Add 200 μl of color reagent solution to both test and blank tube and mix by vortexing at maximum speed for 10 seconds.
4. Add 40 μl of enzyme solution to the blank tube. Incubate both test and blank tubes in a water bath at 90 °C for 4 minutes then cool on ice.
5. Centrifuge the tubes at maximum speed on a tabletop for 60 seconds in order to pellet cell debris. Otherwise remaining cells debris in the medium will contribute to the absorbance reading at 540 nm.
6. Dissolve 100 μl supernatant of test and blank tubes 900 μl of distilled water.
7. Read the absorbance at 540 nm.
8. The change in absorbance due to enzymatic activity ΔA_{540nm} is obtained by using eqn. 3.3.

$$\Delta A_{540nm} = A_{test} - A_{blank} \quad (3.3)$$

Where A_{test} and A_{blank} are the absorbances of the blank and test tubes respectively at 540 nm.

9. On the standard curve using ΔA_{540nm}, read the number of moles of reducing ends released. This is the total number of reducing ends released in 5 minutes by 40 µl of crude enzyme mixture.

A standard curve is plotted as follows;

Add following reagents in a microfuge tube, then incubate at 90 °C for 4 minutes, cool to room temperature on ice

Table 3.1 D-glucose standard preparation

	4 µmole glucose standard	3 µmole glucose standard	2 µmole glucose standard	1 µmole glucose standard	0 µmole glucose standard
0.1 M D-glucose	40 µl	30 µl	20 µl	10 µl	0 µl
dH_2O	-	10 µl	20 µl	30 µl	40 µl
2% starch solution	360 µl	360 µl	360 µl	360 µl	360 µl
Color reagent solution	200 µl	200 µl	200 µl	200 µl	200 µl

Read the absorbance at 540 nm. Plot absorbance vs. glucose concentration. This plot can be used to determine how many µmoles reducing end is released by the amylotic action of the rPFA. One unit of enzyme activity is defined as the µ moles of reducing ends released using D-glucose as standard. Thus, total enzyme activity in 1 µl enzyme preparation can be calculated by dividing this number by incubation time (5mins) and enzyme volume (40 µl). The maximum experimental error in activity measurements were determined as 3%.

3.19 Iodine test

An efficient method for determination of the extend of starch degredation was proposed by Fuwa in 1954. This method is based on blue color development when iodine forms complexes with starch. In standard procedure, 2.5 ml of starch (2% w/v) is incubated with equal volume of α-amylase solution at 37 °C for 30 minutes. The reaction is terminated by adding 5ml 1N acetic acid and the mixture is diluted to 200 ml by adding dH2O and 5 ml of iodine reagent (0.2% iodine and 2% potassium iodide). The amount of color development is measured at 700 nm.

Slightly modified version of Fuwa method was used in our experiments to determine dextrinizing activity. Samples were incubated with equal volume of 2% w/v soluble starch at 90 °C for 4 minutes. The reaction was stopped by cooling on ice, samples were centrifuged at 4000 rcf for 5 minutes and the supernatant absorbances were measured at 492 nm, instead of 700. Dextrinizing activity of the samples are calculated by eqn. 3.4

$$((D_0-D)/D_0)\ x100\ /10 \qquad (3.4)$$

where D is the absorbance of the sample and D0 is the absorbance of the blank sample. The maximum experimental error in activity measurements were determined as 3%.

3.20 Glycerol and methanol assays

Glycerol and methanol concentrations are determined by Waters Allience 2695 High Pressure Liquid Chromatography (HPLC) system with Capital optimal ODS-5μm column (Capital HPLC, West Lothian, UK) installed. Samples are prepared by centrifuging fermentation broth at 4000 rcf for 5 minutes, and then filtering the supernatant from 0.45 nm filters (Acridisc CR PTFR). 5 μl of sample is injected to the column together with 5 mM H_2SO_4 mobile phase flowing at a rate of 0.5 ml/min. Samples are analyzed in Waters 2414 Refractive Index Detector at 410 nm wavelength. Calibration curves for methanol and glycerol are presented in Appendix D.

3.21 Genomic DNA isolation of *P. pastoris*

Genomic DNA isolation is carried out in accordance with the protocol stated by Amberg et al., including slight modifications (Amberg, Burke and Strathern 2000).

1. Cells were grown in 10 ml YPD medium until they reach 6-10 OD.
2. 10 ml of cell broth is centrifuged at 4000 g for 10 minutes to pellet cells.
3. Cell pellet is dissolved in 0.2 ml yeast lysis buffer and 0.2 ml phenol:chloroform:isoamyl alcohol (25:24:1), 0.3 g of glass beads is added and mixture is vortexed vigorously for 4 minutes.
4. 0.2 ml of TE buffer (pH=8) is added and the total mixture is centrifuged at 4000 rcf for 2 minutes in a tabletop centrifuge.
5. Aqueous phase is transferred to a fresh 2ml. eppendorf tube, mixed with 1 ml 100% EtOH by inversion and centrifuged at maximum speed (13200 rcf) for 2 minutes, supernatant is discarded.

6. Cell pellet is re-suspended in 0.4 ml of TE buffer (pH=8) and treated with 10 µl of 10mg/ml RNAse A, and incubated at 37 °C for 5 minutes.

7. After the incubation reaction mixture is mixed with 1 ml of 100% EtOH and 14 µl of 3M ammonium acetate, then DNA is pelleted by centrifugation at 13200 rcf for 2 minutes. Supernatant is discarded and the pellet is air-dried.

8. DNA pellet is dissolved in 50 µl of TE buffer (pH=8)

3.22 Codon bias analysis

Codon Adaptation Index of Sharp and Li (1987) was calculated using the online CAI index calculator (GenScript rare codon analysis tools 2011).

3.23 Nucleotide sequence analysis

The nucleotide sequences are determined by Sagner's method, using ABI Prism 310 Genetic Analyzer in METU Central Laboratory. The results of the nucleotide sequence analysis of the pPICZαA:PFA expression cassette is given in appendix C.

3.24 Wet cell weight measurement

1 ml of cultre broth is pipette in a 1.5 ml Eppendorf tube whose weight is known. Centrifuging at 4000 rcf for 5 minutes cell is pelleted and supernatant is decanted. Weight cell weight in 1 ml of cultre broth is calculated by subtracting the Eppendorf tube weight from the final weight of the tube together with the cell pellet.

CHAPTER 4

RESULTS

4.1 Selection of the expression system

In order to determine the expression efficiency of rPFA in different hosts, CAI (Codon Adaptation Index) was used as a measure. Out of several possible expression hosts, *P. pastoris* got the highest CAI index, which means that rPFA ORF has the greatest resemblance with the proteins from *P.pastoris*, thus has the greatest possibility of being expressed effectively in this host. Table 4.1 shows the CAI scores of several hosts for rPFA expression.

Table 4.1 CAI scores of several expression hosts for rPFA expression and the percentage of low frequency codons

Host	CAI score	Percentage of low frequency (non-optimal) codons, %
E.coli	0.59	12
S. cerevisiae	0.72	2
P. pastoris	0.73	3
Insect	0.63	6
Streptomyces	0.00	63

4.2 pPICZαA:PFA vector construction

P. furiosus has been grown overnight in specified *P. furiosus* growth medium in appendix and genomic DNA isolated as described in Chapter 3.

PFA coding sequence of the *P. furiosus* genome has been amplified by PCR and the amplification has been verified by agarose gel electrophoresis showed in Figure 4.1. PFA coding region is 1308 bp in length.

After verification of the presence of the PFA coding sequence by agarose gel electrophoresis, corresponding DNA sequence has been amplified and thereafter purified from the PCR reaction mixture by Quiagen spin column purification kit.

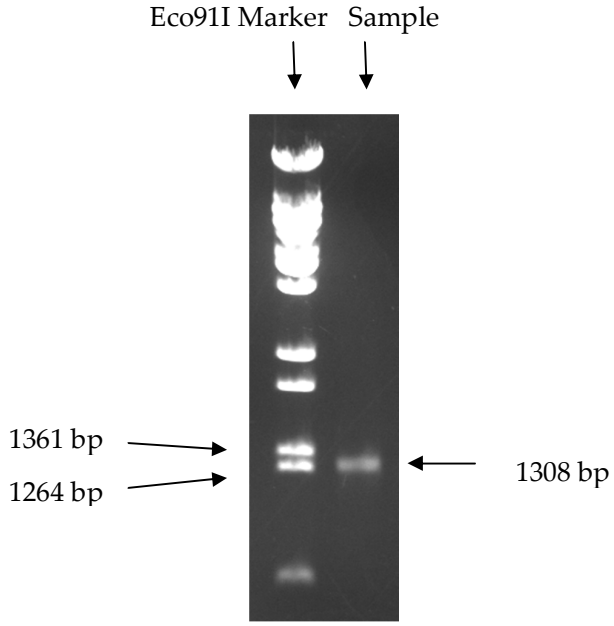

Figure 4.1 Agarose gel electrophoresis image of the PFA coding DNA sequence. Marker; Eco91I, Lane 1; PFA coding DNA sequence of the *P. furiosus*

4.2.1 Isolation of pPICZαA vector

pPICZαA plasmid is isolated from *E. coli* XL1-blue strains using the Alkaline Lysis method. Agarose gel image of the circular and linear pPICZαA plasmid was shown in the 1st and 2nd lanes in Figure 4.2.

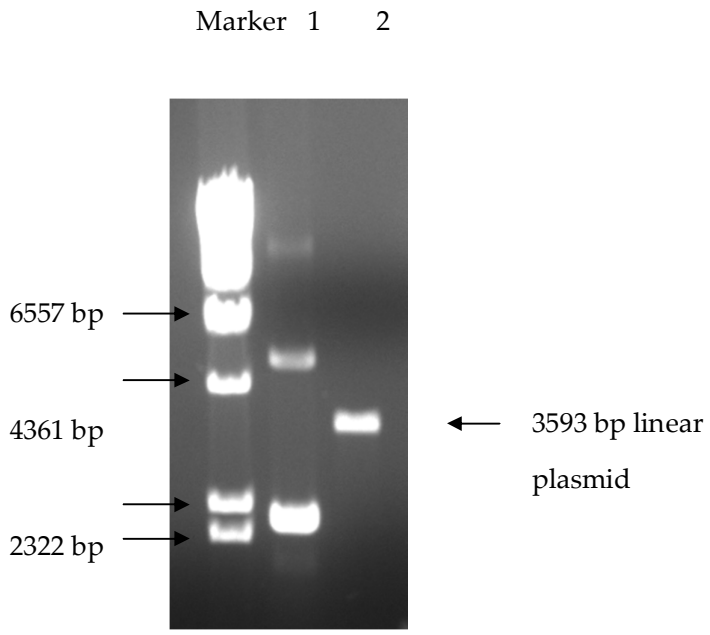

Figure 4.2 Agarose gel electrophoresis image of the pPICZαA vector isolated from *Ecoli* XL1 blue strains. Marker; Lamda DNA/HindIII, Lane 1; undigested, circular vector Lane 2; EcoRI digested, linear vector at expected 3593 bp band.

4.2.2 Insertion of the PFA cDNA into the expression cassette of the pPICZαA vector.

Amplified PFA sequence is single digested with EcoRI whereas pPICZαA plasmid is double digested from EcoRI and SacII restriction sites. After 4 hours of digestion, DNAs are purified by spin column purification and the concentrations are determined at 260 nm in a spectrophotometer.

Purified digests are ligated with each other in a ligation mixture composed of 1:1 molar ratio of PFA and pPICZαA. Products of the ligation mixture are shown on figure 4.3.

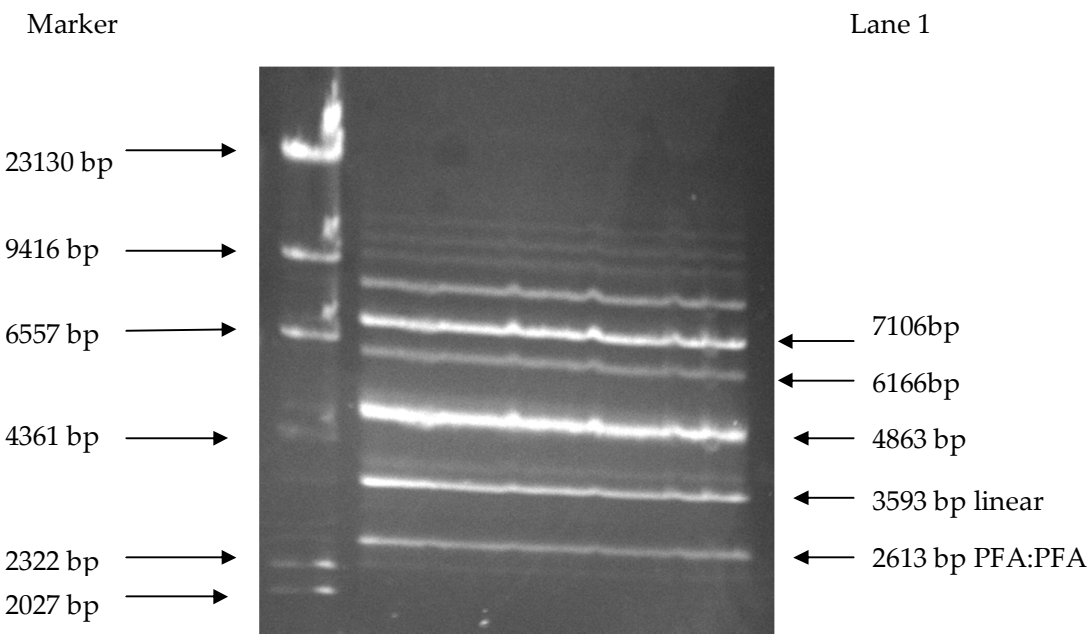

Figure 4.3 Agarose gel electrophoresis image of the ligation products. Marker; Lamda DNA/HindIII, Lane 1; ligation products

pPICZαA:PFA construct is extracted out of the gel by cutting 4863 bp band out and purified via Qiagen's Gel Elution kit. In the T4 ligase buffer first SacII digestion is carried out in order to create SacII site on the insert side, in order to match with the vector end of the construct. SacII is heat deactivated and T4 ligase is added to the same buffer. The vector is circularized by self circularization reaction.

4.3 Transformation

pPICZαA::PFA is amplified in *E. coli* before it is transformed into *P. pastoris.*

4.3.1 Transformation into *E. coli*

For long term storage of positive transformants and easier screening, ligation mixture was first transformed to *E. coli* XL-1 blue strains by $CaCl_2$ transformation method. Positive transformants are screened on LB agar slants with 200 μg/ml zeocin™. Positive transformants carrying rPFA expression cassette was verified by Genome Sequence analysis experiments carried out by METU Central Laboratory. Selected colonies are stored in 13% glycerol solution at -80 °C.

4.3.2 Transformation into *P. pastoris*

pPICZαA:PFA plasmid is propagated in *E. coli* cells by growing in LB broth strain carrying the pPICZαA:PFA is grown on LB agar then inoculated in LB broth for high density cultivation. pPICZαA:PFA plasmid was isolated using Alkaline lysis method and transfected into *P. pastoris* X-33 genome by LiCl

transformation. If the native AOX gene carrier strains of *P. pastoris* (X-33, GS115 or SMD 1168H) is used as the host for pPICZα construct, zeocin-resistant transformants will be Mut^+ phenotype (Invitrogen 2010).

Presence of the rPFA expression cassette in *Pichia* genome was verified by "Polymerase Chain Reaction" in which forward and reverse AOX1 primers were used. As native AOX1 gene is still present in the genome of Mut^+ phenotypes, normally two bands are expected, one corresponding to native 2105 bp AOX1 ORF and the other to the 1859 bp rPFA expression cassette Agarose gel image of the PCR results are shown in Figure 4.4.

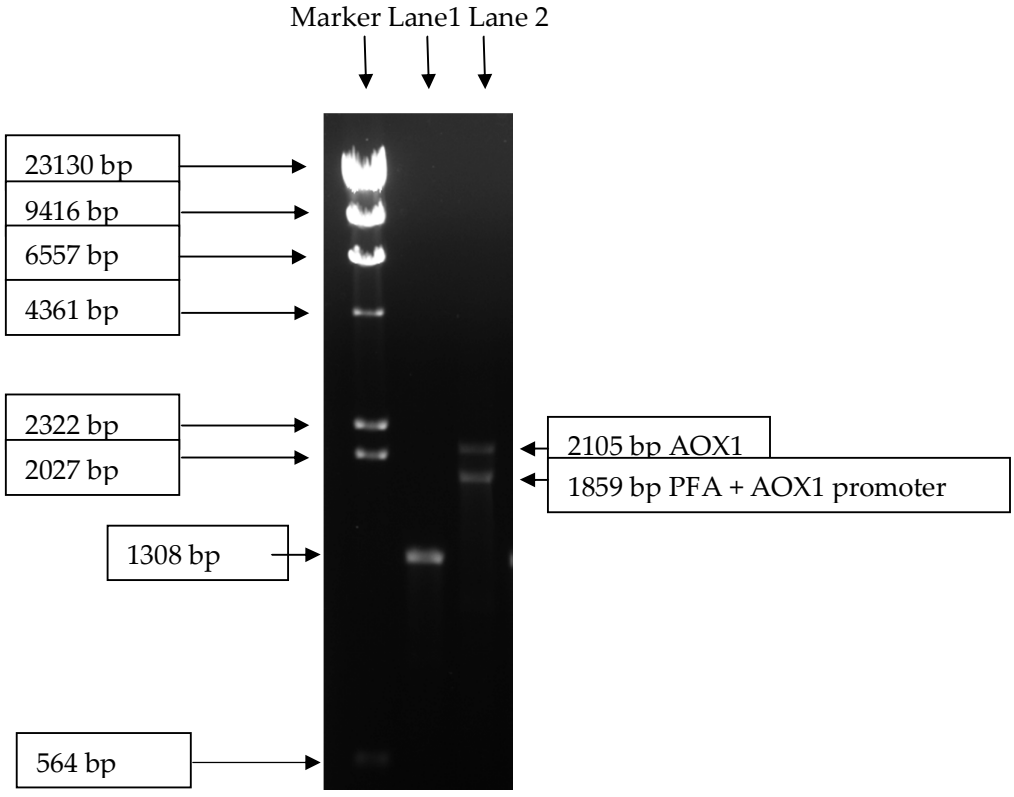

Figure 4.4 Agarose gel image of PCR products. Marker; Lamda DNA/HindIII, Lane 1; PFA reverse primer+ AOX forward, Lane 2; *P. pastoris* genome as template, AOX forward and reverse complementary oligonucleotides as primers

4.4 Isolation and handling of rPFA

Screened mutant *P. pastoris* strains were incubated on agar slants and inoculated into 20 ml BMGY medium. When their OD_{600} reaches 2-6, cells were harvested and transferred into 50 ml of pH6 buffered BSM medium supplemented with 2% MeOH. At the end of 48 hours induction phase in Erlenmeyer flasks, existence of the amylotic activity was assayed in two phases of the production medium, in supernatant and in cell pellet. rPFA is isolated from cell pellet according to the crude enzyme preparation guidelines set in Chapter 3, and cell supernatant is used as it is until the BSM interference to the activity assay and rPFA activity was detected, then supernatant is used after ultrafiltration of the salts.

4.4.1 Intracellular and extracellular production of rPFA

DNS method was first planned to be used as the activity assay however, later on, while drawing calibration curves it was observed that BSM medium interferes DNS method. Independent of the sugar concentration in the standard, at the end of the assay a constant bright yellow color with low absorbance is observed if sugar is dissolved in BSM. Although DNS is a more precise method, we suggested that the assessment of the relative amylotic activities in both phases of the production medium can better be achieved via iodine test. Results of the iodine test are given in Table 4.2. Iodine test did also confirm that there's no significant amylotic activity in the supernatant. Dextrinizing activity of the intracellular medium is nearly 15 fold higher than that is measured in supernatant.

Table 4.2 Dextrinizing activities in cell pellet lysate and in supernant

Localization of enzyme	Dextrinizing Activity
pellet lysate	4.6±0.1
supernatant	0.3±0.2

One might question here whether the BSM directly interferes to the activity assay or it inhibits the enzymatic activity of the PFA. To address it, an active extract of the PFA is mixed with the BSM medium of equal volume and assayed for the amylotic activity with the iodine test. Dextrinizing activities in different assay mixtures are presented in Table 4.3.

Table 4.3 Effect of BSM existence in the reaction medium to the starch degradation

in 400 ml assay mixture	Dextrinizing Activity
40 ml PFA+40ml BSM	0.6±0.2
40 ml PFA + no BSM	4.5±0.2
40 ml BLA + 40 ml BSM	0.2±0.1
40 ml BLA + no BSM	9.6±0.1

Results showed that the BSM not only inhibits the enzymatic activity of PFA but also the BLA. Although a calibration curve with BSM for iodine test can be drawn, enzymatic activities were seriously inhibited when included in assay mixture. In order to detect any hindered α-amylase activity in supernatant, from

that point on, supernatant of the production medium was assayed after dilution of the salts in it via ultrafiltration. Results show us that dilution of the BSM salts increases the α-amylase activity, however even if 100 fold diluted, dextrinizing activity of the supernatant remains 14% of that is at intracellular medium extract as shown in Table 4.4. So it can be stated that most of the amylotic activity is at the intracellular medium. As a consequence, in the rest of this study, only intracellular α-amylase activities were assayed.

Table 4.4 Relative activities of the PFA in cell pellet and filtrated supernatant

Phase	Dextrinizing Activity
Cell lysate	10.0±0.2
10 fold diluted supernatant	0.3±0.1
100 fold diluted supernatant	1.4±0.2

4.4.2 Storage

In order to determine best storage condition for PFA following experimental setup has been designed. 4 separate test tubes including 100 µl crude PFA extracts were exposed to different conditions. After 24 hours, DNS activities were assayed and the relative residual activities were determined. Results are summarized in Table 4.5.

Table 4.5 Effects of various storage conditions on residual activity of PFA

Storage condition	Relative residual activity after 24 hours
At -80 °C	100±6
At room temperature	106±2
At room temperature after treatment at 90 °C for 20 minutes	96±2
At room temperature after clean up of the supernatant by centrifugation	98±3

PFA seems to be very durable as one might expect looking at its thermostability data from previous studies. In addition to this, it doesn't seem to be effected by the proteolytic degradation as keeping at room temperature with the intracellular extract didn't decrease its residual activity. This is a well known behavior of the inclusion bodies. In this study, crude rPFA extract is always stored at T= -80°C.

4.5 Biochemical Characterization of the rPFA

rPFA enzyme was characterized by assaying relative activities within 52-95 °C temperature and 2.4-10.4 pH ranges. Thermal stability data at 90°C is also presented.

4.5.1 Optimum working temperature of rPFA

DNS analysis indicates that the optimum working temperature for the rPFA is 95 °C. This is 3°C lower than that of determined by Dong et al. ((G. Dong, C. Vieille, et al. 1997).

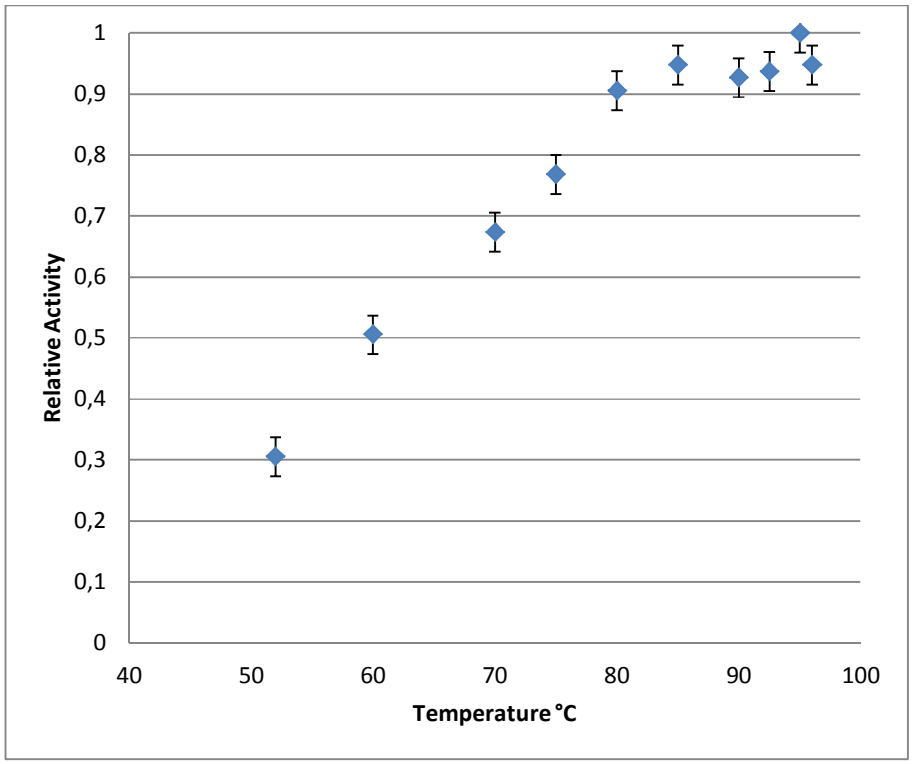

Figure 4.5 Effect of temperature on rPFA activity

4.5.2 Optimum working pH for rPFA

Optimum working pH for the rPFA was determined by adjusting the pH of the 2 % starch solution by 2N phosphoric acid and 2N NaOH. rPFA seems to have its optimum at pH=5.6, and its activity is almost constant over pH 4.5-pH 6.5 range. In previous studies diverse results regarding pH optimum of rPFA was found, ranging from pH=4.5 to pH=6. This can be due to the flat relative activity curve in this range as well as use of different buffer solutions in different studies as rPFA seems to be affected by divalent metal ions. pH vs. relative activity plot is shown in Figure 4.6.

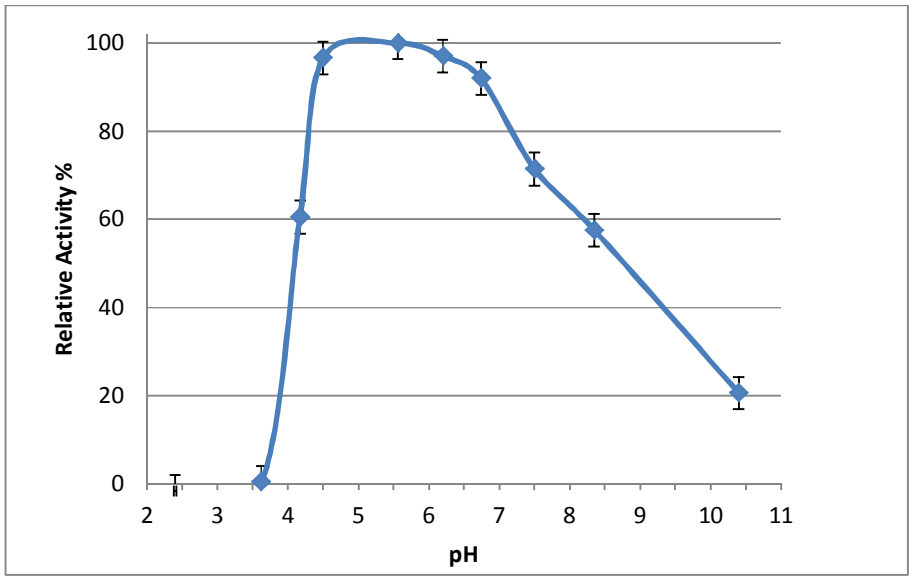

Fig 4.6 Effect of pH on rPFA activity, incubation at 90°C

4.5.3 Thermostability of the rPFA

1 ml of cell lysate is incubated at 90 °C and at different time intervals samples are withdrawn and tested with DNS for the remaining α-Amylase activity. Half life of the rPFA at 90°C is determined to be 13.2h as opposed to 13 h. determined by Dong et al. at 98 °C (G. Dong, C. Vieille, et al. 1997). Residual relative activity vs. time plot at T=90°C is shown in Figure 4.7.

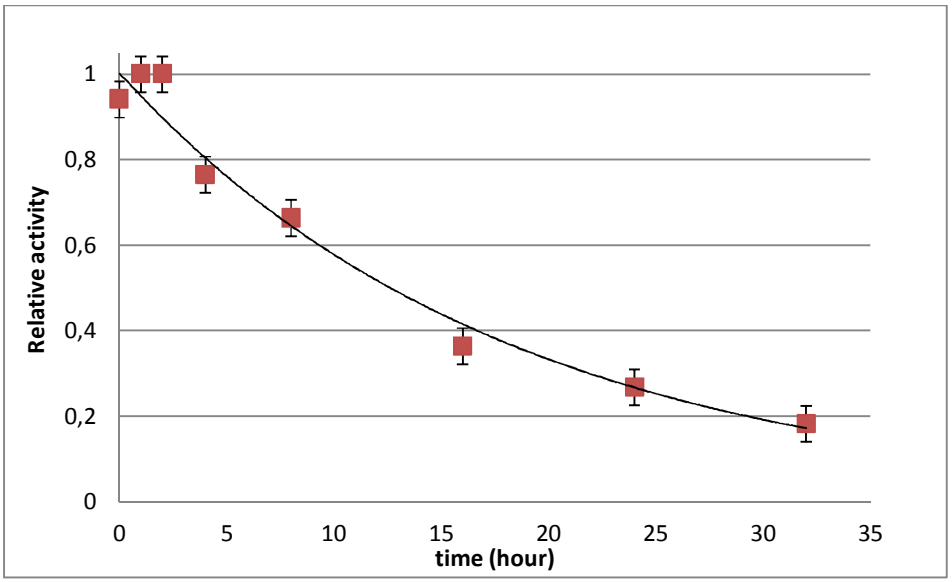

Figure 4.7 Residual relative activity of rPFA vs. time at 90°C

4.6 Erlenmeyer Flask Experimets for determination of the pH Effect on rPFA production

The mutant *P. pastoris* cells were inoculated into un-buffered and pH 6 buffered production media in order to see the effect of pH on rPFA production. Both production media were supplemented with 2% MeOH and 1ml of MeOH is added per 50 ml of production medium at 24th and 48th hour of the induction phase. Flasks are shaken at N=225 min^{-1} and kept at 30°C throughout the whole process. Variation of pH, wet cell density and rPFA activity curves against time plots are given in Figures 4.8, 4.9 and 4.10.

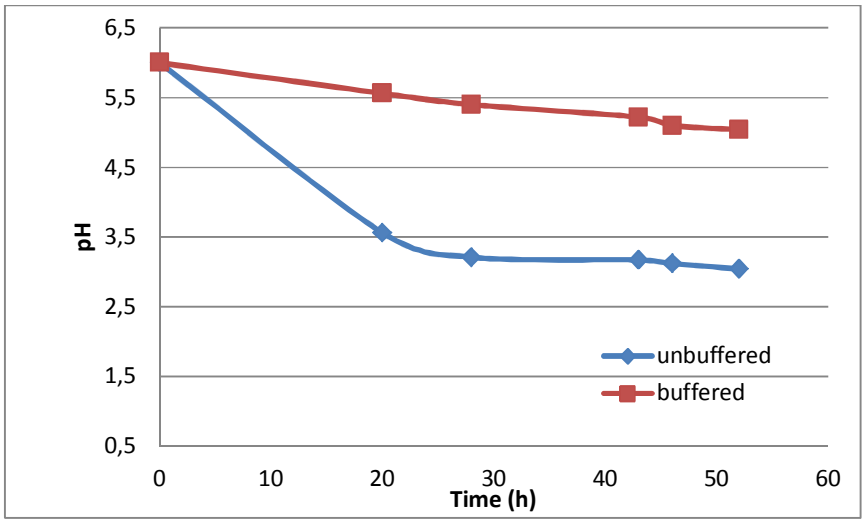

Fig. 4.8 Time vs. pH plot of buffered and un-buffered production media. Growth at T=30°C and N=225 rpm

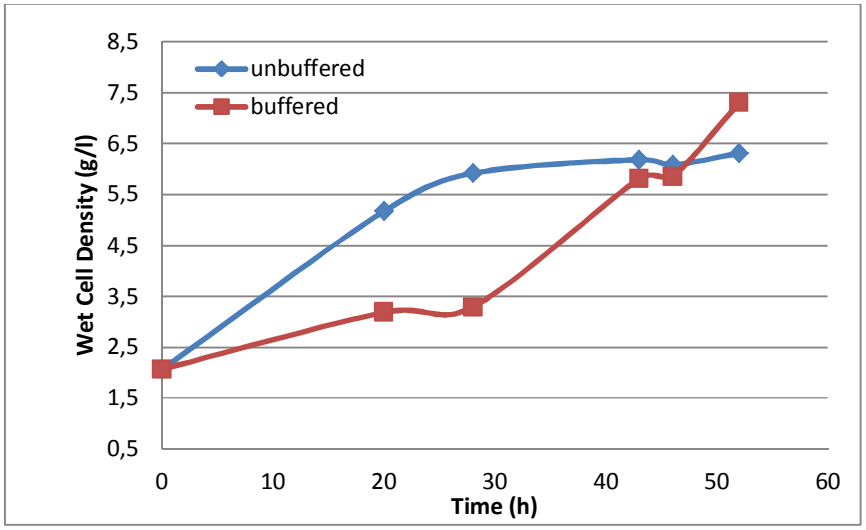

Fig. 4.9 Time vs. wet cell density plot of buffered and un-buffered production media. Growth at T=30°C and N=225 rpm

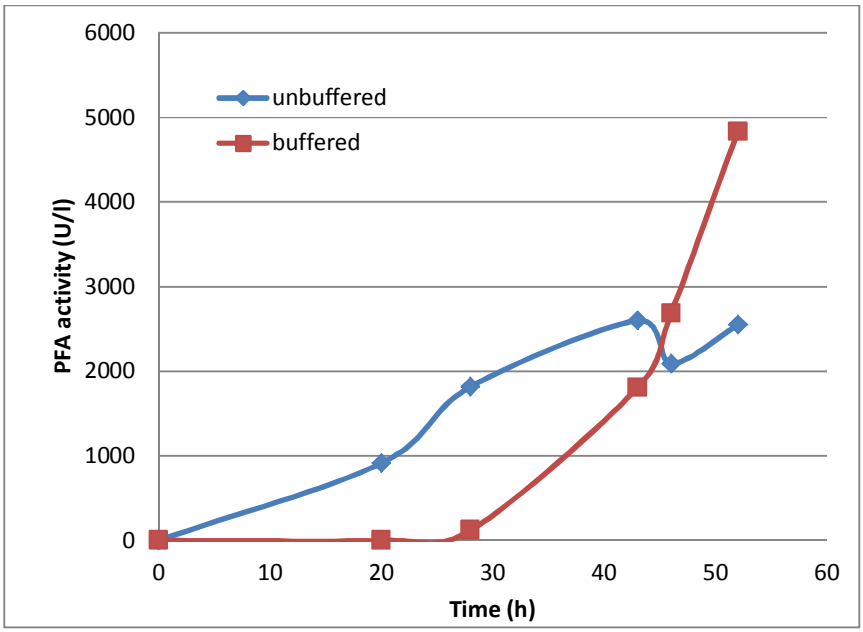

Figure 4.10 Time vs. PFA activity graph for buffered and un-buffered media. Growth at T=30°C and N=225 rpm

Between 0th and 30th hours, specific cell growth rate is higher in unbuffered medium, while there is no rPFA is observed in buffered medium. pH of the unbuffered medium rapidly drops to 3.5 and that of buffered medium is measured as 5.5 in 30th hour. After that time, *P. pastoris* in buffered medium grows faster, when the pH is between 5.5 and 5.0, as opposed to pH≈3 in unbuffered medium. The rPFA expression seems to be direcly related to the cell growth rate as evidenced from the increasing α-amylase activities. The maximum α-amylase activity was measured as 4,832 U/l in buffered flask. As a result, rPFA seems to be efficiently expressed between pH=5.5 and pH=3.5.

4.7 Overexpression of the recombinant α-amylase via fed-batch methanol feeding strategy in bioreactor

Under controlled pH, temperature, stirring rate and dissolved oxygen conditions production of rPFA has been examined at 2 different pH. In all experiments temperature was kept at 30 °C, stirring rate at 900 rpm and dissolved oxygen tension over 20%.

4.7.1 rPFA expression at pH=5

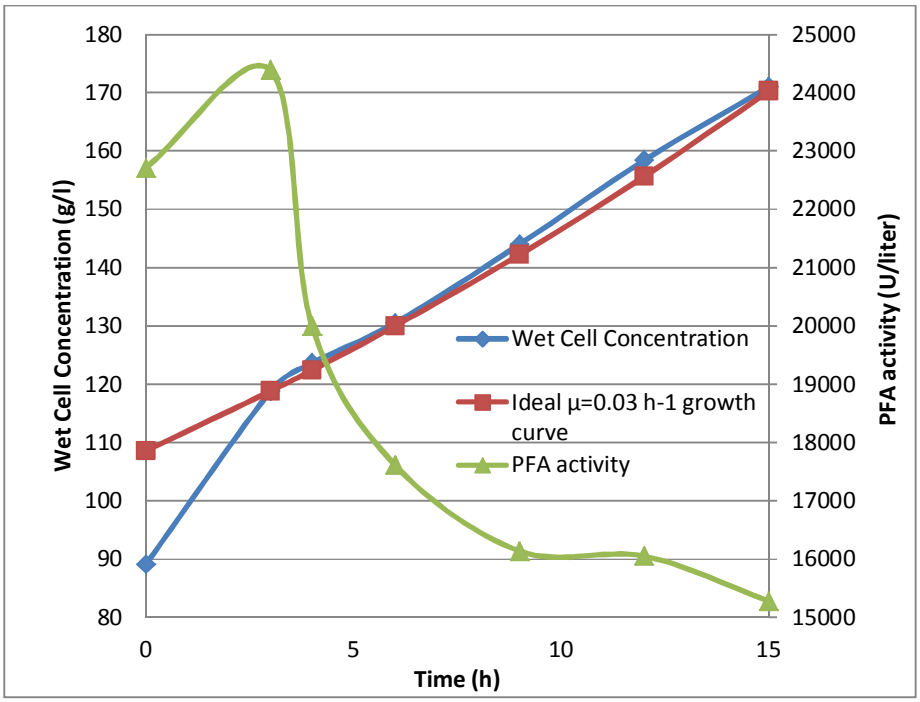

Figure 4.11 Variation of wet cell density and rPFA activity vs. time in the production phase at pH=5.0, T=30°C, N=900 rpm, DO tension over 20%

In the first few hours of the production phase, specific cell growth rate was seen to be higher than the predetermined cell growth rate, 0.03 h^{-1}. Although cell growth is limited to 0.03 h^{-1} by MeOH feeding, residual glycerol in the medium triggers cell growth to a higher level. rPFA production seems to be highest at the initial stages of the production, where glycerol is assumed to be present in the medium. However there seems a continuous decrease in rPFA concentration after the 3rd hour of the operation. This can be explained by the increased proteolytic activity. There has no extracellular rPFA activity been detected at any stage of pH=5 operation condition.

4.7.2 rPFA expression at pH=4

Since alkaline and neutral proteases of *P. pastoris* are inhibited at relatively low pH, production at more acidic medium has been planned in order to avoid degradation of the product by neutral proteases (Invitrogen 2002).

Starting with the transition phase, residual glycerol has been consumed at the 1.5th hour and MeOH accumulation has been observed. AOX promoter is derepressed as a result of glycerol depletion at 1.5th hour and induction is started by MeOH accumulated in the medium. rPFA activity goes as high as 20,922 U/L in this phase. The maximum rPFA activity was observed at the same time when specific MeOH consumption rate is at its highest. Variation of the carbon source concentrations during the course of MeOH transition can be seen in figure 4.12.

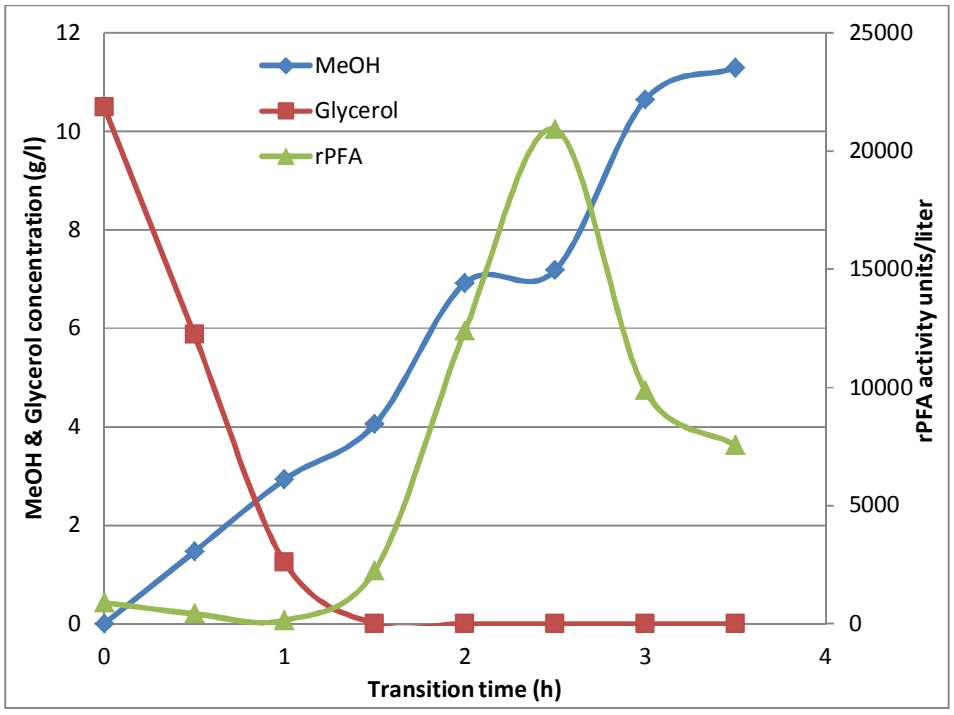

Figure 4.12 Variation of MeOH, Glycerol concentrations & rPFA activities versus time at pH=4.0, transition phase. T=30°C, N=900 rpm, DO tension over 20%

Table 4.6 Yield coefficients and specific rates at transition phase

Time h	q_{GLY} mg g^{-1} h^{-1}	q_M mg g^{-1} h^{-1}	C_{Gly} g L^{-1}	C_{MeOH} g L^{-1}	rPfa activity U L^{-1}
0	0.056	0.001	10.49	0	897
0.5	0.056	0.001	5.87	0.15	422
1	0.015	0.003	1.25	0.29	149
1.5	0	-0.008	0	0.41	2242
2	0	0.008	0	0.69	12404
2.5	0	-0.011	0	0.72	20922
3	0	0.006	0	1.06	9863
3.5	0	-0.005	0	1.13	7547

When MeOH transition phase ended, predetermined MeOH feeding was started where predetermined specific growth rate was μ=0.03. As MeOH fed batch feeding starts, MeOH concentration gradually increases from 4% to 6% until the 30th hour of production, when the exponential phase of growth ends. Since the methanol feeding profile is not well suited for the stationary phase, MeOH accumulation starts beginning with 27th hour of production and this causes further cell inhibition. Maximum rPFA activity was observed at 27th hour of production, when MeOH concentration is 6%.

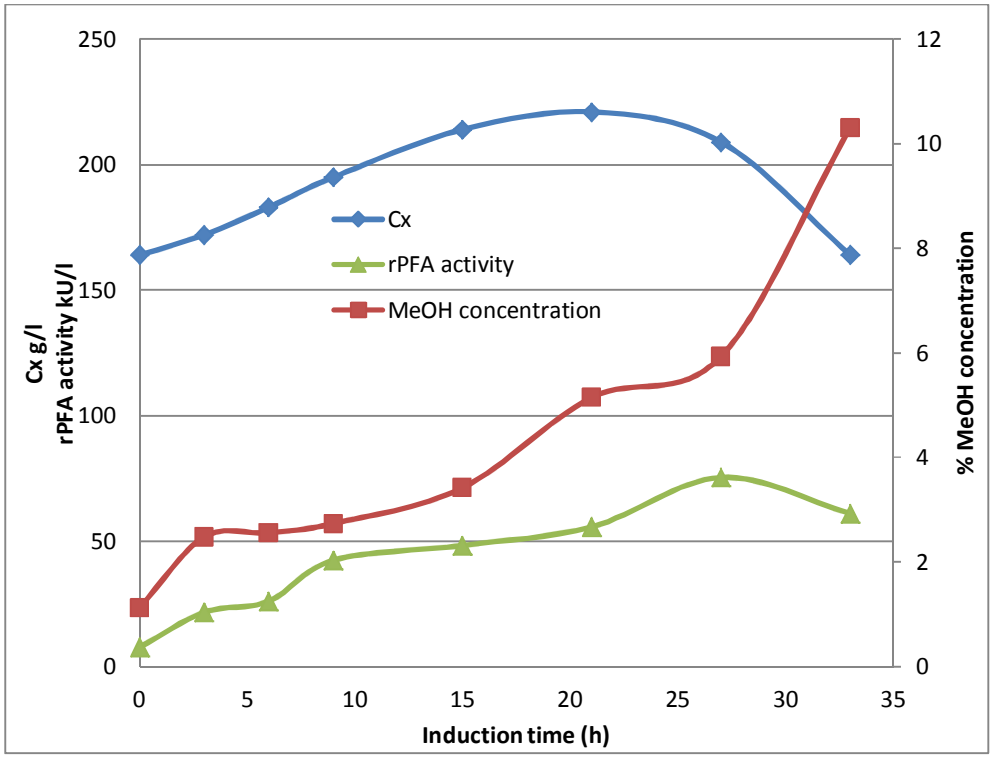

Fig 4.13 Variation of wet cell density, MeOH concentration and rPFA activities versus time at pH=4.0, production phase. T=30°C, N=900 rpm, DO tension over 20%

Table 4.7 Transient Yield coefficients and specific rates at induction phase

Time h	μ' h^{-1}	q_M $\left(\frac{g}{h.g_{wetcell}}\right)$	rPFA activity $\left(\frac{U}{L}\right)$	$Y^I_{X/M}$ $\left(\frac{g_{wetcell}}{g_{MeOH}}\right)$	$Y^I_{P/M}$ $\left(\frac{units_{rPFA}}{g_{MeOH}}\right)$	C_{MeOH} $\left(\frac{g}{L}\right)$
0	0.022	-0.005	7547	-3.91	-5206	1.13
3	0.028	0.022	21146	1.26	418	2.48
6	0.032	0.021	25405	1.41	1520	2.56
9	0.022	0.019	41321	0.84	342	2.74
15	0.014	0.012	47074	0.44	646	3.42
21	0.001	0.024	54322	-0.37	798	5.14
27	-0.024	-0.005	73451	7.33	2052	5.93
33	-0.032	0.017	59552			10.3

Looking at specific rates, one can easily observe the decrease in rPFA activity and specific cell growth rates once MeOH concentration exceeds 2.56%. These results comply well with the literature data which signifies a cell inhibition at MeOH concentrations higher than 31 g/l (Katakura, et al. 1998). Specific cell growth rate also sharply decreases from 0.014 to 0.001 at MeOH concentrations exceeding 34 g/l.

Table 4.8 Overall yield coefficients

	Production phases	$Y_{P/X}$ $\left(\frac{units_{rPFA}}{g_{wetcell}}\right)$	$Y_{P/S}$ $\left(\frac{units_{PFA}}{g_{MeOH}}\right)$	$Y_{X/S}$ $\left(\frac{g_{wetcell}}{g_{MeOH}}\right)$
Preculture		0	N/A	2.11
Induction	Transition	0	760	0
	Exponential	836	418	0.51
	Stationary/Death	-76	38	-0.56
Overall		365	266	0.052

CHAPTER 5

CONCLUSIONS

In this study, it was aimed to produce *P. furiosus* extracellular α-amylase in *P. pastoris* expression system under the control of AOX1 promoter.

As opposed to previous studies in which PFA is produced in prokaryotic hosts, in this research for the first time PFA was expressed in an eukaryotic host. *P. pastoris* is selected as expression host since, the archaeal domain is evolutionarily more closely related to eukaryotes than prokaryotes. Better codon adaptation index scores for PFA in *P. pastoris* than the others', also confirms a better translational compatibility between *P. pastoris* and the PFA.

Although the *S. cerevisiae* α-factor secretion signal was fused to the N terminal of the recombinant protein, recombinant protein was unintentionally expressed intracellularly.

Biochemical characterization of the recombinant protein was assessed within the temperature range of T=40-96 °C, pH range of pH=2.4-10.4. rPFA performs optimally at 95 °C and within the pH range of 4.5-6.5. Furthermore, it doesn't require metal ions as co-factors; additionally, its activity is decreased under the presence of some divalent metal ions present in production medium.

In shake flask bioreactors it was observed that the cell growth is inhibited over pH=5.5 and less than pH=3.5 together with the rPFA activities.

Maintaining the dissolved oxygen above 20%, at high cell density cultivations in a bioreactor envirome nearly 15-fold increase in α-amylase activity was observed. 73,400 U/l of α-amylase activity in recombinant *P. pastoris* expression system is twice of that is previously reported for recombinant *E.coli* system, although it is possible to triple the rPFA activity in *E. coli* by co-expressing thioredoxin in a more refined production medium (G. Dong, C. Vieille, et al. 1997).

In bioreactor experiments, it was evidenced that glycerol is fully depleted in the t=1.5th hour of the phase, thus transition phase might have been kept shorter to decrease process time.

In the production phase, MeOH is fed on the basis of exponential growth curve, accumulation of the MeOH occurs once cells enter stationary phase. Via feed-back control of the MeOH concentration in the medium, accumulation of the MeOH in the medium at the stationary phase can be avoided. Exponential fed-batch feeding regime will be altered to linear feeding or MeOH stat control may be applied manually. In absence of a MeOH detector outcome of the HPLC data may be evaluated to adjust the flow regime of the MeOH feed.

Excessive utilization of MeOH by cells leads to accumulation of toxic byproducts formaldehyde and H_2O_2. Replacement of MeOH with an non-repressing C source will often yield better results in terms of productivity. Mut- & Mut^s strains might be used in order to decrease the MeOH metabolism of the cells. Reducing the MeOH catabolism will remove the oxygen limitation problems occurring at high cell densities, moreover repressed MeOH metabolism will reduce the

formaldehyde and H_2O_2 byproduct which have toxic effects to the cells. Oxygen limited fed-batch strategy can also be applied to decrease the utilization rate of methanol.

A parallel experiment with a protease deficient strain transformed with the same expression vector can be carried out in order to assess the effects of intracellular proteolytic activity which might be the reason for the limited intracellular expression.

In addition to wet cell weight measurement, cell viability is also an important factor affecting the product yield. Viable cell count may be monitored in the succeeding experiments.

PFA is known with its hyrophobicity and due to its nature recombinant protein might have formed inclusion bodies or stacked to cell membrane and result in intracellular expression. Recent studies showed that expression of the rPFA as inclusion body complex is a common problem. Inclusion bodies can be solubilized by treatment with Britton-Robinson buffer at pH 10.5 in expense of decreased specific activity. However intracellular expression still remains as a problem as inclusion bodies are potential inhibitors of cell growth. Soluble and insoluble fraction of the cell extract might be analyzed on SDS gel and it can be determined whether the formation of inclusion bodies causes the accumulation of the rPFA in the intracellular medium. SDS/Strach/PAGE can be used for detection of the rPFA on SDS gel. Later on, PFA may be solubilized by fusing a soluble yeast protein to the N-terminal, that may increase the extracellular expression figures.

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APPENDIX A

PREPERATION OF BUFFERS AND SOLUTIONS USED IN EXPERIMENTS

10X YNB

3.4 g of Yeast Nitrogen Base (YNB) without amino acids and 10 g of Ammonium Sulphate is dissolved in 100 ml. dH2O, heat sterilized and stored at 4 °C.

500X Biotin (0.02% Biotin)

20 mg. biotin is dissolved in 100 ml dH2O, filter sterilized. This solution can be stored for one year at 4 °C.

20% Dextrose

20 g. of D-glucose is dissolved in 100 ml dH2O, either filter sterilized or heat sterilized for 15 min. at 121.5 °C. Can be stored at least one year at room temperature.

10% Glycerol

10ml of Glycerol is dissolved in 90 ml. of water, either filter or heat sterilized. Can be stored at least one year at room temperature.

1M Potassium Phosphate Buffer (pH 6.0)

112.96 g KH_2PO_4 and 29.6 g K_2HPO_4 is dissolved in 1 L distilled H_2O

TNE buffer

100 mM Tris-HCl pH 8.0

50 mM NaCl

50 mM EDTA pH 8.0

0.2% (w/v) Glucose standard solution

0.2 grams of analytical grade glucose is dissolved in 100 ml of distilled water.

10 mM Glucose standard solution

1.8016 grams of analytical grade glucose is dissolved in 1 liter of distilled water.

2% (w/v) Soluble starch solution

Dissolve 3 gram of soluble potato starch in 135 ml cold water in a glass beaker. Heat the solution while stirring until all the starch dissolves. Color of the solution turns to transparent from white when all the starch dissolves. After cooling down the solution add water to bring the volume to 150 ml.

Sodium potassium tartarate solution

30.0 grams of Sodium Potassium Tartrate Tetrahydrate is dissolved in 20.0 ml of 2 M NaOH with constant stirring and heating without boling.

96 mM 3,5-Dinitrosalicylic Acid Solution

In 50 ml of deionized water dissolve 0.0048 moles of 3,5-Dinitrosalicylic Acid with constant stirring and heating. Do not boil.

Color reagent solution

While stirring slowly add the sodium potassium tartarate solution to the 50 ml of 96 mM 3,5-Dinitrosalysilic acid solution. Add distilled water to add the volume up to 100 ml.

PCI

50 ml equilibrated phenol pH 8.0
48 ml chloroform
2 ml isoamyl alcohol

TE buffer

10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

5X TBE Buffer

Dissolve the following in 1L distilled water

54 g. Tris base
27.5 g. Boric acid
3.722 g. EDTA

Yeast lysis Buffer

Dissolve;

2 g. Tritron X-100

1g. SDS

0.1 moles NaCl

0.01 moles Tris-Cl

0.001 moles Na_2 EDTA

in 1 L of distilled water.

Alkaline lysis I solution

50 mM glucose

25 mM Tris-HCl (pH=8)

10 mM EDTA

Alkaline lysis II solution

0.2 N NaOH

1% SDS

Alkaline lysis III solution

5 M potassium acetate

11.5% (v/v) acetic acid

APPENDIX B

GROWTH and PRODUCTION MEDIA

***P. furiosus* Growth Medium (*Pyrodictium* medium)**

NaCl 13.850 g

MgSO4 x 7 H2O 3.500 g

MgCl2 x 6 H2O 2.750 g

KCl 0.325 g

NaBr 0.050 g

H3BO3 0.015 g

SrCl2 x 6 H2O 7.500 mg

(NH4)2SO4 10.000 mg

Citric acid 5.000 mg

KI 0.050 mg

CaCl2 x 2 H2O 0.750 g

KH2PO4 0.500 g

NiCl2 x 6 H2O 2.000 mg

Trace elements (see medium 141) 10.000 ml

Resazurin 1.000 mg

Yeast extract:

for P. occultum 0.200 g

for P. brockii 2.000 g

Sulfur, powdered 30.000 g

Na2S x 9 H2O 0.500 g

Fill with distilled water up to 1liter.

Low Salt LB medium with Zeocin

In 1 L of distilled water, dissolve;

10 g. Tyrptone

5 g. NaCl

5 g. Yeast Extract

Adjust the pH to 7.5 with 1N NaOH

Add 15 g/L agar before autoclaving in case preparing agar plates.

After sterilization finishes, allow medium to cool at least 55 °C before adding Zeocin™ to 25 μg/ml final concentration.

Plates containing Zeocin™ are stable up to 2 weeks if stored in dark at +4 °C.

YPD (+zeocin)

In 900 ml of distilled water, dissolve;

10 g. yeast extract

20 g. peptone

20 g. agar if making YPD plates

Heat sterilize in a autoclave at 121.5 °C for 20 minutes.

After heat sterilization finishes, add 100 ml of filter sterilized 20% dextrose.

Cool the solution at least to 55 °C before adding required amount of Zeocin™. In expression studies Zeocin™ may be omitted. While colony selection 400 μg/ml final Zeocin™ concentration is used.

YE+P (Yeast extract + peptone)

1.5 g yeast extract + 3 g peptone + 105 ml distilled H_2O

Sterilize at 121.5 °C under steam cycle for 20 minutes.

1M potassium phosphate buffer, pH 6.0

1.695 g KH_2PO_4 + 0.442 g K_2HPO_4

Sterilize at 121.5 °C under steam cycle for 20 minutes.

10x YNB

0.51 g Yeast nitrogen base + 1.5 g $(NH_4)_2SO_4$

Sterilize at 121.5 °C under steam cycle for 20 minutes.

10% glycerol solution

1.5 g glycerol + 12.81 ml distilled H_2O

Sterilize at 121.5 °C under steam cycle for 20 minutes.

***Pichia pastoris* precultivation medium (BMGY)**

Dissolve 105 ml YE+P, 15 ml 1M potassium phosphate buffer, 15 ml 10X YNB and 15 ml 10% glycerol solution in a capped bottle.

add;

0,3 ml 500X biotin

0,15 ml chloramphenicol

***Pichia pastoris* production medium**

Dissolve 4.35 g ammonium sulphate in 100 ml dH_2O, heat sterilize

Dissolve 14.9 g $MgSO_4.7H_2O$ and 1.17 $CaSO_4.2H_2O$ in 500 ml dH_2O, heat sterilize

Dissolve 30 g glycerol in 100 ml dH_2O, heat sterilize

Heat sterilize 100 ml of 1M pH=6 potassium phosphate buffer

Mix these contents and fill the volume up to 1 l with sterilized dH_2O

Add 4.35 ml PTM1 and 0.36 mg chloramphenicol.

***Pichia pastoris* basal salts medium (BSM)**

Mix following ingredients in a 1 L volumetric flask and fill up with dH_2O

85% H_3PO_4	26.7 ml
$CaSO_4.2H_2O$	1.17
$MgSO_4.7H_2O$	14.9
KOH	4.13
K_2SO_4	18.2
Glycerol	40 g/L

Autoclave at 121.5 ° for 20 minutes.

Cool down to room temperature, add 4.35 ml PTM1 trace salts solution and 0.36 mg chloramphenicol.

PTM_1 trace salts solution

Mix the following ingredients in a beaker, add dH_2O up to volume of 1L. Filter sterilize.

Cupric sulfate.5H2O	6.0 g
Sodium iodide	0.08 g
Manganese sulfate.H2O	3.0 g
Sodium molybdate.2H2O	0.2 g
Boric Acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate-.7H2O	65.0 g
Biotin	0.2 g
Sulfuric Acid	5.0ml

This mixture can be stored at room temperature in a capped bottle with sealed tap.

APPENDIX C

NUCLEOTIDE SEQUENCES

Nucleotide sequence of *P. furiosus* extracellular α-amylase

RBS

CGCTATGTGTGTCGAGTTGCTGTTTACAGTATTCCCTGTTAAAAGTTCATCACCTACGGT
AATTATTTCCGCGAACATTGGTTCTCCCCAGGAATTGTTTTTATCAAGAGTTTATTAGAT
TTTGACGTGCGTTGATGAACATTTATGTTCACATGATCATAACAGAAAAATTTATATGTA
TCATCACCAGTGATACATTATGAGACTTTGGTGTATGGAGGTGATCAC

Secretion Signal

GTGAACATAAAGAAATTAACACCCCTCCTAACTCTATTACTGTTTTTTATAGTACTAGCA
AGTCCAGTAAGTGCA

PFA

GCAAAATACTTGGAGCTTGAAGAGGGAGGAGTTATAATGCAAGCATTCTATTGGGATGTT
CCAGGGGGAGGAATTTGGTGGGATCATATAAGATCGAAGATTCCTGAATGGTATGAAGCT
GGAATCTCTGCAATATGGCTACCTCCACCAAGCAAGGGGATGAGTGGAGGATATTCAATG
GGCTACGATCCCTATGATTACTTTGATCTCGGCGAGTACTACCAGAAGGGAACTGTAGAG
ACGCGTTTTGGATCAAAAGAAGAACTAGTGAGATTGATACAAACTGCCCATGCCTATGGA
ATAAAGGTAATCGCCGATGTAGTTATAAACCACAGGGCTGGTGGTGACCTAGAATGGAAC
CCCTTCGTTGGAGATTACACATGGACAGACTTTTCTAAAGTTGCCTCAGGGAAATATACA
GCTAACTATCTGGACTTCCATCCAAACGAGCTTCATTGTTGTGACGAAGGAACCTTTGGA
GGATTTCCAGATATATGTCATCACAAAGAGTGGGATCAGTACTGGCTATGGAAGAGCAAT
GAGAGTTATGCTGCTTATTTAAGAAGCATAGGATTTGATGGTTGGAGATTTGACTATGTT
AAGGGCTATGGAGCTTGGGTTGTCAGAGACTGGCTTAATTGGTGGGGAGGTTGGGCAGTT
GGAGAGTACTGGGACACAAATGTAGATGCACTACTAAGCTGGGCATATGAGAGTGGTGCA
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CCAGCATTAGTCTATGCCCTACAAAACGGACAAACTGTAGTTTCGAGAGATCCATTTAAG
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GCGTTCATATTGACATATGAGGGACAGCCAGTAATATTCTACAGGGACTTTGAGGAATGG
CTGAACAAGGATAAGCTAATTAACCTCATTTGGATCCATGATCATTTGGCAGGAGGAAGC
ACAACAATTGTCTACTACGACAACGATGAGCTCATATTTGTGAGAAATGGAGATTCTAGA
AGGCCTGGGCTTATAACTTACATTAACTTGAGCCCTAACTGGGTTGGTAGGTGGGTATAC
GTTCCAAAGTTTGCAGGGGCTTGTATTCATGAATACACTGGAAACCTAGGAGGATGGGTA
GATAAAAGAGTAGATAGTAGCGGATGGGTATACCTAGAGGCACCACCTCACGATCCAGCT
AACGGCTACTATGGGTACTCCGTATGGAGTTATTGTGGTGTTGGGTGA

STOP

CTTTTTCTTTTTTCTTTTTAACAATGGGAGAAGTGCAAATACTGCGACAATTCCTGGGCC
GCATACAGGAGTTTCTGGAGCGGCTTCATTCAATATTACTGTTTTATTTCCAATTCCATA
CAACGTGAC

Nucleotide sequence of the multiple cloning site at pPICZαA

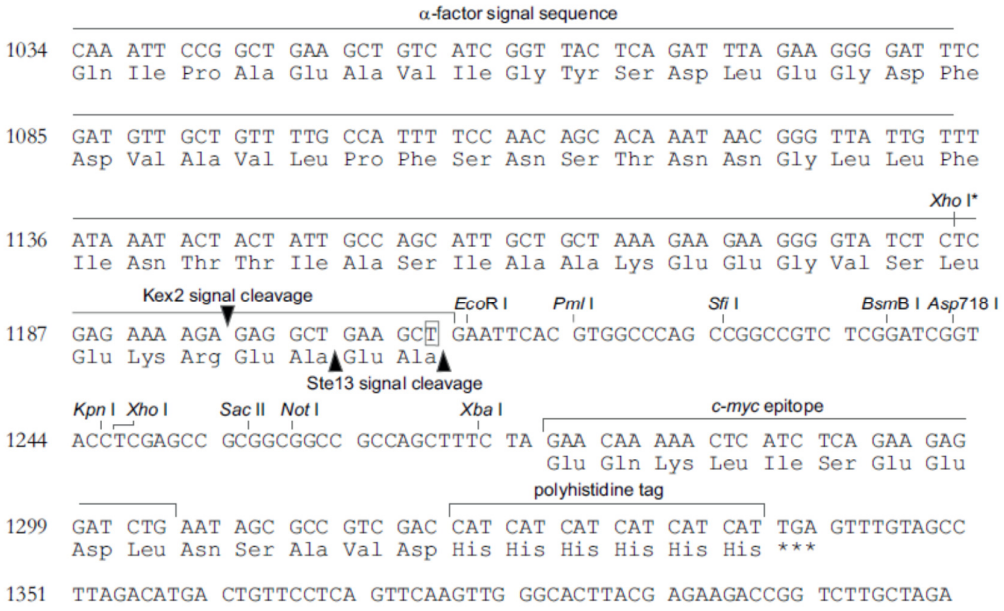

Fig. C.1 Nucleotide sequence of the multiple cloning site at pPICZαA plasmid

Nucleotide sequence analysis results of the expression casette of pPICZαA:PFA construct

Fig. C.2 Nucleotide sequencing chromatogram of the pPICZαA:PFA with the forward primer

Fig. C.3 Nucleotide sequencing chromatogram of the pPICZαA:PFA with the reverse primer

Nucleotide sequence alignment of the forward nucleotide sequencing results with the native PFA cDNA sequence

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 Strand=Plus/Plus

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Sbjct  61   CCAGGGGGAGGAATTTGGTGGGATCATATAAGATCGAAGATTCCTGAATGGTATGAAGCT  120

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Query  570  GACCCGTTTTGGATCAAA-GAA-AACTAGTGAGATTGATACAAACTGCCCATGCCTATG-  626
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Query  800  TTGGAGGATTTCC-GATTTATGTCCTCCCACAG--TGGGATC-GTAC  842
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Sbjct  476  TTGGAGGATTTCCAGATATATGTCATCACAAAGAGTGGGATCAGTAC  522
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Nucleotide sequence alignment of the reverse nucleotide sequencing results with the native PFA cDNA sequence

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 Strand=Plus/Minus

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Query  131   AGGTGGTGCCTCTAGGTATACCCATCCGCTACTATCTACTCTTTTATCTACCCATCCTCC  190
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APPENDIX D

STANDARD CURVES for DNS ASSAY and HPLC ANALYSIS

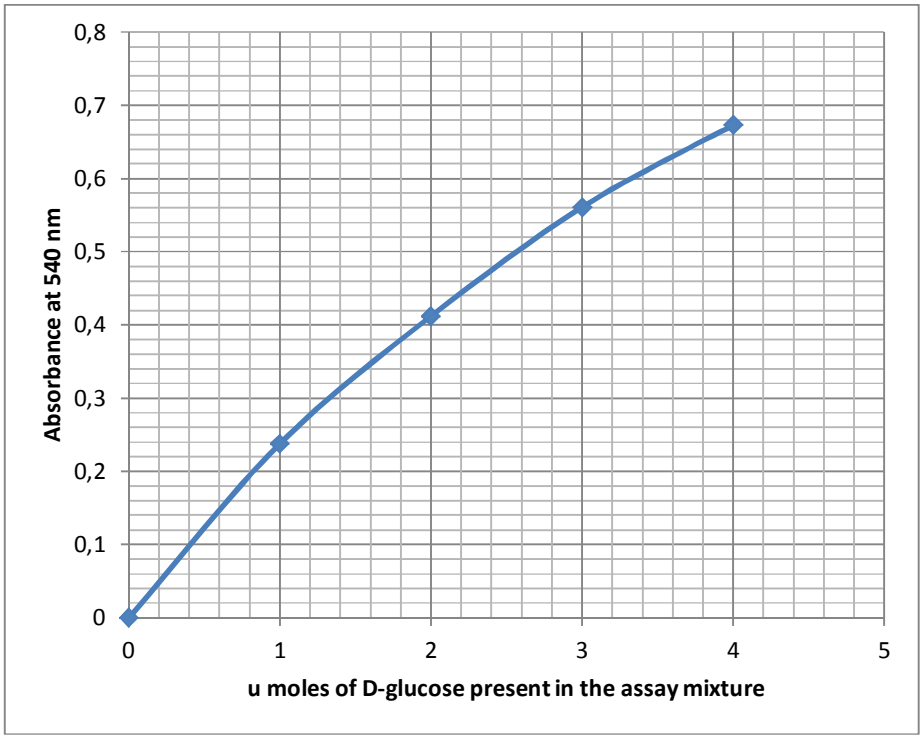

Fig D.1 Standard curve for the glucose concentration vs. absorbance at 540 nm

Methanol and glycerol standard curves for HPLC analysisses

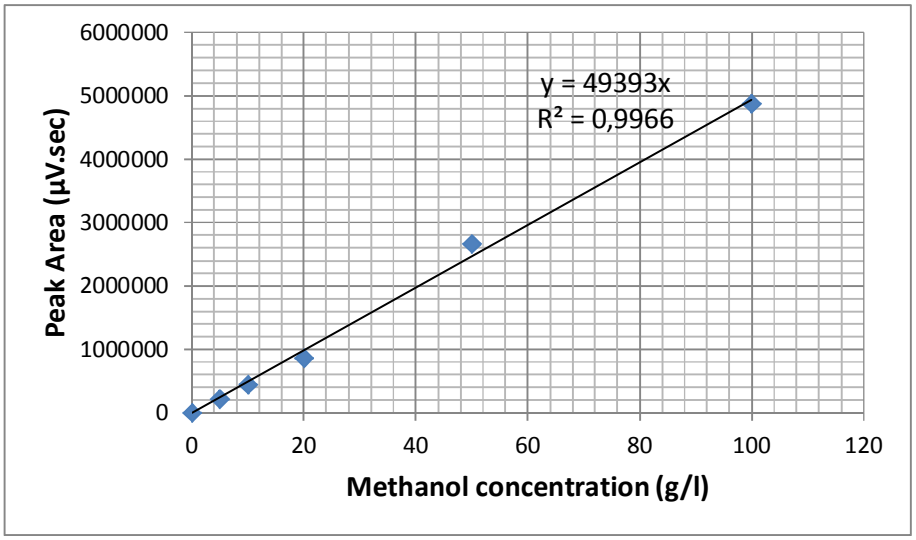

Fig. D.2 Standard curve of the methanol concentration to be used in HPLC analysis

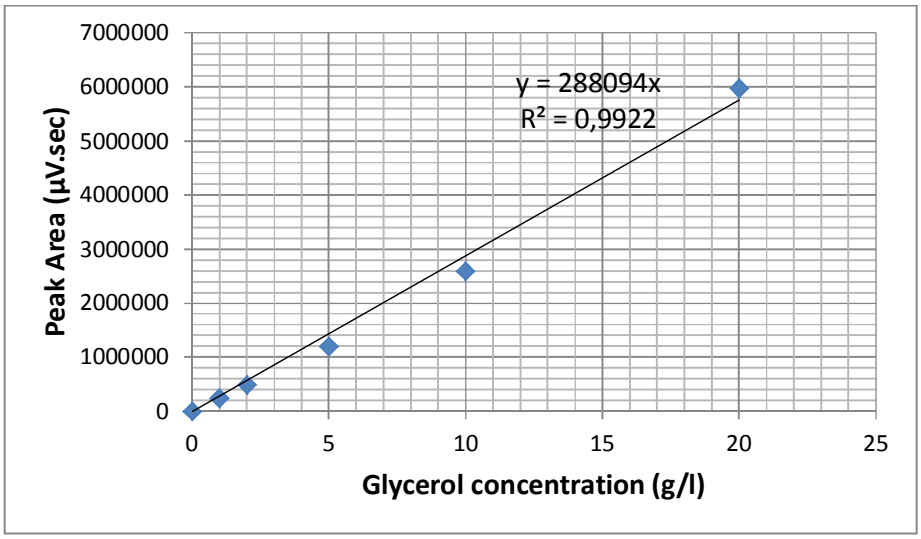

Fig. D.3 Standard curve of the glycerol concentration to be used in HPLC analysis

APPENDIX E

CALCULATIONS

Determination of DNA concentration by using UV spectrophotometer

DNA concentration in a solution is determined by measuring the absorbance at 260 nm in a UV spectrophotometer. DNA concentration in terms of μg/ml is simply calculated by multiplying the absorbance with 50.

APPENDIX F

DNA MARKERS

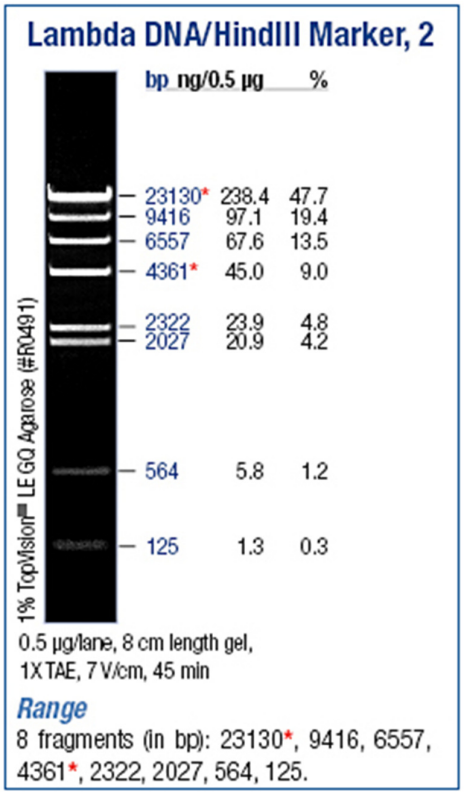

Fig F.1 Lamda DNA/HindIII marker

(http://www.fermentas.com/en/products/all/dna-electrophoresis/lambda-dna-markers/sm010-lambda-dna-hindiii, 23.04.2011)

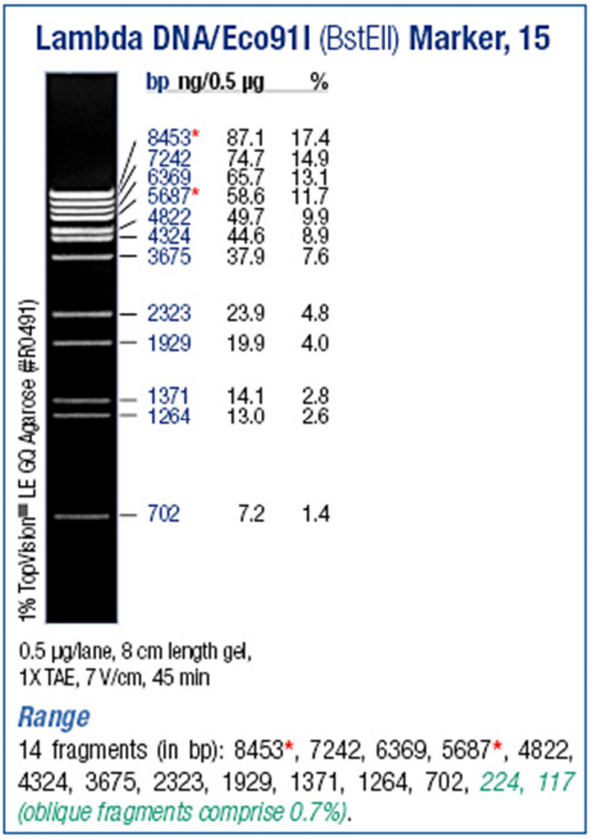

Fig F.2 Eco91I marker (http://www.fermentas.com/en/products/all/dna-electrophoresis/lambda-dna-markers/sm011-lambda-dna-eco91i, 24.04.2011)