OPTIMIZATION OF THE PRODUCTION OF *TRICHODERMA REESEI* ENDOGLUCANASE I IN *PICHIA PASTORIS*

by
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Key words: Endoglucanase, *Trichoderma reesei*, *Pichia pastoris*, Fermentation

Abstract

The aim of this study is to produce *Trichoderma reesei* endoglucanase I in *Pichia pastoris*. *Pichia pastoris* is the host of the production since it has many advantages like high level production of foreign proteins and possession of a tightly regulated promoter, alcohol oxidase I.

Pichia cells expressing heterologous endoglucanase I was grown in fermenter with different strategies. The effects of temperature and methanol concentration on EG I production were tried to be understood. Especially the effect of methanol level in fermenter was investigated since the induction of the promoter needs methanol. Batch and fed-batch fermentations were performed and analyses of the samples were done. Sorbitol was used as a co-substrate to methanol to keep cells growing during production. Protein concentrations, activity analysis of EG I, SDS-PAGE and growth rate results were compared and high level production of endoglucanase I in *Pichia pastoris* was tried to be optimized. For the production of EG I in *Pichia pastoris*, less methanol level and low fermentation temperature were found to be best conditions. Results obtained in this study can be used for further studies and applications especially in detergent, pulp and paper industry.

*PICHIA PASTORIS'*TE *TRICHODERMA REESEI* ENDOGLUKANAZ I ÜRETİMİNİN OPTİMİZASYONU

Aslı Çalık

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Özet

Bu çalışmanın amacı *Trichoderma reesei* endoglukanaz I'inin *Pichia pastoris* organizmasında üretilmesidir. *Pichia pastoris*, yabancı proteinleri yüksek düzeyde üretebilmesi, sıkı regüle edilebilen bir promotöre (alkol oksidaz I) sahip olması dolayısıyla üretim için seçilen organizmadır.

Heterolog endoglukanaz I üreten *Pichia* hücreleri farklı stratejiler uygulanarak fermentörde üretilmiştir. EG I üretiminde sıcaklık ve metanol konsantrasyonunun etkileri anlaşılmaya çalışılmıştır. Özellikle, promotörün indüksiyonu metanol gerektirdiğinden, fermentör içerisindeki metanol miktarının etkisi incelenmiştir. Kesikli ve kesikli beslemeli fermentasyonlar yapılarak örneklerin analizleri yapılmıştır. Kesikli beslemeli fermentasyonlarda, hücrelerin protein üretimi sırasında büyümelerine yardımcı olmak amacıyla metanole ek substrat olarak sorbitol kullanılmıştır. Protein konsantrasyonları, EG I'in aktivite analizleri, SDS-PAGE ve büyüme hızı sonuçları karşılaştırılarak endoglukanaz I'in *Pichia pastoris*'te yüksek üretimi optimize edilmeye çalışılmıştır. Endoglukanaz I'in Pichia pastoris'te üretilmesi için en iyi koşulların düşük metanol seviyesi ve düşük sıcaklık olduğu tespit edilmiştir. Bu çalışmada elde edilen sonuçlar özellikle deterjan ve kağıt endüstrisinde kullanılabilir.

“ to my family and Deniz ”

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TABLE OF ABBREVIATIONS

4-MU	4-Methylumbelliferyl
AOX	Alcohol oxidase
BG	β-glucosidase
BSA	Bovine Serum Albumin
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolase
CBP	Consolidated Bioprocessing
DCW	Dry Cell Weight
DO	Dissolved oxygen
EC	Enzyme Commission
EG	Endoglucanase
FLD	Formaldehyde Dehydrogenase
GAP	Glyceraldehydes 3-Phosphate Dehydrogenase
GBP	Glycerol Batch Phase
GFP	Glycerol Fed-batch Phase
HIS4	Histidine Dehydrogenase
KOH	Potassium Hydroxide
MeOH	Methanol
MFP	Methanol Fed-batch Phase
Mut^s	Methanol Utilization Slow
Mut^-	Methanol Utilization Minus
Mut^+	Methanol Utilization Plus
NaOAc	Sodium Acetate
NMR	Nuclear Magnetic Resonance
PTM1	Trace salts solution
RFU	Relative Fluorescence Unit

ROL	*Rhizopus oryzae* Lipase
RPM	Revolutions Per Minute
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SSF	Simultaneous Saccharification and Fermentation
VVM	Volume of oxygen (liters) per Volume of fermentation culture (liters) per Minute
w/o	Without
w/v	Weight per Volume
YPD	Yeast Peptone Dextrose

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1 INTRODUCTION

Cellulases (EC 3.2.1.4) are a group of enzymes which catalyze the hydrolysis of cellulose. Since cellul**o**se is the most abundant renewable carbon resource on earth, it is important to recycle this polysaccharide ecologically, environmentally and commercially.

Cellulases are composed of three major enzymes which are cellobiohydrolases, endoglucanases and β-glucosidases. Each enzyme has specific roles. Cellobiohydrolases (EC 3.2.1.91) are responsible for releasing cellobiose as main product from crystalline cellulose; endoglucanases (EC 3.2.1.4) attack towards soluble cellulose derivatives with high affinity and β-glucosidases (EC 3.2.1.21) catalyze cellobiose into glucose (Bisaria & Mishra, 1989).

Cellulases have many important roles in industry. Some of the many application areas can be listed as in food, animal feed, textile and chemical industries. Also they can be used in the paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment (Bhat & Bhat, 1997). But most importantly, cellulases are attractive for their use in fuel industry. since the need for renewable alternatives to fussil fuels is increasing, studies on producing liquid fuel by enzymatically hydrolyzing carbohydrate polymers in biomass to sugars and fermenting them to ethanol is also increasing. Cellulases are able to hydrolyze cellulose to cellobiose which is a substrate for β-glucosidase for converting it to glucose (Wilson, 2009).

One of the most important cellulolytic organisms is *Trichoderma reesei*. This fungus is capable of producing a complete set of cellulases which can convert cellulose to soluble sugars. In the presence of glucose, cellulases are repressed and when the organism starves, they are expressed. The enzyme endoglucanase I (EG I) is highly produced in *T. reesei* and has been widely used in textile, pulp and paper industries.

Several organisms, including; *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Aspergillus niger*, *Aspergillus oryzae* have been used for the expression and production of *T. reesei* EGI under different promoters and different conditions (Park, Chang, & Ryu, 2000; Rose & van Zyl, 2002; Takashima, et al., 1998; Vanarsdell, et al., 1987), but according to their EGI production levels and lack of proper post-translational modifications, these organisms are less suitable when compared with the yeast *Pichia pastoris*.

The methylotrophic yeast *Pichia pastoris* is a well-characterized host system that has been used for the expression of a wide variety of heterologous proteins (Macauley-Patrick, Fazenda, McNeil, & Harvey, 2005; Sreekrishna, et al., 1997). The advantages of this host system can be summarized as the simplicity of methods for molecular genetic manipulation, high level production of foreign proteins, the ability to perform higher eukaryotic protein modifications including proteolytic processing, disulfide bond formation and glycosylation and possession of a tightly regulated (methanol-inducible) promoter, alcohol oxidase I (*AOX1*) (G. P. L. Cereghino, Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002; J. L. Cereghino & Cregg, 2000).

For an efficient production of heterologous proteins by *P. pastoris*, many fermentation strategies have been investigated. While the use of methanol as sole carbon source allows a high yield of protein, it causes the slower growth rate and cell yield on methanol limiting productivity. In search of carbon sources that support growth but do not repress the methanol induction of *AOX1* promoter, sorbitol has been found to be a good alternative for glycerol (Sreekrishna, et al., 1997). Many studies including sorbitol as co-substrate have showed that sorbitol not only does not repress the *AOX1* promoter but also helps to overcome the stress caused by some proteins' production like ROL (*Rhizopus oryzae* lipase) overexpression in Mut^{s} cells. Sorbitol has been successfully used in many studies in *Pichia* like production of ROL and avidin.

The aim of this study is to successfully produce *Trichoderma reesei* EG I in *Pichia pastoris* and to find the best condition for this purpose. Different temperatures and methanol feeding rates were investigated on the production of EG I and with analyses like SDS-PAGE, Bradford and activity, optimum conditions were tried to be found. Detailed information about the properties of EG I and *Pichia pastoris* were explained in further sections of this study. Also the methods applied and the results can

be found in Materials & Methods and Results section. In the Discussion and Conclusion sections, the comments on the results can be found.

2 BACKGROUND

2.1 General properties of cellulase

Cellulose, which can also be produced by some animals like tunicates and a few bacteria), is the most abundant component of plant biomass found in plant cell walls in nature (Lynd, Weimer, van Zyl, & Pretorius, 2002). At the molecular level, cellulose is a linear polymer which is composed of β-1-4 glucan linked anhydoglucose units. According to the source, the number of glucose units in the cellulose changes and degree of polymerization ranges from 250 to 10000 (Klemm, 2005). Cellulases are responsible for hydrolyzing cellulose, breaking the β-1-4-D-glucan linkages and producing primary products as glucose, cellobiose and cello-oligosaccharides. There are three major types of cellulase enzymes: Cellobiohydrolase (CBH or 1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), β-glucosidase (BG, EC 3.2.1.21) and Endo-β-1,4-glucanase (EG or endo-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) (Schulein, 1988).

These complex cellulase system (CBH, EG and BG) synergistically act to convert crystalline cellulose to glucose (Table 2.1). The cellobiohydrolases and the endoglucanases are responsible for degrading cellulose to smaller units, releasing cellobiose. Then β-glucosidase act on these cellobiose units subsequently to hydrolyze glucose (Bhat & Bhat, 1997). As it can be seen in Figure 1.1, CBHs attack cellulose molecules from reducing or non-reducing ends, and EGs attack substrates at random sites.

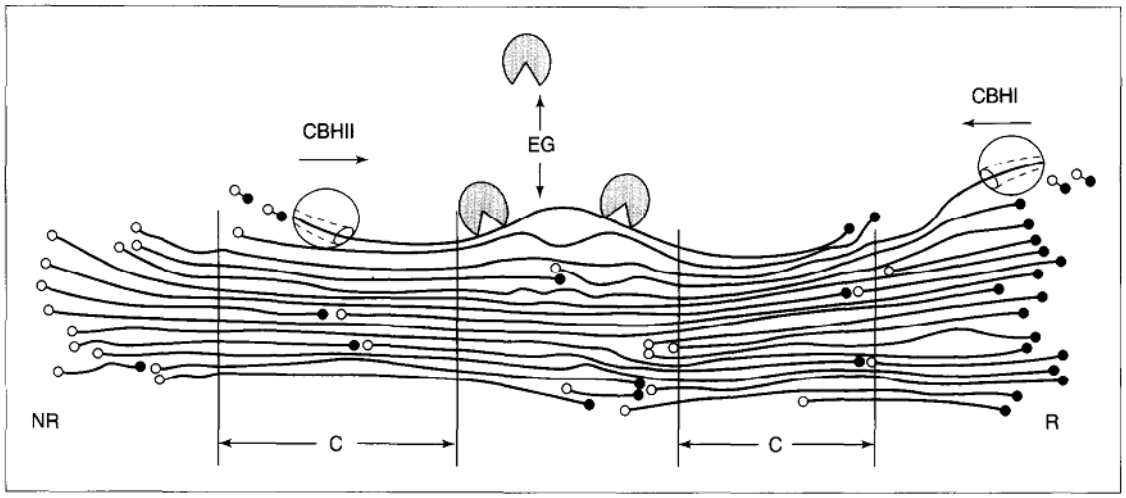

Figure 2.1 Schematic representation of the morphology of crystalline cellulose and reaction mechanism of CBH and EG (T.T. Teeri, 1997)

2.1.1 Properties of cellulase system

As mentioned above, there are three major components of cellulase system. Detailed information about these components is written below.

Table 2.1 The complete cellulase system and their properties (Bhat & Bhat, 1997)

Enzyme Type	EC Code	Synonym	Mode of Action
Endo-(1-4)-β-D-glucanase	EC 3.2.1.4	Endoglucanase or endocellulase	--G--G--G--G--- ↑ ↑ Cleaves linkages at random
Exo-(1-4)-β-D-glucanase	EC 3.2.1.91	Cellobiohydrolase or exocellulase	G--G--G--G--G- ↑ Releases cellobiose either from reducing or non-reducing end
Exo-(1-4)-β-D-glucanase	EC 3.2.1.74	Exoglucanase or glucohydrolase	G--G--G--G--G- ↑ Releases glucose from non-reducing end
β-Glucosidase	EC 3.2.1.21	Cellobiase	G-G, G-G-G-G ↑ ↑ Releases glucose from cellobiose and short chain cello-oligosaccharides

2.1.1.1 Cellobiohydrolase

Cellobiohydrolases (or exoglucanases) are processive enzymes which act on the ends of the cellulose chains. They are responsible for attacking and degrading c rystalline parts of the substrate, producing primarily cellobiose and decreasing the substrate degree of polymerization very slowly. The combined acts of endoglucanases and cellobiohydrolases result in synergy, enhancing the activity over the added activities of the individual enzymes (Teeri, 1997).

2.1.1.2 β-glucosidase

B-glucosidase enzyme is responsible for the hydrolytic cleavage of β-glycosidic linkage between two glycone residues or between glucose and an alkyl or aryl aglycone. Since this enzyme has been isolated from members of all three domains (Eucarya, Archaea, and Bacteria) of living organisms, it is one of the major group among glycoside hydrolases. Research on this enzyme, in cellulolytic microorganisms, have increased since cellulose is the most abundant substrate on earth and is a promising renewable energy resource in the future (Turan & Zheng, 2005).

2.1.1.3 Endoglucanase

Endoglucanases are responsible for breaking the internal glucosidic bonds of the cellulose polymers randomly and producing multiple cellulose chains with different lengths as well as oligosaccharides. More attach possibilities are created for CBHs when more chain ends are produced by endoglucanases (Bata, 2004). Endoglucanases are classified as non-specific enzymes due to releasing reducing sugars from amorphous phosphoric acid-swollen cellululose, hydroxyethyl cellulose, and carboxymethyl cellulose, as well as xylans (KlemanLeyer, SiikaAho, Teeri, & Kirk, 1996). Endoglucanases are generally used in textile, pulp and paper industries.

2.1.2 *Trichoderma reesei* cellulolytic system

Trichoderma reesei is one of the most studied cellulolytic organisms whose mutant strains secrete large amounts of cellulases, up to 40 g/L. This fungus is able to produce a set of cellulases which are used in cleaving the β-1,4-glycosidic bonds present in cellulose or cellulose derivatives (Ortega, Busto, & Perez-Mateos, 2001). Since now, it has been reported that *Trichoderma* possesses two CBH genes, cbh1-2, and eight EG genes, egl1-8, and that CBH I-II and EG I-VI are secreted proteins. It is not well known why this organism produces many kinds of EG to degrade cellulosic biomass (Nakazawa, et al., 2008).

All *Trichoderma reesei* cellulases, instead of small endoglucanase EG III, show similar structural organization composed of two functionally and structurally distinct domains: a small cellulose binding domain (CBD) and a larger catalytic core domain. A long, heavily *O*-glycosylated linker region keeps these domains together. Locations of the CBDs' also change according to the protein. While CBDs of of CBH II and EG II are located at the N terminus, those of CBH I, EG I and EG V are located at the C terminus (Kleywegt, et al., 1997). CBH I and EG I's catalytic domains' structures are also known and have two general site topologies: the tunnel of the cellobiohydrolases and the cleft of the endoglucanases (Eriksson, Karlsson, & Tjerneld, 2002).

Many researches about the purification of *Trichoderma* cellulases revealed that it is difficult to purify these enzymes because of the great number of isoforms (Kleywegt, et al., 1997). There is no doubt about the number of genetically different cellulases, due to the cloning of major cellulases, but the question of isoforms remains. Possible reason for the difference between isoforms is thought to be differences in glycosylation (KlemanLeyer, et al., 1996) (all *Trichoderma* cellulases, except EG III are glycoproteins). Also since the catalytic domains may appear in culture filtrates separated from the CBD, it causes doubts about the number of cellulases. These problems combined with the overall similarity of cellulases make this enzyme hard to purify and perform their quantitative analysis (Medve, Lee, & Tjerneld, 1998).

Endoglucanase I (EG I) is highly produced in *T. reesei* and is the main endoglucanase of this organism. About 5 to 10% of the total amount of cellulases produced by this organism is EG I and it is responsible for releasing reducing sugars

from cellulose. Especially in textile, pulp and paper industries, this enzyme is widely used (Collen, Ward, Tjerneld, & Stalbrand, 2001; KlemanLeyer, et al., 1996). EG I produced by *T. reesei* has a molecular mass around 50 kDa and its isoelectric point is 4,5. While high activity is observed on soluble cellulose, on crystalline substrates degradation levels get very slow.

2.1.3 Applications of cellulases

Since their introduction, cellulases have become one of the largest group enzymes that are used in the industry. In the early 1980s, cellulases were begun to be used in animal feed followed by food applications. Subsequently, many application areas like textile, laundry also pulp and paper industries started to use these enzymes for specific purposes. Today, together with hemicellulases and pectinases, cellulases account for approximately 20% of the world enzyme market, mostly produced by *Trichoderma* and *Aspergillus* (Bhat, 2000).

2.1.3.1 Textile industry

Using pumice stones while washing the garments to acquire a soft handle with an attractive worn look was a common technique that has been used by denim jeans manufacturers. Unfortunately, removal of residual pumice from processes clothing items was difficult and also the overload of tumbling stones was damaging the equipment. Furthermore, machine drainage passages, the drains and sewer lines at the machine sites were getting clogged by pumice stones and particulate material (Heikinheimo, 2000). Due to the problems with pumice, alternative methods like use of cellulases have been used. Now, 80% of denim washing is done by cellulases (Buchert, 1998). To produce the faded look of denim, cellulases act on the cellulose fiber releasing the indigo dye used for coloring the fabric. Denim washing with cellulases provides an environmentally friendly process to acquire an attractive appearance and soft handle for fabrics. Cellulases can also be used in softening, defibrillation and in

processes to provide localized variation in the color density of fibers (Heikinheimo, 2000).

Table 2.2 Cellulases in textile and laundry biotechnology (Bhat, 2000)

Enzyme	Function	Application
Cellulase, preferably neutral and endoglucanase rich	Removal of excess dye from denim fabrics; soften the cotton fabrics without damaging the fibre	Bio-stoning of denim fabrics; production of high quality and environmentally friendly washing powders
Cellulase, preferably acid and endoglucanase rich	Removal of excess microfibrils from the surface of cotton and non-denim fabrics	Bio-polishing of cotton and non-denim fabrics
Cellulase, preferably endoglucanase rich	Restoration of softness and colour brightness of cotton fabrics	Production of high quality fabrics

2.1.3.2 Laundry and detergents

Enzymes have been used in laundry detergents for over 50 years due to their property of removing proteinaceous materials in stains like grime, blood and milk. Especially proteolytic and amylolytic enzymes have mostly been used in laundry products because of the decreased use of phosphate in detergents which results in poorer performance at lower washwater temperatures (Ito, 1989). Cellulase is one of the enzymes that can be used in laundry and detergent industries (Table 2.2). To improve the color brightness, hand feel and dirt removal from garments, cellulases, which are capable of changing the structure of cellulose fibrils, can be added to laundry detergents. Even the amount of cellulase used is approximately 0.4% of the total detergent cost, it is regarded as rather expensive so, alternative cellulase preparations should be done to attract worldwide laundry market (Bhat, 2000).

2.1.3.3 Food and animal feed

Some of the many applications of cellulases in food industry are extraction and clarification of fruit and vegetable juices, production of fruit nectars and purees, and its use in the extraction of olive oil (Galante, 1998). In addition, cellulases can be used in carotenoid extraction in the production of food coloring agents. Also, to enhance the nutritive quality of forages, enzyme preparations can be prepared containing hemicellulase and pectinase with cellulases. In feed processing for animals, increased feed digestibility and animal performance were observed with the use of cellulases (Beauchemin, Rode, & Sewalt, 1995).

2.1.3.4 Pulp and paper industry

Cellulases have importance in the pulp and paper industry since they are used for biomechanical pulping for modification of the coarse mechanical pulp and hand sheet strength properties, de-inking of recycled fibers and for improving drainage and runnability of paper mills. Microbial cellulases are also used for bio-characterization of pulp fibers. Another application is for the manufacturing of soft paper including paper towels and sanitary paper, and to remove adhered paper, preparations containing cellulases are also used (R. K. Sukumaran, Singhania, R.R., Pandey, A., 2005).

2.1.3.5 Biofuel

The most important application area of cellulases is their use in the biofuel industry. For the production of renewable transportation fuels like fuel ethanol, the lignocellulosic residues show the most abundant resource to mankind. Different steps, which are pretreatment, hydrolysis (saccharification) and ethanol recovery, are involved in the production of ethanol from lignocellulosic biomass (R. K. Sukumaran, Singhania, R.R., Mathew, G.M., Pandey, A., 2009). Cellulases are used in the conversion of cellulosic materials to glucose and other fermentable sugars which can be used as substrates for the production of fermentation products like ethanol (R. K. Sukumaran,

Singhania, R.R., Pandey, A., 2005). For efficient production of ethanol, several methods are investigated. Species like *Neurospora*, *Monilia*, *Paecilomyces* and *Fusarium* are able to ferment cellulose directly to ethanol by simultaneous saccharification and fermentation (SSF). Consolidated bioprocessing (CBP), which is a method that features cellulase production, cellulose hydrolysis and fermentation in one step, is also a promising approach with outstanding potential (Nigam, 2010).

2.2 General properties of *Pichia pastoris*

Since 1993, after Phillips Petroleum Company in Bartlesville has decided to release the *Pichia* expression to academic research laboratories, *Pichia pastoris* has become one of the most known expression systems as described in numerous publications (J. L. Cereghino & Cregg, 2000). The methylotrophic yeast *Pichia pastoris* is a well-characterized, highly successful host system that has been used for the expression of a wide variety of heterologous proteins. Up to date, using this system, more than 500 proteins have been cloned and successfully expressed (Macauley-Patrick, et al., 2005). The advantages of this host system can be summarized as:

- ✓ A promoter derived from the alcohol oxidase I (AOX1) gene which is well-suited and tightly regulated (methanol-inducible) for the controlled expression of foreign genes
- ✓ Simplicity of methods for molecular genetic manipulation and similarity of techniques required for manipulation of *Pichia* to *S. cerevisiae*
- ✓ The ability to perform higher eukaryotic protein modifications
- ✓ The low level of native secreted proteins, despite having a well developed secretory mechanism, making the purification of many secreted recombinant proteins easier
- ✓ The strong preference of *Pichia* for respiratory growth which makes its culturing at high-cell densities easier than fermentative yeasts (G. P. L. Cereghino, Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002).

Pichia is a single-celled microorganism and this yeast is easy to manipulate and culture. Since it is eukaryotic organism, it has the ability to perform many of the post-translational modifications such as proteolytic processing, folding, disulfide bond formation and glycosylation. Therefore, many proteins can be produced as biologically active molecules in *Pichia* unlike in bacterial systems, which results as inactive inclusion bodies. The Pichia expression system is also advantageous than the other systems like insect and mammalian tissue culture cell systems since it is regarded as being faster, easier, less expensive to use and having higher expression levels (Macauley-Patrick, et al., 2005).

2.2.1 Important promoters of *Pichia pastoris*

The major principle for heterologous protein production in *Pichia* is the fact that enzymes required for the metabolism of methanol can only be produced when cells are grown on methanol. To date, it has been the most successful expression system for this organism. Therefore, the AOX promoters have been the most widely utilized, but, there are also other available promoters for production of heterologous proteins in Pichia since the use of methanol, which is the source for methane being a petroleum-related compound, may not be appropriate for the production of food products (J. L. Cereghino & Cregg, 2000; Macauley-Patrick, et al., 2005).

2.2.1.1 AOX1 promoter

AOX1 promoter, being the most important promoter of *Pichia*, is mainly responsible for the production of alcohol oxidase enzyme involved in methanol metabolism. Induction by methanol results in alcohol oxidase production, which reaches up to 30% of total soluble protein, indicating the power of this promoter (Cregg, 1993). By a carbon source-dependent repression/induction mechanism, activity of this promoter is tightly regulated. During growth of the yeast, in the presence of glucose or glycerol, its expression is fully repressed, but expression is highly induced during growth on methanol. Expression of the AOX1 gene is controlled at the level of

transcription and to induce high levels of transcription, the presence of methanol is highly essential (Tschopp, Brust, Cregg, Stillman, & Gingeras, 1987). In the table below, main advantages and disadvantages of this promoter can be seen:

Table 2.3 Advantages and disadvantages of AOX1 promoter (Daly & Hearn, 2005)

Advantages	Disadvantages
Transcription of the foreign protein is tightly regulated and controlled by a repression/derepression mechanism	Monitoring methanol during a process is often difficult, due to the unreliability of on-line probes and the complications of measuring off-line
High levels of foreign proteins can be expressed, even if they are toxic to the cell	Methanol is a fire hazard; therefore, storing the large quantities required for these processes is undesirable
The repression of transcription by the initial carbon source ensures that good cell growth is obtained before the gene product is overexpressed	Methanol is mainly derived from petrochemical sources, which may be unsuitable for use in the production of certain food products and additives
Induction of transcription is easily achieved by the addition of methanol	Two carbon sources are required, with a switching over from one to the other at a precise time point

2.2.1.2 AOX2 promoter

Alcohol oxidase enzyme can also be produced by the AOX2 promoter but this gene yields 10-20 times less enzyme activity than the AOX1 gene. However, some proteins' production levels, with the use of AOX2 promoter, can be increased by optimization of physicochemical environment and with addition of anti-foam agents, such as oleic acid (Kobayashi, et al., 2000). But, since the expression levels under the control of the AOX1 promoter is much higher than AOX2 promoter, most of the researchers prefer methanol induced AOX1 promoter for foreign protein expression.

2.2.1.3 FLD1 promoter

One of the enzymes in methanol metabolic pathway of *Pichia* is formaldehyde dehydrogenase (FLD) enzyme. This enzyme is also used in protection of the cell from toxic effects because of the formaldehyde (also the sole nitrogen source for the cells) during methylamine metabolism (Shen, Sulter, Jeffries, & Cregg, 1998). In the Figure

2.1, the role of the common intermediate (formaldehyde) can be seen in both the methanol and methylamine pathway (Macauley-Patrick et al., 2005).

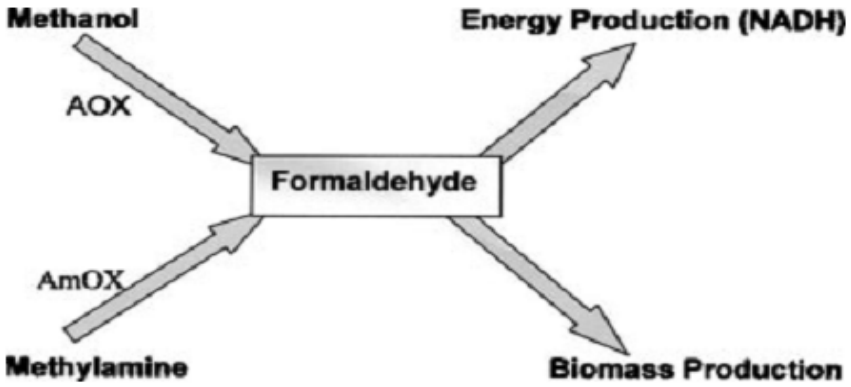

Figure 2.2 For both the methanol and methylamine pathways, the intermediate formaldehyde is common in *Pichia*. Formaldehyde is produced by AOX in the methanol pathway, with the co-production of hydrogen peroxide (H_2O_2); and also by amine oxidase (AmOX) in the methylamine pathway, with the co-production of H_2O_2 and NH_3 (Macauley-Patrick, et al., 2005)

For expression of foreign genes in *Pichia*, one of the alternative promoters is the promoter for the formaldehyde dehydrogenase I (FLD1) gene. This promoter is transcriptionally efficient and has very much alike regulatory properties to AOX1 promoter. Strong induction of this promoter can be by either methanol as a sole carbon source (with ammonium sulphate as a nitrogen source), or by methylamine as a sole nitrogen source (with glucose or sorbitol as a carbon source) (Shen, et al., 1998).

2.2.2 Types and properties of *P. pastoris* strains and phenotypes

Three main steps should be followed in order to express any foreign gene in *P. pastoris*:

1. The insertion of the gene into an expression vector
2. Introduction of the expression vector into the *Pichia* genome
3. Examination of potential expression strains for the foreign gene product (J. L. Cereghino & Cregg, 2000).

According to the desired application, various kinds of strains can be used. Different *Pichia* strains, with their genotype and phenotype characteristics, that are available are shown in the table below:

Table 2.4 Different strains of *Pichia pastoris* with their genotypes and phenotypes (Daly & Hearn, 2005)

Strain	Genotype	Phenotype
SMD1168	*His4, pep4*	Mut^+, His^-, $pep4^-$
GS115	*his4*	Mut^+, His^-
KM71	*his4, aox1: ARG4;arg4*	Mut^S, His^-
X-33	Wild type	—
MP-36	—	—
SMD1165	*His4, prb1*	Mut^+, His^-, $prb1^-$
SMD1163	*his4, prb1, pep4*	Mut^+, His^-, pep4, $prb1^-$
MC100-3	*arg4 his4 aox1Δ:: SARG4 aox2Δ:: Phis4*	Mut^-, His^-

As it can be seen above, SMD1168, GS115, KM71, SMD1165, SMD1163 and MC100-3 do not have the ability to express histidine dehydrogenase gene (HIS4) whose expression allows transformants to be selected depending on their ability to grow in non-histidine containing media.

According to methanol-utilizing properties, three phenotypes can be mentioned. Strains such as SMD1168 and GS115 contain functional copy of the alcohol oxidase I gene (AOX1) which regulates 85% of the alcohol oxidase activity in the cell. These strains with Mut^+ phenotype are "methanol utilization plus" phenotypes and they have the ability to grow on methanol at wild type rate. Strains with Mut^+ phenotype contain functional copies of both alcohol oxidase I and II genes (AOX1 and AOX2). A major concern for *Pichia* Mut^+ phenotype strains in methanol non-limited fed-batch cultures is the oxygen metabolic demand. Since production process requires more oxygen, the bioreactor oxygen transfer capacity is insufficient to overcome the demand, affecting the heterologous protein production (Cos, Ramon, Montesinos, & Valero, 2006).

As in MC100-3 strain, when both the AOX1 and AOX2 genes are disrupted, "methanol utilization minus" (Mut^-) phenotype is obtained. The alcohol oxidase defective strains do not have the ability to utilize methanol as their sole carbon source. Alternate carbon sources should be used for growth of these strains (W. Zhang, Inan,

M., Meagher, M.M., 2000). All of these strains, even the Mut^- strain, retain the ability to induce expression at high levels from the AOX1 promoter (Chiruvolu, Cregg, & Meagher, 1997). For intracellular expression, it is preferable to use Mut^- cells because they will have a lower level of alcohol oxidase protein and the expressed protein can be more readily purified (Sreekrishna, et al., 1997).

Disruption of AOX1 gene by gene/cassette insertion results in obtaining "methanol utilization slow" Mut^s phenotype of *Pichia* strains as in KM71. These kinds of strains contain functional copy of the alcohol oxidase II gene (AOX2) gene for protein production. The AOX2 gene has the same ability as AOX1 instead it has a much lower expression level since it is a weaker promoter (Daly & Hearn, 2005). When compared with Mut^+ phenotype, Mut^s strains can grow on mannitol or sorbitol and be induced with lower amounts of methanol. The slower growth and protein production of Mut^s is especially preferable when the folding of expressed protein is rate-limited (Romanos, 1995). It has been shown that when growing on 0.5% methanol, expression of carboxypeptidase A2 construct by Mut^s phenotype was higher than the Mut^+ phenotype, however when higher methanol concentrations were used, Mut^+ phenotypes were observed to be better (Reverter, Ventura, Villegas, Vendrell, & Aviles, 1998). It can be said that Mut^+ phenotypes are less likely to become poisoned by methanol than Mut^s phenotypes but are more likely to become oxygen limited.

To obtain good cell growth with the expression of the heterologous protein, glycerol (at limiting rates) and methanol (to a small excess) feeding can be used so that these substrates can be utilized simultaneously. However, because of the partial repression of AOX1 promoter even by slight levels of residual glycerol, the maximum level of protein expression cannot be reached even though it leads to increased overall productivity (Thorpe, d'Anjou, & Daugulis, 1999).

2.3 Heterologous protein expression in *Pichia pastoris*

Shake-flask cultures are one of the ways to produce heterologous proteins but since protein levels are typically much higher in fermenter cultures, it is better to use fermenters instead of shake-flasks. Also, since the mineral media for *Pichia* are

economical and well defined, large-scale production of heterologous proteins in fermenters by *Pichia* is ideal. But, most importantly, since parameters like pH, aeration and carbon source feed rate are important for production, it is only fermenters that these parameters can be controlled (G. P. L. Cereghino, Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002).

For the production of heterologous proteins, generally a three stage process is performed in fermenter cultures of P. pastoris. First, engineered yeast is batch cultured in a nonfermentable carbon source such as glycerol to increase biomass. Then, after the glycerol is depleted, a feed-batch transition phase in which glycerol is fed to the culture at a growth limiting rate to further increase the biomass concentration and to prepare the cells for induction. Finally, actual gene of interest driven by the AOX1 promoter is induced by addition of methanol to the fermentation culture (Higgins & Cregg, 1998).

There are many strategies to optimize the conditions of production in Pichia, since there are many factors affecting the production of heterologous proteins in fermenter cultures. Most important factors can be listed as: medium composition, temperature, pH, dissolved oxygen (DO) control, substrate properties and substrate feeding rates. All these factors are discussed below in details.

2.3.1 Fermentation process optimization strategies in *Pichia pastoris*

2.3.1.1 Temperature optimization

One of the important parameters in fermentation is the temperature. Higher temperatures have detrimental effects on proteins. It leads to exposure of hydrophobic surfaces during peptide folding and favor hydrophobic interactions, so leading the proteins to aggregation (Lee, Choi, & Yu, 1990). The misfolded and aggregated proteins are more prompted to proteolytic degradation. There are many studies on expressing heterologous proteins in *Pichia* at low temperatures (Hong, Meinander, & Jonsson, 2002; Whittaker & Whittaker, 2000). *Pichia* can be grown at temperatures as low as 15°C, thus reducing the protease levels and enhancing the protein production. Even the fermentation period is longer than 30°C, low temperature expression can be

used to increase the yields of aggregation-prone and/or unstable gene products in *Pichia*.

2.3.1.2 pH optimization

One of the parameters of fermentation is pH. Finding the optimum pH is important especially when secreting protein into the medium and for optimal growth. Since the optimum pH depends on individual protein properties, especially stability, it is best determined by running a series of fermentations at different pH values. While some proteins are best expressed at low pH, some are best expressed at high pH values. The expression of small multifunctional cytokine, growth blocking peptide (GBP) was successfully performed in *Pichia* at pH 3,0 (Koganesawa, et al., 2002). On the other hand, increasing pH from 6,0 to 7,0 while expressing porcine lactoferrin in *Pichia* resulted in significant improvements and increase in the expression level (Wang, et al., 2002).

2.3.1.3 Dissolved oxygen control

During the fermentation process, dissolved oxygen (DO), which is the relative percent of oxygen in the medium where 100% is O_2-saturated medium, is one of the most important parameters affecting *Pichia* cell growth and heterologous protein expression. Since *Pichia* consumes oxygen while it grows, in batch phase when glycerol is consumed completely, the dissolved oxygen value rises rapidly. Also, since oxygen is required for the first step of methanol catabolism, it is important to maintain the dissolved oxygen concentration at a certain level (>20%). Generally in *Pichia* fermentations, the dissolved oxygen is kept around 30-35% but for different proteins different optimal levels may be needed (Li, et al., 2007). When the dissolved oxygen concentration of a culture is measured accurately, the state and health of the culture can be understood.

2.3.1.4 Mixed feed strategy

To improve the foreign protein production, one of the methods that can be used is mixed feed strategy for the fed-batch phase of *Pichia*. Especially for Mut^s strains, since they utilize methanol slowly, mixed feed of glycerol and methanol is performed during the induction phase. For cell growth, *Pichia* uses glycerol and for target protein production methanol is consumed. Advantages of this strategy can be listed as improvement of cell-culture viability, a shorter induction phase, and a higher recombinant protein production rate. With this strategy, several proteins have been produced successfully (W. H. Zhang, et al., 2003). However there are also some disadvantages. Since excess glycerol is a repressor of the methanol pathway, including AOX1 promoter, this strategy may result in lower yields than the potential maximum on methanol alone. In addition, ethanol and acetate build-up may be triggered by glycerol metabolism repressing the AOX1 promoter and so decreasing or delaying heterologous protein production (G. P. L. Cereghino, Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002). Since glycerol is a repressing carbon source at induction phase, different carbon sources have been investigated. It has been found that molecules such as sorbitol, alanine, mannitol, trehalose are seen to be good substrates, supporting growth but not repressing the methanol induction of AOX1 promoter (Inan & Meagher, 2001; Sreekrishna, et al., 1997). Especially, sorbitol has been widely used as an additional carbon source to methanol for the production of recombinant proteins in *Pichia* strains.

Many researches about this substrate revealed that sorbitol is a non-repressing carbon source to AOX1 promoter and is suitable for use in production in *Pichia* (Celik, Calik, & Oliver, 2009; Ramon, Ferrer, & Valero, 2007). There are several advantages of using sorbitol as a co-substrate. One advantage is that sorbitol accumulation during fed-batch phase does not affect the expression levels of recombinant proteins (Thorpe, et al., 1999), so that residual sorbitol concentration control is less necessary and critical than with mixed feeds of glycerol and methanol. Also, mixed feeds of sorbitol and methanol result in the lower heat production rate and lower oxygen consumption rate due to slower growth on sorbitol than glycerol. Especially for large scale productions, reduction in oxygen consumption rate and heat production rate is very favorable in high cell density cultures with recombinant *Pichia* strains (Jungo, Schenk, Pasquier, Marison, & von Stockar, 2007).

Studies about the sorbitol as a co-substrate also revealed the assimilation characteristics of Mut^S strain. It is understood that sorbitol and methanol are consumed simultaneously in Mut^S strain allowing higher specific growth rate and moreover higher secretion levels of heterologous protein compared with cells grown on sole methanol as a carbon source (Ramon, et al., 2007).

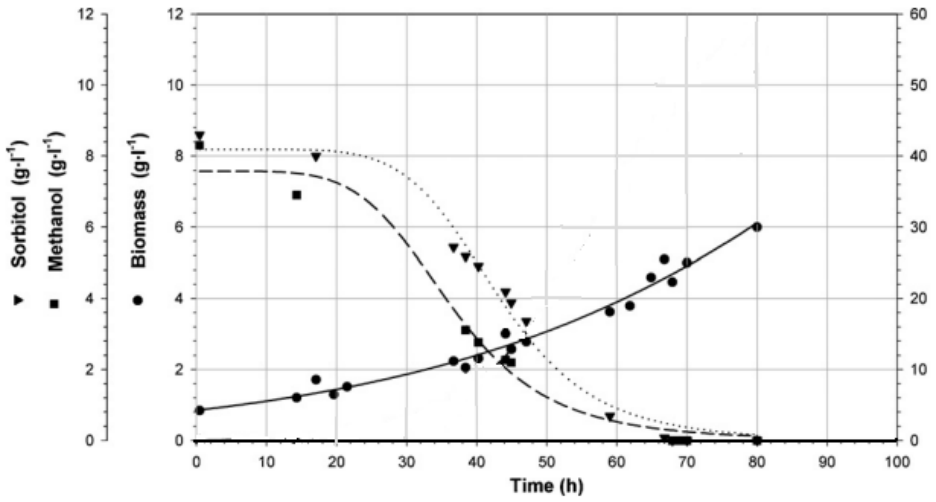

Figure 2.3 Sorbitol and methanol assimilation and biomass evolution with respect to time using methanol and sorbitol (Ramon, et al., 2007)

As it can be seen in Figure 2.3, mixed feed of sorbitol and methanol leads to increase in biomass and also the consumption of methanol can be seen, indicating the promoter to be active.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All of the chemicals used are listed in Appendix.

3.1.2 Equipment

For fermentation New Brunswick Bioflo 310 5L fermenter was used. Other general laboratory equipments that were used are listed in Appendix.

3.1.3 Buffers and solutions

Standard buffers and solutions were prepared according to *Molecular Cloning: A Laboratory Manual, Sambrook et al.,* 2001.

3.1.4 Strain and vector

For production, *Pichia pastoris* KM71H cells, containing *eg1* gene from *Trichoderma reesei*, were used. To insert desired gene to KM71H cells, pPICZαA vectors were purchased from Invitrogen.

3.1.5 Media

3.1.5.1 Inoculation media

After the selection of suitable colony, *P. pastoris* strains were grown on YPD (Yeast Peptone Dextrose) liquid media. This mixture contains 1% yeast extract, 2% peptone, 2 % dextrose (all w/v), which were mixed in appropriate amounts. 50 g of YPD Broth was used for preparation of 1 L liquid medium. The liquid medium was autoclaved at 121°C for 15 minutes before using.

3.1.5.2 Batch fermentation media

For batch fermentation of *Pichia*; 3,4 g/L YNB (Yeast Nitrogen Base w/o amino acids and ammonium sulphate) and 5 g/L ammonium sulphate were mixed and autoclaved inside the fermenter. Since different substrates were used (glycerol, sorbitol, and methanol), according to the type of batch fermentation 10 g/L of substrate was also added before autoclaving. In methanol batch fermentation, filter sterilized methanol was added with syringe after autoclaving. Before inoculation, 2 ml of antifoam and 8,7 ml PTM1, which was prepared according to *Invitrogen, Pichia fermentation process guidelines*, were added with syringe under sterile conditions.

3.1.5.3 Fed-batch fermentation media

For fed-batch fermentation of *Pichia*; fermentation basal salts medium was prepared according to *Invitrogen, Pichia fermentation process guidelines* and autoclaved inside the fermenter. In transition phase, 5 g/L methanol and 10 g/L sorbitol were added with syringe under sterile conditions. In fed-batch phase, methanol and sorbitol were continuously added either together or separately.

For sorbitol-methanol mix, 250 g sorbitol and 250 g ddH_2O were autoclaved together and 250 ml of methanol was added under sterile conditions. 12 ml/L PTM1 was also added to the solution.

When sorbitol and methanol were added separately, 300 g/L sorbitol was autoclaved in 1000 ml of ddH_2O and 12 ml/L PTM1 was added to the solution. For methanol, filter-sterilized 100% methanol was mixed with 12 ml/L PTM1.

3.2 Methods

3.2.1 Selection of the colony

For the production of EG I enzyme, KM71H cells containing egI gene, were given by laboratory member, Günseli Bayram Akçapınar. Best expressing colony was picked from YPD-Agar plates.

3.2.2 Preparation of glycerol stock

To keep the cells alive for long time, glycerol stocks of the same colony were prepared. For this purpose, one colony expressing EGI was selected and grown overnight in YPD, at 250 rpm, 30 °C. Then, cells were centrifuged and pellets were resuspended with YPD and 30% glycerol at a final OD_{600} of 100 (approximately $5x10^9$ cells/ml). To store, cells were put at -80°C.

3.2.3 Growth on YPD medium

For fermenter inoculation; cells, which were kept at glycerol stock, were grown overnight in YPD at 250 rpm, 30 °C. 1L shake flasks were used, each containing 200 ml of YPD. Growth conditions were the same for each inoculation medium.

3.2.4 Fermentation of *Pichia*

3.2.4.1 Batch fermentations of *Pichia*

3.2.4.1.1 Glycerol batch fermentation of Pichia

For batch fermentation with glycerol, fermenter's pH was adjusted to 5,5 (using 5 M KOH) , temperature was set to 30°C. Air flow was set to 1 vvm. After inoculation, in every 2 hours, sampling was done and OD_{600} of cells were measured. Also cells were centrifuged and pellets were used in dry cell weight analysis. Supernatants were also saved for further analysis. Fermentation was ended after the depletion of glycerol.

3.2.4.1.2 Sorbitol batch fermentation of *Pichia*

For batch fermentation with sorbitol, fermenter's pH was adjusted to 5,5 (using 5 M KOH) , temperature was set to 30°C. Air flow was set to 1 vvm. After inoculation, in every 2 hours, sampling was done and OD_{600} of cells were measured. Also cells were centrifuged and pellets were used in dry cell weight analysis. Supernatants were also saved for further analysis. Fermentation was ended after the depletion of sorbitol.

3.2.4.1.3 Methanol batch fermentation of *Pichia*

For batch fermentation with methanol, fermenter's pH was adjusted to 5,5 (using 5 M KOH) , temperature was set to 30°C. Air flow was set to 1 vvm. After inoculation and addition of methanol with syringe, in every 2 hours, sampling was done and OD_{600} of cells were measured. Also cells were centrifuged and pellets were used in dry cell weight analysis. Supernatants were also saved for further analysis. Fermentation was ended after the depletion of methanol.

3.2.4.1.4 Sorbitol-MeOH batch fermentation of *Pichia*

For batch fermentation with sorbitol and methanol, fermenter's pH was adjusted to 5,5 (using 5 M KOH) , temperature was set to 30°C. Air flow was set to 1 vvm. After inoculation and addition of methanol (10 g/L) with syringe, in every 2 hours, sampling was done and OD_{600} of cells were measured. Also cells were centrifuged and pellets were used in dry cell weight analysis. Supernatants were also saved for further analysis. Fermentation was ended after the depletion of methanol.

3.2.4.2 Growth rate and feeding rate calculations

In order to calculate growth rates of cells on different substrates, the equation below was used.

$$\mu = \frac{\ln(X_{i+1}/X_i)}{t_{i+1}-t_i}$$

Eq 3.1 Calculation of growth rate

In this equation; μ stands for growth rate, t stands for time, and X stands for biomass (g). Letter "i" was used in order to indicate different time points.

To calculate the yield of cells on different substrates, equation below was used.

$$Y_{X/S} = \frac{X_f - X_o}{S_o - S_f}$$

Eq 3.2 Calculation of the yield

In Eq 3.2, $Y_{X/S}$ corresponds to the yield, X_f and X_o stands for final biomass and initial biomass and S_o and S_f corresponds to initial substrate concentration nd final substrate concentration, respectively.

After calculating the growth rate and the yield specific to the substrate, to use in fed-batch fermentations, feeding rates of substrates were calculated. The equation used for this purpose can be seen below.

$$F(t) = \frac{\mu * V_O * X_O}{Y_{X/S} * S_O} * e^{\mu t}$$

Eq. 3.3 Feeding rate calculation of substrates

In equation 3.3, F(t) stands for feeding rate at a given time point and V_O corresponds to initial volume.

3.2.4.3 Fed-batch fermentation of *Pichia*

3.2.4.3.1 Sorbitol-MeOH fed-batch fermentation of *Pichia* at 30°C, using 1,1 g/L MeOH level

Fed-batch fermentation with sorbitol and methanol substrates was started with the same conditions as in batch fermentations (pH, temperature, air flow). In batch phase, ammonia 26% was used for pH adjustment. After the depletion of glycerol, transition phase was started with injections of sorbitol and methanol. During this phase, base was changed to 5 M KOH. Following the depletion of both substrates, fed-batch phase was started. Sorbitol-methanol mix was started to add continuously in appropriate amounts. Methanol level was tried to be stabilized around 1 g/L. Sampling was done in every 2 hours and both supernatants and pellets were saved for further analysis.

3.2.4.3.2 Sorbitol-MeOH fed-batch fermentation of *Pichia* at 25°C

Fed-batch fermentation with sorbitol and methanol substrates was started with the same conditions as in batch fermentations. In batch phase, ammonia 26% was used for pH adjustment. After the depletion of glycerol, transition phase was started with injections of sorbitol and methanol. During this phase, base was changed to 5 M KOH, and in every hour, temperature decreased one degree until 25°C. Following the depletion of both substrates, fed-batch phase was started. Sorbitol-methanol mix was started to add continuously in appropriate amounts. Methanol level was tried to be

stabilized around 1 g/L. Sampling was done in every 2 hours and both supernatants and pellets were saved for further analysis.

3.2.4.3.3 Sorbitol-MeOH fed-batch fermentation of *Pichia* at 30°C using 2,3 g/L MeOH level

Fed-batch fermentation with sorbitol and methanol substrates was started with the same conditions as in batch fermentations. In batch phase, ammonia 26% was used for pH adjustment. After the depletion of glycerol, transition phase was started with injections of sorbitol and methanol. During this phase, base was changed to 5 M KOH. Following the depletion of both substrates, fed-batch phase was started. Sorbitol and methanol were added separately and continuously. Methanol level was tried to be stabilized around 2 g/L. Sampling was done in every 2 hours and both supernatants and pellets were saved for further analysis.

3.2.4.3.4 Sorbitol-MeOH fed-batch fermentation of *Pichia* at 30°C using 0,6 g/L MeOH level

Fed-batch fermentation with sorbitol and methanol substrates was started with the same conditions as in batch fermentations. In batch phase, ammonia 26% was used for pH adjustment. After the depletion of glycerol, transition phase was started with injections of sorbitol and methanol. During this phase, base was changed to 5 M KOH. Following the depletion of both substrates, fed-batch phase was started. Sorbitol and methanol were added separately and continuously. Methanol level was tried to be stabilized around 0,5 g/L. Sampling was done in every 2 hours and both supernatants and pellets were saved for further analysis.

3.2.5 OD_{600} measurement of fermentation samples

During each fermentation, after the sampling was done, OD_{600} of samples were measured. Serial dilutions were made in case the value was higher than the range of the

spectrophotometer. For this purpose, dilutions were done with ddH_2O and appropriate amount of sample.

3.2.6 Dry cell weight measurement of fermentation samples

To measure dry cell weight of samples, eppendorf tubes were pre-weighed using sensitive balance and noted. After the sampling was done, 1 ml of sample was taken into pre-weighed eppendorf tubes and centrifuged. For more accurate results, triplicates of each measurement were done. Supernatants were put into other tubes for further analysis and pellets were washed with ddH_2O to get rid of precipitated salts. Until the fermentation was finished, pellets were kept at -20°C. After the fermentation was ended, tubes were taken and put into 70°C for 2 days. Then, each tube was weighed again and dry cell measurements were calculated. This procedure was done for each fermentation.

3.2.7 Total protein analysis

To analyze protein concentration in the samples Bradford reagent was used. For this purpose, master mix containing 5X Bradford reagent and ddH_20 was prepared. For standards to calculate protein amounts, Bovine Serum Albumin (BSA) was diluted in appropriate amounts. 25 µl from each sample and 175 µl from master mix were added together in a 96-well plate and analyzed with Biorad Microplate Reader at 595 nm wavelength. For standards and blanks, same procedure was applied. According to the standard curve, concentrations in samples were calculated.

3.2.8 Endoglucanase activity assay

To measure the activity of secreted EG I, 4-MU cellubioside substrates were used. The supernatants of the final samples of each fermentation were used directly as the source of enzymes in the assays. SpectraMax Gemini XS spectrofluorometer was used to measure fluorescence.

For this purpose, 200 mM NaOAc pH 5 was prepared and 96-well black microtiter plates (Costar) were used. A master mix, which contained 99 µl ddH2O and 50 µl buffer for each sample, was prepared. 1 µl substrate for each sample was added right before the reading. Measurements were done as duplicates and 50 µl from each sample was added to the plate. For the analysis, spectrofluorometer was used at 330 excitation and 456 emission wavelengths at 40°C.

3.2.9 SDS-PAGE analysis

SDS-PAGE gels were prepared and run according to the protocol in *Molecular Cloning: A Laboratory Manual, Sambrook et al*., 2001.

3.2.9.1 Zymogram analysis

To see the activity on the gels, zymogram of gels were done. For this purpose, after running the gels, they were washed with ddH_2O and kept in TritonX100-NaOAc buffer for 10 minutes. For half an hour, buffer was renewed when the time was up. Then, gels were kept in 50 mM pH 4,8 NaOAc buffer for half an hour. Then, gels were put in UV-Transilluminator and 4-MU cellobioside substrates were spreaded all around the gels. When the activity was observed, the pictures were taken and saved.

3.2.10 MeOH level calculation

To calculate the MeOH levels in fed-batch fermentations, nuclear magnetic resonance (NMR) technique was used. By comparing with a sample, which has a known methanol level, the methanol amounts of the samples were calculated by NMR.

4 RESULTS

4.1 OD_{600} and Dry cell weight results

4.1.1 Batch fermentations

To obtain the growth rate yields and also the feeding rates to be used in fed-batch fermentations, the OD_{600} and DCW results of batch fermentations were calculated. The means of OD_{600} results obtained from duplicate measurements and the means of DCW results obtained from triplicate measurements can be seen in Table 4.1 below:

Table 4.1 Values of batch glycerol and sorbitol batch fermentations

Batch Glycerol				**Batch Sorbitol**		
Aging	**lnOD600**	**lnDCW**		**Aging**	**lnOD600**	**lnDCW**
0,00	1,27	0,15		0,00	1,26	0,09
1,00	1,35	0,17		1,85	1,43	0,25
2,00	1,49	0,14		17,83	1,73	0,56
3,00	1,69	0,44		22,62	1,82	0,65
4,00	1,85	0,61		24,73	1,94	0,77
5,00	2,20	0,85		26,62	1,98	0,81
6,00	2,44	1,18		42,08	2,46	1,29
7,00	2,80	1,72		43,95	2,52	1,35
8,00	3,04	1,98		47,22	2,59	1,42
9,00	3,33	2,24		49,22	2,65	1,47
10,00	3,61	2,58		65,63	3,03	1,86
19,28	4,75	3,65		67,78	3,08	1,90
23,05	4,75	3,59		69,98	3,01	1,84
25,05	4,77	3,58		71,87	2,99	1,82
				72,87	2,97	1,80

Table 4.2 Values of batch methanol and MeOH-sorbitol batch fermentations

Batch Methanol				Batch MeOH-Sorbitol		
Aging	**lnOD600**	**lnDCW**		**Aging**	**lnOD600**	**lnDCW**
0,00	1,34	0,17		0,00	1,17	0,26
1,00	1,35	0,17		2,00	1,35	0,41
2,00	1,33	0,16		4,00	1,37	0,71
3,00	1,38	0,21		16,10	1,85	0,86
4,00	1,38	0,21		18,10	1,93	0,94
5,00	1,37	0,20		20,10	1,99	1,13
19,22	1,51	0,34		22,10	2,11	1,14
22,87	1,52	0,35		24,10	2,17	1,40
26,53	1,58	0,41		28,10	2,48	1,53
45,90	1,63	0,46		37,38	3,06	1,87
				40,67	3,21	1,98
				42,67	3,27	2,01
				44,67	3,34	2,09
				46,67	3,36	2,11
				48,67	3,36	2,12
				55,88	3,34	2,13

According to these values, graphs of them were drawn all together. They can be seen in the figures below.

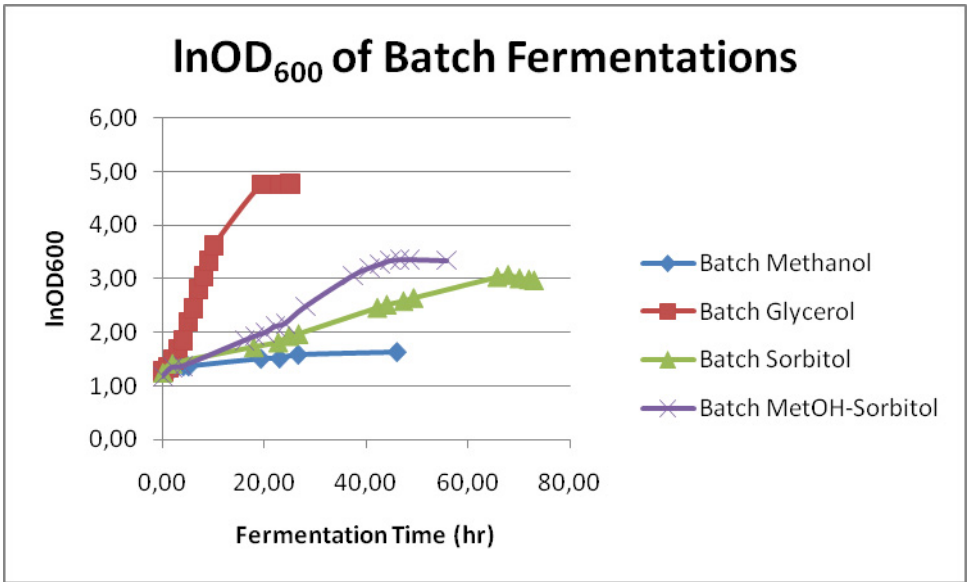

Figure 4.1 $lnOD_{600}$ results of batch fermentations

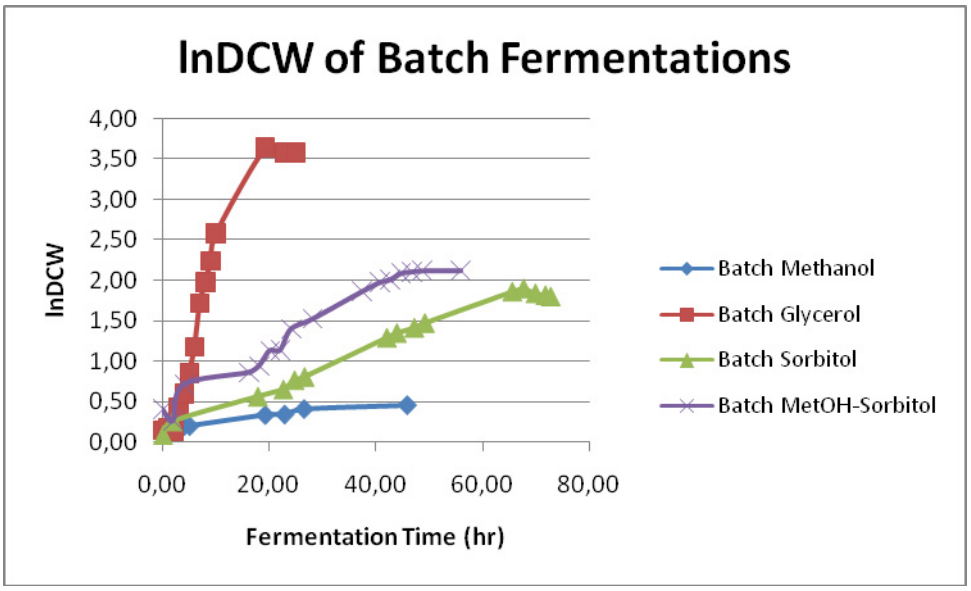

Figure 4.2 Dry cell weight results of batch fermentations

As it can be seen from the Figure 4.1 and Figure 4.2, the best growth was observed in batch glycerol fermentation, due to the fact that *Pichia* cells growing on glycerol as a substrate faster. The second best one was observed to be in MeOH-Sorbitol batch fermentation. The combined effect of methanol and sorbitol was observed to be affecting *Pichia* cells positively, when compared with the results of sole sorbitol batch fermentation. In batch methanol fermentation, almost no growth was observed.

According to these values, in each fermentation, growth rate of *Pichia* cells was calculated. These values were used in fed-batch fermentations to decide pump rates.

4.1.2 Fed-batch fermentations

To observe the growth rate profiles of *Pichia* cells on different fed-batch strategies, OD_{600} (as duplicates) and DCW (as triplicates) results were calculated. The results obtained from all fed-batch fermentations can be seen in tables below:

Table 4.3 OD_{600} and DCW values of fed-batch at 25°C and 30°C using 2,3 g/L MeOH level fermentations

Fed-batch Temp.25°C				**Fed-batch Temp.30°C; using 2,3 g/L MeOH**		
Aging	**lnOD600**	**lnDCW**		**Aging**	**lnOD600**	**lnDCW**
0,00	1,33	0,10		0,00	1,32	0,10
8,45	2,81	2,52		14,25	4,17	3,38
17,45	4,38	3,69		16,25	4,30	3,60
19,45	4,36	3,76		18,25	4,34	3,62
21,45	4,45	3,83		20,25	4,44	3,72
23,45	4,51	3,80		22,25	4,59	3,78
25,45	4,63	3,79		27,12	4,65	3,78
27,45	4,67	3,84		38,08	5,00	3,92
42,82	5,44	4,39		40,08	5,06	4,00
44,82	5,58	4,45		42,08	5,13	4,11
46,82	5,69	4,49		44,08	5,30	4,14
48,82	5,65	4,55		46,08	5,36	4,18
57,53	5,83	4,84		48,08	5,44	4,25
66,20	5,99	4,97		50,08	5,50	4,35
68,20	5,99	5,00		62,05	5,78	4,65
				64,05	5,89	4,71
				66,05	5,98	4,75
				68,05	6,05	4,80
				70,05	6,12	4,77
				72,05	6,19	4,78
				85,83	6,28	4,81
				87,83	6,28	4,81

Table 4.4 OD_{600} and DCW values of fed-batch fermentations with 0,6 g/L and 1,1 g/L MeOH values

Fed-batch Temp.30C, using 0,6 g/L MeOH				**Fed-batch Temp.30C, using 1,1 g/L MeOH**		
Aging	**lnOD600**	**lnDCW**		**Aging**	**lnOD600**	**lnDCW**
0,00	1,29	0,26		0,00	1,32	0,10
16,13	4,28	3,27		14,67	4,28	3,24
18,13	4,35	3,37		16,67	4,30	3,23
20,13	4,44	3,44		18,67	4,37	3,31
22,13	4,52	3,46		20,67	4,47	3,38
24,13	4,52	3,49		21,73	4,45	3,35
36,60	5,02	3,90		23,73	4,51	3,43
38,60	5,13	3,96		27,90	4,66	3,65
40,60	5,16	4,01		37,58	5,14	3,91
42,60	5,21	4,09		39,58	5,20	3,98
44,60	5,28	4,13		41,58	5,34	4,03
46,60	5,32	4,21		43,58	5,48	4,08
60,78	5,82	4,47		45,58	5,52	4,14
62,78	5,94	4,53		47,58	5,56	4,21
65,33	5,96	4,45		61,35	5,81	4,43
67,73	5,96	4,49		63,35	5,90	4,40
83,77	6,01	4,62		65,35	5,95	4,43
				67,35	6,01	4,46
				69,35	5,96	4,46
				87,10	5,98	4,44
				89,47	6,00	4,54

According to these values, graphs of them were drawn all together. They can be seen in the figures below.

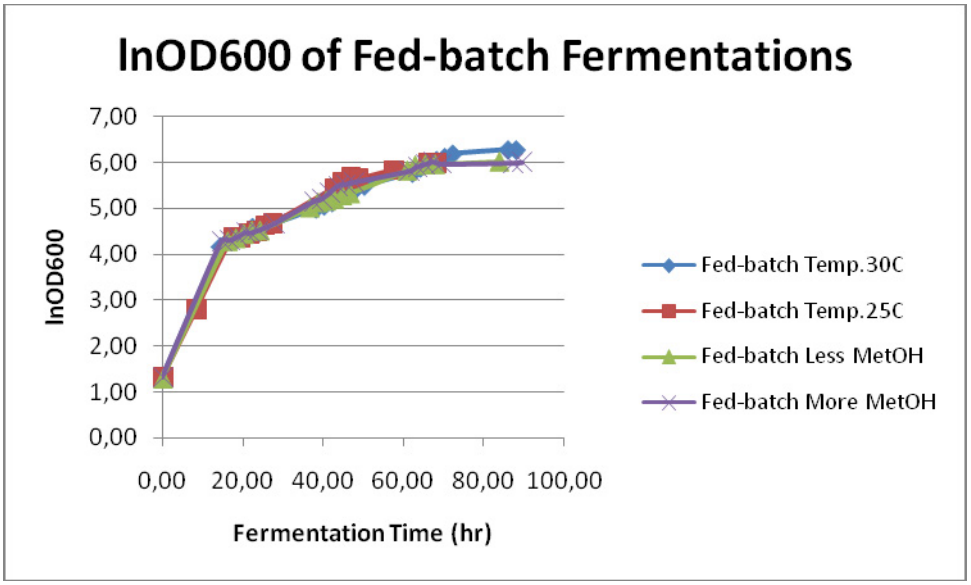

Figure 4.3 lnOD600 results of fed-batch fermentations

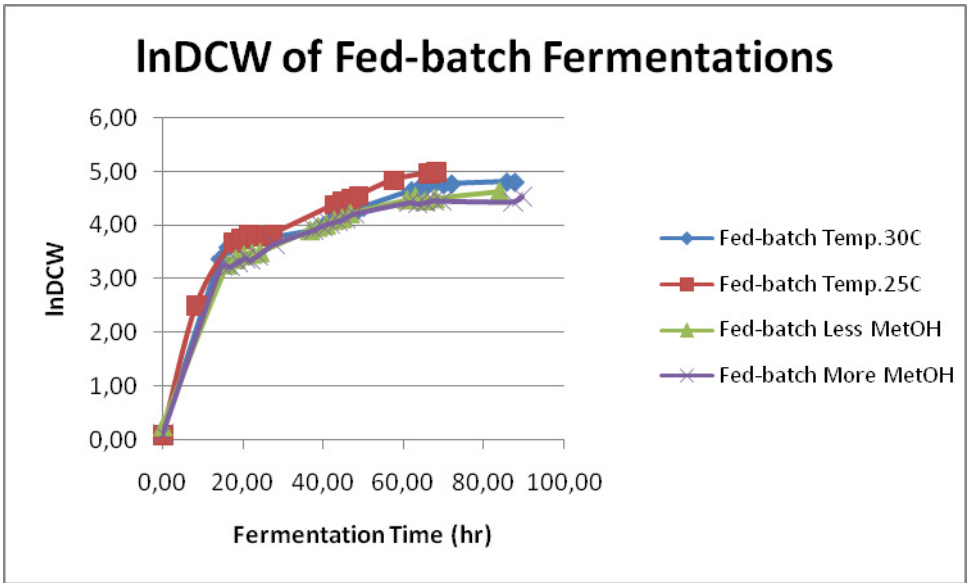

Figure 4.4 Dry cell weight results of fed-batch fermentations

As it can be seen in Figure 4.3 and Figure 4.4, there is almost no difference between the growth curves of all fed-batch fermentations. It can be said that the growth of *Pichia* cells at 25°C was observed to be a little higher when compared with other fermentation strategies. Due to *Pichia* cells consuming substrates faster at 25°C, as it can be seen in the Figure 4.3 and Figure 4.4, the fermentation was ended earlier than the other fermentations. According to these results, it can be said that the feeding rate calculations obtained from batch fermentations were seen to be accurate. After about 60th hour, growths of all fermentations were observed to be stabilize.

4.2 Total protein analysis

For the calculation of protein amounts in fed-batch fermentations, BSA was used as standards. The values of these standards measured at 595 nm and the standard curve can be seen below.

Table 4.5 Standard values of BSA used in total protein analysis

BSA Conc, mg/ml	Absorbance
0	0,0155
0,03125	0,191
0,0625	0,309
0,125	0,5125
0,25	0,862
0,5	1,4205
1	1,5065

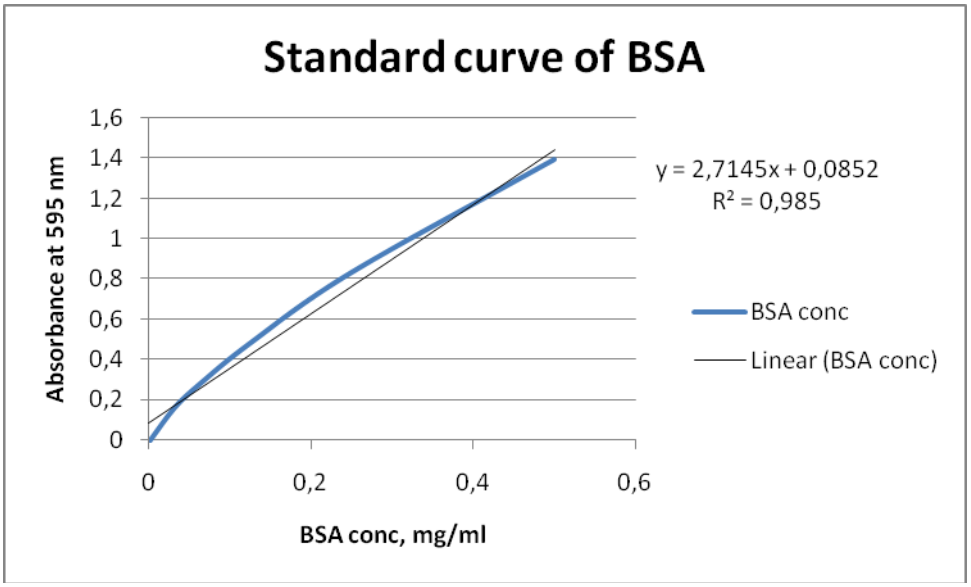

Figure 4.5 Standard curve for Bradford assay

According to the values obtained from standard curve, total protein amounts of the samples were calculated. Using triplicates of the same samples, means of the protein amounts were calculated. Results of the total protein analysis (Bradford) can be seen below.

Table 4.6 Total protein amounts of fed-batch fermentations using 0,6 g/L and 1,1 g/L MeOH and MeOH-Sorbitol batch fermentation

Fed-batch 0,6 g/L MeOH				**Fed-batch 1,1 g/L MeOH**				**Batch MeOH-Sorbitol**		
	Hour	**Total prot. (mg/L)**			**Hour**	**Total prot. (mg/L)**			**Hour**	**Total prot. (mg/L)**
S1	0,00	0,00		**S1**	0,00	0,00		**S1**	0,00	0,00
S3	16,20	145,36		**S3**	15,00	58,27		**S4**	16,10	0,00
S5	20,13	177,17		**S5**	18,67	98,91		**S6**	20,10	0,00
S7	22,97	217,70		**S7**	21,73	124,25		**S8**	24,10	0,00
S9	36,60	376,26		**S9**	27,90	161,95		**S10**	37,38	12,21
S11	40,60	425,02		**S11**	39,58	300,04		**S12**	42,67	34,91
S13	44,60	459,90		**S13**	43,58	319,75		**S14**	46,67	37,35
S15	60,78	525,85		**S15**	47,58	328,80		**S16**	55,88	28,56
S17	65,33	501,17		**S16**	61,35	295,51				
S19	83,77	459,65		**S18**	65,35	308,48				
				S20	69,35	289,88				
				S22	89,47	228,30				

Table 4.7 Total protein amounts of fed-batch fermentations at temperature 30°C, using 2,3 g/L MeOH and 25°C

Fed-batch Temp.30C, 2,3 g/L MeOH				**Fed-batch Temp.25C**		
	Hour	**Total prot. (mg/L)**			**Hour**	**Total prot. (mg/L)**
S1	0,00	0,00		**S1**	0,00	0,000
S3	14,60	97,70		**S4**	17,70	142,962
S5	18,25	141,43		**S6**	21,45	192,078
S7	22,25	174,83		**S8**	25,45	209,385
S8	27,12	170,54		**S10**	42,82	421,207
S9	38,08	269,41		**S12**	46,82	466,789
S11	42,08	346,90		**S14**	57,53	521,633
S13	46,08	379,33		**S16**	68,20	556,977
S15	50,08	440,62				
S17	64,05	372,57				
S19	68,05	352,68				
S21	72,05	339,78				
S23	87,83	354,15				

According to these values, graphs of them were drawn all together. They can be seen in the figure below.

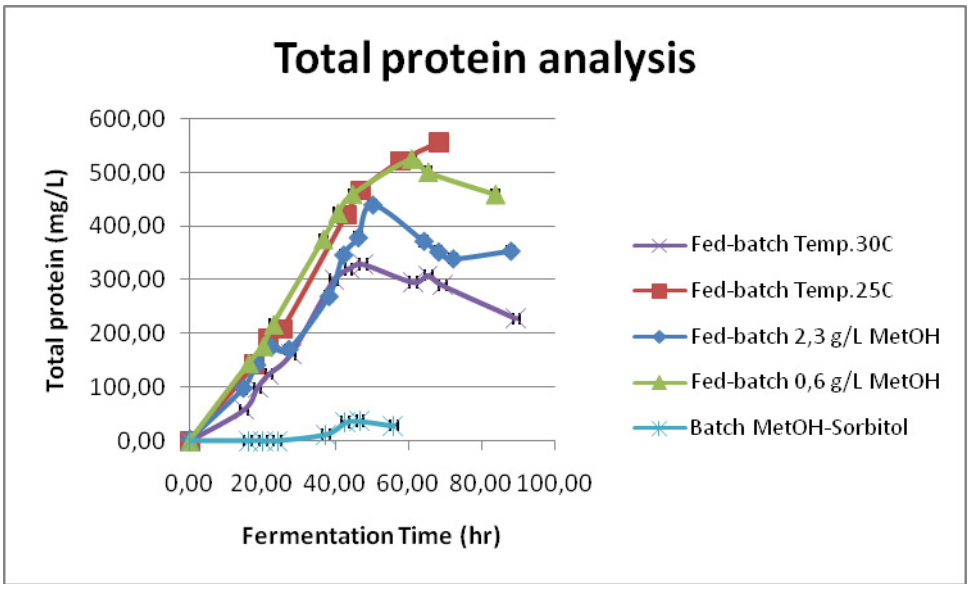

Figure 4.6 Total protein analysis of fed-batch fermentations and MeOH-Sorbitol batch fermentation

As it can be seen in Figure 4.5, less protein amount was observed in MeOH-Sorbitol fermentation as expected, since there was no addition of substrates during the fermentation. The best protein amount was observed to be in 25°C fermentation and also in the fed-batch fermentation using 0,6 g/L MeOH. Protein amounts were started to decrease in all fed-batch fermentations at 30°C but it kept increasing in 25°C fermentation. The least protein amount was observed in fed-batch fermentation using 1,1 g/L MeOH level, however in fed-batch fermentation using 2,3 g/L MeOH level, the protein amounts were observed to be higher. When comparing the effects of MeOH levels on protein concentration, it can be said that low and high levels of methanol seems to be affecting the production positively.

4.3 Endoglucanase I activity analysis

To observe the activity of the produced EG I, fluorescence substrates (4-MU cellobioside) were used. Using duplicates of the same samples, means of the activity analyses were calculated. The results obtained from these calculations can be seen below:

Table 4.8 Activity results of fed-batch fermentations using 0,6 g/L and 1,1 g/L MeOH and MeOH-Sorbitol batch fermentation

Fed-batch 0,6 g/L MeOH				**Fed-batch 1,1 g/L MeOH**				**Batch MeOH-Sorbitol**		
	Time (hr)	**Activity**			**Hour**	**Activity**			**Time (hr)**	**Activity**
S1	0,00	0,00		**S1**	0,00	0,00		**S1**	0,00	0
S3	16,20	50,79		**S3**	15,00	42,92		**S4**	16,10	46,22
S5	20,13	191,60		**S5**	18,67	202,90		**S6**	20,10	72,12
S7	22,97	379,10		**S7**	21,73	393,90		**S8**	24,10	115
S9	36,60	1770,00		**S9**	27,90	800,80		**S10**	37,38	316,3
S11	40,60	2574,00		**S11**	39,58	2327,00		**S12**	42,67	415,8
S13	44,60	3479,00		**S13**	43,58	2810,00		**S14**	46,67	443,2
S15	60,78	4881,00		**S15**	47,58	2912,00		**S16**	55,88	497,7
S17	65,33	5396,00		**S16**	61,35	3456,00				
S19	83,77	6036,00		**S18**	65,35	3526,00				
				S20	69,35	3311,00				
				S22	89,47	3094,00				

Table 4.9 Total protein amounts of fed-batch fermentations at temperature 30°C, using 2,3 g/L MeOH and 25°C

Fed-batch Temp.30C; 2,3 g/L MeOH				**Fed-batch Temp.25C**		
	Hour	**Activity**			**Hour**	**Activity**
S1	0,00	0,00		**S1**	0,00	0,00
S3	14,60	25,82		**S4**	17,70	48,06
S5	18,25	87,82		**S6**	21,45	243,30
S7	22,25	254,30		**S8**	25,45	501,70
S8	27,12	315,90		**S10**	42,82	2598,00
S9	38,08	924,90		**S12**	46,82	3008,00
S11	42,08	1654,00		**S14**	57,53	3835,00
S13	46,08	2533,00		**S16**	68,20	4826,00
S15	50,08	3060,00				
S17	64,05	4264,00				
S19	68,05	4490,00				
S21	72,05	4933,00				
S23	87,83	4755,00				

According to these values, graphs of them were drawn all together. They can be seen in the figure below.

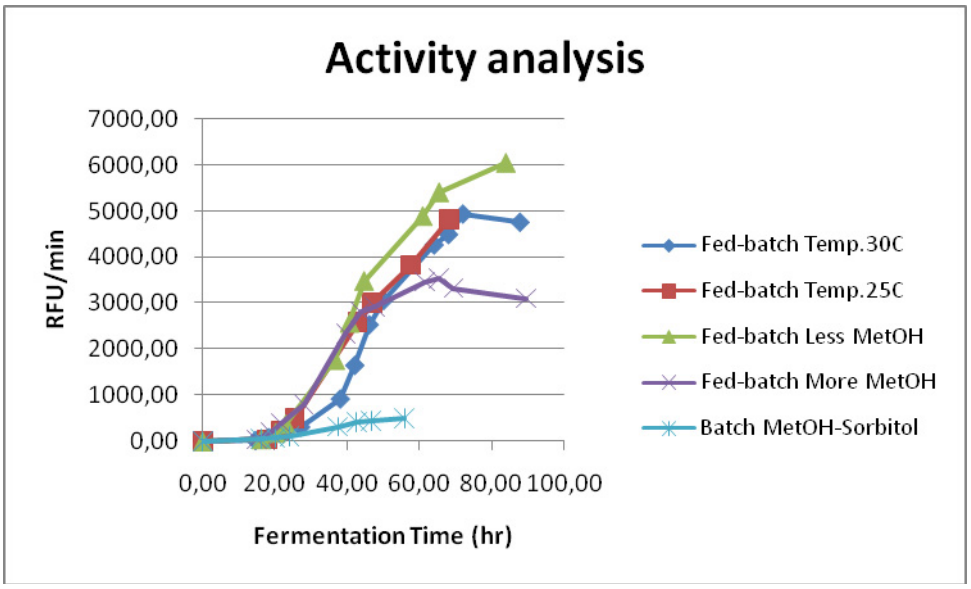

Figure 4.7 Activity analysis of all fed-batch fermentations and MeOH-Sorbitol batch fermentation

As it can be seen in Figure 4.6, the least activity was observed in MeOH-Sorbitol batch fermentation as expected, since there was no addition of substrates during the fermentation. The best activity was observed in the fermentation using 0,6 g/L MeOH level. The activity in fermentation 25°C was seen to be less than expected when compared with the protein amounts results. There is almost no difference between fermentations using 2,3 g/L MeOH level and at 25°C. Activity was observed to be increasing in 0,6 g/L MeOH fermentation, however using 1,1 g/L and 2,3 g/L MeOH levels seems to be affecting the activity of EG I negatively after around 65th hour.

4.4 SDS-PAGE and zymogram analyses

In order to see the expressed EG I and activity, SDS-PAGE and zymogram analyses were performed. Since the results of the SDS-PAGE analyses of the some fed-batch fermentations was not so bright, an ultra-filtrated sample obtained from batch MeOH-Sorbitol fermentation was also run to see the proteins on the gel more clear. From the same ultra-filtrated sample, dilutions with different ratios (Table 4.10) were done and load on the gel. The gel picture (both SDS-PAGE and zymogram) of ultra-filtrated sample can be seen below.

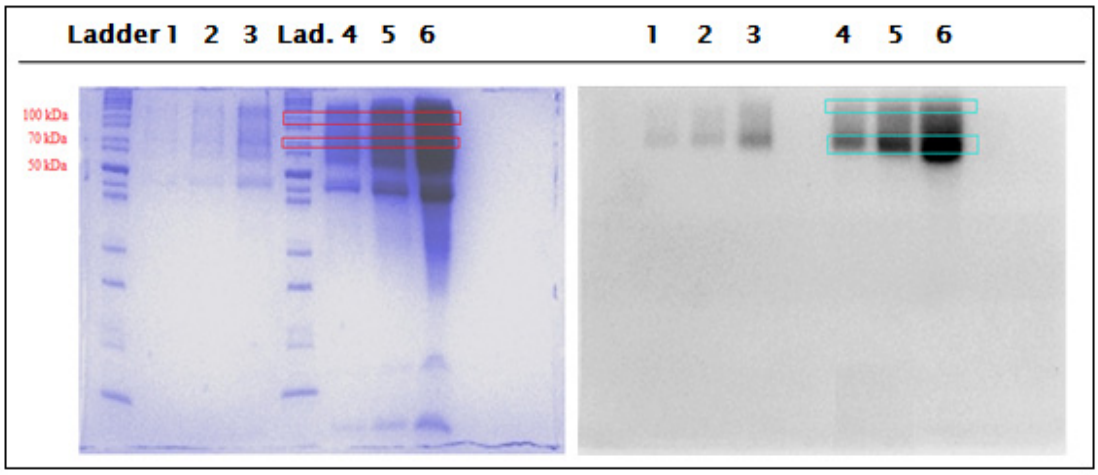

Figure 4.8 SDS-PAGE and zymogram analyses of ultra-filtrated sample

Table 4.10 Dilution ratios of ultra-filtrated sample

Sample	Dilution
1	1/100
2	1/50
3	1/20
4	1/10
5	1/5
6	1/2

As it can be seen in Figure 4.8, EG I was observed to be around 100 kDa and 70 kDa. The difference with the native protein's molecular weight (50 kDa) might be because of the glycosylation, especially for 70 kDa. 100 kDa might be because of the dimerization of the protein.

The results of SDS-PAGE and zymogram analyses of the fed-batch fermentations can be seen below. Samples were loaded according to the sampling hours.

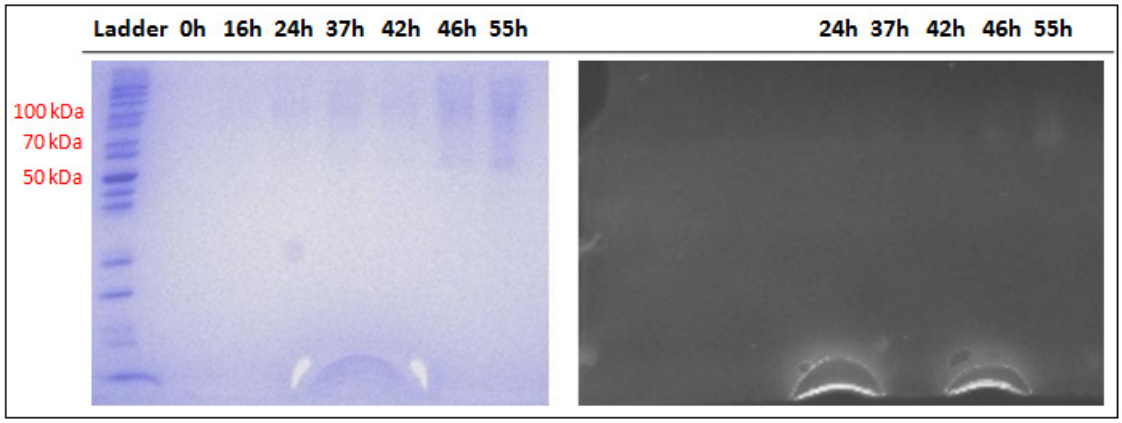

Figure 4.9 SDS-PAGE and zymogram analyses of MeOH-Sorbitol batch fermentation

As it can be seen in Figure 4.6 and Figure 4.7, the amount of the protein and the activity were observed to be really less in MeOH-Sorbitol batch fermentation. As predicted, also in SDS-PAGE and zymogram analyses, the amount of the protein and the activity were seen to be less. The last sample has shown the best amount and the activity. EG I was observed to be around 70 kDa and 100 kDa.

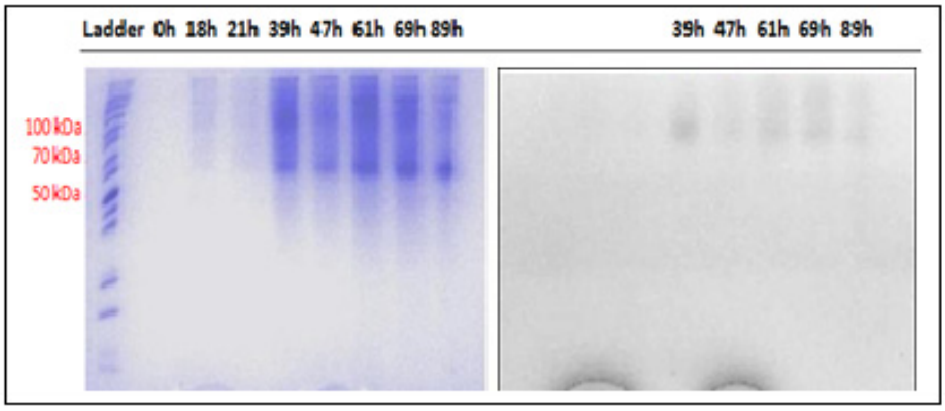

Figure 4.10 SDS-PAGE and zymogram analyses of fed-batch at 30°C fermentation (using 1,1 g/L MeOH)

As it can be seen in Figure 4.10, the amount of the protein and the activity were observed to be less when compared with other fermentations. In zymogram analysis, it can be observed that the activity has remained the same. EG I was observed to be around 70 kDa and 100 kDa.

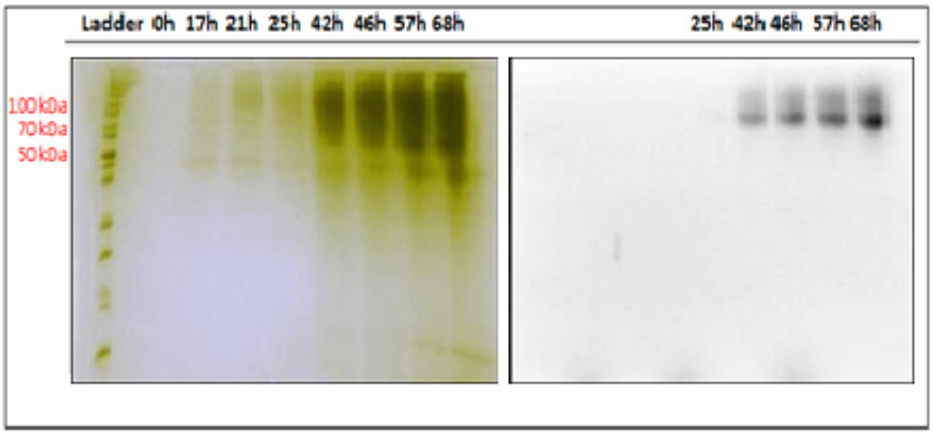

Figure 4.11 SDS-PAGE and zymogram analyses of fed-batch at 25°C fermentation

As it can be seen in Figure 4.11, the amount of the protein and the activity were observed to be high. The highest amount and activity were observed in the last sample. EG I was observed to be around 70 kDa and 100 kDa. Also it can be observed that the amount of the protein and the activity were observed to be stable.

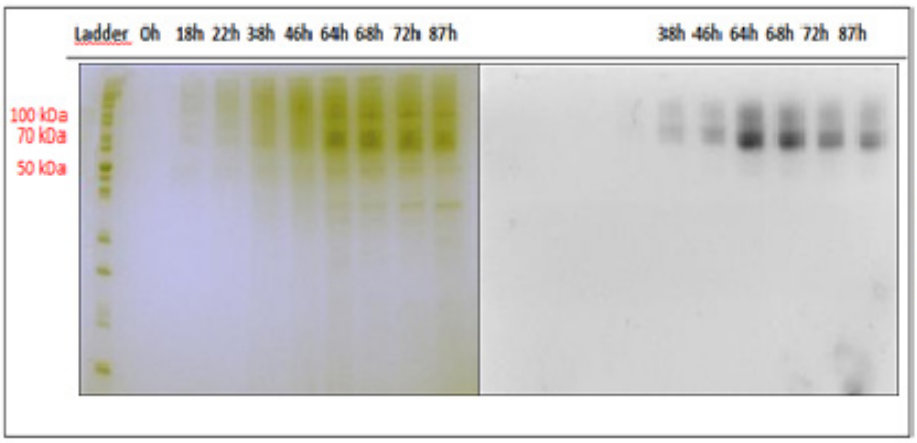

Figure 4.12 SDS-PAGE and zymogram analyses of fed-batch using 2,3 g/L MeOH fermentation

As it can be seen in Figure 4.12, the amount of the protein and the activity were observed to be high. EG I was observed to be around 70 kDa and 100 kDa. Also a little activity was observed around 50 kDa. After 68h, the activity seems to be decreasing.

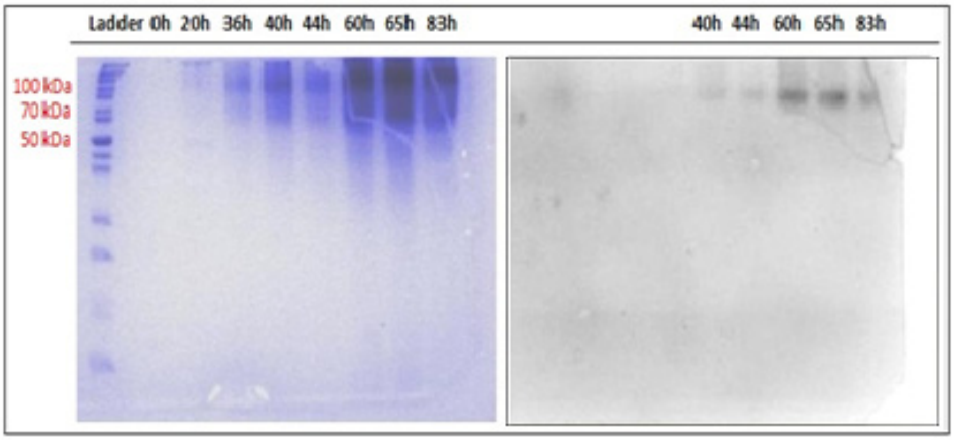

Figure 4.13 SDS-PAGE and zymogram analyses of fed-batch using 0,6 g/L MeOH fermentation

As it can be seen in Figure 4.13, the amount of the protein and the activity were observed to be high. EG I was observed to be around 70 kDa and 100 kDa.

4.5 Comparison of fed-batch fermentation parameters

To better understand the effect of the different strategies applied to produce EG I in *Pichia*, specific activity and specific productivity of the fed-batch fermentations were

calculated. The overall results of the fed-batch fermentations (growth rate, DCW, maximum level of protein, maximum level of activity) can be seen in the table below.

Table 4.11 Overall results of fed-batch fermentations

FERMENTATIONS	μ (1/h)	Max protein (mg/L)	Max activity (rfu/min)	DCW (g/L)	Specific activity (rfu/min/mg)	Specific Productivity (mg/gX/h)
Sorbitol-MeOH fed-batch 2,3 g/L	0,02	440	4933	123	11,21	0,07
Sorbitol-MeOH fed-batch at 25°C	0,02	556	4826	148	8,68	0,08
Sorbitol-MeOH fed-batch, 0,6 g/L	0,02	525	6036	101	11,50	0,1
Sorbitol-MeOH fed-batch at 30°C	0,02	328	3526	94	10,75	0,07

As it can be seen in Table 4.11, the best results were obtained using 0,6 g/L MeOH level fermentation. Specific activity and specific productivity were observed to be higher using low methanol level. The second best one was observed in fed-batch fermentation using 2,3 g/L MeOH, since specific activity level was higher when compared with other fermentations.

5 DISCUSSION

Different carbon sources have different roles in *Pichia* fermentation. While substrates like glycerol and sorbitol are used in growth of cells, substrates like methanol and methylamine are used in expression of several kinds of proteins. Cell growth is the first parameter to be considered during the evaluation of different feeding strategies. As it can be seen from the growth rate results (Figure 4.1 and Figure 4.2), *Pichia* cells grow really fast in the presence of glycerol however in the presence of methanol, there is almost no growth. In the presence of sorbitol, it takes longer time for *Pichia* to grow when compared with glycerol. According to these results, it is showed that for batch part of the fermentations, glycerol is the best source to reach high cell amounts in short time, which is also consistent with the protocol of *Pichia* fermentation.

According to the type of promoter that is going to be used for the expression, different substrates should be selected. AOX promoter, which is the widely used and effective promoter of *Pichia*, needs methanol for the induction but using methanol as sole carbon source has almost no effect on cell growth, affecting the expression level of the protein. So, to increase the expression level, a co-substrate should be used with methanol. However, glycerol cannot be used as a co-substrate since AOX promoter cannot be induced in the presence of glycerol even if it is the best substrate that *Pichia* can grow on. As it is indicated in the Background of this study, sorbitol is found to be a non-repressing carbon source as a co-substrate to methanol. Even in the accumulation of sorbitol, there is no effect on heterologous protein expression. As it can be seen in Figure 4.3 and Figure 4.4, in the presence of methanol and sorbitol, cells both kept growing and expressed heterologous protein (Figure 4.6). Changes in temperature and methanol concentration did not have any effect on cell growth since growing of cells were controlled with limited sorbitol feeding, achieved with the results obtained from batch fermentations.

The optimum conditions for fermentation of *Pichia* change according to the type of the protein. While some proteins can be expressed at lower temperatures more effectively than higher temperatures, some proteins might be affected by the concentration of methanol. For this purpose, different strategies should be considered for optimum expression of the desired protein. For the expression of EG I in *Pichia*, one strategy that has been tried is the change in fermentation temperature. Generally, *Pichia* fermentations are performed at 30°C and since the temperatures above 32°C is detrimental for the protein expression; a temperature value like 25°C was selected as an alternative to 30°C. As it can be seen in Figure 4.6, the total protein amount at 25°C fermentation is higher than 30°C fermentation. Moreover, the expression of the protein seems to be proceeding at 25°C even if it was decreasing at 30°C fermentation after a while. According to these results, it can be said that for the production of EG I, it is better to perform fermentations at 25°C rather than 30°C. This is probably achieved by the reduction of extracellular proteolysis at 25°C due to reduced protease release from cell lysis. Moreover, decrease in protein levels at 30°C might be due to stress situations, like metabolic and environmental stress, of *Pichia* since they have a major effect on productivity during fermentation. Due to these stress conditions, culture viability might be affected resulting in decrease of productivity.

The effects of methanol concentration, which is the other parameter affecting the protein expression, also can be seen in Figure 4.6. Changes in methanol concentration have found to be an important parameter on heterologous protein expression (Arnau, Ramon, Casas, & Valero, 2010). In this study, increase in methanol concentration seems to have a positive effect on protein expression. The decrease of protein level in the fermentation using 1,1 g/L MeOH level may be due to substrate inhibition response to methanol concentration. So, it can be said that for the better expression of EG I in *Pichia*, methanol concentration in fermenter should be either low or high. Decrease in protein levels at different methanol concentrations might be due to stress situations since they all performed at 30°C.

In a fermentation process, if the product is an enzyme, the activity level of this enzyme is as important as its production level. For this purpose, activity analysis with fluorescence substrate 4-MU cellobioside was performed. As it can be seen in Figure 4.7, the best activity was observed in the less methanol fermentation. When results of the total protein and the activity level was compared, although fermentation at 25°C

resulted in more protein, the activity level of fermentation at 30°C showed higher activity especially at last samples. It is known that, EG I shows maximal activity between 40 – 60°C. The secretion of EG I at 25°C might be affecting the enzyme activity, higher degrees like 30°C seems more appropriate for this enzyme's quality. There are some studies showing that the expression of foreign proteins at lower temperatures helps reducing protein misfolding and resulting in more properly folded proteins (Georgiou & Valax, 1996). In our case, the folding of EG I in *Pichia* might be negatively affected by lowering the temperature.

As it can be seen in SDS-PAGE and zymogram results, there are more than one active form of EG I synthesized by *Pichia*. It is well documented that there are many isoforms of EG I. Moreover, foreign proteins expressed in *Pichia* undergo posttranslational processing like glycosylation, affecting the molecular size of the expressed protein. In this study, we observed bands around 70 kDa and 100 kDa in SDS-PAGE results, also shown by zymogram analyses. The molecular weight of EG I that is been used in this study was 50 kDa. These results indicate that EG I undergo glycosylation and hyperglycosylation processes, but as it can be seen in zymogram results, these forms of EG I were observed to be highly active. Also, in zymogram analyses, it can be observed that less protein and activity around 100 kDa was observed in less methanol fermentation compared with more methanol and 30°C fermentation. The glycosylation of the protein might be affected by the methanol concentration level, so it can be said that for purification and structural analysis purposes, it might be better to perform fermentations of EG I with less methanol concentration levels.

6 CONCLUSION

Cellulases are important enzymes since they have many industrial applications and more importantly they can be used in biofuel studies. To our knowledge, for the first time, the high level production of EG I in *Pichia pastoris* was performed in this study. The parameters affecting the expression of EG I were investigated. Therefore the protein expression level and the activity of proteins were observed. No difference in cell amounts in fed-batch fermentations was observed since the growth rate was stabilized with the calculations obtained from batch fermentations. Affect of the temperature on EG I production was analyzed. It is understood that low temperature was better for the production of EG I, but the activity of the enzyme showed better results at higher temperatures. It can be said that according to the production purposes of EG I, low or high temperature values can be used. Also the effect of methanol concentration level was also investigated. It is understood that, for the production of EG I in *Pichia*, low methanol levels were found to be more appropriate than high or average methanol levels. Also, the activity of EG I at low methanol levels was found to be the highest among all applied different fermentation strategies. According to these results, it can be said that for optimum production of EG I in *Pichia*, low methanol levels should be maintained and fermentation should be performed at 30°C.

In this study, we performed to produce EG I in *Pichia pastoris* successfully for the first time. For future prospects, the effect of pH on the production of EG I in *Pichia pastoris* can also be examined. Additionally, for better understanding of the substrate consumption rates and to have stable feeding, online monitoring of sorbitol and methanol concentrations inside the fermenter should be established.

7 REFERENCES

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8 APPENDIX

Chemicals

Chemical Name	Supplier	Catalog Number
4-MU cellobioside	Sigma, Germany	
Acetic acid	Merck, Germany	1.00063.2500
Acrylamide/Bis-acrylamide	Amresco, USA	0254
Ammonia	Tekkim, Turkey	TK.010010.05000
Ammonium persulphate	Riedel-de Haen, Germany	11222
Ammonium sulphate	Merck, Germany	1.01217.1000
Biotin	Calbiochem, Germany	2031
Boric acid	AppliChem, Germany	A2949.1000
Calcium sulphate-2-hydrate	Riedel-de Haen, Germany	31221
Coomassie Brilliant Blue	Merck, Germany	115444
Cobalt chloride-6-hydrate		
Cupric sulphate-5-hydrate	Sigma, Germany	209198
Ethanol	Riedel-de Haen, Germany	32221
Ferrous sulphate-7-hydrate	Riedel-de Haen, Germany	
Glycerol	Merck, Germany	1.04092.2500
Glycine	Duchefa,	G0709.1000
Hydrochloric acid	Merck, Germany	100314
Magnessium sulphate-7-hydrate	Fluka, Switzerland	63142
Manganese sulphate-1-hydrate	Riedel-de Haen, Germany	13255
Methanol	Riedel-de Haen, Germany	24229
Phosphoric acid, 85%	Merck, Germany	1.00563.2500
Potassium hydroxide	Carlo Erba, Italy	362257
Potassium sulphate	Fluka, Switzerland	60533

Sodium acetate	Tekkim, Turkey	1.70500.01000
Sodium dodecyl sulphate	Amresco, USA	0227
Sodium hydroxide	Merck, Germany	1.06462.1000
Sodium iodide	Carlo Erba, Italy	370305
Sodium molybdate-2-hydrate	Merck, Germany	1.06521.0100
D(-) Sorbitol	AppliChem, Germany	A2222.0500
Struktol J673	Schill-Seilacher, Germany	
Sulphuric acid	Carlo Erba, Italy	410306
TEMED	Sigma, Germany	T-7029
Tris	Amresco, USA	0826
Yeast nitrogen base (without amino acids and ammonium sulphate)	rpi, USA	Y20060-250.0
YPD Broth	rpi, USA	Y20090-1000.0
Zinc chloride	Carlo Erba, Italy	393007

Equipment

Air Supply:	Nitto Kohki Co. Ltd., JAPAN
Autoclave:	Hirayama, Hiclave HV-110, JAPAN Nüve, TURKEY
Balance:	Sartorius, BP211D, GERMANY Sartorius, BP221S, GERMANY Sartorius, BP610, GERMANY
Centrifuge:	Eppendorf, 5415C, GERMANY Eppendorf, 5415D, GERMANY Eppendorf, 5415R, GERMANY Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY Hitachi, Sorvall RC5C Plus, USA
Chiller:	Sartorius, Frigomix® 1000, GERMANY
Deepfreeze:	-80°C, ThermoForma, USA -20°C, Bosch, TURKEY
Distilled Water:	Millipore, Elix-S, FRANCE Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biorad Inc., USA
Fermenter:	New Brunswick, USA
Gel Documentation:	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA

Incubator:	Memmert, Modell 300, GERMANY Memmert, Modell 600, GERMANY Nüve, EN 120, TURKEY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY VELP Scientifica, Microstirrer, ITALY
Methanol Sensor:	Raven Biotech. Inc., CANADA
Microliter Pipette:	Gilson, Pipetman, FRANCE Eppendorf, GERMANY
Microplate Reader:	Biorad, Model 680, USA
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA Wealtec, Elite 300, USA
Refrigerator:	4°C, Bosch, TURKEY
Shaker:	Forma Scientific, Orbital Shaker 4520, USA GFL, Shaker 3011, USA
Spectrophotometer:	Schimadzu, UV-1208, JAPAN Schimadzu, UV-3150, JAPAN
Spectrofluorometer:	Molecular Devices, SpectraMax Gemini XS