RECOMBINANT TRANSGLUTAMINASE PRODUCTION BY METABOLICALLY ENGINEERED *Pichia pastoris*

THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

BURCU GÜNDÜZ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SEPTEMBER 2012

Approval of the thesis:

RECOMBINANT TRANSGLUTAMINASE PRODUCTION BY METABOLICALLY ENGINEERED *Pichia pastoris*

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ABSTRACT

RECOMBINANT TRANSGLUTAMINASE PRODUCTION BY METABOLICALLY ENGINEERED PICHIA PASTORIS

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September 2012, 190 pages

Transglutaminases (EC 2.3.2.13) are enzymes that catalyze an acyl transfer reaction between a γ-carboxyamide group of a peptide bound glutaminyl residue (acyl donor) and a variety of primary amines (acyl acceptors), including the amino group lysine. Transglutaminase has a potential in obtaining proteins with novel properties, improving nutritional quality of foods with the addition of essential amino acids, preparing heat stable gels, developing rheological properties and mechanical strength of foods and reducing the applications of food additives.

The aim of this study is to develop intracellular and extracellular microbial protransglutaminase (pro-MTG) producing recombinant *Pichia pastoris* strains by using genetic engineering techniques. In this context first,

protransglutaminase gene (*pro-mtg*) from *Streptomyces mobaraensis* was amplified by PCR both for intracellular and extracellular constructs using proper primers then they were cloned into the pPICZα-A expression vectors, separately. Both intracellular (pPICZαA*::pro-mtg*$_{intra}$) and extracellular (pPICZαA::*pro-mtg*$_{extra}$) constructs were prepared with strong alcohol oxidase 1 promoter which is induced by methanol. *Pichia pastoris* X_{33} cells were transfected by linear pPICZαA*::pro-mtg*$_{intra}$ and pPICZαA*::pro-mtg*$_{extra}$, separately and plasmids were integrated into the *Pichia pastoris* X_{33} genome at AOX1 locus. After constructing the recombinant *P. pastoris* strains, batch shaker bioreactor experiments were performed for each recombinant cell and the best producing strains were selected according to Dot blot and SDS-PAGE analyses. The selected recombinant *P. pastoris* strains, carrying pPICZαA*::pro-mtg*$_{extra}$ gene and pPICZαA*::pro-mtg*$_{intra}$ gene in their genome were named as E8 and I1, respectively.

Afterwards, a controlled pilot scale bioreactor experiment in a working volume of 1 L was performed with E8 clone and produced pro-MTG was activated by Dispase I. The variations in the recombinant MTG activity, cell concentration, total protease activity, AOX activity and organic acid concentrations throughout the bioprocess were analyzed and specific growth rates, specific consumption rates and yield coefficients were calculated regarding to measured data. Maximum MTG activity was obtained as 4448 U L^{-1} and the maximum cell concentration was measured as 74.1 g L^{-1} at t=36 h of the bioprocess. In this study, an active transglutaminase enzyme was produced extracellularly by *P. pastoris* for the first time and the third highest extracellular MTG activity was achieved with E8 clone.

Keywords: Microbial transglutaminase, *Pichia pastoris*, extracellular recombinant protein production

ÖZ

METABOLİK MÜHENDİSLİK YÖNTEMLERİYLE MODİFİYE EDİLMİŞ PİCHİA PASTORİS KULLANILARAK TRANSGLUTAMİNAZ ÜRETİMİ

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Eylül, 2012 190 Sayfa

Transglutaminazlar (EC 2.3.2.13) proteinlerin glutaminil gruplarının γ-karboksil grupları ve lisin de dahil olmak üzere çeşitli birincil aminler arasında gerçekleşen açil transfer reaksiyonunu katalizleyen enzimlerdir. Transglutaminaz enzimi, yeni fonksiyonel özelliklerde protein üretimi, elzem amino asitlerin yapıya eklenmesi ile gıdaların besin değerinin geliştirilmesi, ısıya dayanıklı jellerin oluşturulması, gıdaların reolojik özelliklerinin ve mekanik dayanımının geliştirilmesi ve gıda katkı maddelerinin kullanımının azaltılması için kullanılma potansiyeline sahip bir enzimdir.

Bu yüksek lisans çalışmasının amacı, hücreiçi ve hücredışı mikrobiyal protransglutaminaz (pro-MTG) üreten rekombinant *Pichia pastoris* suşlarının

metabolik mühendislik teknikleri kullanılarak geliştirilmesidir. Bu kapsamda ilk olarak, *Streptomyces mobaraensis*'in protransglutaminaz geni hücreiçi ve hücredışı olarak ayrı ayrı üretim yapmak amacıyla pPICZα-A ekspresyon vektörlerine ayrı ayrı klonlanmıştır. Hücreiçi (pPICZαA*::pro-mtg*$_{intra}$) ve hücredışı (pPICZαA::*pro-mtg*$_{intra}$) üretim için geliştirilen plazmidler *P. pastoris* X_{33} genomunda bulunan AOX1 lokusuna klonlanmış ve *pro-mtg* geninin metanolle indüklenen alkol oksidaz promoter vasıtasıyla ekspresyonu sağlanmıştır. Rekombinant *Pichia pastoris* suşlarının hazırlanmasından sonra, kesikli çalkalayıcılı biyoreaktör deneyleri her bir rekombinant hücre için yapılmış ve en iyi üretim yapan suşlar Dot blot ve SDS-PAGE analizleri ile seçilmiştir. Genomlarında pPICZαA*::pro-mtg*$_{extra}$ ve pPICZαA::*pro-mtg*$_{intra}$ genlerini taşıyan seçilmiş rekombinant hücreler sırasıyla E8 ve I1 olarak adlandırılmışlardır.

Pilot ölçekli 1 L çalışma hacmına sahip kontrollu biyoreaktör deneyi E8 klonu ile gerçekleştirilmiş ve üretilen pro-MTG Dispase I ile aktive edilerek MTG enzimi elde edilmiştir. Biyoproses süresince rekombinant MTG aktivitesi, hücre derişimi, toplam proteaz aktivitesi, AOX aktivitesi ve organik asit derişimlerindeki değişimler analiz edilerek spesifik çoğalma hızı, spesifik tüketim hızı ve verim katsayıları hesaplanmıştır. Maksimum 4448 U L^{-1} MTG aktivitesi ve maksimum 74.1 g L^{-1} hücre derişimi t=36 st'te elde edilmiştir. Bu çalışmayla, *Pichia pastoris* tarafından hücre dışı aktif transglutaminaz enzim üretimi ilk kez gerçekleştirilmiştir ve üçüncü en yüksek hücre dışı MTG aktivitesi E8 klonu ile elde edilmiştir.

Anahtar Kelimeler: Mikrobiyal transglutaminaz, *Pichia pastoris*, Hücredışı rekombinant protein üretimi

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my supervisor Prof. Dr. Pınar Çalık for her support, guidance and help, in all the possible ways, throughout this study.

I am also thankful to my co-supervisor Dr. Remziye Yılmaz for her valuable advices and critics.

I am also thankful to my friends in Industrial Biotechnology and Metabolic Engineering research group for their help and advice. I would like to specially thank to Melda Eskitoros, Erdem Boy, Aslan Massahi, Seda Okumuş, Shaghayegh Kerimzadeh and Gül Güneyligil for their support, great friendship, advice and encouragement throughout my studies.

I would like to express my special thanks to Prof. Dr. Markus Pietzsch and Dr. Karin Buttner for sending me the microbial protransglutaminase gene and helping me with their invaluable guidance throughout this study.

My national M.Sc. scholarship provided by Scientific and Technical Council of Turkey (TUBITAK-BIDEB 2210) is gratefully acknowledged.

Above all, I would like to deeply thank to my family and Onur Ergün for loving, supporting and encouraging me all through my life.

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NOMENCLATURE

C	Concentration in the medium	g L^{-1}
DO	Dissolved oxygen	%
Q	Feed inlet rate	L h^{-1}
q	Specific formation or consumption rate	g g^{-1} h^{-1}
r	Formation or consumption rate	g g^{-1} h^{-1}
t	Cultivation time	h
T	Temperature	°C
U	One unit of an enzyme	
V	Volume of the bioreactor	L
Y	Yield (overall)	g g^{-1}

Greek Letters

μ	Specific growth rate	h^{-1}
μ_t	Total specific growth rate	h^{-1}
λ	Wavelength	nm

Subscripts

0	Refers to initial condition
A	Refers to activity
C	Refers to concentration
AOX	Refers to alcohol oxidase

m	Refers to melting point
M	Refers to methanol
p	Refers to product
S	Refers to sorbitol or substrate
t	Refers to total
X	Refers to cell

Abbreviations

A	Activity
AOX	Alcohol oxidase
CDW	Cell dry weight
DNA	Deoxyribonucleic acid
FTG	Red seabream liver transglutaminase
mtg	microbial transglutaminase gene
MTG	Microbial transglutaminase
pro-mtg	microbial protransglutaminase gene
pro-MTG	Microbial protransglutaminase
rhGH	Recombinant human growth hormone
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
OD	Optical density
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid

CHAPTER 1

INTRODUCTION

Industrial biotechnology, also known as 'white biotechnology' primarily depends on the enzymes as biocatalysts and cell factories and their natural or engineered abilities to transform a wide range of renewable substrates to produce a wide range of products for food, feed, textile, pulp, chemistry and energy sectors. The main purpose of the industrial biotechnology is the designing of environmental friendly production strategies for chemicals, materials, pharmaceuticals and bio-energy. The rapid technological developments such as genetic engineering techniques, process developments, mathematical modeling, high throughput analysis tools etc. as well as the need for sustainable development and the continuous globalization, competition are the main drivers of the industrial biotechnology (Olguin *et al*., 2012). Industrial biotechnology provides advantages with reducing energy consumption and waste production as well as minimizing the costs. Additionally, renewable sources are utilized instead of non renewable ones. Moreover, products whose chemical synthesis is not possible can be produced by industrial biotechnology applications (Olguin *et al*, 2012; Hatti-Kaul *et al*., 2007). In microbial biotechnology approach, synthetic biology, microbial physiology, metabolic and pathway engineering was generally used to supply highly complex natural products. Strain improvement methods have decreased costs by increasing productivity of the desired product and inhibiting unwanted side-products (Wolgmeuth, 2009). To design a bioprocess,

recent developments in genomics, bioinformatics and proteomics provide huge amounts of information to facilitate the choice of suitable host microorganism and interested product.

Transglutaminase catalyzes inter and intramolecular peptide bond formation between proteinaceous compounds and this results change in properties of the proteins. For food applications, MTG improves nutritional value of foods and feeds by incorporating essential aminoacids, changes rheological properties and develops mechanical strength, facilitate production of novel proteins, reduces and even eliminates the application of food additives (Yokoyoma *et al*., 2004). MTG catalyzes the binding of polyamines to alter permanently the surface properties of the target proteins and this reaction modifies functional properties of modified proteins with either loss or gain of function (Beninati *et al*., 2009). Additionally, MTG has potential in constructing collagen or gelatin based scaffold to produce artificial organs, site-specific protein conjugation with DNA for biotechnological researches, material science, textile and leather industry (Zhu *et al*., 2008).

Transglutaminase was discovered by Heinrich Waelsch in 1957. The first standardized procedure to isolate tissue transglutaminase was developed in the laboratory of John E. Folk in 1985. At the beginning, source of the enzyme was guinea pig liver which contains larger amount of enzyme than other tissues (Beninati *et al*., 2009) but tissue transglutaminase's limited supply and unacceptability in some applications led the scientists to find new sources. In 1989 a microorganism with the ability of transglutaminase production was discovered and named as *Streptomyces mobaraensis* (Washizu *et al*., 1994; Yokoyama *et al*., 2004).

Microbial transglutaminase (MTG) was firstly produced by *Streptoverticillium mobaraensis* in 1989 by Ando *et al.* and the first recombinant MTG production was performed with *Streptomyces lividans* in 1994 (Washizu *et al*., 1994). Synthesized active MTG gene was firstly

expressed in *E. coli* but it was observed that active enzyme inhibits the cell growth (Takehana *et al*., 1994). Prosequence of the enzyme blocks the activity while increases enzyme thermostability (Pasternack *et al*., 1998). Then, several host microorganisms such as *Escherichia coli*, *Bacillus*, yeasts and *Aspergillus* were used for recombinant microbial transglutaminase production. Recombinant *C. glutamicum* was developed to produce microbial protransglutaminase (pro-MTG) and *promtg* gene was constructed behind the promoter of *cspB*, a cell surface protein of *C. glutamicum* and the signal peptide of CspA, a cell surface protein of *Corynebacterium ammoniagenes* and 142 mg/l pro-MTG was produced by recombinant *C glutamicum* (Kikuchi *et al*. 2003). Site directed mutagenesis was applied to *pro-mtg* to generate Subtilisin like serine protease (SAM-P45) cleavage site and Subtilisin like serine protease (SAM-P45) gene from *Streptomyces albogriseolus* was co-secreted with the pro-MTG (Date *et al*., 2003). Chimeric proregion in which the C-terminal domain of *S. mobaraensis* proregion is replaced by the *S. cinnamoneus* transglutaminase proregion was used to construct recombinant plasmid for *C. glutamicum* and 881 mg/l pro-MTG was produced (Date *et al*., 2004). Highest pro-MTG production was reported 2.5 g/L over a period of 71 h by *Corynebacterium ammoniagenes* ATCC6872. In that study, the plasmid for *S. mobaraensis* transglutaminase expression was constructed with *cspB* promoter and the CspA signal peptide (Itaya *et al*., 2008).

A mutant transglutaminase gene product carried a single amino acid exchange (serine replaced by proline at position 2) showed higher specific activity of 46.1 U mg^{-1} when compared to native MTG (Sommer *et al*., 2011). A recombinant *E. coli* cell harbors this mutated gene construct produced 500mg/l pro-MTG and 23000 U/L over 16 hours cultivation, which was the highest activity value of MTG produced by *E. coli* (Sommer *et al*.,2011).

Methylotrophic yeasts *Pichia pastoris* and *Candida boidinii* were also used for recombinant MTG production. For this purpose 3 different

expression cassettes were prepared. All of the expression cassettes include α-factor signal sequence of *Saccharomyces cerevisiae* and mature *mtg* gene, *pro-mtg* gene or *pro-mtg* gene with Kex2 cleavage site. Alcohol oxidase promoter (AOX1) of *Pichia pastoris* and alcohol oxidase promoter (AOD1) of *C. boidinii* were used for expression of MTG, separately. MTG has 3 putative N-glycosylation sites. With mutagenesis in the glycosylation sites *C. boidinii* strains produced 1.83 U/ml MTG (approximately 87 mg/l; specific activity 22U/mg protein) after 119 h cultivation. Too low enzyme activity was detected with *Pichia pastoris* and it was reported that, due to high glycosylation, they didn't obtain active MTG with recombinant *P. pastoris* strain *(Y*urimoto *et al*., 2004).

Pichia pastoris is one of the most widely used methylotrophic yeast for heterologous protein expression. Up to now, more than 500 recombinant proteins were expressed by *Pichia pastoris* with high expression levels up to 80% of total secreted protein or up to 30% total cell protein (Potvin *et al*., 2012). *Pichia pastoris* expression system is advantageous for industrial applications due to its strong and tightly regulated methanol inducible alcohol oxidase 1 (AOX1) promoter and its ability of recombinant protein secretion. Moreover *Pichia pastoris* expression system can accomplish post translational modifications of proteins such as glycosylation, disulfide bond formation. *Pichia pastoris* can grow on defined media to large cell mass. It prefers respiratory system instead of fermentative growth. To construct expression cassettes, commercialized expression kits of *Pichia pastoris* are available and the genome of the *Pichia pastoris* is quite simple for genetic manipulations. Additionally, it has GRAS (generally recognized as safe) status and doesn't produce toxic compounds (Potvin *et al*., 2012).

The aim of this study is to develop metabolically engineered *Pichia pastoris* strains for production of extracellular and intracellular microbial protransglutaminase from *Streptomyces mobaraensis* under the control of

AOX1 promoter. In this context firstly, *pro-mtg* genes were cloned into pPICZα-A expression vectors, separately and extracellular (pPICZαA*::pro-mtg*$_{extra}$) and intracellular (pPICZαA*::pro-mtg*$_{intra}$) constructs were developed. Then, these plasmids were integrated into *P. pastoris* genome. The best pro-MTG producing strains were selected with shaker bioreactor experiments. Afterwards, a controlled pilot scale bioreactor experiment was performed with the selected extracellular clone and fermentation characteristics and pro-MTG production were investigated.

CHAPTER 2

LITERATURE SURVEY

In the modern world, people need products and services to the extent that they have never experienced throughout the whole history. To supply the growing need, different sources are investigated while novel production strategies are developed continually. Especially, green technology has come into prominence in industrial processes to protect environment while supplying sustainable economy and production. Enzymes are indispensable components of white biotechnology and bioprocesses. Industrial operations need enzymes in bulk quantities to maintain their activities. For these purposes, researchers design new bioprocesses to obtain enzymes and other proteinaceous compounds such as antibiotics, hormones, vaccines etc. in high amounts.

Industrial bioprocess development comprises many stages. First, detailed product examination in all aspects is performed. Then, proper host cell is determined due to its expression, post-translational modification and growth characteristics. With genetic engineering tools, recombinant cell with the desired product gene is created. After cloning, fermentation strategies are developed to obtain effective growth and expression. Fermentation parameters such as medium composition, pH and temperature are optimized with small scale experiments. During these production experiments, the best producer cells are selected and product analyses are performed. After determination of the optimal conditions, pilot scale production experiments

start with 1 or 2 liter bench top reactor equipped with devices for controlling temperature, pH, dissolved oxygen level, stirring rate etc. Fermentation parameters can be controlled better with pilot scale bioreactor and growth and expression characteristics (oxygen need, foaming and product quantity etc.) are analyzed during fermentation. For modeling of the growth several calculations (mass-transfer coefficient, product formation rate, oxygen uptake and transfer rate, feed consumption rate, etc.) are done. Additionally, optimized bioreaction mode (batch, fed-batch or continuous) should be determined. Finally, product can be processed (for ex: activation of zymogen) if needed.

2.1 Transglutaminase

Transglutaminase (TG; protein-glutamine γ-glutamyl-transferase, EC 2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxyamide group of a peptide bound glutaminyl residue (acyl donors) and a variety o primary amines (acyl acceptors), including the amino group of lysine. When amine substrates absent, TG catalyzes the hydrolysis of the γ-carboxyamide group of the glutaminyl residue, resulting deamidation. If the ε-amino group of a peptide-bound lysyl residue is the substrate, peptide chains are covalently connected to form ε-(γ-glutamyl) lysine (G-L) bonds (Yokoyama *et al*., 2004).

Transglutaminases catalyze intramolecular and intermolecular polymerization of proteins and make them more resistant to proteolytic degradation. TG is found in vertebrates, invertebrates, mollusks, plants and microorganisms. They facilitate some biological functions such as blood clotting, epidermal keratinization, wound healing and stiffening of the erythrocyte membrane in vertebrates (Yang *et al*., 2011; Yokoyama *et al*., 2004). In plants TG has functions in stabilization of photosynthetic complex in the chloroplast, modification of cytoskeletal proteins, aging, abiotic and biotic stress and programmed cell death (Serafini-Fracassini *et al*., 2008).

Transglutaminase was discovered by Heinrich Waelsch in 1957. The first standardized procedure to isolate tissue transglutaminase was developed in the laboratory of John E. Folk in 1985. At the beginning, source of the enzyme was guinea pig liver which contains larger amount of enzyme than other tissues (Beninati *et al*., 2009). TG is potentially useful for creating proteins with novel properties. Limited supply of guine pig liver transglutaminase and unacceptability of its use in food processes led the scientists to find a way to obtain mass production of TG. Firstly, transglutaminase was extracted from tissues and body fluids of food animals but the red pigmentation was detrimental to appearance of the product. In addition thrombin was required to activate the enzyme. Then, transglutaminase producing microorganisms screened to find a good expression agent. In 1989 a microorganism with the ability of formation G-L bonds in proteins was discovered and named as *Streptomyces mobaraensis* (Washizu *et al*., 1994; Yokoyama *et al*., 2004). Microbial transglutaminase (MTG) was obtained from a variant of *Streptomyces mobaraensis* (formerly classified as *Streptoverticillium mobaraense*).

MTG primary structure includes 331 aminoacids. MTG has a single cysteine residue and calculated molecular weight of 37.842 kDa. Its isolecetric point (pI) is 9. MTG is a monomeric, simple protein and it has three potential glycosylation sites (-Thr-Xxx-Asn-). The optimum pH for MTG activity is between 5 and 8, besides it shows some activity at pH 4 or 9. The optimum temperature for MTG activity is 55^{o}C (for 10 min at pH 6.0) and full activity is maintained for 10 min at 40^{o}C. At 70^{o}C MTG activity was lost within a few minutes. It was still active at 10^{o}C and at about freezing temperature it shows some activity. While Factor XIII like transglutaminases (skin transglutaminase, guinea pig liver transglutaminase, tissue transglutaminases etc.) require Ca^{2+} to show activity while MTG is absolutely independent from Ca^{2+}. This feature is very convenient to modify food proteins sensitive to and easily precipitated by

Ca^{2+} ions. Cu^{2+}, Zn^{2+}, Pb^{2+} and Li^{2+} ions are inhibitors of MTG. The mechanism of inhibition was thought that heavy metals are expected to bind the thiol group of the single cysteine residue at the active site (Yokoyama *et al*., 2004, Kanaji *et al*., 1993). MTG reaction rate is higher and it has broader substrate specificity for the acyl donor while showing lower level activity in deamidation reactions than Factor XIII like transglutaminases (Shimba *et al*., 2002). The apparent K_m, V_{max} and K_{cat}/K_m of the activated MTG are found to be 52.66 mM, 49.67µmol/min/mg and 40.42 $mM^{-1}min^{-1}$, respectively and specific activity of 10.59 units/mg, k_{cat} of 2128.57 min^{-1} while the specific activity, V_{max} and K_m values of guine pig liver transglutaminase were 25units/mg, 37 µmol/min/mg and 66 mM, respectively (Yang *et al.,* 2011; Folk *et al.,* 1966).

2.1.1 Structure of Microbial Transglutaminase and Its Zymogen

MTG forms a single, compact domain with dimensions of 65x59x41 Å, folds into a plate like shape with a deep crevice at the edge of the molecule (Figure 2.1). The catalytic residue, Cys^{64}, is at the bottom of the crevice. MTG has 11 α-helices and 8 β-strands and involved in α+β folding class. One of the β-sheets which are clustered into three regions is covered by α-helices. The central β-sheet forms a seven stranded anti-parallel structure, however it is heavily twisted between the β_5 and β_6 strands and only one hydrogen bond is found between the main chains of these strands (Trp^{258} and Thr^{273}). The first cluster of α-helices is comprised of the α_1, α_2 and α_3 helices and Cys^{64} residue is in the loop between the α_2 and α_3 helices. The second cluster including the α_4, α_5, α_{10} helices and the third cluster comprising the α_6, α_7, α_8, α_9 helices (Kashiwagi *et al*., 2002).

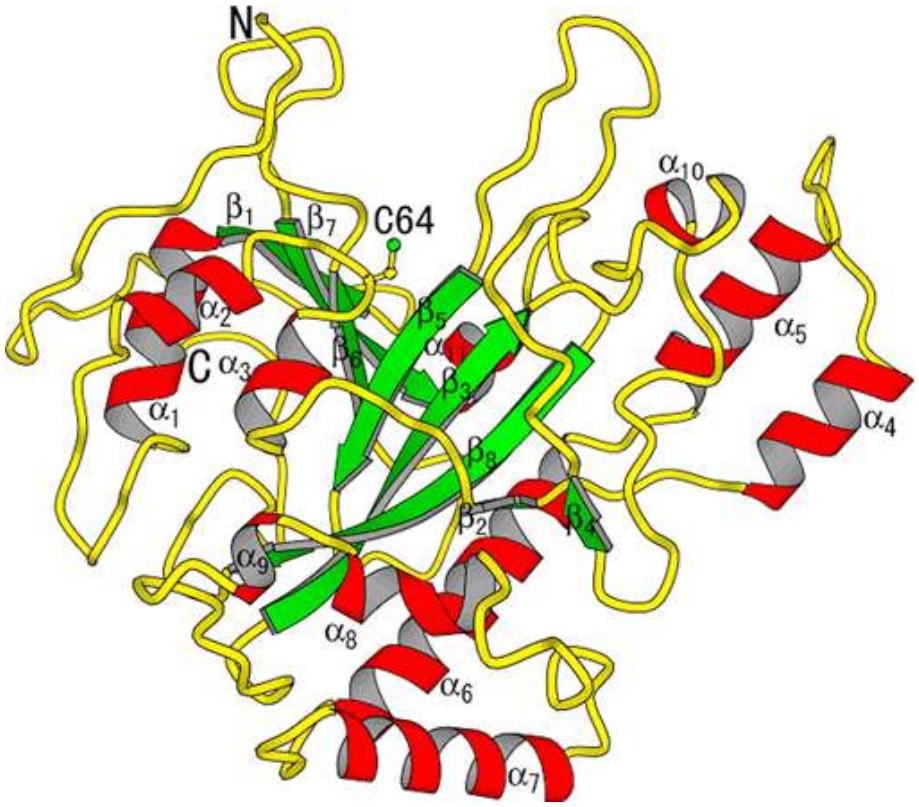


Figure 2.1 Overall structure of microbial transglutaminase: schematic ribbon drawing of the MTG molecule viewed from the above the plate face. The secondary structure is numbered. *Ball-and-stick model* of the side chain of Cys^{64} (Kashiwagi *et al.,* 2002)

The overall structure of MTG is quite different from the factor XIII like transglutaminases, which have a cysteine protease like catalytic triad (Figure 2.2.a). Red sea bream liver transglutaminase (FTG), like human coagulation factor XIII, composed of four sequential domains, these are named as β-sandwich, core, barrel 1 and barrel 2 by Yee *et al.* (1994). The active site of the FTG is in the core domain comprising 334 amino acid residues (Kashiwagi *et al.,* 2002). MTG has a novel catalytic mechanism specialized in cross-linking reactions. On the contrary to the limited solvent accessibility of the active site and complex activation procedure of the Factor XIII like TG, Cys^{64} of MTG is adequately subject to the solvent to catalyze the reaction

rapidly. Additionally, the flexibility of the right side wall of the active site cleft may diminish steric hindrance between enzyme and substrates (Yokoyama *et al.,* 2004).

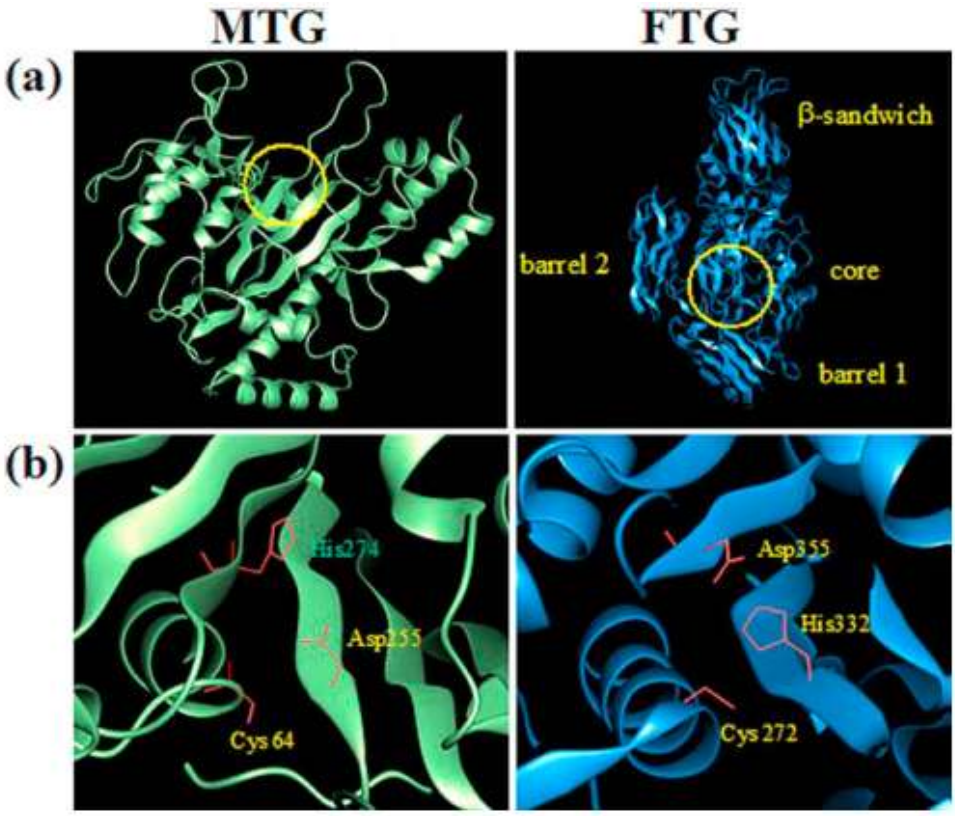


Figure 2.2 a,b Structural comparison of MTG and red sea bream liver transglutaminase (FTG) **a.** Overall structures, **b.** Structure around the active sites of MTG and FTG. Regions in yellow circles are active sites. Red wire model: Catalytic triad of FTG (Cys^{272}, His^{332}, Asp^{355}) and the corresponding position in MTG (Cys^{64},Asp^{255}, His^{274}) (Yokoyama *et al.,* 2004)

The active crevice at the edge of the molecule is filled by the N-terminal amino acids (residues 9-33) in the microbial transglutaminase zymogen (pro-MTG). The Cys^{110}, Asp^{301} and His^{320} catalytic triad is placed at the bottom of the crevice. Prosequence residue mainly folds into an L-shaped

structure and a long α-helix region (residues 9-20) of it intercalates with the side wall of the open end of the active crevice. The other short one turn α-helix (residues 9-20) fits on the other open end of the crevice. Tyr-12 and Tyr-16 residues in the short helix of the prosequence are placed on top of the catalytic triad and prevent access of the acyl donors and acceptors to block the reaction.

Weak and asymmetric interactions are enhanced by few hydrogen bonds and weak van der Waals bonds between the prosequence and the active crevice (Yang *et a*l., 2011).

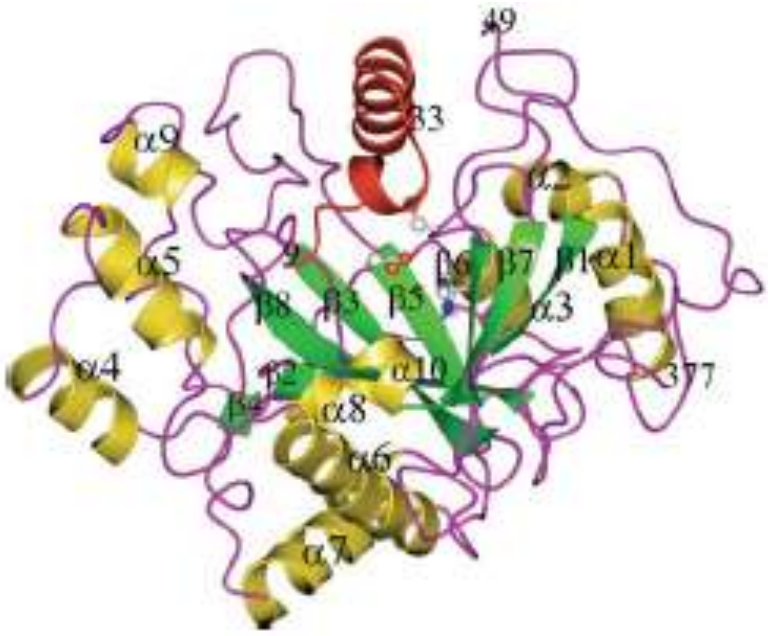


Figure 2.3 Stereo view of the overall structure of the MTG zymogen (pro-MTG). The Cys-Asp-His catalytic traid was shown in ball-and-stick format. The visible portion of the prosquence (residues 9-33) of the zymogen was colored red (Yang *et al.,* 2011)

2.1.2 Activation of Microbial Transglutaminase Zymogen

In vivo, transglutaminase produced as inactive proenzyme includes additional prosequence composed of 45 amino acid residues. Expression of synthetic active transglutaminase gene in microorganism causes cell growth reduction and cell lysis due to cross linking of functional cytosolic proteins (Takehana *et al.,* 1994) .The proenzyme become active after proteolytic cleavage. For activation of microbial protransglutaminase (pro-MTG) several researches were carried out.

Transglutaminase activating metalloprotease (TAMEP) was isolated from *S. mobaraensis* DSM 40587 and used for the activation of pro-MTG. Activation procedure with TAMEP leaved a tetrapeptide on the N-terminus of the MTG but this extension doesn't affect the specific activity (Zotzel *et al*., 2003). In *Corynebacterium glutamicum* co-expression and co-secretion of MTG gene from *S. mobaraensis* and protease SAM-P45 gene from *S. albogriseolus* was performed but the productivity of the bioprocess (approximately 9 U L^{-1} h^{-1}) was relatively low. Additionally, MTG was degraded by protease SAM-P45 after prolonged times of cultivation (Date *et a*l., 2004; Date *et al.,* 2003; Kikuchi *et al.,* 2003). Trypsin was thought as a useful protease for the activation of wild type pro-MTG from *S. mobaraensis* (Pasternack *et al*., 1998) but in recombinant pro-MTG production with His-Tag, enzyme activity was decreased with reaction time due to pro-MTG degredation by trypsin (Marx *et al*., 2008a). Chymotrypsin and proteinase K activate the pro-MTG but at the same time they degrade the pro-MTG and the activity of MTG is utterly depleted after prolonged reaction times. Dispase is a neutral protease isolated from *Bacillus polymyxa.* It's also a strong fibronectinase and type IV collagenase (Stenn *et al.,* 1989). Dispase was used for the first time for the activation skin protransglutaminase of mice (Martinet *et al*., 1988). Cathepsin B, dispase I and thrombin enzymes successfully activate the pro-MTG while Dispase I shows an apparent higher activation rate

than Cathepsin B and thrombin (Marx *et al*., 2008a). Dispase and TAMEP activated MTG with His tag didn't show any activity decrease after 3 hours of reaction times (Marx *et al.,* 2008a). On the other hand, Dispase I cleaves the C-terminal His-tag of the pro-MTG during activation (Sommer *et al.*, 2012).

Consequently, TAMEP can be used for activation of pro-MTG, but its commercial production doesn't exist. Among from commercial enzymes dispase I is a good activating agent without any degradation of MTG.

2.1.3 Reactions of Transglutaminase

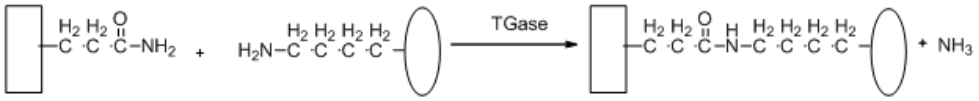
Transglutaminases catalyze the attack of a suitable nucleophile (acyl acceptor) at the carboxyamide group of a glutaminyl residue of a protein or peptide substrate (acyl donor). The acyl acceptor can be the ε-amino group of a lysl residue in a protein or peptide, an amine, diamine or polyamine, water or glutaminyl bound polyamine. Additionally, some esterification reactions are catalyzed by TG. Hydrolysis reactions with water result in deamidation and alterations in protein charge.

ε-amino group of lysine is a primary amine and it's a good substrate of MTG. Other amino acids have either amidated, esterified or decarboxylated α-carboxyl group to eliminate their negative charges. In transglutaminase reactions the lysine including peptides act as acyl acceptors and the proteins act as acyl donors, while glutamine including peptides act as acyl donors and the proteins as acyl acceptors.

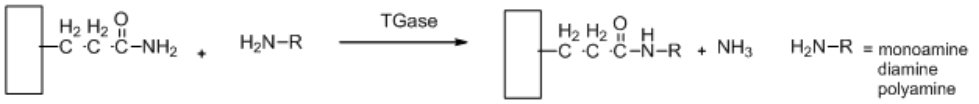
3D structure of substrate proteins are also important for increasing the reactivity of transglutaminase, because denatured proteins are usually better substrates than native ones (Beninati *et al.,* 2009).

Figure.2.4 Reactions catalyzed by transglutaminase (Lajitha and Banik, 2007)

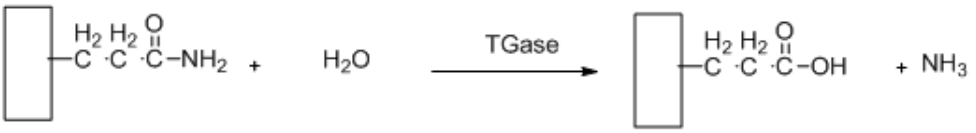
1. **Crosslinking**



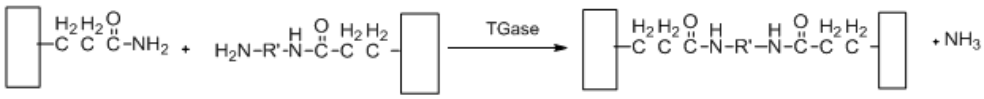
2. **Amine Incorporation**



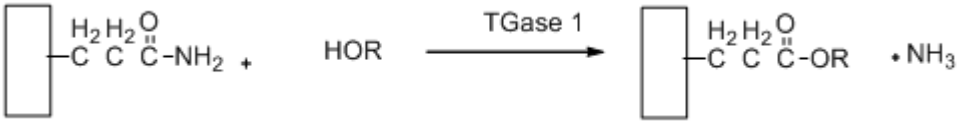
3. **Hydrolysis**



4. **Polyamine Bridging**



5. **Esterification**



2.1.4 Applications of Transglutaminase

Transglutaminases catalyze the binding of polyamines to alter permanently the surface properties of the target proteins as it changes the electrically neutral regions into positively charged region. This reaction modifies functional properties of modified proteins with either loss or gain of function (Beninati *et al*., 2009).

Mutant animals deleted for the gene coding transglutaminase are valuable tools to test the involvement of transglutaminases in the pathogenesis of diseases (Beninati *et al.,* 2009).

Transglutaminases have potential in constructing collagen or gelatin based scaffold to produce artificial organs, site-specific protein conjugation with DNA for biotechnological researches, material science, textile and leather industry (Zhu *et al*., 2008). MTG can incorporate amino acids or peptides covalently into substrate proteins and as a result of this reaction, nutritional value of food and feed proteins is improved (Yokoyama *et al.*, 2004).

2.1.4.1 Meat Products

MTG is useful agent as it creates novel meat binding method to produce novel meat products. Caseinate, when come across with MTG, becomes viscous and sticky and binds different foodstuffs together. Thanks to this method without sodium chloride or phosphates, healthy meat products can be obtained (Carballo *et al*., 2006; Uresti *et al*., 2004). MTG can also be used to obtain protein based cold set gels at temperatures low enough without any changes in the raw flavor or the appearance of the product. Several restructured meat models (fish, pork, beef etc.) prepared with MTG are stable during storage as the protein was almost aggregated and well organized protein structure was formed into more compact from outset (Moreno *et al*., 2010; Romero de Avila *et al*., 2010). Moreover, in protein emulsions MTG act as an emulsifier as well as an emulsion stabilizer via its enzymatic activation and increase the shelf life of the protein emulsion products (Hong *et al*., 2012).

2.1.4.2 Dairy Products

MTG catalyzes the conversion of casein into heat resistant, firm gel which improves texture of the final product. Serum separation problem of

yogurt could be overcomed by the addition of MTG as it improves the water holding capacity of the gel. Gels obtained from goat milk usually appear weak due to the low casein content. Goat milk products treated with MTG have higher viscosity, stiffness and less syneresis problems when compared with untreated milk. Additionally, MTG treatment provides positive effect on the survivability of the probiotic cultures (Ardelean *et al*., 2012; Farnsworth *et al*., 2006). Casein, gelatin and gelatin-casein blend edible films can be produced by MTG incorporation (Chambi *et al*., 2006). Heat treatments during processing often cause partial denaturation of functional whey proteins. MTG cross-linking supplies increased thermal stability to β-lactoglobulin in heat shocked whey protein isolates (Damodaran *et al*., 2013). The MTG provides ice cream with greater overrun, more fat coalescence, melting resistance and hardness. Additionally, MTG can be used for partial replacement for fat in icecream with the same rheological and functional properties (Rossa *et al*., 2012). Properties of the non fat yoghurt could also be improved by MTG treatment instead of extra protein or stabilizer addition (Ozer *et al*., 2007).

2.1.4.3 Cereal Products

MTG modifies chemical and functional properties of glutenin proteins of wheat and improves dough strength and bread volume (Seravalli *et al*., 2011). Insect damages are critical for bread making whet flours because of their proteolytic enzymes. MTG can diminish hydrolytic effect of proteases present in insect damaged wheat flour. By MTG treatment a thermally stable structure was obtained from damaged flours and quality of flour was increased (Caballero *et al*., 2005). Although rice flour is quite valuable for nutritional aspect, its use in fermented baked products was rare due to low gas holding capacity of rice proteins. MTG catalyzed polymerization of rice proteins yield a convenient protein network for holding the gas produced in fermentation (Gujral *et al*., 2004). MTG treatment of noodles and pasta increases the strength of the product and inhibits the texture deterioration

during cooking even though low quality flours are used (Wang *et al*., 2011). Water adsorption, viscoelastic behavior and thermal stability of oat dough was improved with MTG incorporation and free amino acid groups of the dough was decreased as a result of protein cross linking catalyzed by MTG (Huang *et al*.,2010). Tofu is a soy milk curd product. Producing long life Tofu is so difficult as its soft and smooth texture is easily destroyed by autoclaving. In order to enhance long life time, smooth texture of sterilized tofu was obtained with MTG addition.

2.1.4.4 Tissue Engineering

Transglutaminase helps the construction of scaffolds derived from collagen or gelatine. Scaffolds' improved mechanical strength and the short formation time give advantages to MTG in tissue engineering. Collagen scaffold treated with MTG showed increased cell attachment, spreading and proliferation of human osteoblasts and human foreskin dermal fibroblasts and more resistance to cell-mediated endogenous protease degradation when compared with native collagen scaffolds (O'Halloran *et al*., 2006; Chau *et al*., 2005). The enzymatically treated composite collagen-hyaluronan scaffold has potential to create an injectable cell-seeded scaffold for nucleus pulposus treatment in degenerated invertebral disc (O'Halloranan *et al*., 2008). MTG treated collagen scaffolds can also be used as a dermal precursor as they facilitate neovascularisation and epithealisation resisting wound contraction (Garcia *et al*., 2008). Enzymatically stabilized gelatine scaffolds have less cytotoxicity and when hepatocytes were cultered on these scaffolds, they lead more differentiated phenotypes (Barbetta *et al*., 2006).

2.1.4.5 Textile and Leather Processing

MTG treatment of wool reduces the tensile strength loss and shrinkage tendency of wool fabrics while improving the fabric strength and felting properties (Du *et al*, 2007; Cortez *et al*., 2004). With microbial transglutaminase treatment, cheap proteinaceous byproducts such as whey

protein isolate and gelatin can be used to smoot any irregularity on the leather surface instead of expensive filling materials (Hernandez Balada *et al*., 2009).

2.1.4.6 Site Specific Protein Conjugation

Depending on the structure and dynamics of the therapeutic proteins, prediction of the sites of TG mediated modification and PEGylation is possible and also effects of modifications on the functional and physiochemical properties of the proteins can be foreseen. Thus researchers can design useful strategies for TG mediated therapeutic protein drug modifications (Fontana *et al*., 2008). Additionally, specific and desired protein-DNA conjugates can be obtained using TG (Tominaga *et al*., 2007). With the site specific protein modification, thermal stability of proteins and resistance against autolysis at alkaline pH were increased (Villalonga *et al*., 2003).

2.1.5 Production of Microbial Transglutaminase with Different Host Microorganisms

Microbial transglutaminase (MTG) was firstly produced by *Streptoverticillium mobaraensis* in 1989 by Ando *et al*. The first recombinant MTG production was performed with *Streptomyces lividans* in 1994 (Washizu *et al*., 1994). Synthesized active MTG gene was expressed in *E. coli* but it showed that active enzyme inhibits the cell growth (Takehana *et al*., 1994). Prosequence of the enzyme blocks the activity while increases enzyme thermostability (Pasternack *et al*., 1998). Several host microorganisms such as *Escherichia coli*, *Bacillus*, yeasts or *Aspergillus* were used for recombinant microbial transglutaminase production. Recombinant *C. glutamicum* was developed to produce microbial pro-transglutaminase (pro-MTG) and pro-MTG was constructed behind the promoter of *cspB*, a cell surface protein of *C. glutamicum* and the signal peptide of CspA, a cell surface protein of *Corynebacterium ammoniagenes* and 142 mg/l pro-MTG was produced by recombinant *C glutamicum* (Kikuchi *et al*. 2003). Site directed mutagenesis

was applied to pro-MTG gene to generate Subtilisin like serine protease (SAM-P45) cleavage site and Subtilisin like serine protease (SAM-P45) gene from *Streptomyces albogriseolus* was co-secreted with the pro-MTG (Date *et al*., 2003). Chimeric proregion in which the C-terminal domain of *S. mobaraensis* proregion is replaced by the *S. cinnamoneus* transglutaminase proregion was used to construct recombinant plasmid for *C. glutamicum* and 881 mg/l pro-MTG was produced (Date *et al*., 2004).

Highest pro-MTG production was reported as 2.5 g/L over a period of 71 h by *Corynebacterium ammoniagenes* ATCC6872. The plasmid for *S. mobaraensis* protransglutaminase expression was constructed with *cspB* promoter derived from *C. glutamicum* ATCC13869 and the CspA signal peptide derived from a cell surface protein of *C. ammoniagenes* ATCC6872. In that study, the specific activity of the purified and activated MTG was about 23U/ml (Itaya *et al*., 2008).

A mutant transglutaminase gene product carried a single amino acid exchange (serine replaced by proline at position 2) showed higher specific activity of 46.1 U mg^{-1} when compared with other MTGs. A recombinant *E. coli* cell harbours this mutated gene construct produced 500 mg L^{-1} pro-MTG and 23000 U/L over 16 hours cultivation, which was the highest activity value of MTG produced by *E. coli* (Sommer *et al*.,2011).

Methylotrophic yeasts *Pichia pastoris* and *Candida boidinii* were also used for recombinant MTG production. For this purpose 3 different expression cassettes were prepared. All of the expression casettes include α-factor signal sequence of *Saccharomyces cerevisiae* and mature MTG gene, pro-MTG gene or pro-MTG gene with Kex2 cleavage site. Alcohol oxidase promoter (*AOX1*) of *Pichia pastoris* and alcohol oxidase promoter (*AOD1*) of *C. boidinii* were used for expression of MTG. MTG have 3 putative N-glycosylation sites. With mutagenesis in the glycosylation sites *C. boidinii*

strains produced 1.83 U/ml MTG (approximately 87 mg/l; specific activity 22U/mg protein) after 119 h of cultivation. In the same study of Yurimoto *et al*.,(2004) too low enzyme activity was obtained with recombinant *Pichia pastoris* cells and this was related with highly glycosylated MTG product of *Pichia pastoris* (Yurımoto *et al*., 2004).

2.2 Host Microorganism Selection

The second stage of a bioprocess development is the selection of a host microorganism which has efficient recombinant protein expression and secretion capabilities. Microorganisms are more preferable for recombinant protein production when compared with animal and plant cells in terms of less doubling time, simple cultivation conditions and easier purification and isolation steps of the product. Potential host microorganism should grow large cell mass in a reasonable time and produce recombinant product with sufficient yield. Host microorganism's cultivation becomes cheaper as it doesn't require expensive and rare compounds for growth. Microorganism also shouldn't be pathogenic and produce toxic substances. Especially, if the recombinant product is used in food industry applications, microorganism must be in GRAS (generally recognized as safe) status. Additionally, protein secretion capability of a host microorganism simplifies purification steps of the product (Soetaert and Vandamme, 2010; Kirk and Othmer, 1994).

Today, in industry most common microorganisms are *E.coli* and *S. cerevisiae* as recombinant protein production experiments with them began at the very early stages of the recombinant protein production. Although, so much research carried out for their recombinant protein production systems, they have some disadvantages. *E. coli* can't secrete proteins into the extracellular medium and proteins inside cell may aggregate so inclusion bodies are formed. This may cause loss of functionality. Additionally, refolding step of insoluble inclusion bodies is too time consuming and not economically

feasible (Marx *et al*., 2007). *S. cerevisiae* hyper-glycosylates the proteins (Walker *et al*., 1998) and alters protein characteristics, this situation may also cause functionality loss of proteins. In recent times, *Pichia pastoris* become one of the most prominent expression agents due to its advantageous characteristics for recombinant protein production.

2.2.1 *Pichia pastoris*

Pichia pastoris was firstly used in researches done by Philips Petroleum Company and today, it has become one of the most widely used yeast for heterologous protein expression. Up to now, more than 500 recombinant proteins were expressed by *Pichia pastoris* with high expression levels up to 80% of total secreted protein or up to 30% total cell protein (Potvin *et al*., 2012). *Pichia pastoris* expression system is advantageous for industrial applications due to it's strong and tightly regulated methanol inducible alcohol oxidase 1 (AOX1) promoter and it's ability of recombinant protein secretion. Moreover *Pichia pastoris* expression system can accomplish post translational modifications of proteins such as glycosylation, disulfide bond formation. *Pichia pastoris* can grow on defined media to large cell mass. It prefers respiratory system instead of fermentative growth. To construct expression cassettes, commercialized expression kits of *Pichia pastoris* are available and its' genome is quite simple for genetic manipulations (Potvin *et al*., 2012).

Recombinant protein production rate is affected by both genetic and cultivation characteristics. Genetic factors are codan usage bias of *P. pastoris*, GC content of the product gene, gene copy number, promoters and glycosylation sites on the protein. Additionally, high proteolytic acitivity of *Pichia pastoris* affects the product yield. The major cultivation parameters affecting expression are temperature, pH and dissolved oxygen level. Although optimal growth temperature of *P. pastoris* is 30^0C, some studies showed that

up to 15^0C cells can express proteins without a considerable change in cell growth. Furthermore, as temperature decreases the proteolytic activity is reduced so low temperature fermentation can increase the product yield (Wu *et al*., 2008; Siren *et al.*, 2006). Optimal pH values for *P. pastoris* cultivation are between 3 to 7 (Cregg *et al*., 2000). In oxygen limited cultivation, *P. pastoris* may produce fermentative by-products such as ethanol and these may adversely affect protein expression.

2.2.1.1 *Pichia pastoris* Expression System

Pichia pastoris cells can overproduce recombinant proteins with methanol induction of strong and tightly inducible alcohol oxidase 1 (AOX1) promoter. High protein expression capability of *Pichia pastoris* makes it very useful for industrial applications. Two alcohol oxidase enzymes regulate methanol metabolism in *P. pastoris*. These enzymes are AOX1 which comprises up to 95 % of total expressed alcohol oxidase enzymes and AOX2 (Macauley-Patrick *et al*., 2005). Alcohol oxidase is the first enzyme in the methanol utilization pathway and it represents up to 35% of total cell protein in wild type cells grown on methanol. Due to the methanol utilization characteristics, *P pastoris* strains divided into three phenotypes (Potvin *et al*., 2012; Daly and Hearn, 2005):

- Methanol utilization plus phenotype (Mut^+): AOX1 and AOX2 genes are functional; grow on methanol such a wild type rate; but requires high methanol concentration during fermentation.
- Methanol utilization slow phenotype (Mut^S): AOX1 gene is deleted; strain growth rate is limited due to only AOX2 expression; lower sensitivity to methanol
- Methanol utilization minus phenotype (Mut^-): cannot express either AOX1 or AOX2; cannot grow on methanol

Table 2.1 Advantages and disadvantages of *Pichia pastoris*

Advantages	Disadvantages
• High recombinant protein expression capability, high yield and productivity	• Harmful methanol need, it may not be suitable for food industry application
• Recombinant protein secretion capability	• High protease secretion
• Strong and tightly regulated alcohol oxidase 1 promoter (AOX1)	• Non-native glycosylation
• Growth on chemically defined media	• Longer cultivation time compared to bacteria
• Don't need expensive and rare compounds for growth	• Difficult methanol concentration monitoring during bioprocess
• Genomic integration of plasmids, stable production strains	• Methanol storing in industrial scale is dangerous as it is a fire hazard
• Easy purification and isolation steps	
• Eukaryotic post-translational modifications	
• No toxin production	
• GRAS (Generally Recognized as Safe) status	
• Wide pH range: 3-7	
• Methanol utilization ability	
• Respiratory growth preference instead of fermentative, an advantage against *S cerevisiae*	
• Crabtree-negative	
• Hyper-glycosylation doesn't occur as much as *S cerevisiae*	

Pichia pastoris expression system has some drawbacks. As methanol is used for induction of alcohol oxidase 1 promoter, its presence in the production medium may be hazardous for people who work in the

fermentation processes. Large scale storage and utilization of methanol may be risky due to its fire hazard. Besides, acceptability of the recombinant product may be reduced for food and pharmaceutical industry because of methanol (Careghino and Cregg, 2000). To prevent methanol risks, alternative constitutive and inducible promoters were developed for over expression of recombinant proteins with *Pichia pastoris*. These promoters are glyceraldehydes 3-phosphate dehydrogenase constitutive promoter (GAP), glutathione-dependent enzyme formaldehyde dehydrogenase promoter (FLD1), 3-phosphoglycerate kinase promoter (PGK1) and isocitrate lyase promoter (ICL1) (de Almeida *et al*., 2005; Menendez *et al*., 2003; Shen *et al*., 1998).

2.2.1.2 Glycerol, Sorbitol and Methanol Metabolism of *Pichia pastoris*

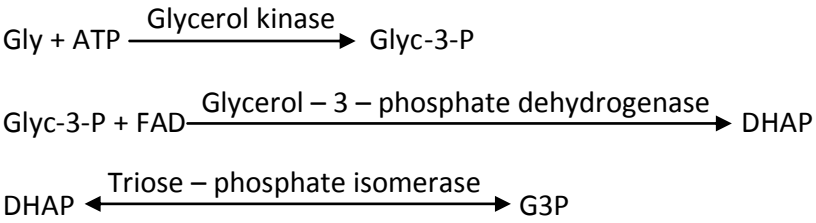
In order to achieve high recombinant cell mass in bioprocess, *P. pastoris* cells are firstly grown on glycerol as maximum specific growth rate and biomass yield of *P. pastoris* are higher on glycerol than other carbon sources such as methanol. Recombinant protein expression is also repressed during cultivation with glycerol. Glucose isn't used as a carbon source since more ethanol is formed during cultivation with glucose than cultivation with glycerol (Macauley-Patrick *et al*., 2005). Even as low levels as 10-50 mg/l, ethanol represses the gene expression by alcohol oxidase promoter (Inan *et al*., 2001a)

In glycerol catabolic pathway, glycerol first phosphorylated to glycerol-3-phosphate by glycerol kinase (EC 2.7.1.30). Then glycerol-3-phospate is oxidated to dihydroxyacetone by FAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.99.5). Dihydroxyacetone phosphate enters into the glycolytic pathway and its isomerization into glyceraldehydes-3-phosphate is catalyzed by triose-phosphate isomerase (EC 5.3.1.1). Rare

glycerol dissimilation pathway is found in a few yeast species, this pathway includes a NAD-dependent glycerol dehydrogenase and a dihydroxyacetone kinase (Nevoigt *et al*., 1997). Glycerol catabolic pathway is given in Figure 2.5.

The first step of methanol dissimilation which is oxidation of methanol with molecular oxygen into formaldehyde and hydrogen peroxide is catalyzed by alcohol oxidase enzyme. Methanol oxidation performed within peroxisomes to avoid toxic effects of hydrogen peroxide (Veenhuis *et al*., 1983). Peroxisomes covers 80% of the yeast cell volume which grown on methanol.

Figure 2.5 Catabolic metabolism of glycerol



Some formaldehyde molecules leave the peroxisome and enter into dissimilatory pathway. After two subsequent dehydorgenase reactions, formate and carbondioxide are formed. Formate formation is catalyzed by glutathione dependent formaldehyde dehydrogenase. Energy generation from NADH has a crucial role in the detoxification of formaldehyde in methylotrophic yeasts (Lee *et al*., 2002). Schematic representation of methanol oxidation is given in Figure 2.6.

The remaining part of formaldehyde takes place in assimilation reactions. In transketolase reaction formaldehyde with xylulose-5-phosphate

converted into dihydroxyacetone and glyceraldehyde-3-phosphate by peroxisomal dihydroxyacetone synthase. The formed C_3 compounds go into several cytosolic reactions and serve as building blocks for biomass synthesis. Phosphorylation of dihydroxyacetone to dihydroxyacetone phosphate is catalyzed by dihydroxyacetone kinase (Lüers *et al*., 1998). The overall methanol metabolism of *P. pastoris* is shown in Figure 2.7.

For sorbitol utilization first, sorbitol is oxidized to fructose by sorbitol dehydrogenase and then phosphorylated to fructose-6-phosphate by sorbitol kinase. Sorbitol enters into the central metabolism of yeast as fructose-6-phosphate.

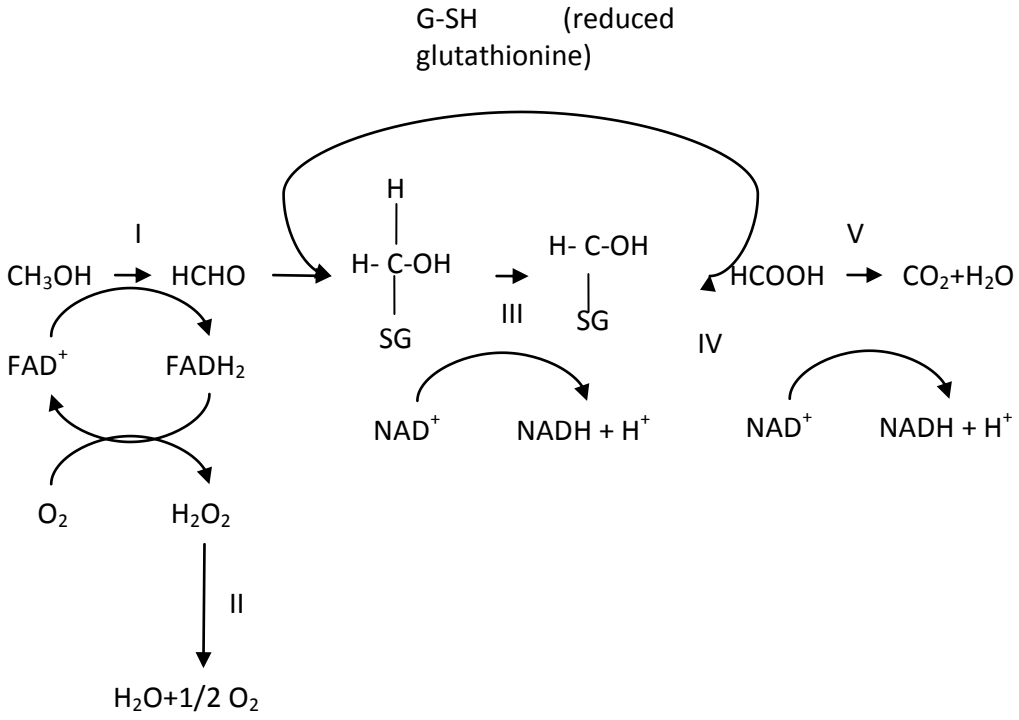


Figure 2.6 Catabolic pathway of methanol in yeast *Pichia pastoris*. I) alcohol oxidase, II) catalase, III) formaldehyde dehydrogenase, IV) S-FG hydrolase, V) formate dehydrogenase (Ellis *et al.,* 1985)

2.2.1.3 *Pichia pastoris* Recombinant Protein Secretion System and Post-translational Modifications

The major advantage of *P. pastoris* over bacteria is the ability of eukaryotic post translational modifications. These modifications include:

- Signal peptide processing
- Protein folding
- Disulfide bond formation
- O- and N- linked glycosylation
- Certain types of lipid addition (Cereghino and Cregg, 2000)

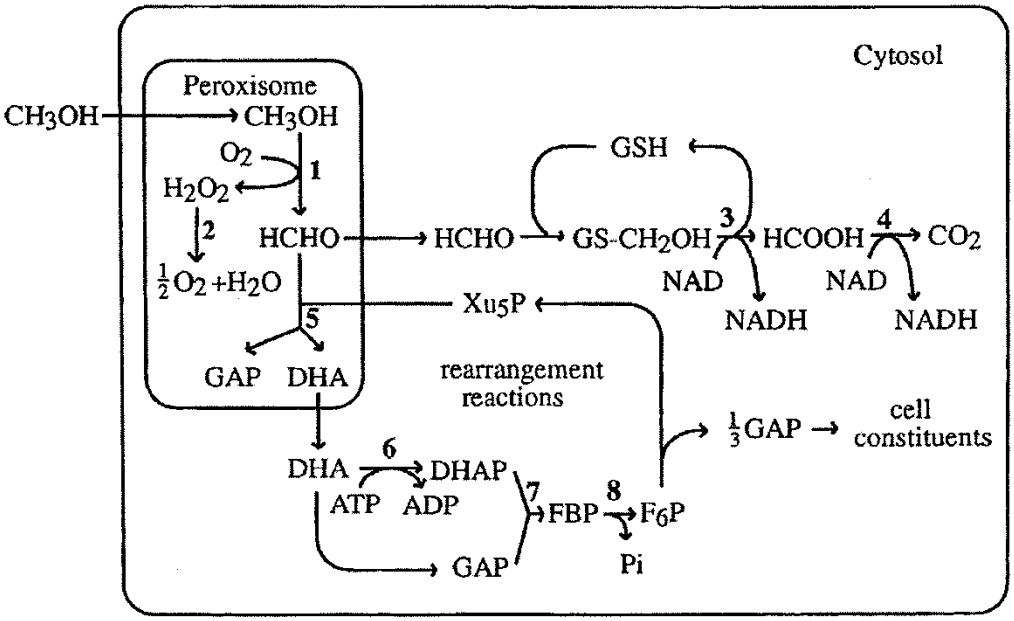


Figure 2.7 Methanol metabolism in *P. pastoris* (Cereghino and Cregg 2000)

In industrial applications, extracellular production methods are generally preferred as they facilitate downstream processing such as isolation and purification. To achieve extracellular recombinant protein production,

signal peptide, a short peptide chain, is incorporated into the protein product. The α-factor prepro peptide of *S. cerevisiae* is one of the secretion signals and used successfully within several microorganisms including *P. pastoris*. The α-factor signal sequence composed of a pre sequence consisting of 19 amino acid residue and a pro sequence consisting of 66 amino acid residue. Signal peptide processing occurs in three steps. Firstly, pre signal is removed via signal peptidase in the endoplasmic reticulum. Then, the pro protein goes through golgi compartment and Kex2 endopeptidase catalyzed cleavage occur at the Arg-Lys site of the pro sequence. Finally, Glu-Ala repeats are cleaved by Ste13 protein (Cereghino and Cregg, 2000). *P. pastoris* acid phosphatase (PHO1) signal and any protein's native signal can be used alternatively (Daly and Hearn, 2005). In this study, pPICZα-A vector which harbors α-factor signal sequence is used for extracellular microbial transglutaminase production.

P. pastoris expression system has the capability of N- and O-linked glycosylation while bacteria can't do this modification. Although *P. pastoris* is a eukaryotic microorganism, it's glycosylation pattern is different from mammalian cells. Yeast expression system usually performs hyperglycosylation with the corporation of N-linked high mannose oligosaccharides into proteins, while P pastoris doesn't cause any hyperglycosylation problem (Cereghio and Cregg, 2000). *P. pastoris* O-linked glycosylation pattern is similar with other yeasts and O-oligosaccharides are usually incorporated into hydroxyl groups of threonine and serine aminoacid residues of proteins.

2.3 Genetic Engineering Techniques and Methodology for Heterologous Protein Production with Microorganisms

Genetic engineering techniques also known as recombinant DNA technology pave the way for creating superior microorganism for industrial applications with modifying their genomes. Thanks to the recombinant DNA

technology we cannot only synthesize valuable proteins by microorganisms but also increase their expression level by modifying the DNA of a microorganism. For industrial applications recombinant DNA technology helps reducing the costs by both increasing the product yield and enhancing easier purification steps (Lui *et al*., 2010). The basic steps in gene cloning are explained below.

The basic steps of Gene Cloning

1. Pure DNA sample from desired organism is isolated and the polymerase chain reaction (PCR) is performed for gene amplification. Appropriate expression vector is also determined.
2. Interested DNA strand and vector DNA were digested using proper restriction enzymes.
3. DNA fragments full cleavage is controlled with agarose gel electrophoresis.
4. Fragmented DNA and vector were joined to each other by DNA ligase enzyme.
5. Construct including the interested gene is introduced into competent cells and the plasmid replicates itself within the cell to produce tens of identical copies.
6. Recombinant cells containing the plasmid are selected by plating the cell suspension onto the selective media and clones are identified with screening techniques.

The schematic representation of gene cloning is shown in Figure 2.8.

In this study, microbial pro-transglutaminase gene is amplified via polymerase chain reaction. As a template DNA, pDJ1-3 plasmid (Marx *et al.,* 2007), including *pro-mtg* gene insert is used. Primers are designed to create restriction sites at the both end of the *pro-mtg* gene. Amplified gene is inserted into pPICZα-A expression vector and first, *E. coli* Top10 cells are

transformed with this construct then the expression host *P. pastoris* is transfected with the recombinant gene. Positive colonies are selected via inoculating on the antibiotic containing medium and then, verification is performed by PCR, agarose gel electrophoresis and the gene sequencing.

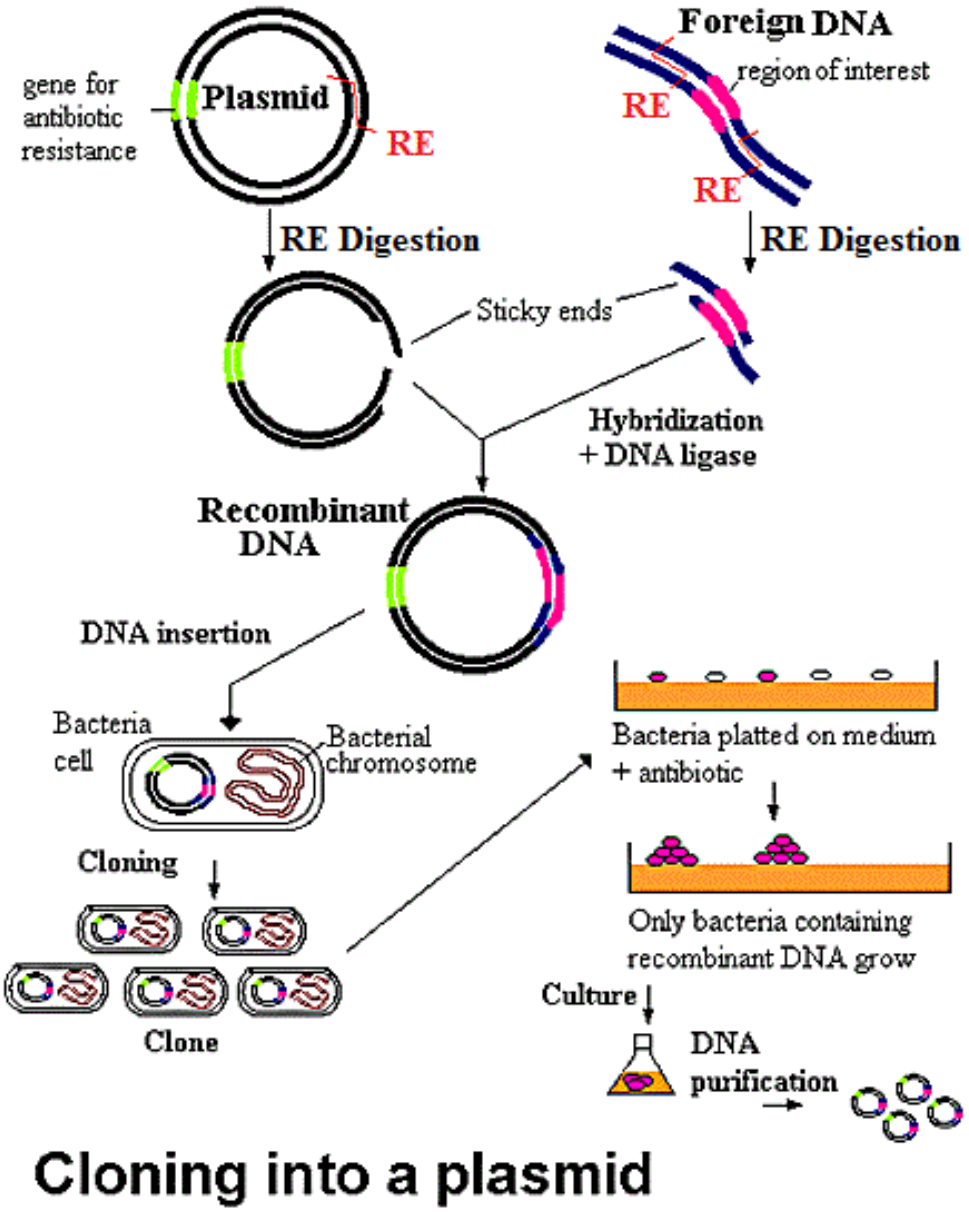


Figure 2.8 The basic steps of Gene Cloning (http://www.bio.davidson.edu)

2.3.1 Polymerase Chain Reaction (PCR)

The first step of a gene cloning process is usually gene amplification via polymerase chain reaction (PCR). Both 5' and 3' ends of the interested gene is known before and complementary oligonucleotides names as primers are designed to initiate the replication of the gene in vitro. Primer design also supply primers with the ability to introduce site specific mutations and restriction sites to the 3' and 5' ends of the interested gene (Lui *et al*., 2010). PCR depends on the ability of DNA polymerase to synthesize new strand of DNA complementary to the interested gene template in the presence of dNTPs and primers. PCR is a repetitive process (30-40 cycles) which composed of denaturation, annealing and extension steps. PCR is performed by an automated cycler device which rapidly cools and heats the mixtures. Major steps of PCR are explained below:

Denaturation: Double stranded DNA molecule is uncoiled with raising the temperature to 94-95^{0}C. Single stranded DNA molecule serves as a template for amplification of the gene. Denaturation steps usually take 45 s. at 94^{0}C but first denaturation step can last up to 5 minutes for complete denaturation of the DNA samples. Additionally, higher temperatures are needed when G+C content of the template is high.

Annealing: Temperature decrease is necessary to facilitate annealing of the primers to the single stranded DNA (ssDNA). The optimum temperature for annealing depends on the melting point of the template-primer hybrid. If temperature is decreased so much, non-specific bindings occur. On the other hand, if temperature is too high, annealing doesn't occur. Annealing temperature is usually 45-55^{0}C which is 5^{0}C below the melting point of the primers.

Extension: In this step, DNA synthesis is carried out by thermostable DNA polymerase. Thermostable enzyme gets PCR procedure easier with catalyzing

amplification reactions at high temperature. Higher specificity, sensitivity and yield were achieved with higher reaction temperature (Saiki *et al*., 1988). *Taq* DNA polymerase (from *Thermis aquaticus*) is the first and most common DNA polymerase among others (Glazer and Nikaido, 1995). Optimum working temperature for *Taq* polymerase is 72^{0}C and it catalyzes the annealing of nucleotides at a rate of 35-100 nucleotides/sec at 75^{0}C (Invitrogen). So, extension time depends on the length of the target gene. Schematic representation of polymerase chain reaction is showed in Figure 2.9.

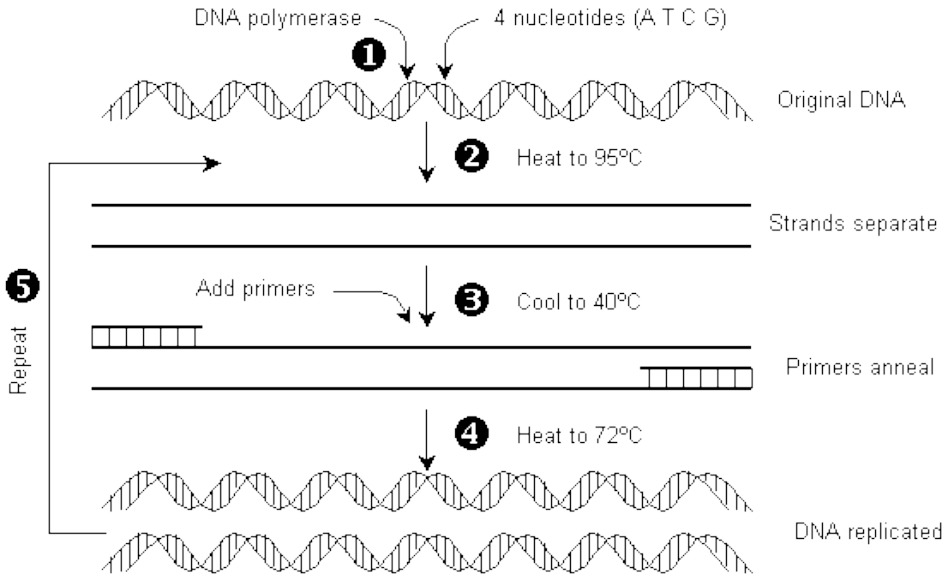


Figure 2.9 Major steps of polymerase chain reaction (PCR) (http://www.biologymad.com)

2.3.2 Selection of an Appropriate Expression Vector

Another important step in gene cloning is the selection of an appropriate expression carrier plasmid DNA, known as expression vector. Plasmid is a circular DNA molecule that can replicate itself independently from

the chromosomal DNA. Commercial expression vectors, modified plasmids, are designed with genetic engineering techniques to optimize them for cloning purpose. A proper cloning vector should harbor some characteristics: it should be able to replicate independently; it's isolation and purification procedures should be easy; transformation of host cell with the vector should be easy; it should include suitable markers (eg. antibiotic resistance gene) for detection and/or isolation of the transformed cells; it should contain multiple cloning sites to insert a DNA molecule (Walker, 1998). Most of yeast expression vectors are shuttle vectors that replicate themselves in bacteria. Yeast integrative plasmid introduces itself into yeast genome via homologous recombination and can't replicate itself independently within yeast. Yeast integrative plasmids are advantageous for maintaining the recombinant gene stably, even if the selectivity agent doesn't apply (Walker, 1998).

2.3.2.1 pPICZα-A Vector

Yeast integrative pPICZα-A vector was manipulated for recombinant protein production in *P. pastoris*. It can replicate itself independently within *E. coli* cells as it's a shuttle vector. It harbors Zeocin resistance marker gene for selection of both recombinant *E. coli* and *P. pastoris* cells. The vector multiple cloning site includes thirteen different restriction sites for gene insertion. Under strong and tightly regulated AOX promoter, high level expression of recombinant protein is performed by methanol induction. α-factor signal sequence of *S. cerevisiae* was introduced into the expression vector to secrete recombinant proteins into the extracellular media. If needed for purification, pPICZα vectors also contain polyhistidine (6XHis) sequence which can be added to the recombinant protein C-terminal end. In this study pPICZα-A expression vector is used to produce recombinant *P. pastoris* cells containing *pro-mtg* gene. Gene map of the pPICZα expression vector is shown in Figure 2.10.

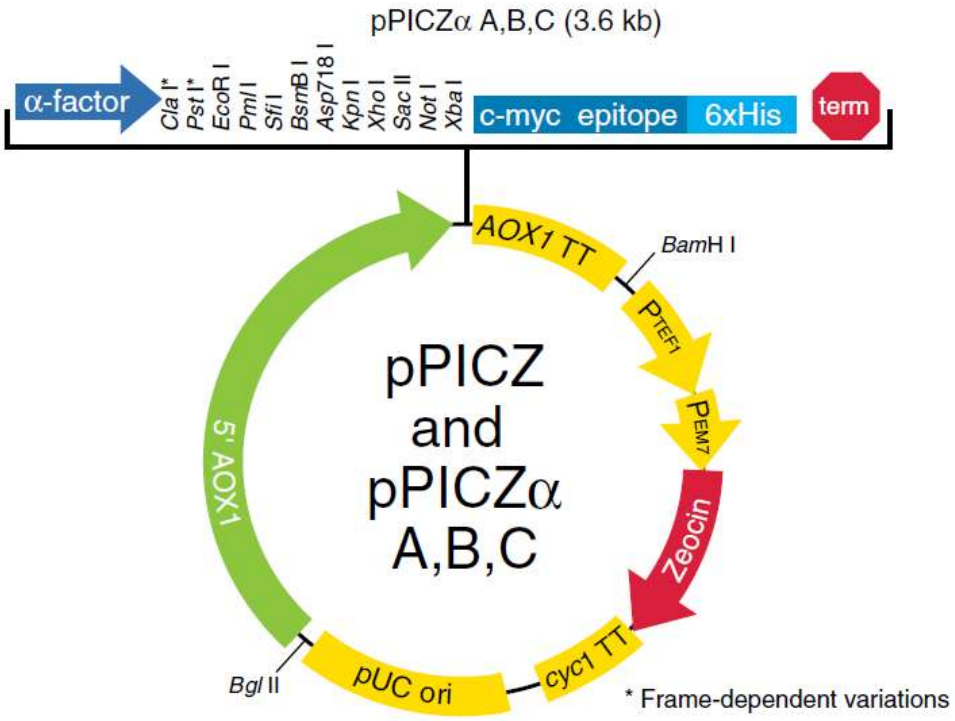


Figure 2.10 pPICZα Expression Vector (http://www.invitrogen.com)

2.3.3 Recombinant Plasmid Construction

For recombinant plasmid construction both the target gene and vector DNA is digested by proper restriction enzymes. Microbial restriction enzymes cut the foreign DNA from outside of the helix while they don't catalyze any reaction on their native DNA due to difference in methylation patterns of the DNA (Scragg, 1998). Restriction endonucleases act as a immune system, protecting bacterial cells against viral infections. Depending on the composition, cofactor requirements, the structure of target sequence and position of restriction site; restriction enzymes are divided into four classes (Types I, II, III and IV). The type II restriction enzymes, the most common used in recombinant DNA technology, have the capability of binding DNA helix at any site. They move through the strand until they find the correct

restriction sequence. Recognition site's length varies from four to twenty nucleotides long. Restriction enzyme digestion results blunt or sticky end formation on the fragmented DNA. Sticky ends facilitate the ligation efficiency as including complimentary single stranded ends and preferred over blunt end. Restriction enzymes and their recognition sequences, used in this study, are shown in Table 2.2.

Table 2.2 Recognition sequences and cleavage sites of restriction endonucleases.

Restriction Enzyme	Recognition Sequence
*EcoR*I	G^AATTC
*Xba*I	T^CTAGA
*Bsp*119I	TT^CGAA

The DNA ligase enzyme catalyzes the phosphodiester bond formation between the 3' hydroxyl group of one nucleotide and the 5' phosphate group of another. In other words, the DNA ligase binds linear digested target gene and vector together with covalent bonds Figure 2.11. The ligation step is the most crucial stage of the recombinant plasmid construction since correct orientation and annealing of the target gene onto vector plasmid is required to prevent self ligation of vector DNA. So, ligation reaction conditions are optimized carefully by adjusting vector and target gene concentrations. Vector self ligation can be prevented by using different restriction enzymes at the 5' and 3' ends of the DNA particle and application of phosphatase enzyme to the linear vector (Çalık *et al*., 1998; Glazer, 1995; Bailey, 1986).

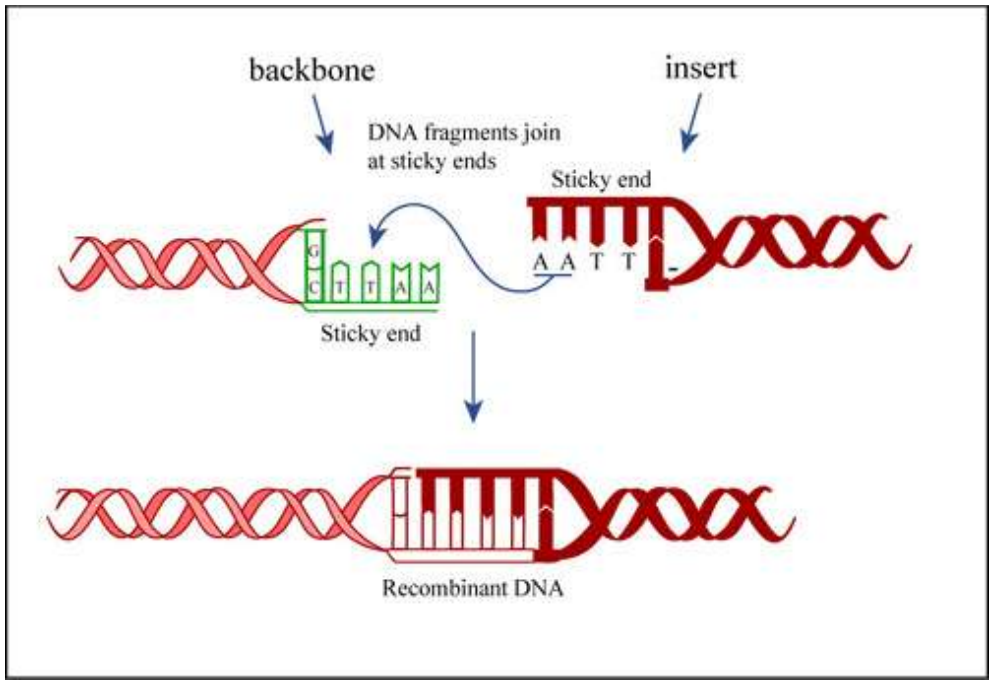


Figure 2.11 Mechanism of DNA ligation (http://ocw.mit.edu)

2.3.4 Transformation

After successful ligation reaction, recombinant plasmid is transferred into the recipient or host cell and this procedure usually performed by transformation. Chemical, biolistic and electrical methods are used for transformation of yeast cells (Walker, 1998). Before yeast transformation, recombinant plasmid is first propagated in *E. coli* to reach high DNA concentrations. Without cloning and manipulating plasmids within *E . coli*, the applications of gene cloning technology to other microorganisms would be blocked (Primrose, Tywan and Old, 2001). In this study chemical method was used to transfer pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA::*pro-mtg*$_{intra}$ plasmids into the *E. coli* and *P. pastoris* cells. Calcium chloride solution is used to permeabilize *E. coli* cells and lithium chloride is used to permeabilize *P. pastoris* cells.

2.3.5 Screening of Transformants

Construction of insert-vector DNA usually leads mixture of self ligated vector DNA, vectors with contaminant gene inserts and correct constructs. To select transformants with the correct plasmid, efficient screening methods are required. Antibiotic resistance gene which is found in some of the expression vectors enhances the recombinant microorganism with the ability of surviving in antibiotic containing media. The antibiotic resistance characteristic is the most commonly used test for screening and selection of recombinant cells because it can be used for an extended host range and supplies more flexibility for changes in growth conditions (Smith, 1995). Following the transformation, cells that participate in transformation process are inoculated on solid medium containing appropriate antibiotic to select the true transformants. In this study, the used pPICZα-A expression vector includes Zeocin resistance gene that is quite strong antibiotic. Since the vector integrated into *P. pastoris* genome, antibiotic is not necessary during fermentation. Figure 2.12 schematically represents the antibiotic resistance test. All the cells grown on medium with antibiotic don't harbor the correct vector-insert DNA combination and they should be further analyzed for verification. Genetic material of transformant cells are usually analyzed by three methods: Southern blotting, restriction mapping, PCR and DNA sequencing (Klug *et al*., 2006). To determine whether the gene of interest is inserted into plasmid or not PCR can be used. Plasmids isolated from transformant cells are used as template DNA with designed primers in PCR. The most reliable method to analyze a transformant's DNA is DNA sequencing, as it reveals even one nucleotide change; on the other hand it is the most complicated one among others. DNA sequencing is used in this study to verify the plasmid after transformation.

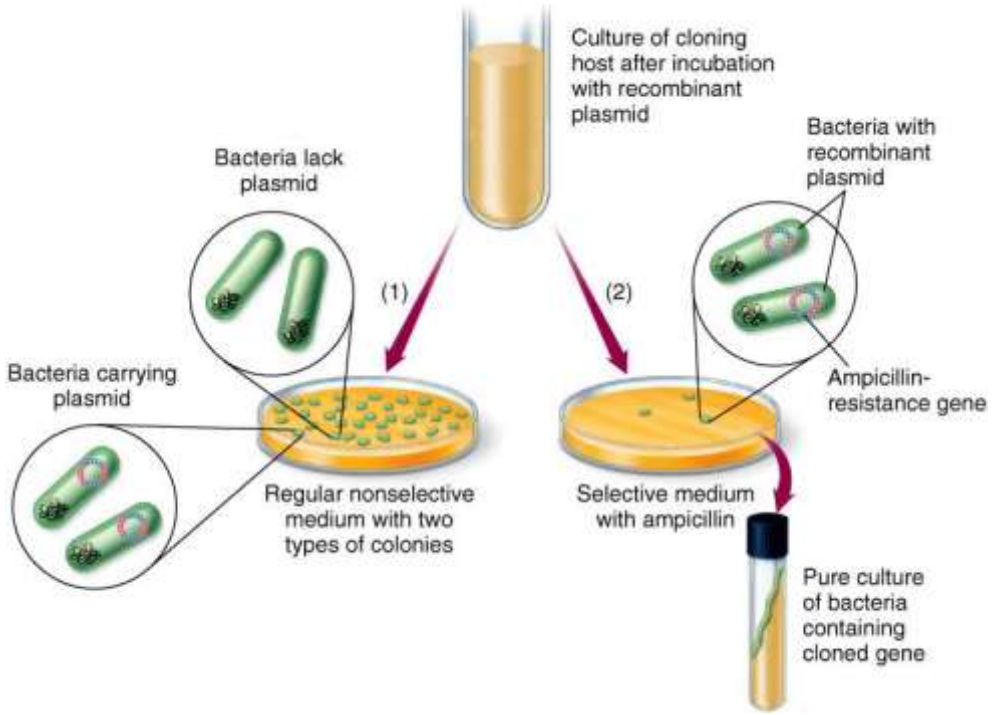


Figure 2.12 Schematic representation of antibiotic test (http://www.worldofteaching.com)

2.4 Medium Design and Bioreactor Operation Parameters

Any activity related with design and optimization of processes to produce products from raw materials via using microorganisms or their products (e.g. enzymes) named as a ''bioprocess'' (Moses and Cape, 1991). The main goal in industrial bioprocess applications is that increasing the yield and productivity of the product through supplying optimal conditions for microorganisms' growth and expression. In terms of bioprocess design, the crucial criteria should be considered are:

1. Medium composition design

2. Bioreactor operation parameters
    - pH
    - Temperature
    - Dissolve oxygen concentration

2.4.1 Medium Composition Design

Theorically, the microorganisms function as semi-batch microbioreactors with volume V, in which the biochemical reactions carry out (Çalık *et al*., 2003). Between the microorganisms and bioreactor medium, named as environment, lots of compounds which have roles in cell metabolic systems are diffused or transported with active or facilitated transport mechanism. In general, product formation within microorganism is a response of environmental conditions such as inducers, growth hormones, nutrients and ions. To optimize the cell growth and product formation, the amount and type of nutrient requirements of the cell should be determined. General composition of a fermentation medium is explained below:

- **Water**: It's the main component of all growth media.
- **Macronutrients**: Oxygen, carbon, hydrogen, nitrogen, sulphur, phosphorous, potassium and magnesium are classified as macronutrients and they comprise over 1% of the cells dry weight. Macronutrients are crucial in the formation of cell organelles, membranes, nucleic acids and amino acids. In the fermentation media their concentration should be over 10^{-4}M.
- **Micronutrients**: Elements such as Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mo^{2+}, Mn^{2+}, Na^{+}; vitamins, hormones and metabolic precursors are micronutrients. Their concentration is needed less than 10^{-4}M. 0.1-1% of the dry cell weight is composed of micronutrients and they employed as primary constituents in proteins' structure.

- **Trace Elements**: They are consumed in too minor levels (less than 0.1 %) and higher concentrations of them may inhibit cell growth. On the other hand, adequate concentrations of trace elements promote cell growth.
- **Buffers**: They are used to adjust the pH of the fermentation medium.
- **Vitamins** and **growth factors**: They are required for some specific bioprocesses.
- **Inducers:** They are required for high protein expression rates which is controlled by an induction mechanism.
- **Antifoams:** In bioreactor experiments when high foam is occurred, antifoams are required. They are surface active agents and reduce the surface tension in the fermentation media. Usually they don't have any metabolic effect but they may reduce the oxygen transfer rate.

Growth media are divided into 2 classes due to their compositions. First one is defined or synthetic medium which contains pure inorganic components with known concentrations. Defined medium is usually preferred to evaluate the effect of a certain element on cell growth rate and product yield (Kampen, 1997). It provides better control over fermentation and facilitates the isolation and purification steps to recover the product. Moreover fermentation experiments in defined media are well documented and reproducible results are obtained. Second one is complex medium, that contains natural compounds and its' exact composition is not known implicitly. Usually higher protein and cell yield are obtained with complex medium regarding to its' rich composition. Complex medium is usually preferred in industrial applications as they found in bulk quantities and are relatively cheap sources of carbon, nitrogen, essential minerals, several growth factors, vitamins and trace elements (Nielsen and Villadsen, 2003).

There isn't any exact way to ensure optimal production, but there are some instructions that enhance the productivity.

Basal salt medium along with its companion trace salt element (PTM1) is the most commonly used fermentation medium of methylotrophic yeast *P. pastori*s to reach high cell densities. Although it's widespread use, it has some problems such as high ionic strength, precipitates and unbalanced composition (Cereghino *et al*., 2002; Cos *et al*., 2006). To optimize the medium composition, several researches were performed (Jungo *et al*., 2006; Brady *et al*., 2001) and the influence of each medium component to *P. pastoris* expression system have been investigated in detail (Plantz *et al*., 2006). Ammonium hydroxide solution is used as a nitrogen source in BSM. Besides, it helps to control the pH of the fermentation medium (Cos *et al*., 2006). Steady ammonium hydroxide addition to medium prevents nitrogen accumulation, which causes growth inhibition and prolonged lag phase (Yang *et al*., 2004). In case of inadequate nitrogen feeding, nitrogen starvation begins and cells' protease secretion increases.

Carbon sources are important for cell growth as they are the main energy sources. Methanol, glycerol, sorbitol, mannitol and trehalose are widely used carbon sources of *Pichia pastoris* (Inan and Meagher, 2001b; Thrope *et al*., 1999; Sreekrishna *et al.,* 1997; Brierley *et al.,* 1990). Among others methanol is not only a carbon source but also the inducer of the AOX promoter to express recombinant proteins. Even so, high methanol concentrations may cause substrate inhibition of growth as it leads rapid formaldehyde and hydrogen peroxide formation within yeast cell (Zhang *et al*., 2000). So that a fed- batch feeding strategy is usually preferred to control the methanol level throughout the fermentation.

P. pastoris recombinant protein production under alcohol oxidase promoter is usually performed in three stages (Stratton *et al*., 1998). First of

all, cells are grown batchwise in defined medium including excess amount of glycerol in order to reach high cell densities quickly. In this stage, recombinant gene expression under AOX promoter is repressed by rapid cell growth on glycerol (Tschopp *et al*., 1987). Then, limited glycerol is fed in order to increase the cell concentration further. Glycerol fed-batch stage also depresses the enzymes used in methanol utilization gradually and time needed for the cells to adapt methanol is reduced (Chiruvolu *et al*., 1997). Finally, recombinant protein production under the control of AOX1 promoter is performed by *P. pastoris* via inducing the cells with methanol, continuously. Since elevated concentrations of methanol may be toxic to *P. pastoris* cells, keeping its' concentrations below toxic limits, 4 g L^{-1} is important. This is supplied with methanol fed-batch feeding strategy.

The use of multi-carbon sources besides methanol is another strategy to increase the cell density, increase productivity and reduce the induction time. Multi-carbon source strategy has been mostly applied to Mut^S strains, since they have low capacity to utilize methanol as a result of genetic modifications and this results in prolonged induction times over 100 h (Ramon *et al*., 2007). Mixed substrate feeding strategy applications of Mut^+ strains have been increasing, too; regarding to its several advantageous.

The first mixed substrate feeding strategy, glycerol and methanol, of *P pastoris* was reported by Brierley *et al*. (1990). Several researches were designed to increase the volumetric protein productivity by increasing the cell density and feeding rate with growth on glycerol (Zhang *et al*., 2003; Katakura *et al*., 1998; Loewen *et al*., 1997; McGrew *et al*., 1997; Cregg *et al*., 1993). However, some scientists thought that optimal protein expression with glycerol-methanol mixtures can't be achievable due to partial repression of the AOX1 promoter by glycerol that may lead to lower the specific productivities of recombinant proteins (Xie *et al*., 2005; Hellwig *et al*., 2001; Sreekrishna *et al.,* 1997).

In contrast with glycerol, sorbitol is a non-repressing carbon source for alcohol oxidase promoter, so high sorbitol concentrations during production phase don't affect the recombinant protein expression level. These advantageous make sorbitol an invaluable co-carbon source for Mut^+ strains. This idea was firstly introduced by Sreekrisha *et al*. (1997) while the first experimental results, showing the advantageous of sorbitol when it used in induction phase of Mut^+ strains, was reported by Inan *et al*., (2001b). Then, consumption mechanism of sorbitol and methanol were investigated in a batch bioreactor and sequential consumption of the carbon sources by the cells was observed (Ramon *et al*., 2007). Afterward, sorbitol content of the production medium was optimized in a continuous bioreactor experiment and optimized feeding ratio was tested with two fed-batch bioreactor experiments using specific growth rates as 0.03 h^{-1} and 0.05 h^{-1} (Jungo *et al*., 2007). In recently, the effect of methanol exponential feeding rate, calculated with different specific growth rate values, on recombinant human growth hormone production by recombinant *Pichia pastoris* Mut^+ strain was investigated (Çalık *et al*., 2010a). It was shown that, when specific growth rate values were over 0.03 h^{-1}, sorbitol consumption developed independently from methanol feed rate. The highest recombinant human growth hormone production was achieved in methanol-sorbitol mixed feed system where methanol feeding performed at a specific growth rate of μ=0.03 h^{-1}.

2.4.2 Bioreactor Operation Parameters

Bioprocesses are usually affected by various parameters containing: pH, temperature, dissolved oxygen level, agitation rate, nature and composition of the medium, ionic strength and shear rates in the bioreactor. Changes in these parameters may lead different effects on cell and product yield via influencing metabolic pathways and changing metabolic fluxes (Soetaert and Vandamme, 2010; Çalık *et al*., 1999). In order to achieve high

productivity, bioprocess should be performed under controlled conditions of temperature, pH, dissolved oxygen and other possible parameters.

2.4.2.1 Temperature

In order to achieve efficient microbial growth and recombinant protein production, it is crucial to keep temperature constant at microorganisms' optimal value throughout the bioprocess (Nielsen *et al*., 2003). Microorganisms can't regulate their internal temperature. Therefore, cell temperature and biochemical reaction rates depend directly on the environment. Although temperature affects the microbial growth and product formation rate, their optimal values may be different. Temperature affect on specific growth rate of microorganism is like that the affect on enzymatic activity of an enzyme: as temperature increases specific growth rate increases, too up to an exact point where protein denaturation begins, and a rapid decrease is observed beyond this point. Up to microorganisms' optimum temperature, the specific growth rate display a trend like a chemical rate constant, explained by Arrhenius equation (Nielsen *et al*., 2003). When temperature is over optimum point, the maintenance needs of cells increase. Additionally, nutritional requirements, metabolic regulations, yield coefficients and biomass composition are also influenced with temperature.

Bioprocesses with *P. pastoris* are usually performed at an optimum growth temperature of 30^0C (Wegner, 1983). Temperatures above 32^0C may be harmful to recombinant protein expression and may cause cell death (Invitrogen, 2002) and temperatures higher than 30^0C are not convenient for recombinant protein production as high temperatures cause cell death which results cell lysis and higher proteolytic activity in bioreaction medium.

Low fermentation temperature leads less cell death, as a result of this protease release to the media decreases and the yield of recombinant

proteins produced by *Pichia pastoris* is influenced as the degradation of recombinant proteins by proteases are diminished (Macauley-Patrick *et al*., 2005). Additionally, fermentations at low temperature reduce protein misfolding while facilitate proper folding of proteins to be secreted into the cultivation medium (Georgiou and Valax, 1996). Many studies reported that the product yield was increased at lower temperatures (Wu *et al*., 2008; Siren et al., 2006) while other studies reported that lowering the temperature below 30^0C does not have a significant effect on the recombinant protein production by *Pichia pastoris* strains (Potvin *et al.,* 2012).

In this study, production of microbial protransglutaminase by recombinant *P. pastoris* cells is performed at the optimal temperature value of P. pastoris, which is 30^0C.

2.4.2.2 pH

Hydrogen ion concentrations (pH) is another important bioprocess parameter as it affect the activity of enzymes, intracellular and extracellular reactions and transport mechanism, so microbial growth rate. Microorganisms can keep their intracellular pH at a constant level at the expense of energy. Changes in pH value due to the formation of organic acids, utilization of acidic or basic compounds or production of alkali compounds is a common process during fermentation. In order to keep pH at a constant level throughout the fermentation, buffer solutions together with active pH controller system are generally used in bioreactor experiments.

P. pastoris has the capability of growing across a wide pH range from 3.0 to 7.0. Growth isn't affected by pH changes significantly within this pH range, thus pH adjustments of the media can be done due to the recombinant protein production and protein stability (Macauley-Patrick *et al*., 2005). Optimal pH values of proteins differ regarding to nature and stability of the recombinant protein. Optimum pH value for recombinant mouse

epidermal factor and human serum albumin production was found 6.0 (Kobayashi *et al*., 2000b; Clare *et al*., 1991); while optimum pH value for recombinant cytokine growth blocking peptide was found 3.0 (Koganesawa *et al*., 2002). In the recombinant human growth hormone production by *P. pastoris*, the maximum product yield was obtained at pH 5.0 (Çalık *et al.*, 2010b); whereas in the recombinant human erythropoirtin production by *P. pastoris* Mut^{+} strain, the maximum product yield was obtained when the cultivation medium was operated at pH 5.0 for the glycerol and glycerol fed-batch stages and at pH 4.5 for production phase (Soyaslan *et al*., 2011). In general applications, the pH value of the medium has been kept around 5.5 to improve the stability of the recombinant proteins and to prevent the affect of proteases in the medium (Cos *et al*., 2006).

2.4.2.3 Oxygen Transfer Rate

In aerobic fermentation, oxygen influences product formation in various ways by affecting metabolic pathways and altering metabolic fluxes (Çalık *et al*., 1999). Oxygen requirements of a bioprocess rely on the microbial species, the cell mass, the rheological properties of the medium and type of substrate utilized in fermentor. In aerobic bioprocesses, oxygen supply should be more than oxygen demand of the cells, otherwise microbial growth is restricted by inadequate oxygen (Soetaert and Vandamme, 2010).

Gaseous oxygen is brought into the growth media by surface aeration or by sparging air. Oxygen transfer rate can be manipulated either changing the agitation rate or air feed rate.

P. pastoris prefers respiratory system instead of fermentative mode (Cereghino *et al*., 2002), therefore inhibitory byproducts such as ethanol and acetic acid aren't produced during fermentation. When methanol found in the environment, it becomes obligatory aerobic and it requires high oxygen concentration throughout the production phase. One of the disadvantageous

of *P. pastoris* expression system is that in order to achieve high cell densities with methanol, aeration of the medium with pure oxygen is required. On the other hand, oxygen limited fed-batch processes were investigated (Trentmann *et al*., 2004; Trinh *et al*., 2003). For recombinant Thai Rosewood β-glucosidase production, oxygen limited fed batch (OLFB) process is compared with methanol limited fed-batch (MLFB) process and 35 % higher oxygen uptake rate, higher productivity and specific activity was determined in OLFB when compared with MLFB. Consequently, in most of the fermentation processes with *P. pastoris,* pure oxygen is supplied into the media to keep the oxygen level above 20-30% throughout the induction phase on methanol (Jahic *et al*., 2006).

2.5 Structural Analysis of Recombinant Protein

After creating recombinant cells and producing the product, structural protein analysis should be performed to prove the structure. In order to determine primary structure of the recombinant protein, there are several methods. In this study, SDS-PAGE and dot blot analysis are used to determine the presence and molecular weight of the recombinant protein.

2.5.1 Dot-Blot Analysis

Dot-Blot analysis is used to determine the presence of a specific protein in a protein mixture. In this technique the antibody-protein interaction is used to detect a specific protein. First protein samples are loaded onto a nitrocellulose membrane and then antibody-antigen reactions are performed within solutions. Finally, protein-antibody complex is visualized by treating with chromagenic dye.

2.5.2 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE is used to separate the proteins according to their size via running the denatured protein particles through a polyacrylamide gels

under electrical field. Proteins are dissociated into their individual polypeptide subunits before analyses begin. Combination of the strong anionic detergent SDS (sodium dodecyl sulfate) and a reducing agent (generally 2-mercaptorthanol) is used to denature the proteins via heat treatment. SDS charges all the denatured proteins negatively without depending on the sequence of the proteins. Therefore, denatured protein particles migrate through polyacrylamide gels according to the size of the proteins. After separation of the proteins within the gel, protein bands are detected by staining, either with silver salts or Coomassie Brilliant Blue. Coomassie Brilliant Blue binds to proteins nonspecifically, so proteins are visualized as discreet blue bands within the translucent gel matrix. Although it's complicated procedure, silver staining is more sensitive when compared with Coomassie Brilliant Blue staining as it allows the detection of protein concentrations nearly 100-fold lower than detected by Coomassie Brilliant Blue staining. (Sambrook and Russel, 2001).

2.6 Computation of Bioprocess Characteristics

Specific rates of growth, substrate consumption and product formation and yield coefficients are important values of fermentation operations in order to characterize and investigate the bioprocess.

2.6.1 Specific Growth Rate

In order to understand and manipulate cell growth, specific growth rate (μ) is a key parameter. The biomass formation or cell growth rate, r_x, is defined as the multiplication of specific growth rate (μ) and cell concentration (C_x) i.e.,

$$r_X = \mu C_X \qquad (2.1)$$

where μ is the specific growth rate (h^{-1}). With the assumption of no loss is occurred through sampling, general mass balance for biomass in a fed batch bioreactor is written as:

$$r_x V = \frac{d(C_x V)}{dt} \tag{2.2}$$

where C_x is the cell concentration (kg m^{-3}), V is the volume of the bioreactor (m^3) and r_x is the cell growth rate (kg m^{-3} h^{-1}). With the combination of equations (2.1) and (2.2), a new equation is obtained:

$$\frac{d(C_X V)}{dt} = \mu C_X V \tag{2.3}$$

As the bioreactor is operated in a fed-batch mode throughout the bioprocess, volume of the bioreactor changes with time:

$$Q = \frac{dV}{dt} \tag{2.4}$$

Where Q is the volumetric flow rate (m^3 h^{-1}). With evaluating equation (2.3) and then inserting equation (2.4) into it, equation (2.5) is obtained:

$$\frac{dC_X}{dt} = \left(\mu - \frac{Q}{V}\right) C_X \tag{2.5}$$

an equation for μ is obtained by rearragngin the equation (2.5):

$$\mu = \frac{dC_X}{dt}\frac{1}{C_X} + \frac{Q}{V} \tag{2.6}$$

With equation (2.6), specific growth rate can be calculated with known cell concentration and volume data throughout the bioprocess.

2.6.2 Methanol Consumption Rate

The major carbon source of the bioprocess is methanol and it is added in fed-batch mode into the production medium. Methanol mass balance for fed-batch process is written as:

$$QC_{MO} - 0 + r_M V = \frac{d(C_M V)}{dt} \tag{2.7}$$

where, the substrate consumption rate, r_M (kg m^{-3} h^{-1}), is defined as:

$$r_M = q_M C_X \tag{2.8}$$

r_M is the product of cell concentration (C_x) and specific substrate consumption rate, q_M (h^{-1}). Via inserting the equation (2.8) into equation (2.7);

$$QC_M + q_M C_X V = V\frac{dC_M}{dt} + C_M\frac{dV}{dt} \tag{2.9}$$

In fed-batch bioprocess, as the cells consume almost all the methanol fed to the system, the system can be assumed to perform at quasi-steady state. According to this assumption and negligible volume change of the media the second term on the right side is neglected.

$$C_M\frac{dV}{dt} \sim \mathbf{0} \tag{2.10}$$

By appliying equation (2.10) into the equation (2.9) and dividing all the terms by V; equation (2.11) is obtained:

$$\frac{dC_M}{dt} = \frac{Q}{V}C_{M\mathbf{0}} + q_M C_X \tag{2.11}$$

By rearranging the eqauation, an equation for the specific consumption rate of methanol, q_M (h^{-1}) is obtained:

$$q_M = \frac{Q}{V}\frac{C_{M0}}{C_X} - \frac{1}{C_X}\frac{dC_M}{dt} \tag{2.12}$$

2.6.3 Sorbitol Consumption Rate

In this study, co-carbon source sorbitol is fed to the system batch-wise, the mass balance equation for sorbitol can be written as:

$$r_S V = \frac{d(C_S V)}{dt} \tag{2.13}$$

where, C_s is the sorbitol concentration in the medium (kg m^{-3}). The substrate consumption rate of sorbitol, r_s (kg m^{-3} h^{-1}), is defined as:

$$r_S = q_S C_X \tag{2.14}$$

in which q_s is the specific subsrate consumption rate (h^{-1}). Combining equation (2.13) and (2.14), and then by dividing all the terms by V, equation (2.15) is found:

$$\frac{dC_S}{dt} = -\frac{Q}{V}C_S + q_S C_X \tag{2.15}$$

The equation for the specific sorbitol consumption rate (q_S) is obtained via rearranging the equation (2.15):

$$q_S = \left(\frac{dC_S}{dt} + \frac{Q}{V}C_S\right)\frac{1}{C_X} \tag{2.16}$$

2.6.4 Overall Yield Coefficients

The yield coefficient (Y_{ij}) is obtained, when any particular rate, q_i or r_i, is scaled with another rate, q_j or r_j (Nielsen *et al*., 2003). The biomass yield on substrate and the product yield on substrate are the other important parameters in ivestigation of bioprocess as they demonstrate the efficiency of conversion of the substrate into biomass and product. Frequently used yield coefficients are given in Table 2.3.

Table 2.3 Symbols and descriptions of the yield coefficients

Symbol	Definition	Unit
$Y_{X/S}$	Mass of cells produced per unit mass of substrate consumed	g cell g^{-1} substrate
$Y_{X/O}$	Mass of cells produced per unit mass of oxygen consumed	g cell g^{-1} oxygen
$Y_{S/O}$	Mass of substrate consumed per unit mass of oxygen consumed	g substrate g^{-1} oxygen
$Y_{P/X}$	Mass of product formed per unit mass of cells produced	g product g^{-1} cell
$Y_{P/S}$	Mass of product formed per unit mass of substrate consumed	g product g^{-1} substrate

The biomass yield ($Y_{x/s}$) and the product yield ($Y_{p/s}$) are important bioprocess values as they show the process efficiency for the product and biomass formation. $Y_{X/S}$ is defined as the ratio of produced biomass amount per consumed substrate amount while $Y_{P/S}$ is defined as the ratio of the formed product amount per consumed substrate amount within the same time interval. These coefficients are written as:

$$Y_{X/S} = -\frac{\Delta X}{\Delta S} \tag{2.17}$$

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \tag{2.18}$$

Where X is biomass, P is product and S is substrate, $Y_{X/S}$ is the overall biomass yield coefficient, $Y_{P/S}$ is the overall product yield coefficient, ΔX is the mass of cell produced, ΔP is the mass of product produced and ΔS is the mass of substrate consumed.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals and solutions used in this study are analytical grade and obtained from Merck&Co. Inc, Sigma Aldrich Co., Fluka, Difco, Fermentas, Fitzgerals Inc. and Zedira Gmbh.,

3.2 Buffers and Stock Solutions

All buffers and stock solutions used are listed in Appendix A. All of them were prepared with distilled water. The sterilization of solutions was carried out either by autoclaving at 121^{o}C for 20 min or by filtering through 0.20 µm filters (Sartorius Stedim Biotech GmbH, Gottingen, Germany).

3.3 Strains, Plasmids and Maintenance of Microorganisms

The microbial protransglutaminase gene was amplified from pDJ1-3 (Marx *et al*., 2007). The pDJ1-3 plasmid was carrying the *Streptomyces mobaraensis* protransglutaminase gene and antibiotic resistance gene against ampicillin. For propagation of plasmids One Shot TOP10 *E.coli* cells were used. *Pichia pastoris* X-33 strain, One Shot TOP10 *E. coli* strain and pPICZα-A shuttle vector were purchased from Invitrogen. E. coli strain carrying the pDJ1-3 vector was named as *E.coli* pDJ1-3, the strain carrying the pPICZα-A vector was

named as E.coli pPICZαA. The *E.coli* strains carrying the vector with extracellular and intracellular *pro-mtg* gene, were named as *E.coli* pPICZαA-pro-mtg_{extra} and *E.coli* PPICZαA-pro-mtg_{intra}, respectively. *Pichia pastoris* strains expressing microbial protransglutaminase gene extracellularly and intracellularlly were named as *P. pastoris* pPICZαA-pro-mtg_{extra} and *P. pastoris* pPICZαA-pro-mtg_{intra}, respectively. The sequence and schematic representation of the plasmids are given in Appendix C. For long term storage of microorganisms, the microbanks (PRO-LAB) were prepared with young colonial growth cells. Cells were inoculated into cyropreservative solution and mixed gently to enhance adsorption of cells onto the porous beads dispersed in the solution. After that, by pipetting excess amount of cryopreservative solution was removed. Inoculated microbanks were stored at -80^{o}C.

3.4 *E. coli* and *Pichia pastoris* Growth Media

E. coli pDJ1-3 cells were grown on LB and Low Salt LB Media containing 100 μg ml^{-1} Ampicillin. LB and Low Salt LB medium supplemented with 25 μg ml^{-1} Zeocin were used both for propogation of *E.coli* pPICZαA cells and for selection of transformed *E. coli* pPICZαA-pro-mtg_{extra} and *E. coli* pPICZαA-pro-mtg_{intra}. Wild type *Pichia pastoris* was grown at YPD, BMGY and BMMY media without any antibiotic while *Pichia pastoris* pPICZαA-pro-mtg_{extra} and *Pichia pastoris* pPICZαA-pro-mtg_{intra} were grown at YPD, BMGY and BMMY media supplemented with 150 μg ml^{-1} Zeocin. The composition and preparation procedures of all the media used were given in Appendix B.

Table 3.1 Strains and plasmids used in this study

Genus	Species	Strain	Genotype/Plasmid	Source
Escherichia	*coli*	pPICZαA	pPICZαA	Invitrogen
Escherichia	*coli*	pDJ1-3	pDJ1-3	Marx *et al*., 2007
Escherichia	*coli*	pPICZαA-pro-mtg_{extra}	pPICZαA*::pro-*mtg_{extra}	This study
Escherichia	*coli*	pPICZαA-pro-mtg_{intra}	pPICZαA*::pro-*mtg_{intra}	This study
Pichia	*pastoris*	X-33	wild type	Invitrogen
Pichia	*pastoris*	pPICZαA	pPICZαA	This study
Pichia	*pastoris*	pPICZαA-pro-mtg_{extra}	pPICZαA*::pro-*mtg_{extra}	This study
Pichia	*pastoris*	pPICZαA-pro-mtg_{intra}	pPICZαA*::pro-*mtg_{intra}	This study

3.5 Genetic Engineering Techniques

3.5.1 Enzymes, Kits and Other Genetic Engineering Tools

Taq DNA polymerase, protease free Ribonuclease, T4 DNA ligase, dNTP mixture, restriction enzymes (*Eco*RI, *Xba*I, *Bsp*119I) and their buffers, Lambda DNA/HindIII Marker, Gene Ruler 50bp DNA ladder and 6x Loading Dye were purchased from Fermentas INC. Gene Elution Kit was obtained from Gene Molecular Biology Tools. Plasmid Purification Kit and PCR Purification Kit were provided by Fermentas Inc.

3.5.2 Plasmid DNA Isolation from E.coli with Alkaline Lysis Method

Plasmid DNA (pDJ1-3 and pPICZα-A vector) from *E. coli* cells was extracted with Alkaline Lysis Method (Sambrook and Russell, 2001). For this purpose, the procedure given below was followed.

Preparation of Cells

1. 10 ml LB medium with appropriate antibiotic was inoculated with a single colony. Culture was incubated overnight at 37^{o}C with vigorous shaking.
2. Bacteria were recovered by centrifugation at 2000 g for 10 minutes at 4^{o}C.
3. Medium was removed and the pellet was left as dry as possible.

Lysis of Cells

4. Bacterial pellet was resuspended in 200 µl ice-cold Alkaline lysis solution I by vigorous vortexing, then suspension was transferred to a microfuge tube.
5. 400 µl of freshly prepared Alkaline lysis solution II was added to each bacterial suspension. The contents were mixed by inverting the tubes for five times. Tubes were stored on ice.
6. 300 µl of Alkaline lysis III solution was added and mixed by inverting several times. Tubes were stored on ice for 3 to 5 minutes.
7. Bacterial lysates were centrifuged at maximum speed for 5 minutes at 4^{o}C in a microfuge. The supernatant of each tube was transferred to a fresh tube.
8. Equal volume of phenol:chloroform:isoamylalcohol was added and organic and aqueous phases were mixed by vortexing. Emulsion was

centrifuged at maximum speed for 2 minutes at 4°C in a microfuge. Aqueous upper layer was transferred to a fresh tube.

Recovery of Plasmid DNA

9. Nucleic acids were precipitated with the addition of 600 µl isopropanol at room temperature. Solution was mixed by vortexing and then mixture was allowed to stand for 2 minutes at room temperature.
10. Nucleic acids were precipitated by centrifugation at maximum speed for 5 minutes at room temperature.
11. Supernatant was removed by pipetting. Tube was standed in an inverted position to remove any drops of fluid adhering to the walls of tube.
12. 1 ml 70% ethanol was added to the DNA pellet and DNA was recovered via centrifugation at maximum speed for 2 minutes at room temperature.
13. All the supernatant was removed as described step 11. The open tube was standed at room temperature until all the ethanol has been evaporated. (2-10 minutes)
14. Nucleic acids were dissolved in 100 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A. The solution was mixed gently by vortexing for a few seconds. DNA solution was stored at -20°C.

3.5.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualize the DNA fragments to determine their size and their concentration. 0.8% (w/v) agarose gel was prepared by solving 0.8 g agarose in 100 ml 1X TBE buffer and solution heated up to boiling point. After solution was cooled to approximately 55°C, 5 µl of ethidium bromide (Sigma-10mg/ml) was added to gel solution, final concentration of ethidium bromide was 0.5 µg/ml. Gel was poured into a suitable tray and appropriate comb was inserted into the gel. Gel was allowed to cool. Agarose gel electrophoresis tank was filled with 1X TBE buffer. 5 µl of

DNA samples were mixed with 1 µl of 6X loading dye and were loaded into the wells, together with the DNA ladder (Appendix E) for size estimation. Electrophoresis was performed at 100 V for 45-75 min and DNA fragments were visualized under UV illumination and photographed.

3.5.4 DNA Extraction from Agarose Gels

After agarose gel electrophoresis, to extract a specific DNA fragment from the gel, Gel Elusion Kit (GeneMark) was used. This procedure was designed to eliminate other DNA fragments, salts and agarose. For this purpose, interested DNA fragments was excised from the gel under UV illumination and weighed in a microcentrifuge tube (up to 350 mg). Gel slices were melted in 500 µl Binding Solution at 60^{o}C until complete melting accomplished. Solution was loaded into spine column. Manufecturer's instructions were followed. At the end of the procedure, 30-50 µl of dH_2O was used to elute the DNA from spin column. Extracted DNA was stored at -20^{o}C for further applications.

3.5.5 Primer Design

Five different primers were designed in this study. Primers and their sequences showed in Table 3.2. Restriction enzyme recognition sites were analyzed by the help of ABD Molecular Biology Resources' Restriction Mapper web-site (http://www.restrictionmapper.org/).

Primer thermodynamic properties, melting points, G+C base composition, the possibility of primer dimer structure and self complementarity formation of primers were explored by the Oligo Analyzer 1.2 program. The properties of the designed primers are given in Appendix D. Extra nucleotides, especially G or C bases were added to the 5' end of the restriction enzyme recognition sites to facilitate recognition of the sequence by the restriction enzyme with high probability. In the selection of nucleotide

sequences corresponding to start and stop codons, the codon usage bias of *Pichia pastoris* was taken into account, wherever possible.

Table 3.2 Primers used in this study and their sequences

Name	**Sequence**
MTG_{extra}-Forward	5'GGAATTCATGGACAATGGCGCGGGGGAAG 3'
MTG_{intra}-Forward	5'GTTCGAAATGGACAATGGCGCGGGGGAAG 3'
MTG-Reverse	5'CCTCTAGATCACGGCCAGCCCTGCTTTACC 3'
5'AOX	5'GACTGGTTCCAATTGACAAGC 3'
3'AOX	5'GCAAATGGCATTCTGACATCC 3'

Primers MTG_{extra}-Forward, MTG-Reverse designed regarding to the protransglutaminase gene from *Streptomyces mobaraensis* (Accession no: Y1835) to adjoin *Eco*RI restriction site (6bp) and start codon to the 5' end of *pro-mtg* gene sequence during amplification to produce rpro-MTG extracellularly. To construct intracellular rpro-MTG construct, primer MTG_{intra}-Forward was designed and used in combination with MTG-Reverse. For intracellular production *Bsp*119I restriction site (6bp) and start codon were included to the 5' end. *Bsp*119I restriction enzyme removed α-factor signal sequence from pPICZα-A expression vector. Primer MTG-Reverse with the *Xba*I restriction site and stop codon was used for the amplification of microbial protransglutaminase gene both for extracellular and intracellular construct formation. Restriction enzyme recognition sites in the primers are underlined. 5'AOX and 3'AOX primers were designed to control *pro-mtg* integration after transformation.

3.5.6 Polymerase Chain Reaction (PCR)

Microbial protransglutaminase gene amplification via PCR carried out by thermal cycling program (Techgene, Flexigene). The PCR reaction mixture was prepared on ice containing the following materials:

10x PCR Reaction Buffer (with Mg^{2+})	: 5 µl
dNTP mixture (1mM stock)	: 10 µl
Forward primer (10 µM stock)	: 1.5 µl
Reverse primer (10 µM stock)	: 1.5 µl
Template DNA	: 100 ng (1-3 µl)
Taq DNA Polymerase (1U μl^{-1})	: 1 µl
Sterile dH_2O	: to 50 µl

The PCR program used was

94 °C	**3 min**	**x 1cycle**
94 °C	1 min	
60 °C	1 min	x 32 cycle
72 °C	1 min 30 s	
72 °C	5 min	x 1 cycle
4 °C	∞	

3.5.7 Microbial Protransglutaminase Gene Purification after PCR

The purification of *pro-mtg* gene after polymerase chain reaction was performed by Fermentas PCR Purification kit according to manufacturer's instructions. The aim of this procedure was to purify DNA from *Taq* DNA Polymerase, primers, nucleotides and salts. At the end of the procedure, 20-50 µl dH_2O was used to elute DNA from spin columns.

3.5.8 Digestion of DNA using Restriction Enzymes

Restriction digestion of template gene (*pro-mtg*) and plasmid DNA (pPICzαA) were performed by proper restriction enzymes (REs). The composition of the digestion reaction coctail was given in the Table 3.3 and 3.4.

The reaction mixtures were incubated at 37°C water bath 2-16 hours. After digestion of DNA accomplished the mixture contained *Eco*RI and *Xba*I was incubated at 65°C for 20 min and the mixture contained *Bsp*119I and *Xba*I was incubated at 80°C for 20 min to inactivate REs.

Table 3.3 Composition of reaction coctail of restriction digestion with *Eco*RI and *Xba*I REs.

Components	Amounts
10X Tango Buffer	4 µl
DNA Fragment	0.1 to 5 µg
*Eco*RI RE (10 U μl^{-1})	0.5 µl
*Xba*I RE (10 U μl^{-1})	1 µl
Sterile dH_2O	to 20 µl

Table 3.4 Composition of reaction coctail of restriction digestion with *Bsp*119I and *Xba*I REs.

Components	Amounts
10X Tango Buffer	4 µl
DNA Fragment	0.1 to 5 µg
*Bsp*119I RE (10 U μl^{-1})	0.5 µl
*Xba*I RE (10 U μl^{-1})	0.5 µl
Sterile dH_2O	to 20 µl

3.5.9 Gel Elution of DNA after Digestion

After digestion of pPICZα-A and *pro-mtg*; removal of primers, enzymes, salts, unincorporated nucleotides and digested gene parts was performed by Gel Elusion Kit (GeneMark) according to manufacturer's instructions. At the end of the procedure, 30-50 µl of dH_2O was used to elute the DNA from spin column. Extracted DNA was stored at -20^oC for further applications.

3.5.10 Ligation

PCR amplified *pro-mtg* gene and pPICZαA vector had sticky ends after digestion with restriction enzymes. The amount of insert DNA (*pro-mtg*) for ligation reaction was calculated such that insert:vector ratio of 1:3, as given in the equation 3.1.

$$\text{amount of vector (ng)} \times \frac{\text{size of insert (bp)}}{\text{size of vector (bp)}} \times \frac{3}{1} = \text{amount of insert DNA (ng)} \qquad (3.1)$$

The components of ligation reaction mixture were shown in Table 3.5 and 3.6. The ligation reaction was performed by incubating of 20 µl final reaction volume at 22^oC for 1 hour and 5 µl of the ligation mixture was used for transformation of chemically competent *E. coli* cells.

Table 3.5 Composition of ligation mixture for construction of extracellular *pro-mtg* construct.

Components	Amounts
Linear vector DNA (3547 bp)	100 ng
Insert DNA (1147 bp)	97 ng
10X T4 DNA Ligase Buffer	2 µl
T4 DNA Ligase (5 U μl^{-1})	0.5 µl
Sterile dH_2O	to 20 µl

Table 3.6 Composition of ligation mixture for construction of intracellular *pro-mtg* construct.

Components	Amounts
Linear vector DNA (3252 bp)	100 ng
Insert DNA (1146 bp)	106 ng
10X T4 DNA Ligase Buffer	2 µl
T4 DNA Ligase (5 U μl^{-1})	0.5 µl
Sterile dH_2O	to 20 µl

3.5.11 Transformation of *E. coli* by $CaCl_2$ Method

For propagation of constructed plasmids after the ligation, *E. coli* TOP10 chemically competent cells (3.5.11.1) were used. Freezed chemically competent cells were thawed on ice and 5 µl of the ligation mixture was added into the vial.

3.5.11.1 Preparation of Chemically Competent *E. coli* cells

1. Wild type *E.coli* TOP10 cells were incubated on a LB agar plate for 16-20 h. One single colony was selected and inoculated into LB Broth. Culture was incubated at 37°C with vigorous shaking until OD_{600} 0.35-0.40.
2. 50 ml of bacterial cell culture was transferred to an ice-cold polypropylene tube and tubes were stored on ice for 10 minutes.

3. Cells were harvested by centrifugation at 2700 g for 10 minutes at 4^{o}C.
4. Supernatant was discarded and tubes were standed in an inverted position for 1 min to allow the last traces drain away.
5. Each pellet was resuspended in 30 ml of ice-cold 20 mM $CaCl_2$ solution by swirling or gentle vortexing.
6. Cells were harvested by centrifugation 2700 g for 10 minutes at 4^{o}C.
7. Supernatant was discarded and tubes were standed in an inverted position for 1 minute to allow the last traces drain away.
8. Each pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$.
9. At this point, cells were either used directly or dispensed into aliquot and freezed at -70^{o}C.

3.5.11.2 Transformation of Chemically Competent *E. coli* TOP10 Cells

10. 200 µl of chemically competent cell suspension was transferred to ice cold polypropylene tube using ice chilled micropipette tip. Add DNA (no more than 50 ng in a volume of 10 µl or less) to each tube. The content of tubes was mixed by swirling gently. Tubes were stored on ice for 30 minutes.
11. Tubes were transferred to preheated 42^{o}C water bath and they were stored in water bath for exactly 90 seconds without shaking.
12. Tubes were transferred to an ice bath rapidly. Cells were allowed to chill for 1-2 minutes.
13. 800 µl of LB medium was added to each tube. Culture was incubated for 45 minutes in water bath set at 37^{o}C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
14. 25-200 µl of transformed competent cells were transferred onto agar LB medium containing Zeocin.

15. Plates were stored at room temperature until the liquid has been adsorbed.
16. Plates were incubated at 37^{o}C in an inverted position. Transformed colonies should appear in 12-16 hours.

3.5.12 DNA Sequencing After Transformation

Plasmids were isolated from the transformed *E. coli* cells with Alkaline Lysis method. The sequence of the *pro-mtg* insert within pPICZαA plasmid was sequenced using both primers complementary to the *pro-mtg* and primers complementary to the AOX promoter region. The gene sequence was analyzed by Sagner's method, using ABI Prism 310 Genetic Analyzer in METU Central Laboratory. The results of the gene sequence analysis of pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ expression cassettes were given in Appendix C. The provided data was analyzed using Blast sequence alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=blast2seq&QUERY=&SUBJECTS=).

3.5.13 Linearization of pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$

For transfection of *Pichia pastoris,* linearized plasmid DNA was needed. For this purpose, restriction digestion of pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ were carried out by *Pme*I restriction enzyme. The composition of the restriction digestion mixture was given in Table 3.7. Restriction mixture was mixed gently and spinned down for a few seconds. Mixture was incubated at 37^{o}C for 2 hours.

Table 3.7 Composition of reaction mixture of restriction digestion with *Pme*I.

Components	Amounts
Nuclease free dH_2O	16 µl
10X Buffer B	2 µl
DNA (0.5-1 µg $µl^{-1}$)	1 µl
*Pme*I (5 U $µl^{-1}$)	1 µl

Full digestion was verified by agarose gel electrophoresis. Then, *Pme*I enzyme was inactivated by incubation at 65°C for 20 minutes. Linearized plasmid DNA phenol/chloroform extracted once was precipitated with the addition of 1/10 volume of 3 M sodium acetate and 2.5 volume of pure ethanol. DNA was harvested by centrifugation at 4°C and maximum speed for 2 minutes. Pellet was washed with 80% ethanol and centrifuged again. Pellet was air dried and resuspended in 10 µl of dH_2O.

3.5.14 Transfection of *Pichia pastoris* by LiCl Method

Transfection of *Pichia pastoris* was accomplished by LiCl method according to manufacturer's instruction (Invitrogen).

3.5.14.1 Preparing *Pichia pastoris* Cells

1. *Pichia pastoris* X_{33} was inoculated into YPD plate and incubated for 48 hours at 30°C. 50 ml YPD was inoculated with a single colony and culture was grown at 30°C with shaking to an OD_{600} of 0.8 to 1.0.
2. Cells were harvested at 4000 g for 5 min, washed with 25 ml sterile water and centrifuged at 1500 g for 10 minutes at room temperature.
3. Supernatant was decanted and cells were resuspended in 1 ml of 100 mM LiCl.
4. Cell suspension was transferred to a 1.5 ml microcentrifuge tube.
5. Cells were pelletted at maximum speed for 15 seconds and LiCl was removed with a pipet.

6. Cells were resuspended in 400 µl of 100 mM LiCl.
7. 50 µl of the cell suspension was dispensed into a 1.5 ml microcentrifuge tube for each transformation and cell suspension was used immediately.

3.5.14.2 Transformation

8. 1 ml sample of single-stranded DNA (2 mg/ml denatured and fragmented herring sperm DNA) was boiled for five minutes and then quickly chilled on ice.
9. LiCl-cell solution from Step 7 was centrifuged and LiCl was removed with a pipet.
10. For each transformation sample, following reagents were added in the order given to the cells.

    240 µl 50% PEG

    36 µl 1 M LiCl

    25 µl single stranded DNA

    Plasmid DNA (5-100 µg) in 50 µl sterile water

11. Each tube was vortexed vigorously until the cell pellet was mixed completely.
12. Tube was incubated at 30^{o}C for 30 minutes without shaking.
13. Heat shock was applied in a water bath at 42^{o}C for 20-25 minutes.
14. Tubes were centrifuged at 6000 rpm for 15 seconds and supernatant was removed with a pipette.
15. Pellet was resuspended in 1 ml of YPD gently and incubated at 30^{o}C with shaking.
16. After 2 hours incubation, 25-100 µl of the suspension was spread on YPD + Zeocin plates and incubated for 2-3 days at 30 ^{o}C.

3.5.15 Isolation of Genomic DNA from *Pichia pastoris*

Pichia pastoris genomic DNA isolation was performed in accordance with the procedure stated by Amberg *et al*., with slight modifications (Amberg, Burke, and Strathern, 2000).

1. Yeast cells were grown in 10 ml YPD medium to an OD_{600} 6-10.
2. 10 ml of culture was centrifuged at 4000 g for 10 minutes and supernatant was discarded.
3. Cell pellet was dissolved in 0.2 ml yeast lysis buffer and 0.2 ml phenol:chloroform:isoamylalcohol (25:24:1), 0.3 g of glass bead was added, then mixture was vortexed vigorously for 4 minutes.
4. 0.2 ml of TE buffer (pH 8.0) was added and the mixture was centrifuged at 4000 rpm for 2 minutes in a tabletop centrifuge.
5. Aqueous phase was transferred to a fresh 2 ml eppendorf tube, mixed with 1ml of 100% EtOH by inversion, then mixture was centrifuged at maximum speed (13200 rpm) for 2 minutes, supernatant was discarded.
6. Pellet was resuspended in 0.4 ml of TE buffer (pH 8.0) and treated with 10µl of 10mg/ml RNase A. Mixture with RNase A was incubated at 37^{o}C for 5 minutes.
7. 1ml of 100% EtOH and 14µl of 3M sodium acetate were added to solution and then DNA was pelleted by centrifugation at 13200 rpm for 2 minutes. Supernatant was discarded and the pellet was air dried.
8. DNA pellet was dissolved in 50 µl of TE buffer (pH 8.0).

3.6 Recombinant Protansglutaminase Production by *Pichia pastoris*

Protransglutaminase production was performed either in air filtered, baffled Erlenmayer flasks or pilot scale bioreactors.

3.6.1 Solid Media Inoculation

Recombinant microbial protransglutaminase producing *Pichia pastoris* cells were inoculated on YPD agar containing 150 µg ml^{-1} Zeocin. The composition of YPD solid medium was given in Table 3.8. *P pastoris* cells were incubated at 30°C for 48-72 hours on solid media.

3.6.2 Precultivation

Pichia pastoris pPICZαA-pro-mtg_{extra} and *Pichia pastoris* pPICZαA-pro-mtg_{intra} cells, propagated on the solid medium were inoculated into BMGY (Buffered Glycerol-complex Medium) precultivation medium. The precultivation medium composition was given in Table 3.9. Cultivation of recombinant *Pichia pastoris* cells was carried out in orbital shakers (B. Braun, Certomat BS-1 and B. Braun, Certomat BS-T) with stirrer rate and temperature controller. For *Pichia pastoris* cells optimum growth condition is 30°C and 225 rpm. *Pichia pastoris* cells were grown for 20-24 hours to reach OD_{600} 2-6. Then, the recombinant cells were harvested by centrifugation at 2000xg and 4°C for 10 min. To initiate recombinant protein production, the pellet was resuspended in production medium and the initial OD_{600}=1 was adjusted.

Table 3.8 The composition of the YPD Agar

Compound	**Concantration, g L^{-1}**
Yeast Extract	10
Peptone	20
Glucose	20
Agar	20
Zeocin	1.5 ml

Table 3.9 The composition of the BMGY

Compound	Concentration, g L^{-1}
Yeast extract	10.0
Peptone	20.0
Potassium phosphate buffer pH 6.0	0.1 M
YNB	13.4
$(NH_4)SO_4$	10.0
Biotin	$4x10^{-4}$
Glycerol (ml)	10.0
Chloramphenicol* (ml)	1

* Chloramphenicol was prepared as 34 mg/ml stock solution in pure ethanol, kept in sterile dark bottle at -20^{o}C.

3.6.3 Protransglutaminase Production in Laboratory Scale Air Filtered Shake Bioreactors

Batch microbial protransglutaminase production was carried out in baffled and air filtered Erlenmayer flasks (250 ml volume) having working volume capacities of 50 ml. For recombinant protransglutaminase production, a defined medium which contains sorbitol together with methanol, basal salt solution and nitrogen sources was used. The composition of production medium was reported by Jungo *et al*. (2006) but in this study ammonium sulfate was used instead of ammonium chloride and the amount of ammonium sulfate was calculated such that Carbon/Nitrogen ratio and Methanol/Nitrogen ratio are 4.57 and 2.19 in the medium (Jungo *et al*, 2006) . Sorbitol was supplemented to the medium as optimized in thesis study by Açık (2009). The composition of the defined production medium and PTM1 (trace alt elements) is given in Table 3.10 and 3.11. Production was carried out batchwise at 30^{o}C and 225 rpm to 72 hours and 1% (v/v) methanol was added to medium at every 24 hours for induction. The aim of laboratory scale batch fermentation was to get a foresight before starting pilot scale bioreactor.

3.6.4 rpro-MTG Production in the Pilot Scale Bioreactor

The pilot scale 3.0 liter bioreactor (B Braun, Biostat CT2-2) having a working volume of 0.5-2.0 liter was used in pilot scale bioreactor experiments. The bioreactor vessel has 4 baffles, 2 sensor ports, 3 feed inlet port, sparger and exhaust filter. During production temperature, pH, dissolved oxygen, foam, stirring rate, feed inlet rate controls of the bioreactor were used to control the process. In order to increase oxygen transfer and provide homogeneity within the vessel, fermentor was stirred with two four bladed Rushton turbines. The bioreactor was equipped with a jacket, an external cooler and steam generator for sterilization and temperature control.

Oxygen need of microorganisms was supplied with a compressor and inlet air was enriched by a pure oxygen tube. Pure oxygen flow into bioreactor was regulated by mass flow controller. Feed solutions were transferred through inlet ports aseptically using peristaltic pumps.

Table 3.10 The composition of the defined production medium

Compound	**Concentration g L^{-1}**
Methanol (ml)	10
Sorbitol	30
Potassium phosphate buffer pH 6.0	0.1 M
$(NH_4)_2SO_4$	21.75
PTM1 (ml)	4.35
1M KiP pH 6.0 (ml)	100
$MgSO_4.7H_2O$	7.30
$CaSO_4.2H_2O$	0.57
Chloramphenicol	1

The schematic representation of the pilot scale fermentation and cultivation steps for production were given in Figure 3.1. BSM (basal salt

medium) was used in pilot scale fed-batch fermentation experiments. The composition of BSM is given in Table 3.12.

Table 3.11 The composition of PTM1 (trace salt solution)

Compound	**Concentration, g L^{-1}**
$CuSO_4.5H_2O$	6
NaI	0.08
$MnSO_4.H_2O$	3
$Na_2MoO_4.2H_2O$	0.2
H_3BO_3	0.02
$ZnCl_2$	20
$FeSO_4.7H_2O$	65
$CoCl_2$	0.5
H_2SO_4 (ml)	5
Biotin*	0.2

* Biotin stock solution was prepared as 0.2 g/L and stored in a light proof bottle at 4 ^oC.

Table 3.12 The composition of basal salt medium (BSM)

Compound	**Concentration g L^{-1}**
85% H_3PO_4 (ml)	26.7
$CaSO_4.2H_2O$	1.17
$MgSO_4.7H_2O$	14.9
KOH	4.13
K_2SO_4	18.2
Glycerol	40.0
Chloramphenicol (ml)	1
10% Antifoam (ml)	1
PTM1 (ml)	4.35

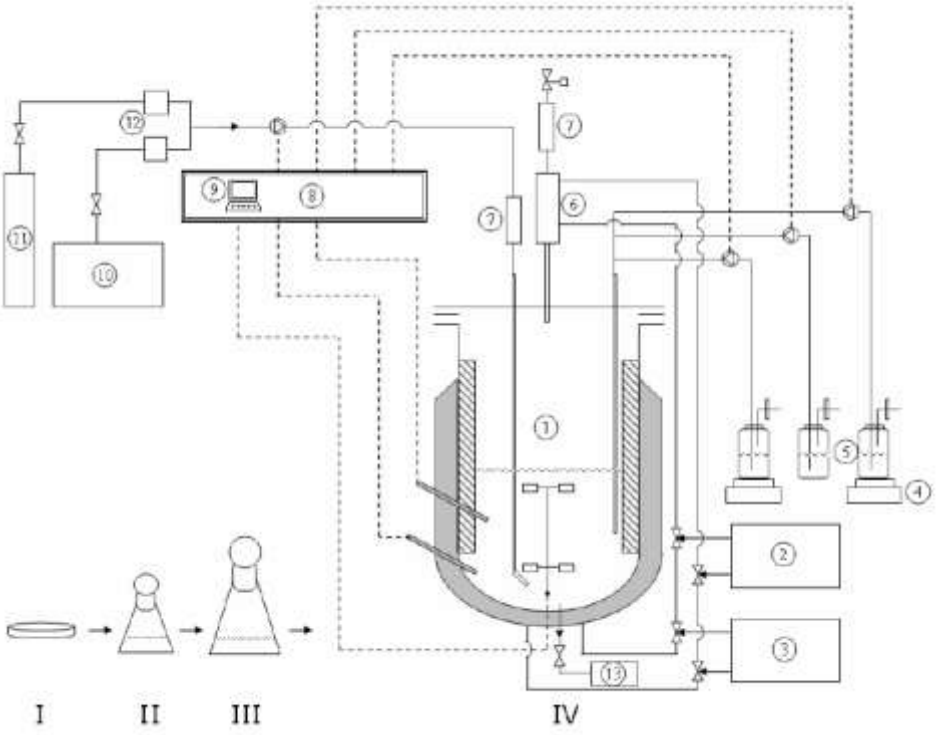


Figure 3.1 Scale up steps and the pilot scale bioreactor system. I: Solid medium inoculated from stock culture; II: 1st Precultivation medium, V = 10 ml; III: 2nd Precultivation medium, V= 50 ml; IV: Pilot scale bioreactor system, Vo=0.9 L, which is composed of (1) Bioreaction vessel, Biostat CT2-2 (2) 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) sampling bottle. (Çelik, 2008)

3.6.4.1 Bioreactor Operation Parameters

Controlling of the bioreactor operation parameters was so important to achieve high protein yield and increase productivity. PI controller of the bioreactor system was used to keep temperature constant at 30°C during fermentation. The pH of production medium was adjusted with ammonium hydroxide solution (25% NH_3OH) (Çelik *et al*., 2009). The pH of the fermentation medium was automatically controlled by using the PI controller

of the bioreactor with parameters as Xp=30% and T_I=30s and 10% base-pump-valve opening.

AOX1 promoter was induced by methanol and methanol utilization pathway requires oxygen. During recombinant protein production as *Pichia pastoris* cell density increases, oxygen consumption increases, as well. In pilot scale bioreactor experiments to prevent oxygen limitation, dissolved oxygen (DO) level was kept above 20% saturation in accordance with the literature. (Xie *et al*., 2005; Çelik *et al.*, 2009). When the DO was below 20%, inlet air was enriched with pure oxygen. PID controller of the bioreactor system controlled the dissolved oxygen level with regulating valve opening.

The agitation rate was kept constant at 900rpm to accomplish effective oxygen transfer, homogeneity through medium and to prevent aggregation. (Thorpe *et al*., 1999; Çelik *et al*., 2009)

High foam formation was prevented with the 0.01% (v/v) antifoam addition to the initial production medium. During fermentation a few drops of 10% antifoam was supplied to the fermentor when needed.

3.6.4.2 Fed-Batch Pilot Scale Bioreactor Operations

For recombinant microbial protransglutaminase production in *Pichia pastoris* modified version of standard protocol (Stratton *et al*., 1998, Çelik *et al.,* 2009) was performed. After precultivation step, cells were harvested and resuspended in BSM such that initial production medium OD_{600}=1. The four phased protocol was performed for rpro-MTG expression.

3.6.4.2.1 Glycerol Batch Phase (GB)

The aim of glycerol batch phase was to increase cell density up to OD_{600}=30. This phase was lasted approximately 14-17 h. Glycerol was used as a carbon source as it has higher specific growth rate on *Pichia pastoris* when compared to methanol. The initial glycerol concentration was 40 g L^{-1} in BSM.

Regarding to literature, higher glycerol concentration was inhibitory to growth (Cos *et al*., 2006).

3.6.4.2.2. Glycerol Fed-Batch Phase (GFB)

50% glycerol solution including 12 ml L^{-1} PTM1 was fed into the fermentor in harmony with pre-determined exponential feeding profile. Fed batch addition of glycerol prevents accumulation within the bioreactor while cell density increases. Additionally, yeast cells were prepared to another carbon source, methanol. Glycerol feeding was applied till the OD_{600}=90-100.

3.6.4.2.3 Methanol Transition Phase (MT)

3.6 ml h^{-1} L^{-1} methanol (100% methanol including 12 ml L^{-1}PMT1) was supplied to the fermentation medium for four hours (Invitrogen 2002). The aim of this step was to enhance cellular adaptation to methanol.

3.6.4.2.4 Methanol Fed-Batch Phase (MFB):

The main recombinant protein expression under Alcohol oxidase1 promoter was achieved in methanol fed-batch phase. At the beginning of the phase sorbitol was fed batch-wise into the fermentor such that initial sorbitol concentration was 50 g L^{-1}. MFB phase was carried out by feeding 100% methanol including 12 ml L^{-1} PTM1 to induce AOX1 Promoter. A predetermined exponential feeding profile for methanol was applied during this phase. The predetermined exponential feed rate was calculated by equation 3.2, specific growth rate assumed constant. C_{so}

$$F(t) = \frac{\mu_0 V_0 C_{xo}}{Cso\ Y_{X/S}} \exp(\mu_0 t) \qquad (3.2)$$

In the equation 3.2; μ_O (h^{-1}) is the desired specific growth rate, V_0 (L) is the initial volume, C_{x0} (g L^{-1}) is the initial cell concentration and $Y_{x/s}$ (g g^{-1}) is the cell yield on the substrate. Specific growth rate and the cell yield due to carbon source was given in Table 3.13.

The predetermined feeding profiles for glycerol and methanol were plotted in Figure 3.2 and Figure 3.4 according to the parameters given in Table 3.13.

Table 3.13 Specific growth rate and cell yield for Glycerol and Methanol

Parameter	Glycerol Fed Batch Feeding	Methanol Fed-Batch Feeding
μ_0 (h^{-1})	0.18	0.03**
$Y_{x/M}$ (g g^{-1})	0.5*	0.42**

* Cos *et al*., 2005, ** Jungo *et al.,* 2006

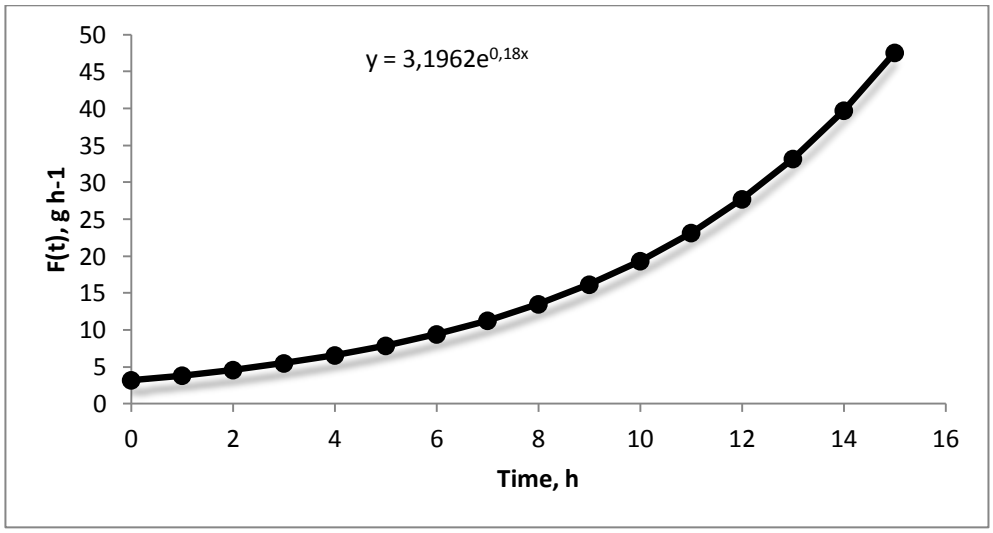


Figure 3.2: The predetermined feeding profile for glycerol according to specific growth rate was 0.18 h^{-1}

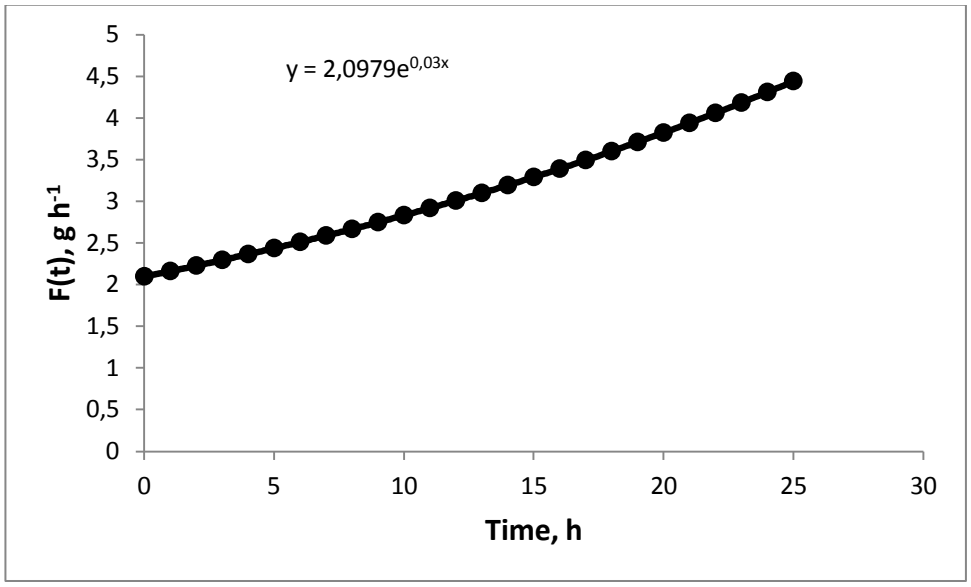


Figure 3.3: The predetermined feeding profile for methanol according to specific growth rate was 0.03 h^{-1}

3.7 Analyses

As laboratory scale shake experiments and pilot scale bioreactor experiment were carried out samples were taken with certain time intervals. Samples were centrifuged (10 min, 2000 g, 4^{o}C), then supernatant, the filtrate and the cell pellet were stored separately at -80^{o}C for analysis. The supernatants were used in dot-blot analysis, transglutaminase activity assay, SDS Gel Electrophoresis, protease activity assay and the filtrates were used in determination of sorbitol, methanol, organic acid concentrations while the cell pellet was used in AOX activity assay.

3.7.1 Cell Concentration

Cell concentration through fermentation was measured using a UV-Vis Spectrophotometer (Thermo Spectronic, Heλiosa) at 600 nm. Samples were diluted with dH_2O to read the OD values within the ranges of

Spectrophotometer (0.1-0.9). For convertion of optical density (OD) to the cell concentration,C_x (kg m^{-3}) equation 3.3 (Çelik *et al.*, 2009) was used.

$$C_x = 0.3 \times OD_{600} \times \text{Dilution Ratio} \quad (3.3)$$

3.7.2 Dot-Blot Analysis

Dot-Blot Analysis was performed to detect pro-MTG by using Monoclonal antibody to bacterial (microbial) transglutaminase (Zedira). Buffers and stock solutions used in Dot-Blot Analysis were given in Appendix A. Detailed procedure was given below.

1. The membrane (0.45 µm PVDF membrane) was pre-wetted with methanol and washed with dH_2O.
2. 5 µl of the supernatant was dropped on the membrane for three times.
3. Membrane was immersed in TBS-T Milk and incubated for 1 hour at room temperature with shaking.
4. The membrane was washed 3 times (shaking 15 min, 5min, 5min with fresh changes) with large volumes of TBS-T, at room temperature.
5. Primary antibody was diluted as 1:1000 (Monoclonal antibody to bacterial (microbial) transglutaminase, Zedira, Darmstadt, Germany)
6. The membrane was incubated in the diluted primary antibody for overnight at 4^oC, on shaker platform.
7. The membrane was washed 3 times (shaking 15 min, 5min, 5min with fresh changes) with large volumes of TBS-T, at room temperature.
8. Secondary antibody was diluted in TBS-T as 1:10 000 (antimouse IgG horseradish peroxidase-linked whole antibody, Amersham Bioscience, Uppsala, Sweden)
9. The membrane was incubated in the diluted secondary antibody for 1 hour at room temperature with shaking.

10. The membrane washed three times (10 min/wash) with fresh changes of large volumes of TBS-T buffer at room temperature.
11. Recombinant protransglutaminase was visualized by using the Diaminobenzidine Chromogen Kit-Substrate-Chromogen Kit (S10 HRP, BioMeda, ABD)

3.7.3 Transglutaminase Activity Assay

An acyl transfer reaction between the γ-carboxyamide group of peptide bound glutamine residue and a variety of primary amines was catalyzed by transglutaminase enzymes.

In the activity assay Z-Glutaminylglycine (Z-Gln-Gly) was used as the amine acceptor substrate and hydroxylamine was used as the amino donor substrate. Microbial transglutaminase catalyzes isopeptide bond formation between these substrates and Z-glutamyl-hydroxamate-glycine was being formed. Z-glutamyl-hydroxamate-glycine develops a colored complex with iron (III), this colored complex was detected at 525nm. Detailed activity procedure was given below:

3.7.3.1 Activation of Protransglutaminase

Protransglutaminase was expressed extracellularly by *Pichia pastoris* pPICZαA-pro-mtg_{extra}. Though, pro enzyme didn't catalyze any reaction, to start activity assay, the pro sequence of the enzyme had to be cleaved first. For this purpose, pro sequence of the enzyme was digested with Dispase I. Protransglutaminase activation mixture was given in Table 3.14. For digestion of pro sequence, the activation mixture was incubated at 37^{o}C for 30 minutes.

3.7.3.2 Reaction Catalyzed by Transglutaminase

Activated transglutaminase catalyzes isopeptide bond formation between substrates. The composition of transglutaminase reaction cocktail, stop solution were given in Table 3.15 and 3.16. Firstly reaction cocktail was

equilibrated to 37^{o}C, and then transglutaminase solution was added into reaction cocktail and mixed by inversion. Mixture was incubated at 37^{o}C for exactly 10 minutes. Stop solution was added into the mixture to cease transglutaminase activity. Colored mixture was measured using a UV-Vis Spectrophotometer (Thermo Spectronic, Heλiosa) at 525 nm. Negative controls were done with the fermentation products of *Pichia pastoris* pPICZαA.

Table 3.14: The composition of the protransglutaminase activation mixture

Components	Amounts
Protransglutaminase solution (supernatant)	160 µl
Dispase (0.2 µg ml^{-1})	20 µl
Tris-HCl Buffer pH 8.0 (350mM)	20 µl

Table 3.15: The composition of transglutaminase reaction coctail

Compound	Amount
Z-Gln-Gly	101 mg
Tris Buffer ph 6.0 (1 M)	2 ml
200mM Hydroxylamine with 20 mM Glutathione	5 ml
Sodium hydroxide solution (0.1 M)	3 ml

Table 3.16: The composition of the stop solution

Compound	Amount
Hydrochloric Acid Solution (3 M)	1 volume
12 % (v/v) Trichloroacetic Acid Solution	1 volume
5 % (w/v) Ferric Chloride Solution	1 volume

3.7.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The aim of SDS-PAGE was to separate proteins according to their size. First, proteins were denatured with SDS and due to negative charged sulfate ions proteins were negatively charged. Therefore, proteins migrate towards the positive pole when placed in an electrical field. Polyacrylamide is a polymer of acrylamide monomers and different sized proteins are allowed to move at different rates depending on the structure of polyacrylamide gels.

SDS-PAGE analysis was carried out as described by Laemmli (1970). The protein samples and the buffer were mixed in 2:1 ratio and proteins were denatured at 95^{o}C for 4 minutes. After denaturation, mixtures were put into ice immediately. 15 µl of the samples were loaded into gel wells together with 3µL of a dual color prestained protein MW marker (PageRuler Prestained Protein Ladder, Appendix E).

Protein samples were run simultaneously under 40 mA electrical filed for 40 to 60 minutes. The buffers and solutions used in SDS-PAGE was given in APPENDİX A. The SDS-PAGE procedure was given below.

3.7.4.1 Preparing the Gel

1. Glasses were cleaned and gel cast was set-up according to manufacturers' instructions.
2. Resolving and stacking solutions were prepared separately in erlenmeyer flasks using the values given in Appendix A. NNN'N'-Tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium persulfate (APS) addition starts polymerization of gel mixtures so, these were added after others.
3. TEMED and APS were added into Resolving Gel. Solution was mixed by inversion and pipetted into gel cast quickly. Sufficient place was leaved

for the stacking gel. Isopropanol was poured into the left space fully to form smooth surface.

4. Gel was left to polymerize for at least 45 minutes.
5. Isopropanol was discarded and gel was washed 3 times by dH_2O. The upper part of the gel cast was made dry with filter paper. After TEMED and APS addition, stacking gel was poured into gel cast. Teflon combs were inserted into gel. Gel was polymerized at least 20 minutes.
6. Comb was removed and gel was washed 3 times by dH_2O. Gels were dired as much as possible with filter paper. The gels can be used immediately or kept at 4^0C covered with wet towels for 1 week.

3.7.4.2 Sample Preparation and Electrophoresis

7. Samples and sample buffer were mixed in 2:1 ratio and proteins were denatured at 95^0C for 4 minutes. After denaturation samples were taken onto ice immediately and kept on ice for 5 minutes.
8. Gels were placed in electrophoresis apparatus and reservoir was filled with running buffer. (1x SDS)
9. 15 µL of each samples and 3 µL prestained protein MW marker were loaded into the wells and running of protein samples was carried out under 40 mA electrical field for 40 to 60 minutes.

3.7.4.3 Staining the SDS-PAGE Gels

After electrophoresis finished the gels were taken out the glasses. Silver staining procedure was performed with using silver salts for gel staining. Procedure was given in Table 3.17.

Table 3.17 Silver Staining Procedure

	Step	Solution	Treatment period	Implementations
1	Fixing	Fixer	> 1 h	Overnight incubation is acceptable
2	Washing	50 % Ethanol	3 x 20 sec	Fresh
3	Pretreatment	Pretreatment Solution	1 min	Fresh
4	Rinse	Distilled water	3 x 20 sec	
5	Impregnate	Silver nitrate solution	20 min	
6	Rinse	Distilled water	2 x 20 sec	
7	Developing	Developing Solution	Time was settled according to the color development	After color formation starts water should be added to slow down the reaction.
8	Washing	Distilled water	2 x 20 min	
9	Stop	Stop solution	-	The gels can be stored in this solution for a long time.

3.7.5 Methanol and Sorbitol Concentrations

Methanol and sorbitol concentrations were measure by reversed phase HPLC (Waters HPLC, Alliance 2695, Milford, MA) on Capital Optimal ODS- 5µm column (Capital HPLC, West Lothian, UK). 5mM H_2SO_4 was used as the mobile phase at a flow rate of 0.5 ml min^{-1} and sorbitol and methanol were detected with refractive index detector (Waters 2414) at 30^0C. In reversed phase HPLC system, samples' methanol and sorbitol concentrations were determined from the chromatogram in accordance with the chromatogram of the standard solutions. Standard methanol and sorbitol

solutions were prepared with mobile phase in different concentrations. All the samples (supernatants) were filtrated with 0.45 µm cellulose acetate filters (Sartorius, Gottingen, Germany) and then diluted 100 fold before loading to the HPLC system. The analysis was carried out according to the optimized conditions (Çelik *et al*., 2009) specified given in Table 3.18. Calibration curves for sorbitol and methanol were given in Appendix F and Appendix G, respectively.

3.7.6 Organic Acid Concentrations

Organic acid concentration measurement was based on reversed phase HPLC and analysis was performed with organic acid analysis system (Waters, HPLC, Alliance 2696). Organic acid concentrations of the samples were calculated according to the standard solutions chromatogram. Supernatants were filtered through 0.45 µm filters and diluted with the mobile phase. 3.12 % (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4 solution (İleri and Çalık, 2006) was used as mobile phase in organic acid analysis. Calibration curves of organic acids were given in Appendix H. The organic acid analysis conditions were specified in Table 3.19.

Table 3.18 HPLC conditions for sorbitol and methanol analysis (Çelik *et al*., 2009)

Column	**Capital Optimal ODS, 5 µm**
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Mobile phase and flow rate	5 mM H_2SO_4, 0.5 ml min^{-1}
Column temperature	30 ^oC
Detector	Waters 2414 Refractive Index detector, 214 nm
Detector temperature	30 ^oC
Injection volume	5 µl
Analysis period	10 min
Space time	5 min

Table 3.19 HPLC conditions for organic acid analysis (İleri and Çalık, 2006)

Column	**Capital Optimal ODS, 5 µm**
Column dimensions	4.6 x 250 mm
System	Reversed phase chromatography
Mobile phase	3.12 % (w/v) NaH_2PO_4 and 0.62x10^{-3} % (v/v) H_3PO_4
Mobil phase flow rate	0.8 ml min^{-1}
Column temperature	30 ^oC
Detector	Waters 2487 Dual absorbance detector
Detector wavelength	210 nm
Detector temperature	30 ^oC
Injection volume	5 µl
Analysis period	15 min
Space time	5 min

3.7.7 Protease Activity Assay

Proteolytic activity of the proteases was measured by hydrolysis of casein. To determine acidic, basic and neutral protease activities, three different buffer solutions were used. Hammerstein casein solution (0.5 % w/v) was prepared in either 0.05 M borate buffer pH 10, 0.05 M sodium acetate buffer pH 5.0 or 0.05 M sodium phosphate buffer pH 7 (Appendix A).0. 2 ml of the casein solution were added to 1 ml of diluted supernatant and proteases catalyze the reaction at 30 ^oC, 200rpm for 20 minutes. 10% (w/v) trichloroacetic acid (TCA) solution was added to the mixture to stop the reaction. Mixture was kept on ice for 20 min and centrifuged at 10500 rpm for 10 min at 4 ^oC. Then mixtures were kept at room temperature for 5 minutes. 1 ml of the mixture supernatant was taken into quartz cuvettes and the absorbance of the final mixture was measured at 275 nm with UV-Vis Spectrophotometer. Dilution must be done if the absorbance value over 0.6.

One unit of protease activity was defined as the amount of enzyme, which releases 4 nmole tyrosine per minute (Moon and Parulekar, 1991). The

equation which was used for calculating the activity of proteases (U ml^{-1}) was given in Equation 3.3 (Çalık, 1998).

$$A = \left(\frac{\text{Absorbance}}{0.8x1/\mu mol.cm^{-3}}\right)\left(\frac{1\ U}{4\ nmolmin}\right)\left(\frac{1}{20\ min}\right)\left(\frac{1000\ nmol}{1\ \mu mol}\right)\left(\begin{matrix}\text{Dilution}\\ \text{Ratio}\end{matrix}\right) \quad (3.3)$$

3.7.8 AOX Activity Assay

The cell pellet of 1 ml fermentation medium was used to determine AOX activity of the *Pichia pastoris* cells. AOX activity assay was carried out after cell disruption as the native AOX enzyme was intracellular.

3.7.8.1 Disruption of Yeast Cells

In order to get intracellular medium, yeast cells were disrupted with using yeast lysis buffer (Appendix A). For this purpose, 500 µl of yeast lysis buffer added to cell pellet and cells were vortexed three times for 20 seconds and after each mixing they were kept on ice for 30 seconds. 300 µg of glass beads were added into mixture, these mixtures were vortexed 20 seconds for three times and after each cycle they were kept on ice for 30 seconds. Finally cells were centrifuged at 3000rpm for 2min at 4^0C and supernatant was taken for second centrifugation at 12500 rpm for 5 min. After second centrifugation supernatant was taken for AOX activity assay.

3.8.8.2 AOX Activity

A consecutive reaction including alcohol oxidase (AOX) and horse radish peroxidase (HRP) was used to observe the oxidation of methanol to formaldehyde by AOX. At the first step of the reaction methanol was oxidized to formaldehyde and H_2O_2 by AOX.

$$\text{Methanol} + O_2 \xrightarrow{\text{AOX}} \text{Formaldehyde} + H_2O_2$$

$$2H_2O_2 + \ PSA + 4 - AAP \xrightarrow{HRP} Quinoneimine\ dye + 3\ H_2O + NaHNO_3$$

The colorimetric method based on the combination of phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) was used to determine the concentration of H_2O_2 produced by AOX. In this second reaction, two moles of H_2O_2 react with one mole of PSA and one mole of 4-AAP, yielding three moles of water, one mole of hydrogenosulfate and one mole of a quinoneimine dye.

The quinoneimin dye has a characteristic magenta color with maximum absorption around 500 nm. The AOX activity was measured by monitoring the increase in absorbance at 490 nm using UV-Vis spectrophotometer. Absorbance value was directly proportional to the rate of H_2O_2 production and the rate of methanol consumption.

All activity measurements were performed at 25^0C using a standard assay reaction mixture, including 0.4 mM 4-AAP, 25mM PSA and 2 U ml^{-1} HRP in 0.1 M phosphate buffer, at pH 7.0. One unit of AOX activity (U) was defined as the amount of enzyme, which causes the formation of number of µmol of H_2O_2 per minute at 25^0C. (Azevedo *et al*., 2004)

The procedure for the analysis was as follows; 3 ml of the standard assay reaction mixture was put into a cuvette and 30 µl of HRP, 375 µl of methanol and 75 µl of sample were added into mixture and they were mixed. The increase in absorbance at 500 nm was monitored for 4 minutes and values were recorded every 30 sec of time intervals. For conversion of absorbance value to specific AOX activity value the equation 3.4 was used. Equation 3.4 was formed according to the calibration curve given in Appendix I.

$$C_{AOX}\left(\frac{U}{gCDW}\right) = 15.67\left(\frac{UmL^{-1}}{Absorbance}\right) * OD_{500} * \frac{1}{C_X} \qquad (3.4)$$

CHAPTER 4

RESULTS AND DISCUSSION

In this study, novel expression systems for the production of extracellular and intracellular microbial protransglutaminase (MTG; EC 2.3.2.13) by *Pichia pastoris* were designed and investigated. In the first part of the study, the recombinant microbial protransglutaminase (pro-MTG) producing *P. pastoris* cells were developed by genetic engineering techniques. In this context, pro-*mtg* genes were inserted into the pPICZα-A expression vectors, separately and extracellular and intracellular plasmids were constructed. Then, plasmids were transferred into *P. pastoris* X_{33} cells and pro-*mtg* genes were integrated into the genome of the yeasts, separately. The transformants were selected via antibiotic test and then verification was carried out by PCR. In the second part of the study, recombinant cells with the highest pro-MTG expression capacity were selected via laboratory scale air filtered shake bioreactors. Dot-blot and SDS-PAGE analyses were performed for selection of the best pro-MTG producing recombinant strain. In the final step of the study, using the data from the literature and other studies, a pilot scale bioreactor experiment was designed and performed; the bioprocess characteristics were explored.

4.1 Development of the Recombinant Microbial Protransglutaminase Producing *Pichia pastoris* Strains

For the development of microbial protransglutaminase producing recombinant microorganisms, the extracellular and intracellular recombinant *Pichia pastoris* expression systems were constructed. Construction of recombinant microbial protransglutaminase producing *P. pastoris* strains was performed mainly in three steps. Firstly, pPICZαA vector DNA and pDJ1-3 plasmid carrying the *pro-mtg* gene were isolated after they had been propagated in *E. coli*. The *pro-mtg* genes were amplified depending on the two different approaches. Extracellular gene (*pro-mtg*$_{extra}$) and intracellular gene (*pro-mtg*$_{intra}$) were amplified using pDJ1-3 plasmid as a template DNA. Afterwards, *pro-mtg* genes were cloned into pPICZα-A expression vectors, separately. Both intracellular pPICZαA*::pro-mtg*$_{intra}$ and extracellular pPICZαA*::pro-mtg*$_{extra}$ constructs were verified by PCR and DNA sequencing. pPICZα-A vector carries AOX1 promoter region to be induced by methanol; α-factor signal sequence to secrete the recombinant proteins into the extracellular media and Zeocin resistance gene to select the transformants. Finally, pPICZαA*::pro-mtg*$_{intra}$ and pPICZαA*::pro-mtg*$_{extra}$ constructs were transferred into *P. pastoris* X_{33} cells and *pro-mtg*$_{extra}$ and *pro-mtg*$_{intra}$ genes were integrated into the *P. pastoris* genomic DNA. Expression of active pro-MTG was achieved by transformants and it was verified by evaluating MTG activities and Dot-Blotting. The research outlines are schematically summarized in Figure 4.1 and Figure 4.2.

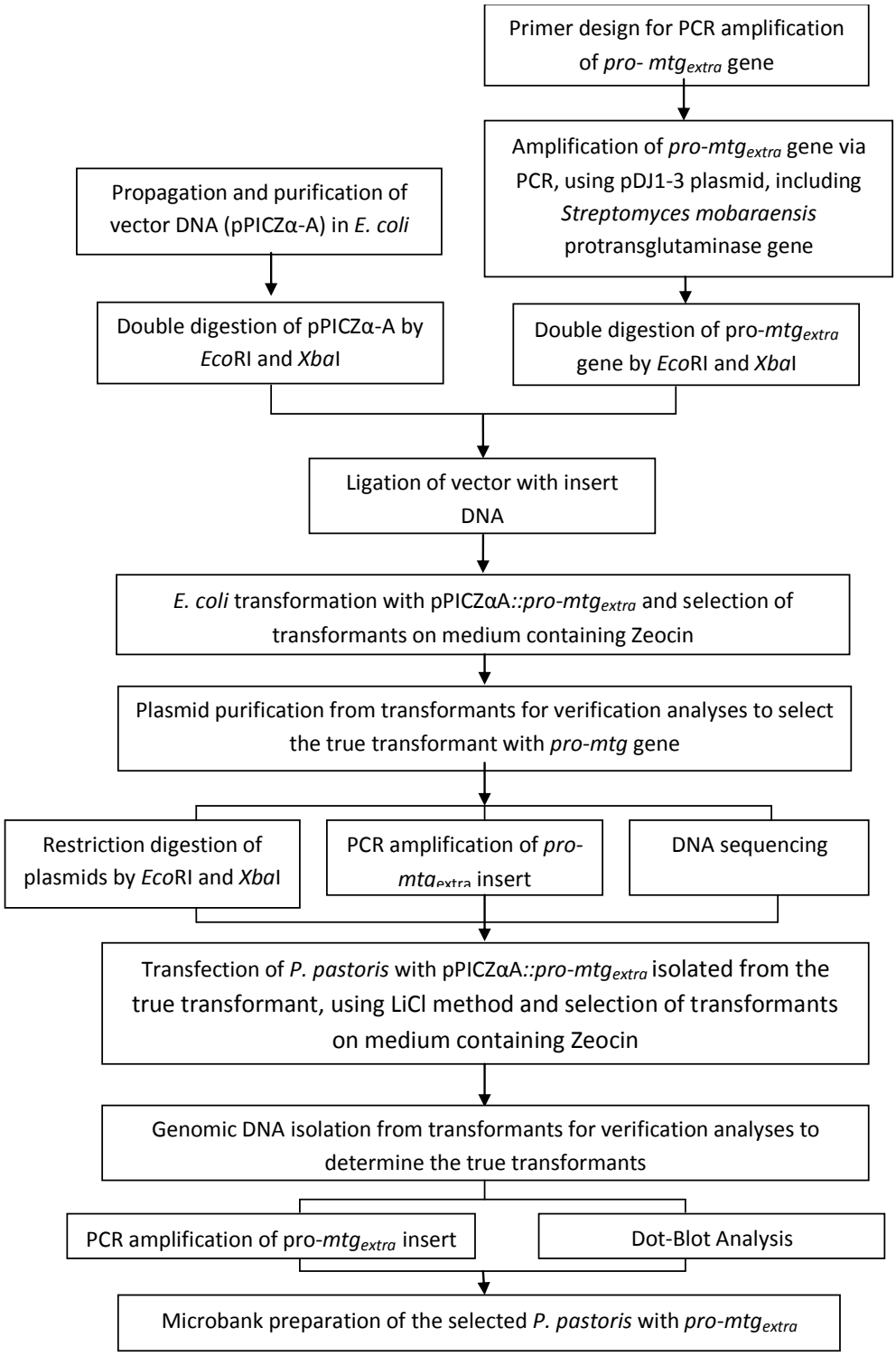


Figure 4.1 Flowchart of the research plan, for the development of recombinant extracellular pro-MTG producing *Pichia pastoris.*

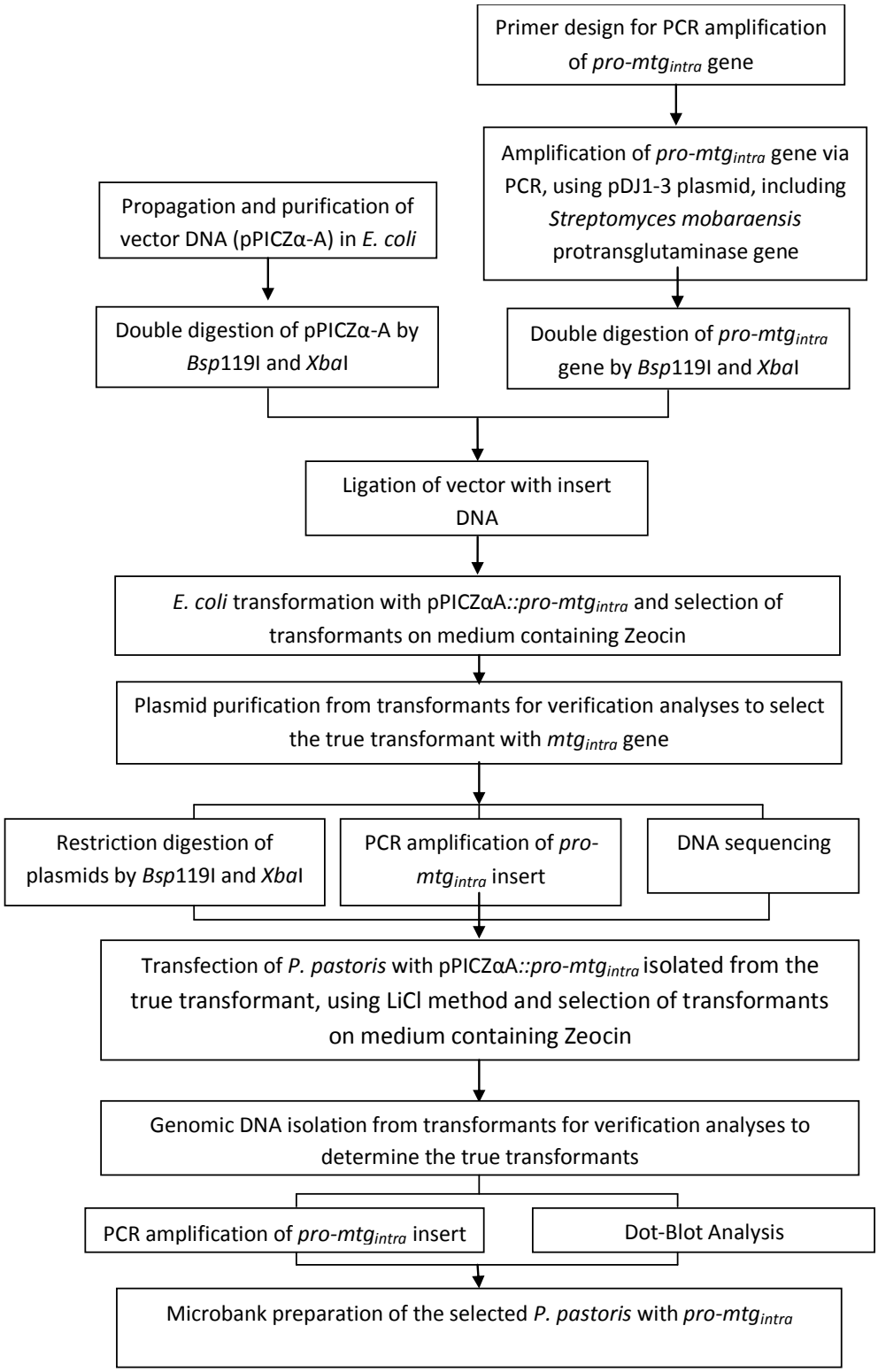


Figure 4.2 Flowchart of the research plan, for the development of recombinant intracellular pro-MTG producing *Pichia pastoris*.

4.1.1 Propagation and Purification of pPICZα-A

Lyophilized powder of pPICZα-A vector DNA was dissolved in sterile water and the concentration was adjusted to 10 ng/µl. Competent *E. coli* Top10 cells were transformed via vector DNA solution and *E. coli* pPICZαA strain was obtained. After incubation of transformants on a selective media, microbanks were prepared using a young single colony. The pPICZα-A expression vector used in this study was isolated from recombinant *E. coli* pPICZαA strain. The vector is composed of 3593 bp. In order to visualize circular and linearized vector, agarose gel electrophopresis was applied (Figure 4.3). Vector DNA was linearized by *Xba*I digestion. As the circular vector has more compact structure, it runs faster than linearized DNA and its position doesn't give exact information about the length of the plasmid.

4.1.2 Primer design for Amplification of mtg_{intra} and mtg_{extra} Genes

pDJ1-3 plasmid including pro-*mtg* gene of *Streptomyces mobaraensis* (Marx *et al*., 2007) was obtained as lyophilized powder, 6µg of pDJ1-3 was dissolved in 500 µl sterile water and the final DNA concentration was adjusted to 12 ng/µl. 5µl of the pDJ1-3 solution was used for *E. coli* transformation. pDJ1-3 was propagated within *E. coli* and then it was isolated to be used as a template for amplification of *pro-*mtg_{extra} and *pro-*mtg_{intra} genes.

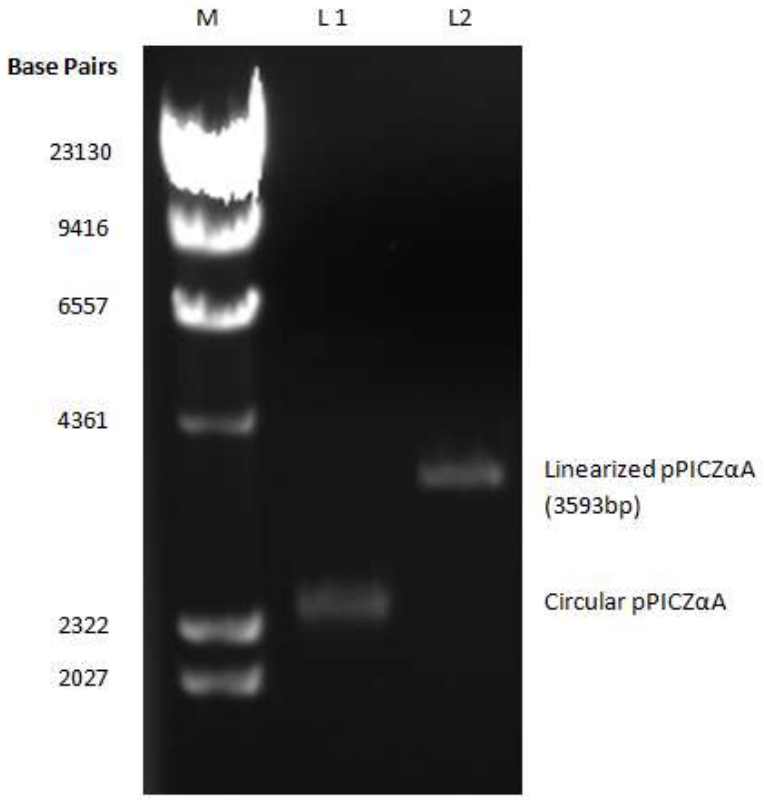


Figure 4.3 Agarose gel electrophoresis image of the circular and linearized pPICZα-A expression vector. M: Lambda DNA/HindIII Marker, Lane 1: circular pPICZα-A, Lane 2: linearized pPICZα-A.

Two different forward (MTG_{extra}-Forward and MTG_{intra}-Forward) primers were designed according to the sequence of the pro-*mtg* gene from *Streptomyces mobaraensis* for the amplification of extracellular pro-mtg_{extra} and intracellular pro-mtg_{intra} genes. *Eco*RI restriction site (6bp) and sequence of start codon (3bp) were combined to the 5' end of *pro-*mtg_{extra} sequence while *Bsp*119I restriction site (6bp) and sequence of start codon (3bp) were combined to the 5' end of *pro-*mtg_{intra} sequence. For intracellular construct design, *Bsp*119I restriction site was selected in order to remove α-Factor signal sequence from pPICZα-A expression vector map during the restriction digestion reaction. Both extracellular and

intracellular designed primers contain start codon, ATG, which is more likely to be preferred by yeasts to facilitate efficient transcription. The reverse primer (MTG-Reverse) was designed as complementary to the 3' end of the *pro-mtg*; stop codon sequence and *Xba*I restriction site were incorporated into 3' end of the *pro-mtg*$_{extra}$ and *pro-mtg*$_{intra}$ genes. In primer design, thermodynamic properties, dimer and loop formation, melting temperatures (T_m) of primers were analyzed via OligoAnalyzer program. The sequences of the primers were optimized considering these characteristics. Primers used for amplification of *pro-mtg*$_{extra}$ and *pro-mtg*$_{intra}$ genes were given in Table 3.2 in the previous chapter. Properties of the primers used in this study are given in Appendix D.

4.1.3 Amplification of *pro-mtg*$_{extra}$ and *pro-mtg*$_{intra}$ Genes by PCR

The important points in designing successful PCR program for specific amplification of a target gene are the annealing temperature and time of elongation which depend on the sequence of the target gene. As too low annealing temperatures lead nonspecific binding and consequently nonspecific amplification, optimum annealing temperature was determined as 60^0C. Under the same conditions, different DNA Polymerases were tested to obtain specific PCR products (Figure 4.4). *Taq* DNA polymerase has higher amplification efficiency than *Pfu* DNA polymerase as it can be seen in Figure 4.4 and Taq DNA polymerase was used in PCR to amplify *pro-mtg*$_{extra}$ and *pro-mtg*$_{intra}$. The time required for elongation was calculated by the formula of 1000 bp= 1min according to the *Taq* DNA Polymerase Manual (Fermentas) and 1.5 min was determined sufficient for elongation of 1131 bp length of the gene. Thermostable DNA polymerase (*Taq* DNA polymerase) used in the study has optimal temperature value as 72^0C and so, gene extension was performed

at 72^{0}C. The optimized parameters of the polymerase chain reaction and mixture composition were given in the Section 3.5.6.

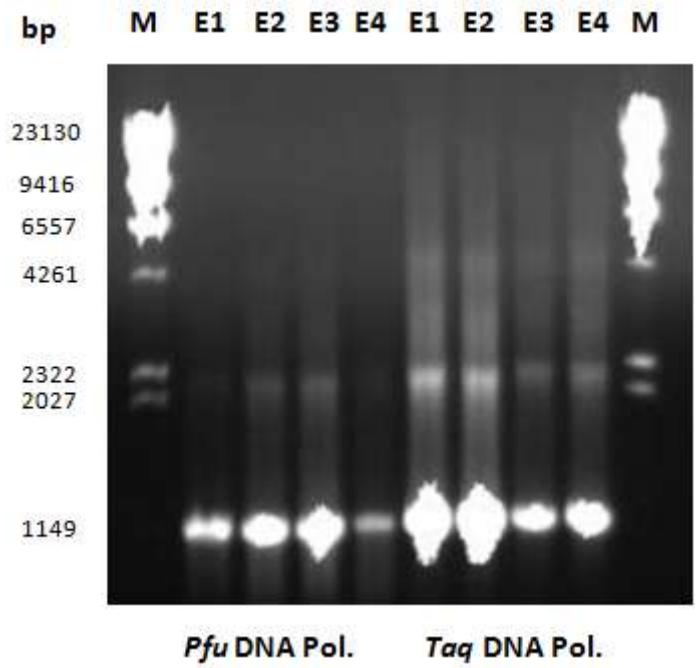


Figure 4.4 Agarose gel electrophoresis image of amplified *pro-mtg*$_{extra}$ genes with PCR by using different DNA polymerases. The first four PCR products (left side) were amplified by *Pfu* DNA polymerase; other four ones (right side) were amplified by *Taq* DNA polymerase. M: Lambda DNA/HindIII Marker

pro-mtg$_{extra}$ gene with *Eco*RI and *Xba*I restriction recognition sites (expected final length of the PCR product= 1149 bp) was amplified after synthesis of MTG$_{extra}$-Forward and MTG-Reverse primers. For intracellular construct, *Bsp*119I and *Xba*I restriction sites were selected and integrated into 5' and 3' end of the *pro-mtg*$_{intra}$ gene, respectively. *Bsp*119I integration aimed to remove α-Factor signal sequence from pPICZα-A expression map during restriction digestion reaction. 1µl of plasmid DNA (pDJ1-3, including *pro-mtg* gene) isolated from *E. coli* pDJ1-3 was used as template to amplify *pro-mtg*$_{extra}$

and *pro-mtg*$_{intra}$ genes. The amplification reactions were carried out at as described in Section 3.5.6. The amplified PCR products (*pro-mtg*$_{extra}$ and *pro-mtg*$_{intra}$) were purified with PCR purification kit (Fermentas) and they were run on agarose gel to visualize (Figure 4.5).

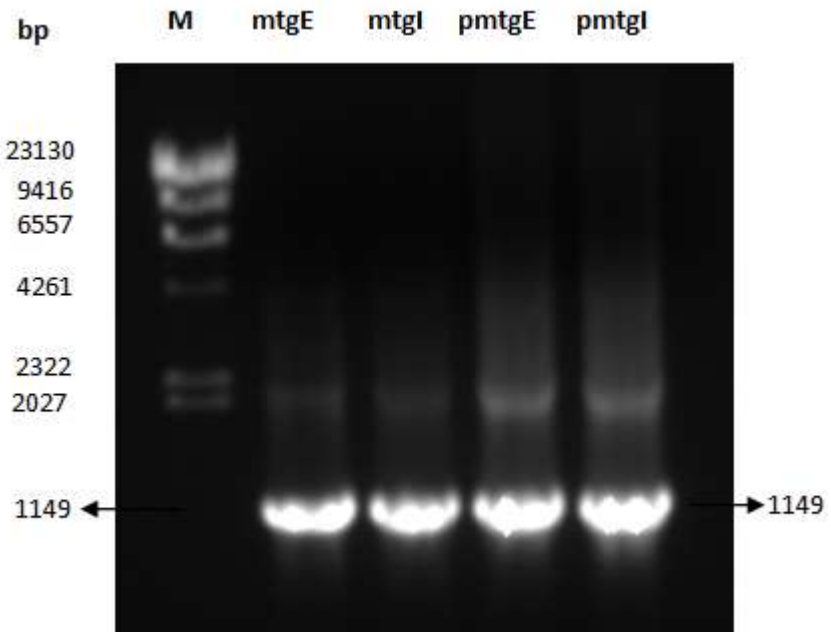


Figure 4.5 Agarose gel electrophoresis image of amplified *pro-mtg* genes (expected size: 1149 bp). M: Lambda DNA/HindIII Marker; mtgE: extracellular PCR product (*pro-mtg*$_{extra}$); mtgI: intracellular PCR product (*pro-mtg*$_{intra}$); pmtgE: purified extracellular PCR product (*pro-mtg*$_{extra}$); pmtgI: purified intracellular PCR product (*pro-mtg*$_{intra}$)

4.1.4 Restriction Enzyme Digestion of *pro-mtg* genes and pPICZα-A

The restriction digestion enzymes used in this study were determined with considering the following criteria:

- *Eco*RI and *Xba*I restriction enzyme recognition sites are normally found in the multiple cloning site of pPICZα-A, so there wouldn't be any extra nucleotides in the sequence.
- *Bsp*119I and *Xba*I restriction sites were selected for intracellular *pro-mtg* expression as *Bsp*119I remove the α-Factor signal sequence from the pPICZα-A vector without any need to add nucleotide in the sequence.
- All restriction enzymes have no restriction sites within the sequence of *pro-mtg* gene as verified by restriction mapper version 3.0 (http://www.restrictionmapper.org/).
- Selected restriction enzymes are readily available and relatively inexpensive enzymes.

pro-mtg genes were amplified via PCR and then purified by PCR Purification Kit (Fermentas) while pPICZα-A vector DNA was propagated within *E. coli* and isolated by Alkaline Lysis Method. Then *pro-mtg* genes and pPICZα-A vector were single and double digested by restriction enzymes. Digestion reactions were performed at 37^{0}C for 2 hours and then heat treatment was applied to reaction mixtures for thermal inactivation of restriction enzymes. For this purpose, extracellular digestion mixtures were kept at 65^{0}C for 20 min while intracellular digestion mixtures were kept at 80^{0}C for 20 min as *Bsp*119I has higher denaturation temperature. After digestion reactions were performed, mixtures were run on agarose gel electrophoresis to determine the accomplishment of the digestion (Figure 4.6, Figure 4.7).

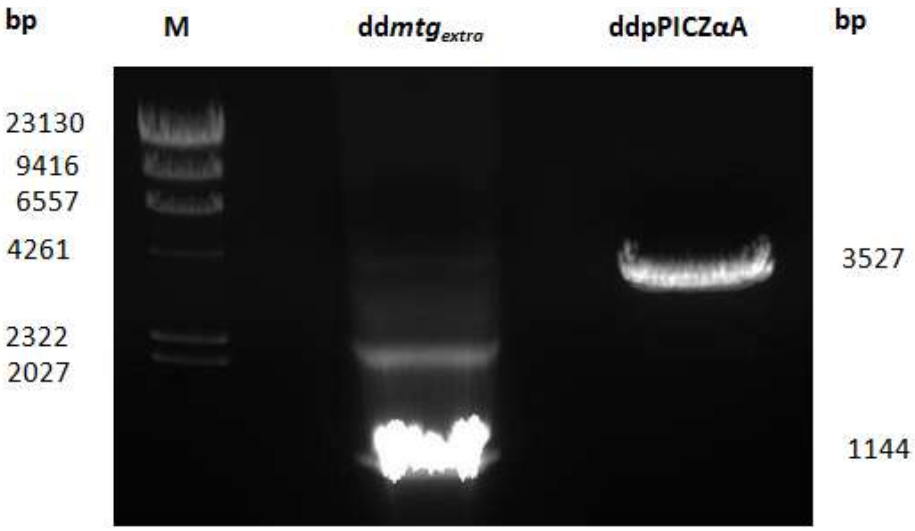


Figure 4.6: AGE images of double digested *pro-mtg*$_{extra}$ gene and pPICZα-A vector DNA by *Eco*RI and *Xba*I. M: Lambda DNA/HindIII Marker; ddmtg$_{extra}$: double digested *pro-mtg*$_{extra}$ gene (expected size: 1144bp); ddpPICZαA: double digested pPICZα-A (expected size: 3527bp)

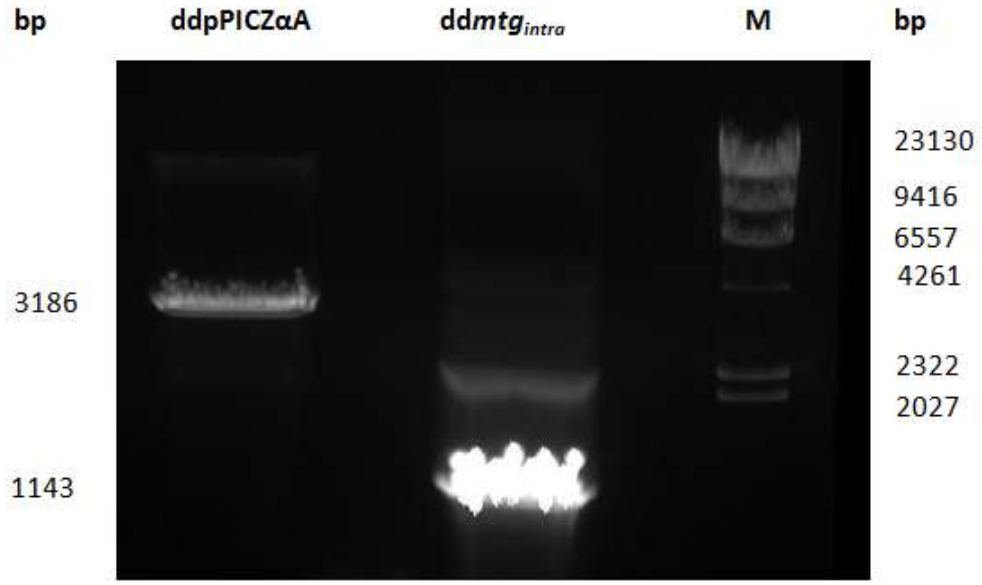


Figure 4.7 AGE images of double digested *pro-mtgi*$_{intra}$ gene and pPICZα-A vector DNA by *Bsp*119I and *Xba*I. ddpPICZαA: double digested pPICZα-A (expected size: 3186 bp); ddmtg$_{intra}$: double digested *pro-mtg*$_{intra}$ gene (expected size:1143bp); M: Lambda DNA/HindIII Marker

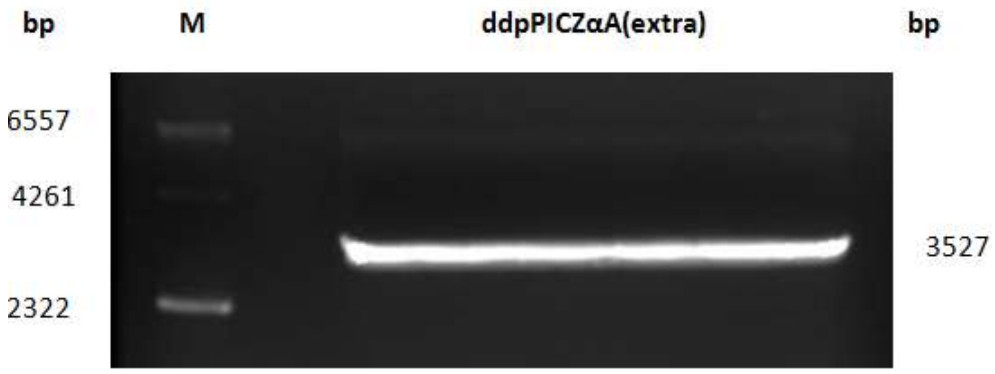


Figure 4.8: AGE images of the double digested pPICZα-A vector DNA by *Eco*RI and *Xba*I (expected size: 3527bp). Bulk quantities of ddpPICZαA was digested and run on the gel before gel elution. M: Lambda DNA/HindIII Marker

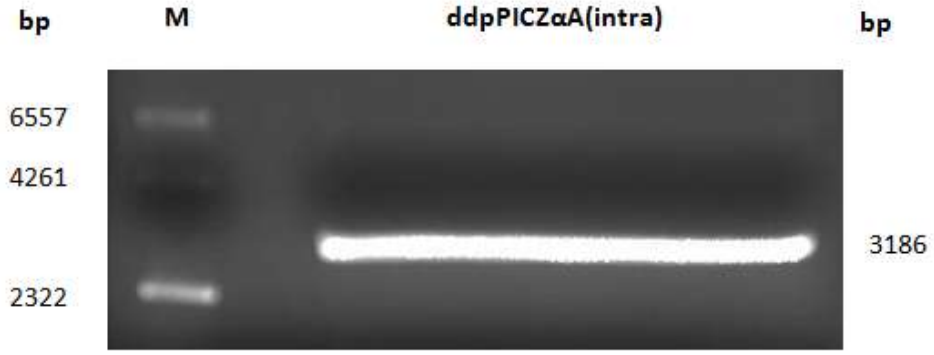


Figure 4.9 AGE images of the double digested pPICZα-A vector DNA by *Bsp*119I and *Xba*I (expected size: 3186 bp). Bulk quantities of ddpPICZαA was digested and run on the gel before gel elution. M: Lambda DNA/HindIII Marker

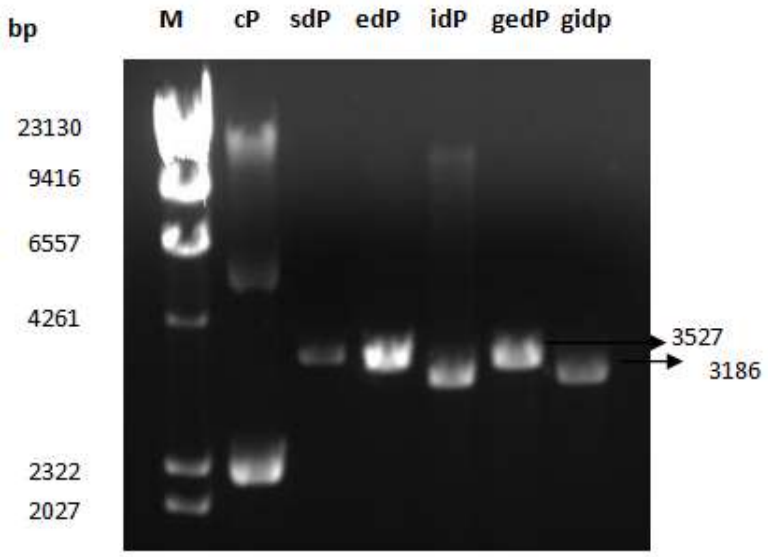


Figure 4.10: AGE image the gel eluted and digested pPICZα-A. M: Lambda DNA/HindIII Marker, cP: circular pPICZαA; sdP: single digested pPICZαA (expected size: 3593bp); edp: double digested pPICZαA for extracellular construct (expeted size: 3527bp); idp: double digested pPICZαA for intracellular construct (expected size: 3186); gedp: gel eluted double digested pPICZαA for extracellular construct; gidp: gel eluted double digested pPICZαA for intracellular construct

The double digestion of *pro-mtg* genes can't be visualized obviously since the DNA fragment digested by restriction enzymes is only a couple of nucleotides.

Before ligation reaction, bulk quantities of the double digested vector DNA along with the double digested insert genes (*pro-mtg*$_{intra}$, *pro-mtg*$_{extra}$) were purified to eliminate the reaction reagents and other DNA fragments resulted from the digestion reaction. For this purpose vector DNA and *pro-mtg* inserts were run on agarose gel and then interested bands were ceased under UV illumination (Figure 4.8 and Figure 4.9). Gel elution was performed to extract pure and properly digested vector DNA and *pro-mtg* genes. Final DNA concentrations were determined by spectrophotometric measurement.

4.1.5 Ligation Reaction

Ligation reactions were performed by mixing the double digested insert DNA with double digested vector DNA as described in Section 3.5.10. Reactions were performed at insert:vector molar ratios of 3:1, 2:1 and 1:1 and ligation was accomplished both for intracellular and extracellular constructs at molar ratios of 3:1 and 2:1 while 3:1 had higher efficiency. The ligation mixture compositions are given in Table 4.1.

Table 4.1 Composition of the ligation mixtures with different insert:vector molar ratios.

Components	**Amounts (3:1)**	**Amounts (2:1)**	**Amounts (1:1)**
Linear vector DNA (3547 bp)	100 ng	100 ng	100 ng
Insert DNA (mtgextra:1144bp; mtg_{intra}:1143 bp)	97 ng	65 ng	32 ng
10X T4 DNA Ligase Buffer	2 µl	2 µl	2 µl
T4 DNA Ligase (5 U μl^{-1})	0.5 µl	0.5 µl	0.5 µl
Sterile dH_2O	to 20 µl	to 20 µl	to 20 µl

Reaction was carried out at $22^{O}C$ for 1 hour and thermal inactivation of the T4 DNA ligase enzyme was achieved at $65^{O}C$ for 20 minutes. Products of ligation reactions are named as pPICZαA*::pro-*mtg_{extra} and pPICZαA*::pro-*mtg_{intra} (Figure 4.11).

4.1.6 Transformation of *E. coli* cells with pPICZα*::pro-mtg*$_{extra}$ and pPICZα*::pro-mtg*$_{intra}$ and Selection of the True Transformants

In order to propagate the pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids, ligation products were transformed into *E. coli* Top10 strain via $CaCl_2$ method as described in Section 3.5.11. The fresh transformants were inoculated on LSLB-agar plates including 25 µg ml^{-1} Zeocin. After 18-20 hours of incubation, fifteen to twenty five single colonies were randomly selected among lots of colonies to verify proper plasmids.

Selected colonies were inoculated into new agar plates and their plasmids were isolated by Alkaline Lysis Method as described in Section 3.5.2. 5 out of 24 transformant cells which probably carried the pPICZαA*::pro-mtg*$_{extra}$ had bigger plasmids than the pPICZα-A vector (Figure 4.12) while 7 out of 19 transformant cells that probably carried the pPICZαA*::pro-mtg*$_{intra}$ had bigger plasmids than pPICZα-A. Five putative extracellular and seven putative intracellular recombinant constructs were utilized in PCR and restriction enzyme digestion analyses to verify the constructs in terms of both accurate length of the plasmids and correct orientation of the *pro-mtg* genes.

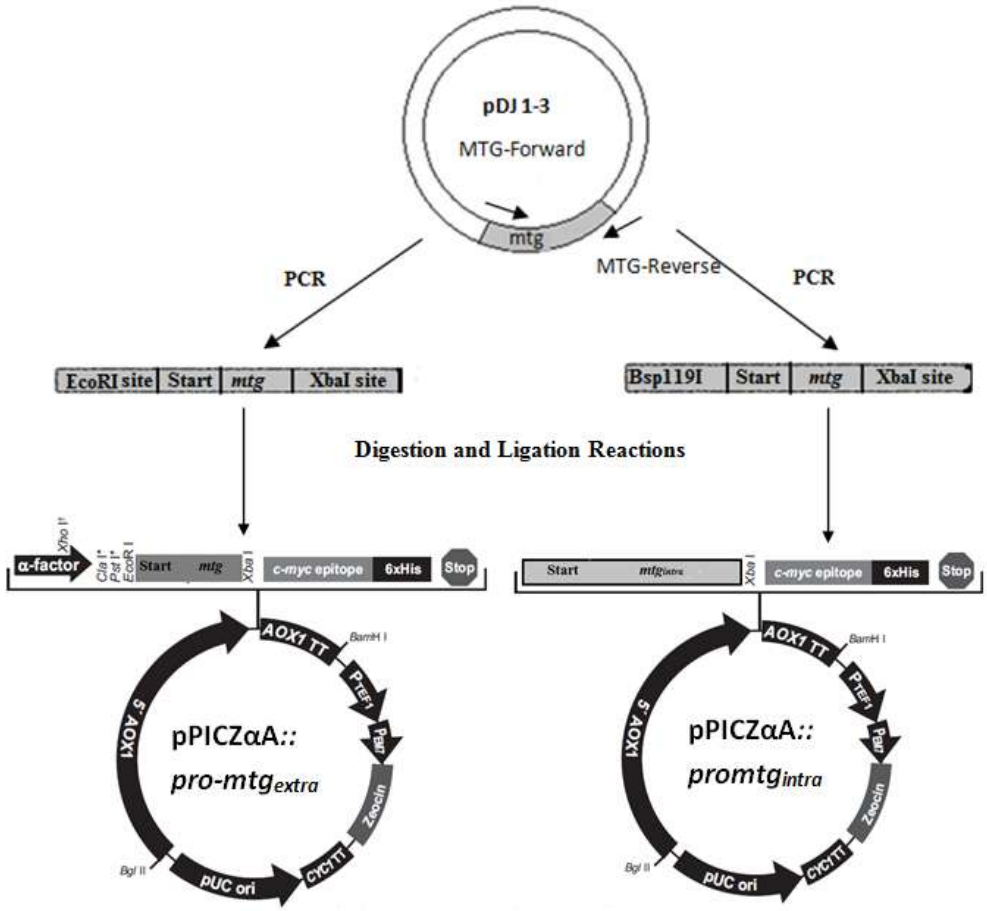


Figure 4.11 Schematic representation of the amplification of *pro-mtg*$_{intra}$ and *pro-mtg*$_{extra}$ genes, integration of the *pro-mtg* genes into the pPICZα-A vector via restriction digestion and ligation reactions and finally construction of pPICZαA*::pro-mtg*$_{intra}$ (expected size: 4329 bp) and pPICZαA*::pro-mtg*$_{extra}$ (expected size: 4671 bp) plasmids.

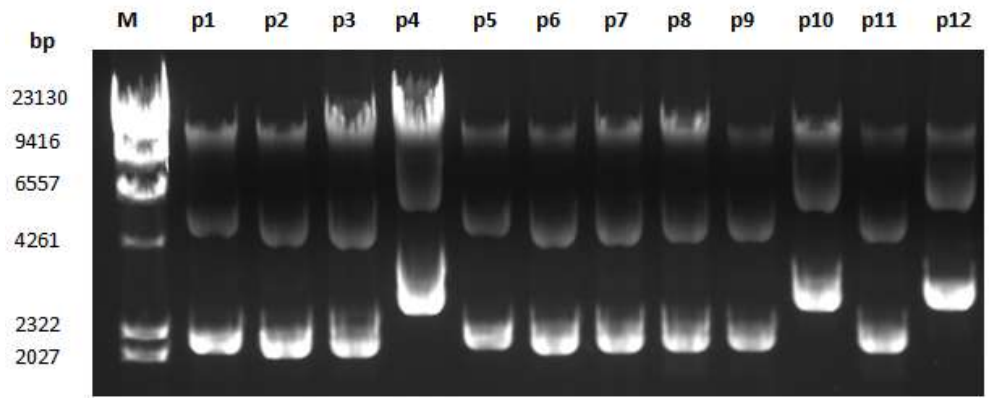


Figure 4.12 Agarose gel electrophoresis image of the isolated plasmids after extracellular transformation of *E. coli* cells. M: Lambda DNA/HindIII Marker; p4, p10 and p12: plasmids isolated from positive colonies containing *pro-mtg*$_{extra}$ gene.

Putative extracellular expression vectors were first single digested by *Eco*RI (Figure 4.13). Then, for double digestion control they were digested by both *Eco*RI and *Xba*I but double digested plasmids were seen in the same position with single digested plasmids in their AGE images. Then digestion reactions were performed by *Xba*I RE. Although *Xba*I restriction enzyme recognition site was found in the recombinant plasmids, it can't cleave the plasmid DNA. I can conclude that, due to three dimensional conformation of the plasmids, *Xba*I RE can't catalyze the digestion reaction. Then *Sal*I was used instead of *Xba*I, whose position was 42 nuclotide distant from *Xba*I restriction recognition site. Double digested plasmids with *Eco*RI and *Sal*I was represented in Figure 4.14.

As it was shown in Figure 4.14, all colonies had a band in the exact size of extracellularly double digested vector DNA and *pro-mtg*$_{extra}$ insert gene. Single digested plasmids gave a band in the size of *pro-mtg*$_{extra}$ gene and extracellular vector combination. Single and double digestion controls were also applied to putative intracellular expression vectors. Single digestion was performed by *Bsp*119I RE while double digestion was performed by *Sal*I and

*Bsp*119I REs. As a result of double and single digestion a band in the exact size of *pro-mtg*$_{intra}$ gene and a band in the exact size of intracellular vector and vector insert combination were obtained, respectively.

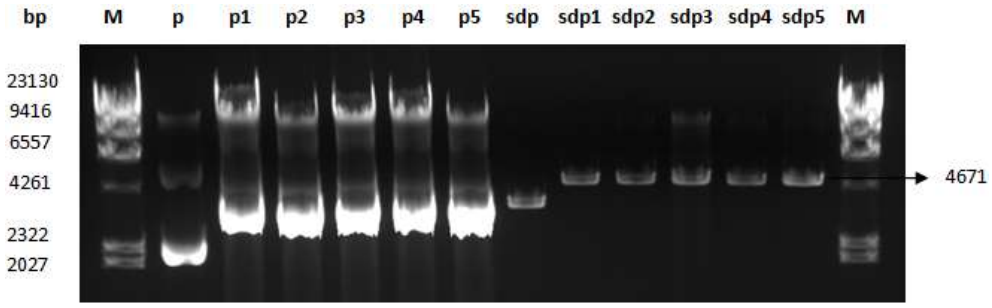


Figure 4.13: AGE image of the pPICZαA*::pro-mtg*$_{extra}$ plasmid. M: Lambda DNA/HindIII Marker; p: circular empty pPICZα-A vector; p1, p2, p3, p4, p5: isolated circular recombinant pPICZαA*::pro-mtg*$_{extra}$ plasmids; sdp: single digested pPICZα-A vector; sdp1, sdp2, sdp3, sdp4, sdp5: single digested pPICZαA*::pro-mtg*$_{extra}$ plasmids (expected size: 4661bp).

Figure 4.14: AGE image of the pPICZαA*::pro-mtg*$_{extra}$ plasmid. M: Lambda DNA/HindIII Marker; sdP: single digested pPICZαA vector; sdE1, sdE2, sdE3, sdE4, sdE5: single digested pPICZαA*::pro-mtg*$_{extra}$ plasmids (expected size: 4661bp), ddP: double digested pPICZαA; ddE1, ddE2, ddE3, ddE4, ddE5: double digested pPICZαA*::pro-mtg*$_{extra}$ plasmids (expected size of the ddE: 3527, expected size of the insert: 1144bp).

The putative plasmids were also controlled with PCR, using MTG_{extra}-Forward and MTG-Reverse primers for verification of *pro-mtg*$_{extra}$ gene insertion, and MTG_{intra}-Forward and MTG-Reverse primers for verification of *pro-mtg*$_{intra}$ gene. Successful amplification of *pro-mtg* genes were accomplished at both cases. Additionally, 5'AOX and 3'AOX primers were used to control the orientation of the insert *pro-mtg* genes. In this context four different primer combinations (5'AOX and 3'AOX; 5'AOX and MTG-Reverse; MTG-Forward and 3' AOX; MTG-Forward and MTG-Reverse) were used for both extracellular and intracellular expression plasmids (Figure 4.15). Finally, the nucleotide sequences of the *pro-mtg* insert genes were further verified by automatic DNA sequencers (Refgen, Turkey). DNA sequencing results confirmed that cloning was performed successfully for both intracellular and extracellular expression vectors.

4.1.7 Transfection of *Pichia pastoris* cells with Recombinant pPICZαA*::pro-mtg*$_{intra}$ and pPICZαA*::pro-mtg*$_{extra}$

For the genomic integration of pPICZαA*::pro-mtg*$_{intra}$ and pPICZαA*::pro-mtg*$_{extra}$ plasmids into the genome of the *P. pastoris* separately, at the *AOX1* locus, (Figure 4.16 and Figure 4.17), recombinant plasmids were firstly linearized in their *AOX1* promoter regions. Therefore double integration event can occur at *AOX* locus. For this purpose, *Pme*I was selected as a single-cutter restriction enzyme in this region and it was also non-cutter of *pro-mtg* gene insert. Full digestion of the recombinant plasmid is important to increase the integration efficiency and to eliminate the false positives. So, digestion of the recombinant plasmids were verified by agarose gel electrophoresis, and 2 hours of incubation along with *Pme*I RE was found to be sufficient for full digestion (Figure 4.18).

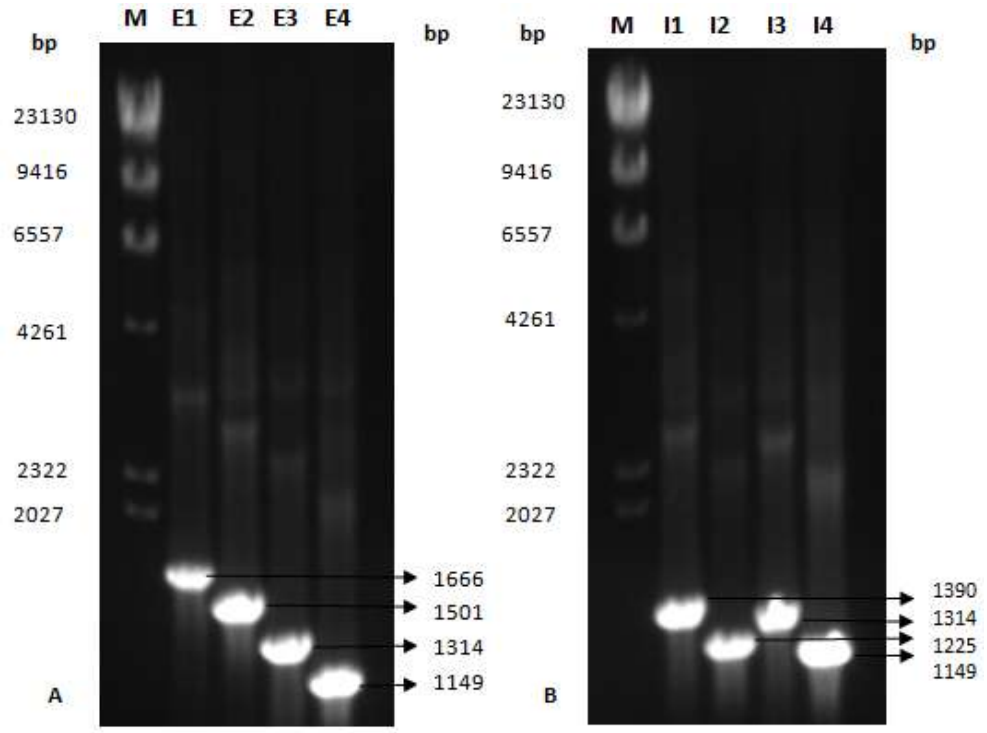


Figure 4.15 AGE images of the amplified PCR products of extracellular and intracellular plasmids. M: Lambda DNA/HindIII Marker; **A)** E1: amplified gene product of pPICZαA*::pro-mtg*$_{extra}$ using 5'AOX and 3'AOX primers (expected size: 1666 bp); E2: amplified gene product of pPICZαA*::pro-mtg*$_{extra}$ using 5'AOX and MTG-Reverse primers (expected size: 1501 bp); E3: amplified gene product of pPICZαA*::pro-mtg*$_{extra}$ using MTG$_{extra}$-Forward and 3'AOX (expected size: 1314 bp); E4: amplified gene product of pPICZαA*::pro-mtg*$_{extra}$ using MTG$_{extra}$-Forward and MTG-Reverse (expected size: 1149 bp). B) I1: amplified gene product of pPICZαA*::pro-mtg*$_{intra}$ using 5'AOX and 3'AOX primers (expected size: 1390 bp); E2: amplified gene product of pPICZαA*::pro-mtg*$_{intra}$ using 5'AOX and MTG-Reverse primers (expected size: 1225 bp); E3: amplified gene product of pPICZαA*::pro-mtg*$_{intra}$ using MTG$_{intra}$-Forward and3'AOX (expected size: 1314 bp); E4: amplified gene product of pPICZαA*::pro-mtg*$_{intra}$ using MTG$_{intra}$-Forward and MTG-Reverse (expected size: 1149 bp).

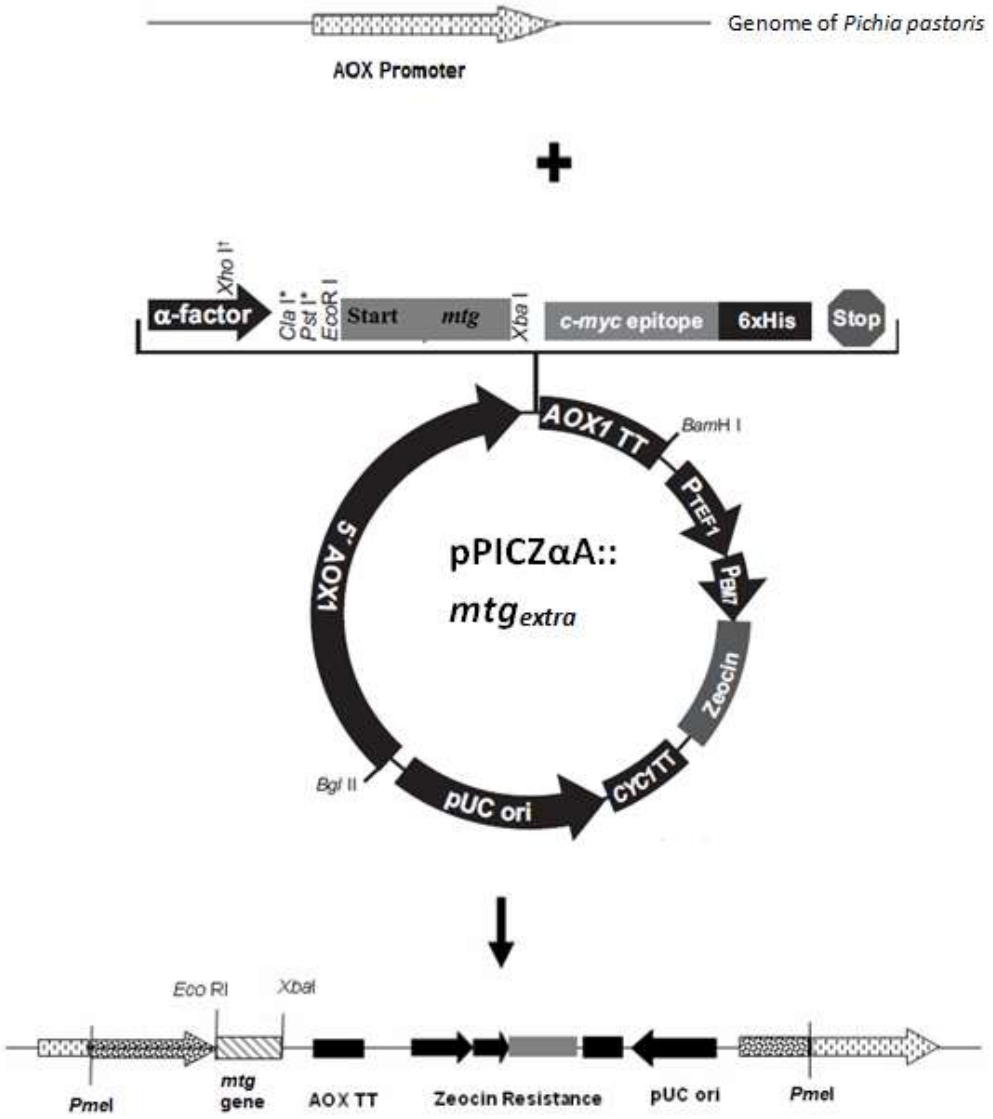


Figure 4.16 Schematic representation of pPICZαA*::pro-mtg*$_{extra}$ plasmid integration into *P. pastoris* genome. The pPICZαA*::pro-mtg*$_{extra}$ plasmid, including *pro-mtg* gene insert was digested with *Pme*I from *AOX*1 promoter region, yielding a linearized plasmid having homologous recombination region to the *AOX*1 promoter region in the genome of *P. pastoris* at both ends. After integration of the pPICZαA*::pro-mtg*$_{extra}$ to the genome, there are two functional copies of the *AOX*1 promoter in the genome.

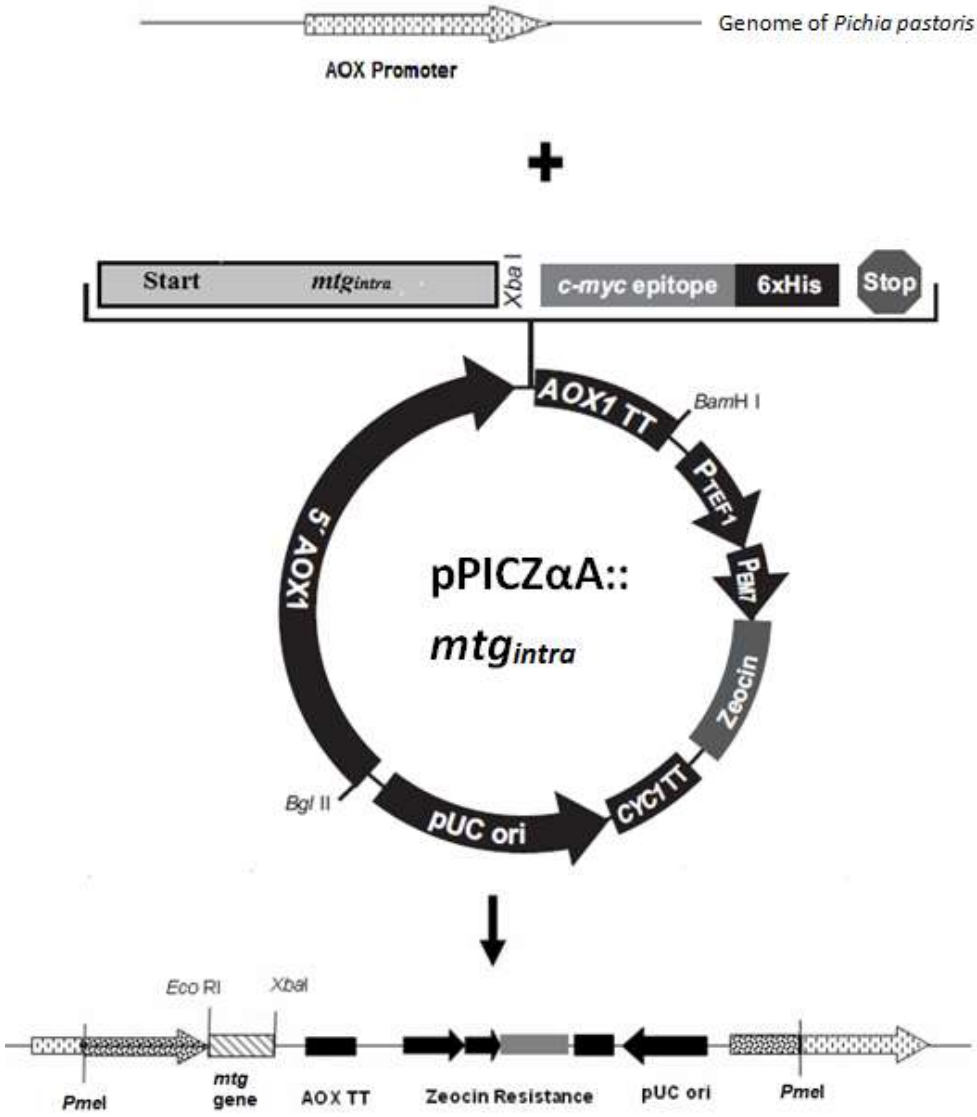


Figure 4.17 Schematic representation of pPICZαA*::pro-mtg*$_{intra}$ plasmid integration into *P. pastoris* genome. The pPICZαA*::pro-mtg*$_{intra}$ plasmid, including *pro-mtg* gene insert was digested with *Pme*I from *AOX*1 promoter region, yielding a linearized plasmid having homologous recombination region to the *AOX*1 promoter region in the genome of *P. pastoris* at both ends. After integration of the pPICZαA*::pro-mtg*$_{intra}$ to the genome, there are two functional copies of the AOX1 promoter in the genome.

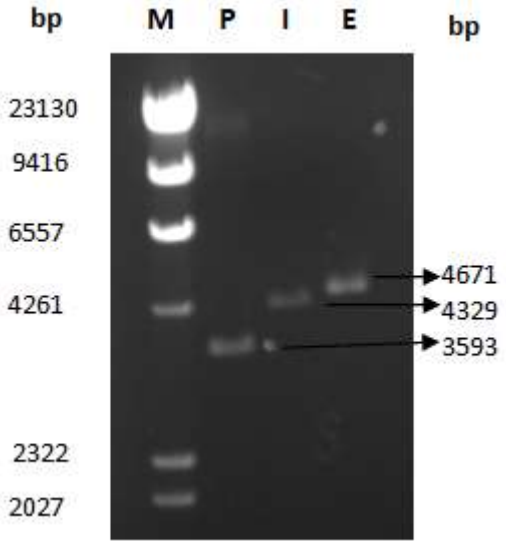


Figure 4.18 AGE images of digested recombinant plasmids by *Pme*I restriction enzyme for 2 hours. M: Lambda DNA/HindIII Marker; P: single digested pPICZα-A vector (expected size: 3593 bp); I: single digested pPICZαA*::pro-mtg*$_{intra}$ (expected size: 4329bp); E: single digested pPICZαA*::pro-mtg*$_{extra}$ (expected size: 4671 bp)

After digestion was accomplished, *Pme*I restriction enzyme was inactivated by heat treatment at 65^{o}C for 20 minutes. First, linearized plasmid DNA was extracted with phenol/chloroform. Then, it was precipitated with the addition of 1/10 volume of 3M sodium acetate and 2.5 volume of pure ethanol. DNA was harvested by centrifugation at 4^{o}C and at maximum speed for 2 minutes. Pellet was washed with 80% ethanol and centrifuged again. Pellet was air dried and resuspended in 10 µl of dH_2O. Although pPICZα-A user manual (Invitrogen) recommends using plasmid DNA at a concentration of 100-200 ng µl^{-1}, in this study, I found that recommended DNA concentration wasn't sufficient for successful transfection. Additionally, DNA concentration was found to be directly proportional to the transfection efficiency. *Pichia pastoris* transfection with recombinant pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids were achieved at 2000 ng µl^{-1} plasmid DNA concentration.

Transfection of *P. pastoris* was carried out as explained in the Section 3.5.14. After 48h of incubation period, ten to twenty single colonies were randomly selected among intracellular and extracellular transfected *Pichia pastoris* cells and inoculated onto YPD + Zeocin (0.150 kg m^{-3}) agar plates for further controls. Genomic DNA isolation of transfected *Pichia* cells were performed as described in the Section 3.5.15 to be used in PCR controls (Figure 4.19, Figure 4.20). Nineteen colonies were selected from transfected ones with extracellular construct and eleven of them were carrying the *pro-mtg* gene. For intracellular transfection, thirteen colonies were selected and one of them was carrying the *pro-mtg* gene. Pichia pastoris strains carrying the pPICZαA*::pro-mtg*$_{extra}$ named as E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11 and *Pichia strains* carrying the pPICZαA*::pro-mtg*$_{intra}$ named as I1. After transfection, native AOX1 region was still present in the genome of Mut^+ phenotypes, therefore two bands were expected as a result of PCR. One of them related to native AOX1 open reading frame (expected size: 2105 bp) and the other one was the product of integrated expression cassette (expected size 1667 bp for extracellular construct, 1398 bp for intracellular construct).

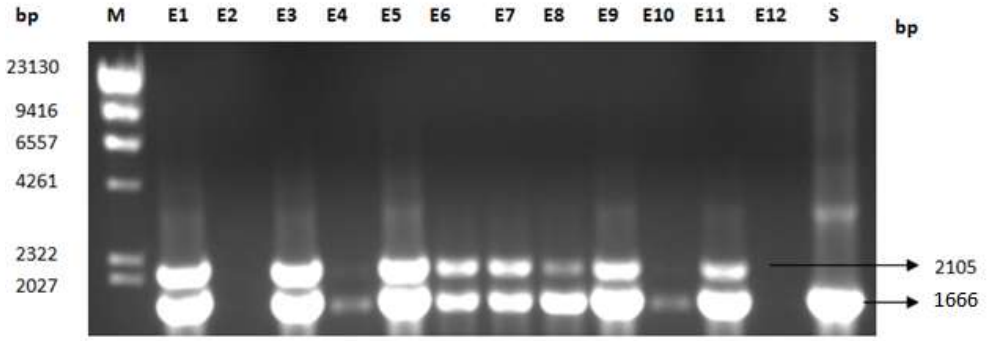


Figure 4.19 Agarose gel electrophoresis image of *pro-mtg$_{extra}$* gene amplification from genomic DNA of *P. pastoris* transformants by PCR. M: Lambda DNA/HindIII Marker E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12: PCR amplification of *pro-mtg$_{extra}$* (expected size: 1666 bp) from ten extracellular colonies by using 5'AOX and 3'AOX primers; S: PCR product of pPICZαA*::pro-mtg$_{extra}$* plamid as positive control.

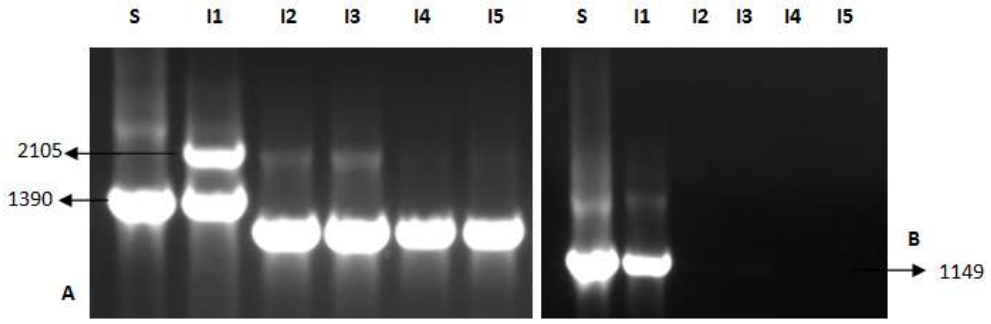


Figure 4.20 Agarose gel electrophoresis image of *pro-mtg$_{intra}$* gene amplification from genomic DNA of *P. pastoris* transformants by PCR. **A)** PCR amplification of *pro-mtg$_{intra}$* (expected size: 1390 bp) from intracellular colonies (five of them were shown in the figure) by using 5'AOX and 3'AOX primers; S: PCR product of pPICZαA*::pro-mtg$_{intra}$* plasmid as positive control. B) PCR amplification of *pro-mtg$_{intra}$* (expected size: 1149 bp) from intracellular colonies (five of them were shown in the figure) by using MTG$_{intra}$-Forward and MTG-Reverse primers; S: PCR product of pPICZαA::mtg$_{intra}$ as positive control.

4.2 Expression of pro-MTG in Recombinant *Pichia pastoris* in Laboratory Scale Air Filtered Shake Bioreactors

After construction of recombinant *Pichia pastoris* strains expressing the *pro-mtg* gene of *Streptomyces mobaraensis* in both intracellular and extracellular manners, the protransglutaminase production capacity of recombinant cells were compared to each others to select the most suitable strains producing the recombinant microbial protransglutaminase enzyme at high expression levels. In this context, ten colonies of positively transfected cells of extracellular *Pichia pastoris* and one positively transfected intracellular *Pichia pastoris* strains were inoculated onto fresh YPD agar including Zeocin and incubated for 48 to 60 hours. Then, all strains were transferred into precultivation medium and incubated at 30^{o}C, 225rpm for 24 hours using air filtered Erlenmeyer flasks. At the end of the precultivation step, cells were harvested by centrifugation at 4000rpm for 10 minutes and resuspended in BMMY production medium. Throughout the production stage, in every 24 hour, 1% (v/v) methanol was added into the medium to induce the AOX promoter. Additionally sorbitol was used as a co-carbon source. Recombinant Pichia cells were incubated at 30^{o}C, 225 rpm for 120 hours using air filtered, baffled Erlenmeyer flasks that have working volume capacity of 50 ml. Every 24 h, 1 ml of sample was taken and the most suitable *Pichia* strain expressing pro-MTG at the highest level was selected by SDS-PAGE and Dot-Blot analyses as described in Section 3.7.4.3 and 3.7.2, respectively. As it can be seen in dot blot analysis view (Figure 4.21) E8 produces pro-MTG in higher amounts when compared to other extracellular strains. SDS-PAGE analyses are consistent with the dot blot analysis results (Figure 4.22, Figure 4.23). In SDS-PAGE results, E8 has broader and darker bands at the microbial protransglutaminase region. Additionally, due to the increase in residence time of the bioprocess, pro-MTG bands of E8 get darker

while other strains pro-MTG bands get lighter. That decrease in the recombinant protein content may be due to the proteolytic cleavage of proteins. As the residence time increases cell mass and protease content also increase which causes more proteolytic degradation (Daly and Hearn, 2005). From these results, E8 has the highest recombinant microbial protransglutaminase production capacity when compared to other extracellular recombinant *Pichia* strains. Moreover, other extracellular strains may secrete higher amounts of proteases so their recombinant protein products may degrade easily. To conclude, E8 is more suitable for extracellular protransglutaminase production in large scale and long time bioprocess operations.

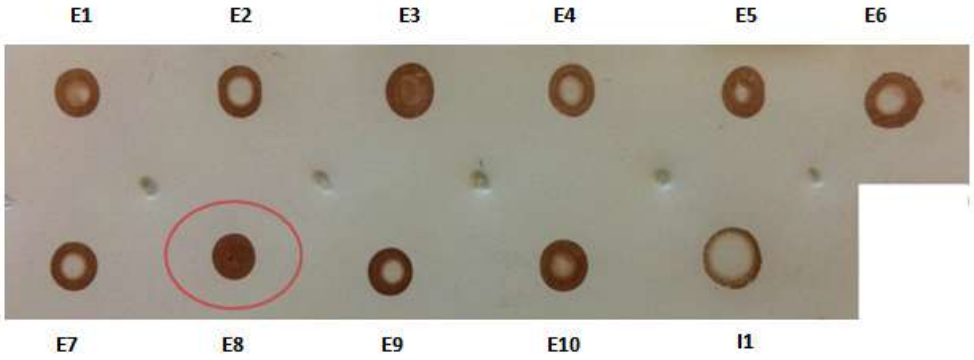


Figure 4.21 Dot blot analysis view of 15 µl supernatant samples taken from different recombinant *Pichia* strains cultivation media at t=85 h of the bioprocess; E1, E2, E3, E4, E5, E6, E7, E8, E9, E10: *Pichia pastoris* strains carrying the pPICZαA*::pro-mtg*$_{extra}$ plasmid; I1 *Pichia pastoris* strain carrying the pPICZαA*::pro-mtg*$_{intra}$ plasmid.

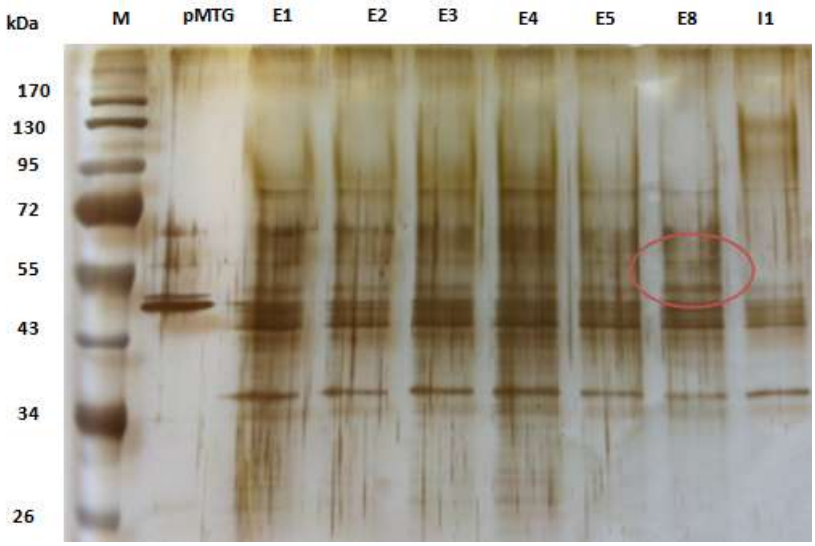


Figure 4.22 SDS-PAGE gel view of laboratory scale shake bioreactors with different recombinant *Pichia* cells at t=85 h of the bioprocess. M: PageRuler Prestained Protein Ladder; pMTG: microbial protransglutaminase; E1, E2, E3, E4, E5, E8: *Pichia pastoris* strains carrying the pPICZαA*::pro-mtg*$_{extra}$ plasmid; I1 *Pichia pastoris* strain carrying the pPICZαA*::pro-mtg*$_{intra}$ plasmid

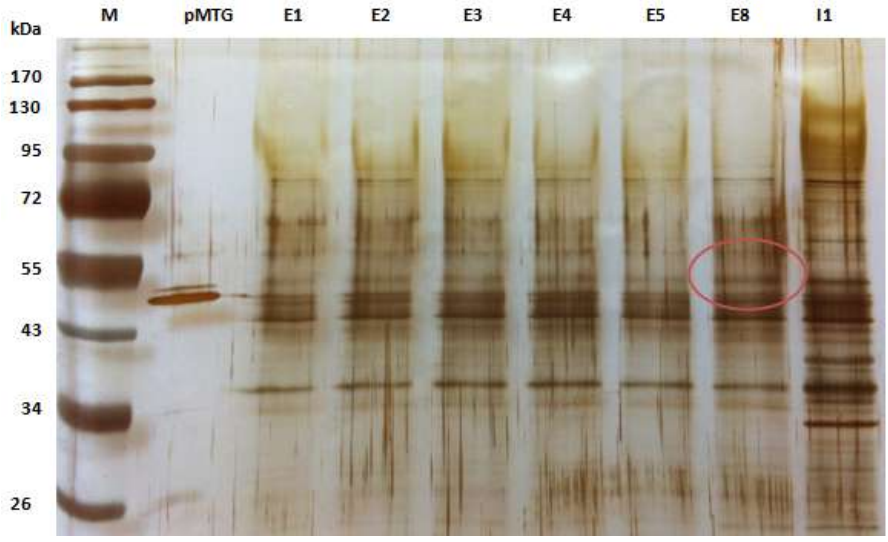


Figure 4.23 SDS-PAGE gel view of laboratory scale shake bioreactors with different recombinant *Pichia* cells at t=96 h of the bioprocess. M: PageRuler Prestained Protein Ladder; pMTG: microbial protransglutaminase; E1, E2, E3, E4, E5, E8: *Pichia pastoris* strains carrying the pPICZαA*::pro-mtg*$_{extra}$ plasmid; I1 *Pichia pastoris* strain carrying the pPICZαA*::pro-mtg*$_{intra}$ plasmid.

4.3 Expression of Microbial Protransglutaminase in Recombinant *Pichia pastoris* in Pilot Scale Bioreactor

The pilot-scale bioreactor used in this study has 1.0-2.2 dm^3 working volume (total volume=3 dm^3) and equipped with temperature, oxygen, pH, stirrer rate and high foam controllers.

Sorbitol as the co-carbon source was found effective for both cell concentration increase and recombinant protein production with recombinant *Pichia pastoris* cells (Çelik *et al.,* 2009; Jungo *et al.,* 2007). Non-inhibitory sorbitol limit for the cell growth and recombinant protein production was reported as 50 g L^{-1} sorbitol by Çelik *et al*., (2009). In the study of Çalık *et al*. (2010a) sorbitol batch and methanol fed batch strategies were investigated and the maximum recombinant human growth hormone production was obtained with 50 g L^{-1} initial sorbitol concentration and fed-batch methanol feeding to the system at the predetermined feedig rate of μ=0.03 h^{-1}. Another study of Çalık *et al*. (2010b), pH effect on the recombinant protein production was investigated and pH 5.0 was found optimum for recombinant protein production with *Pichia pastoris*. According to the literature, in this study to promote cell growth and extracellular recombinant protein production, bioreactor operation parameters were determined to 50 g L^{-1} sorbitol pulse feed at the beginning of the production phase and predetermined methanol feed regarding to the μ=0.03. pH of the production media was adjusted to 5.0 throughout the fermentation.

First, E8 strain was inoculated to YPD-Zeocin agar and after 48 hours of incubation transferred into precultivation medium. To OD_{600}= 4.0-5.0, cells were cultivated at 30^{o}C, 200 rpm using baffled shaker bioreactors with working volume of 50 ml. Then, cells were harvested by centrifugation at 1500g, 4^{o}C for 10 minutes and resuspended in production medium and the initial OD_{600} was adjusted to 1.0. After inoculation of the cells, four-phase

bioprocess was carried out as explained in Section 3.6.4.2. The bioreactor dissolved oxygen level, pH and temperature parameters were set to, 20 %, 5.0 and 30^{o}C, respectively. At the beginning of the production phase sorbitol was added batch-wise as the initial concentration of 50 g L^{-1}.

4.3.1 Cell Growth and Sorbitol Consumption Profile of the Recombinant *Pichia pastoris* pPICZαA-*pro-mtg*$_{extra}$ Cell

Cell concentration values of the recombinant *Pichia* cells with respect to the cultivation time in the production phase are given in Figure 4.24. As, shown in the figure, the maximum cell concentration was obtained as C_x=74.1 g L^{-1} at t=36 h. Addition of the co-carbon source sorbitol besides methanol makes the lag phase shorter than that of the single substrate methanol cultivation (Çalık *et al.,* 2010a). Sorbitol consumption profile with respect to cultivation time is given in Figure 4.25. As can be seen in Figure 4.25, sorbitol concentration in the production medium was decreased with similar trend regarding to time and it was totally utilized at 30th hours of the production stage, while sorbitol was consumed at t=15 h for other bioprocess operations with different recombinant *Pichia pastoris* cell (Çalık *et al.,* 2010a). As sorbitol was added into production medium at the beginning of the methanol induction phase, *Pichia* cells utilize sorbitol and methanol simultaneously. Simultaneous consumption of methanol and sorbitol by *Pichia pastoris* was also studied by Çalık *et al.* (2009), Çelik *et al.* (2009) and Jungo *et al.* (2007). Due to co-carbon source sorbitol consumption, bioprocess was performed with sole carbon source of methanol after t=30 h and this led a characteristic decrease in the cell concentration at 36th hours of the bioprocess as shown in Figure 4.25. In the previous study by Çalık *et al*. (2010a) for recombinant human growth hormone production by *P. pastoris,* the same feeding strategy was applied and maximum cell concentration was obtained at 24th hour as 42.3 g L^{-1}; while in this study, the cell concentration was 66.9 g L^{-1} at 24th hours of the bioprocess. In this study, the maximum cell

concentration is also achieved at 36th hour of the bioprocess as 74.1 g L^{-1} which is 1.8 fold higher than the previous study by Çalık *et al*. (2010a). To conclude, recombinant *Pichia pastoris* strains producing different recombinant proteins display different growth and protein expression profiles although the same feeding strategy is applied.

Table 4.2 shows the calculated instantaneous specific growth rate, specific sorbitol consumption rate and specific methanol consumption rates. Until t=30 h of the bioprocess both substrates, i.e., sorbitol and methanol, were used simultaneously, and then bioprocess was carried out only with the substrate methanol. At the the first 30th hours of the bioprocess, specific growth rate is the combination of specific growth rates on methanol and sorbitol. After that point specific growth rate values are determined by only methanol. As can be seen in the Table 4.2, the specific growth rate values are higher when sorbitol was found in the medium. According to specific growth rate decrease after sorbitol consumption, it should be concluded that, sorbitol as co-carbon source in the media enhances the cell growth.

In the previous studies of *Pichia pastoris* with the predetermined methanol feeding rate, methanol accumulation wasn't observed (Çalık *et al.,* 2010a, Çalık *et al,* 2009, Çelik *et al.,* 2009) while in this study, methanol accumulation began at t=24 h. This situation should be explained with the growth rate profile. As the methanol was fed to the system regarding to the specific growth rate value of μ=0.03 h^{-1}, after t=24 h specific growth rate values are lower than 0.03 h^{-1} so, excess amount of methanol began to accumulate.

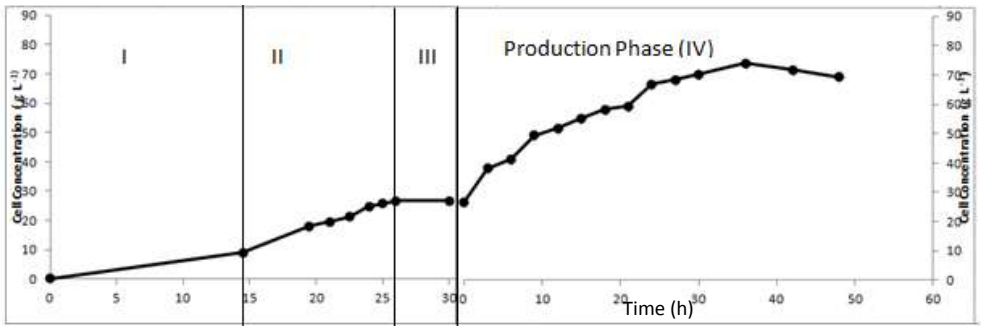


Figure 4.24 The varitions in cell concentration throughout the bioprocess: Precultivation phases (I, II, III) and production phase. I: Glycerol barch phase; II: Glycerol fed-batch phase; III: Methanol transition phase

Table 4.2 The variations in the instantaneous specific growth rate, specific sorbitol consumption rate, sorbitol consumption rate, specific methanol consumption rate and methanol consumption rate throughout the bioprocess

t (h)	μ_t (h^{-1})	q_s ($g\ g^{-1}\ h^{-1}$)	r_s ($g\ L^{-1}\ h^{-1}$)	q_m ($g\ g^{-1}\ h^{-1}$)	r_m ($g\ L^{-1}\ h^{-1}$)
0	0.14	0.00	0.04	0.07	1.91
3	0.03	0.07	2.49	0.06	2.12
6	0.07	0.05	2.03	0.06	2.35
9	0.02	0.01	0.64	0.05	2.62
12	0.02	0.06	2.86	0.06	2.92
15	0.02	0.02	1.22	0.06	3.25
18	0.01	0.03	1.56	0.06	3.61
21	0.05	0.04	2.57	0.07	4.07
24	0.01	0.03	1.94	0.07	4.40
27	0,02	0,02	1,63	0,07	4,92
30	0,02	0,00	0,00	0,08	5,32
36	0,00	0,00	0,00	0,08	6,20
42	0,00	0,00	0,00	0,11	7,69
48	0,01	0,00	0,00	0,14	9,51

For the analysis of bioprocess, the yield coefficients which are the overall yield of biomass generated per mass of sorbitol consumed ($Y_{X/S}$) and the overall yield ofbiomass generated per mass of total methanol consumed ($Y_{X/M}$) were calculated at t=36 h of the bioprocess. Hence, $Y_{X/S}$ and $Y_{X/M}$ were calculated as 1.482 and 2.48, respectively.

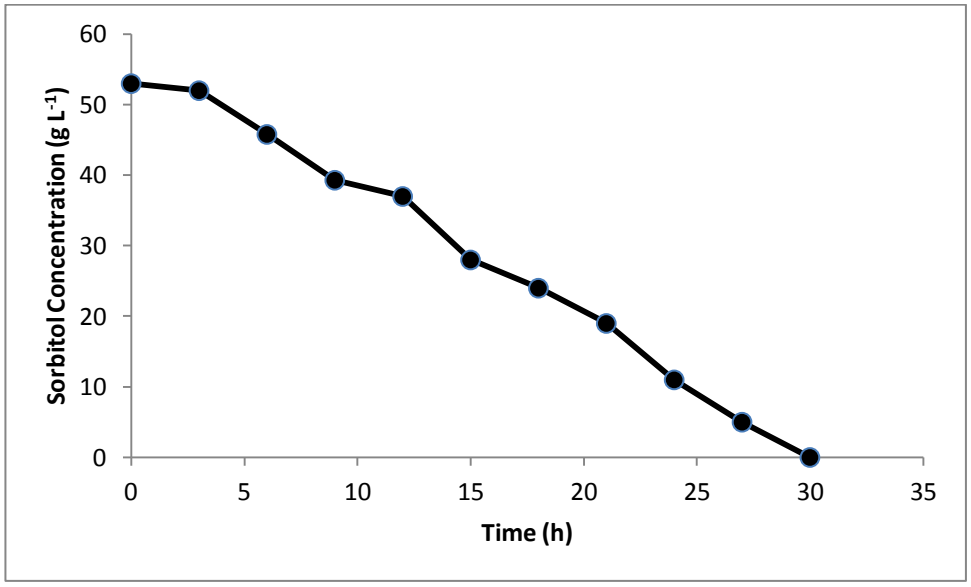


Figure 4.25 The varitions of sorbitol concentration with the cultivation time

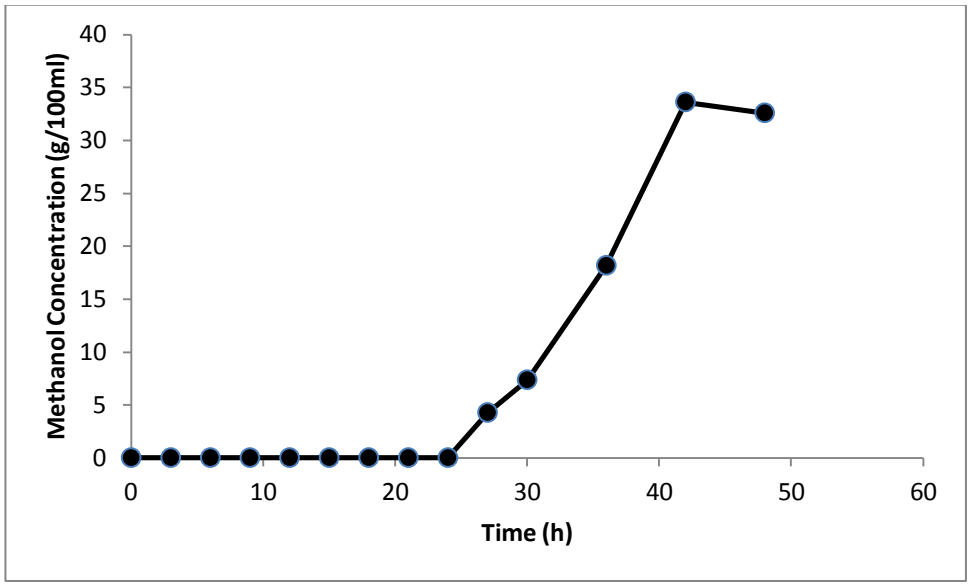


Figure 4.26 The variations of methanol concentration with the cultivation time

4.3.2 Microbial Protransglutaminase Activity and Production

In order to determine the microbial protransglutaminase production capacity of the recombinant *Pichia pastoris* E8 strain carrying *pro-mtg* gene within its genome, a pilot scale bioreactor operation was performed under controlled conditions. The calculated enzyme activity values throughout the bioprocess are shown in Figure 4.27.

First, microbial protransglutaminase was activated by Dispase I for 30 minutes, as explained in section 3.7.3.1 and then activity assay was performed. As shown in Figure 4.27, the maximum MTG activity was obtained at t=36 h of the bioprocess as 4448 U L^{-1}. After t=36 h of the bioprocess, decrease in the enzyme activity was observed. This reduction is compatible with the reduction in cell concentration but after t=48 h although cell concentration showed a slight increase while enzyme activity decreased continuously. This may be related with the increase in proteolytic activity of

the cultivation medium according to the *Pichia pastoris* proteases which is an important drawback of *Pichia pastoris* expression system (Potvin *et al*., 2010). For this reason, protease activities of the cultivation medium were investigated and the results were presented in Section 4.3.4.

As can be seen in Figure 4.27 after t=24h MTG activity value shown an increase, which is compatible with the methanol accumulation in the production media. Actually, after t=24 h of the bioprocess methanol accumulation began and so methanol facilitated the expression of *pro-mtg* gene more effectively but after t=36 h regarding to high proteolytic activity MTG activity started to decrease.

In order to calculate the amount of MTG produced during cultivation, the specific activity of 22.6 U mg^{-1} was used for the *Streptomyces mobaraensis* transglutaminase (Sommer *et al.,* 2011). Calculated concentration values of MTG throughout the bioprocess were shown in Figure 4.28. The highest MTG concentration was obtained at t=36 h of the bioprocess as 197 mg L^{-1}. As reviewed in section 2.1.5 *E. coli* expression system produces more active recombinant transglutaminase after site directed mutagenesis on the *pro-mtg* gene but it can synthesize only intracellular pro-MTG and intracellular enzymes downstream processes need higher costs. To conclude, the extracellular pro-MTG enzyme produced by the constructed recombinant *Pichia pastoris* E8 clone carrying *pro-mtg* gene its genome. For the first time, the active microbial transglutaminase was produced by recombinant *Pichia pastoris* cells. Amongst recombinant yeasts, *Pichia pastoris* E8 produced 197 mg L^{-1} MTG which is 2.26-fold higher than *Candida boidinii,* a methylotrophic yeast (Yurimoto *et al.*, 2004). Depending on the present literature, after genetically engineered *Corynebacterium ammoniagenes* (2500mg L^{-1}) and *Corynebacterium glutamicum* (881 g L^{-1}), the third highest extracellular recombinant pro-MTG production is achieved by *P. pastoris* E8 clone (Itaya *et al*., 2008; Date *et al*., 2004).

Total pro-MTG production was investigated via using SDS-PAGE and variation of pro-MTG concentration throughout the bioprocess was shown in Figure 4.29. To determine the pro-MTG exact position on the SDS-PAGE gel, supernatants taken from the bioreactor experiments were treated with Dispase I. Therefore prosequence of the pro-MTG was cleaved by Dispase I and mature transglutaminase was obtained. As can be seen in Figure 4.30, microbial protransglutaminase and activated transglutaminase have different bands on the SDS-PAGE gel view. Microbial protransglutaminase has 2 distinct bands and smear like view at the region between 43-55 kDa whereas for activated samples nothing is seen at that position. It should be concluded that, this smear like view and two distinct bands may be due to the glycosylation of the enzyme as pro-MTG has three putative glycosylation sites.

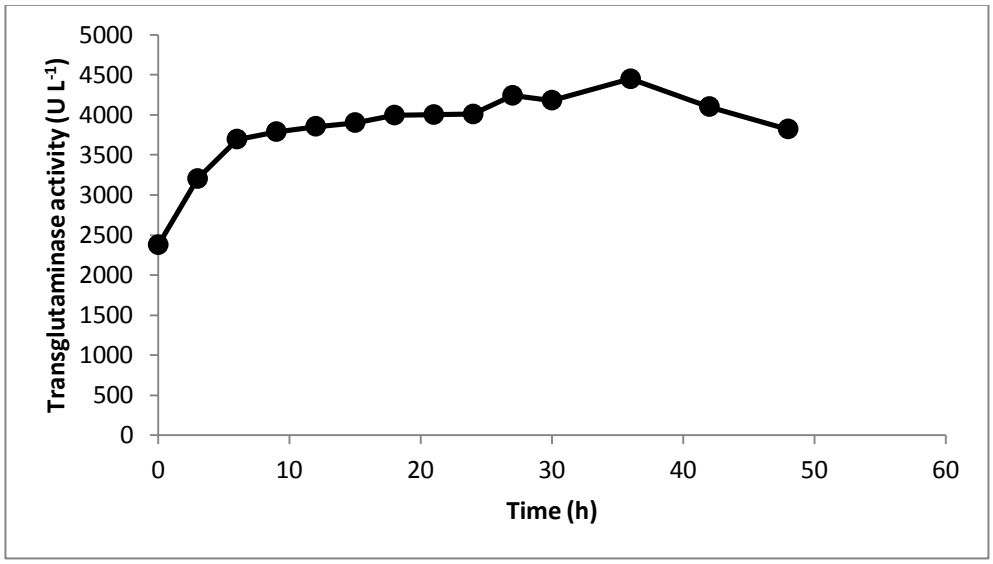


Figure 4.27 The variations of microbial transglutaminase activity with the cultivation time

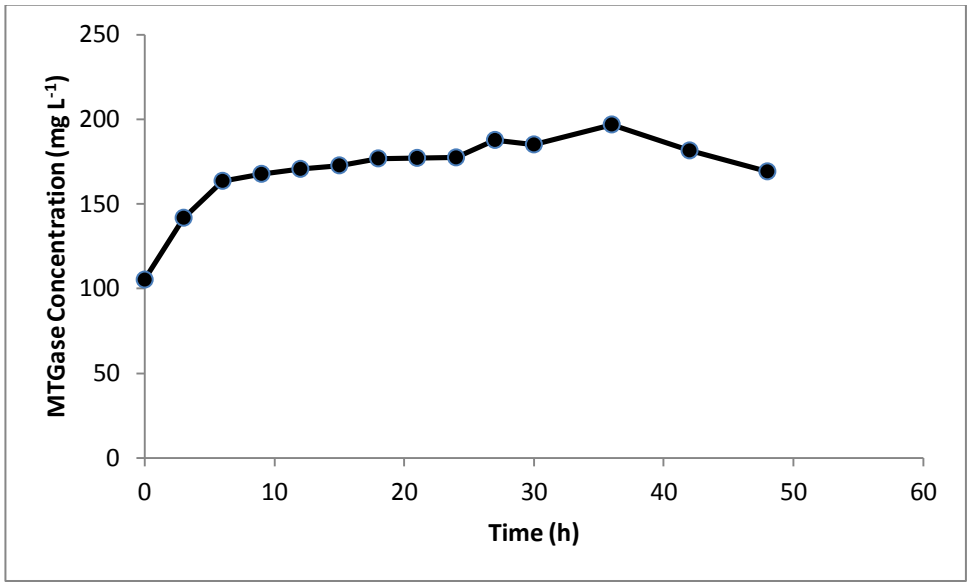


Figure 4.28 The variations of microbial transglutaminase concentration with the cultivation time

Activated samples have 2 distinct bands at the region between 34-43 kDa which is compatible with the native transglutaminase weight, 38kDa. Two distinct activated transglutaminase bands may result from different glycan contents of the enzyme. According to the Figure 4.30, the maximum pro-MTG content is at 42nd hour of the bioprocess and after that pro-MTG concentration begins to decrease. Decrease in the pro-MTG concentration is possibly the result of increased proteolytic activity of the cultivation media. In order to determine the proteolytic activity of the media, proteases of *Pichia pastoris* are investigated and results are presented in Section 4.3.4.

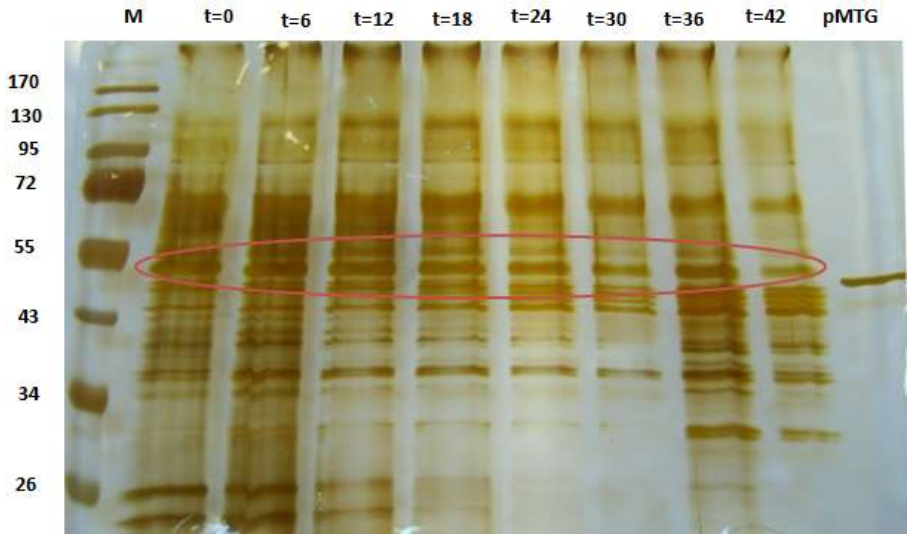


Figure 4.29 SDS-PAGE gel view of the pilot scale bioreactor throughout the bioprocess. M: PageRuler Prestained Protein Ladder; pMTG: microbial protransglutaminase. Other lanes refer to time of sampling.

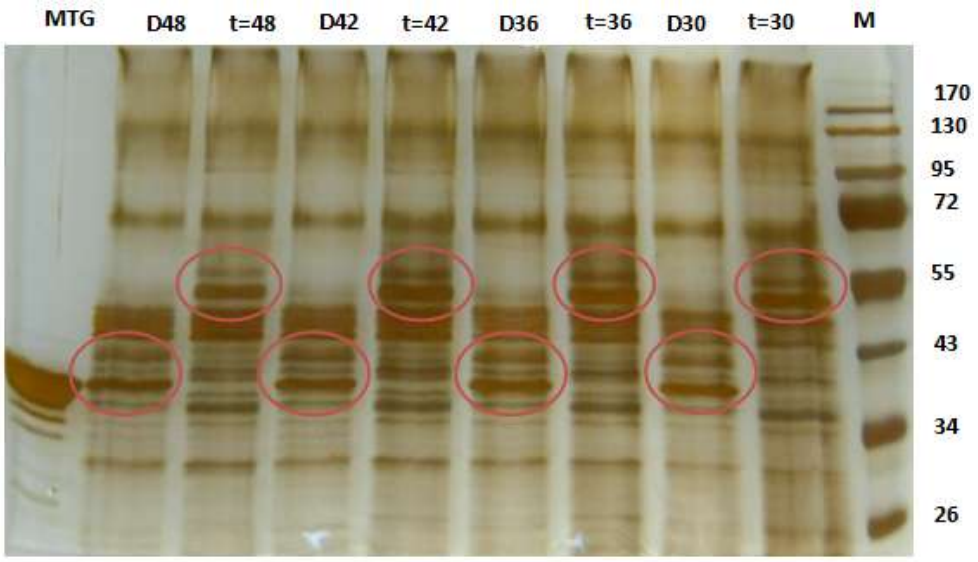


Figure 4.30 SDS-PAGE gel view of the pilot scale bioreactor. M: PageRuler Prestained Protein Ladder; MTG: microbial transglutaminase. t=30, t=36, t=42, t=48 lanes refer to time of sampling; D30, D36, D42, D48 lanes refer to activated reactor samples by Dispase I.

4.3.3 Alcohol Oxidase Activity

In this study, microbial protransglutaminase gene is expressed under control of *AOX1* promoter therefore; AOX activity of the recombinant cells throughout the bioprocess can help to determine the relationship between AOX activity and recombinant pro-MTG production. AOX activities of the *Pichia pastoris* E8 strain regarding to time throughout the bioprocess are given in Figure 4.31.

As can be seen in Figure 4.31, the highest AOX activity was calculated as 38.7 U g CDW^{-1} at the beginning of the production phase. After that value, AOX activity began to decrease and fluctuate between the activity values of 2.2 and 12.6 untill whole sorbitol was consumed. After the total consumption of sorbitol, slight increase in the AOX activity was observed and then, AOX activity decreased again. Fluctuation of AOX activity was the outcome of methanol feeding profile, as methanol wasn't fed to the system continuously. Feeding profile of the methanol sometimes has some intervals between two methanol feeding.

As can be seen in Figure 4.26, after t=24 h of the bioprocess, methanol accumulation within the cultivation medium began. After t=27 h AOX activity didn't fluctuate for 15 hours, which is compatible with the methanol concentration increase. It should be concluded that, gradual increase in AOX activity after t=27 h is due to the methanol accumulation. The maximum methanol concentration was observed at 42^{nd} hour of the bioprocess. According to the SDS-PAGE results maximum pro-MTG content was observed at 42^{nd} hour of the bioprocess, as well.

In this study, AOX activity results of the pilot scale bioreactor experiment did not exhibit a similar trend with the study of Çalık *et al*., (2010b). In previous study of Çalık *et al*. (2010b), maximum AOX activity was obtained at 15^{th} hour of the bioprocess as 40.7 U g CDW^{-1} after total

consumption of sorbitol by recombinant human growth hormone producing *Pichia pastoris* cells. Unlike previous study, maximum AOX activity was obtained at the beginning of the production phase. This distinction was the result of modification of methanol transition phase, which was carried out by feeding methanol to the bioreaction medium at a rate of 2.8 g L^{-1} h^{-1} for 4 hours instead of transferring a pulse methanol feed, C_{M0}=1.5 g L^{-1} for 6 hours as the study of Çalık *et al*., (2010b). So, higher AOX activity values were obtained at the beginning of the production phase.

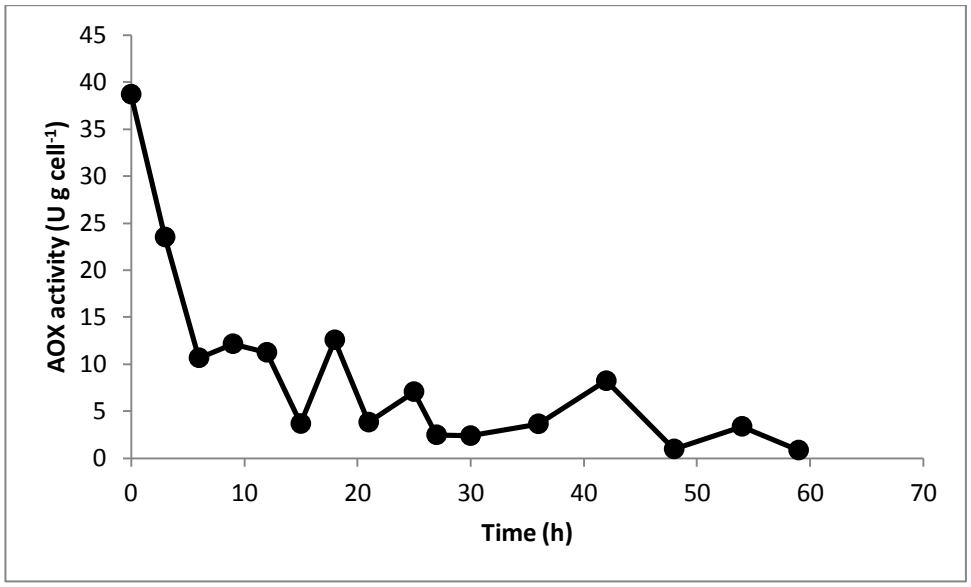


Figure 4.31 The variations of AOX activity with the cultivation time

4.3.4 Protease Profile

Proteolytic activity of the cultivation media is an important drawback of *Pichia pastoris* expression system for recombinant protein production. Therefore, total protease activity of *Pichia pastoris* E8 containing basic, neutral and acidic proteases in the extracellular medium was analyzed

to determine the effect of proteases on recombinant microbial protransglutaminase production. In Figure 4.32 total proteolytic activity of the fermentation medium throughout the bioprocess is given. As shown in the figure, at the beginning of the bioprocess total proteolytic activity has its minimum value which is 134.9 U cm^{-3}; protease activity increases throughout the bioprocess and reaches its maximum value at 59th hour of the bioprocess as 1444.2 Ucm^{-3}.

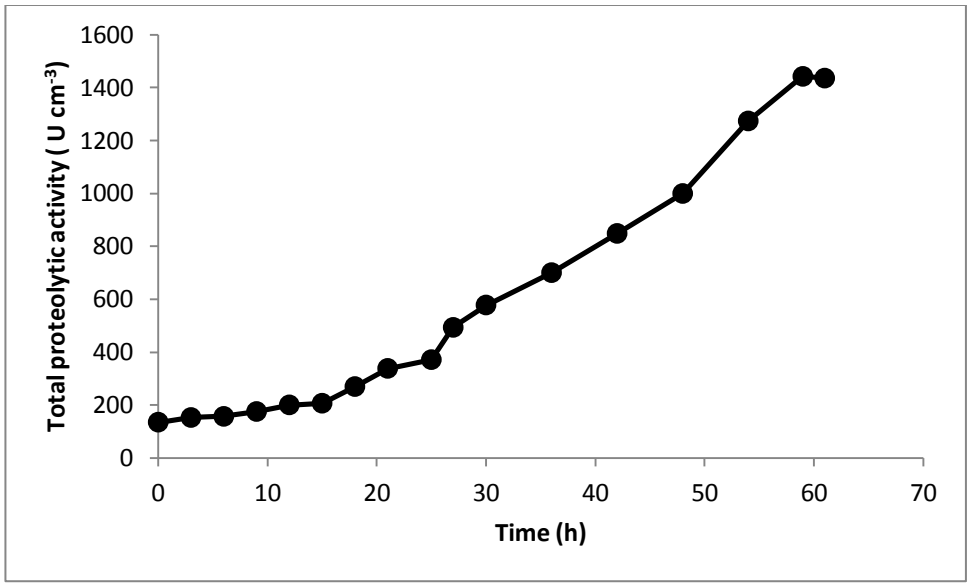


Figure 4.32 Variation in total protease activity with the cultivation time

Figure 4.33 exhibits the relationship between the proteolytic activity and recombinant MTG activity. As can be seen in the figure, increase in the microbial transglutaminase activity is higher in the earlier times of the bioprocess while proteolytic activity of the medium is relatively low. Up to 36th hour of the bioprocess transglutaminase activity increases and the maximum MTG activity is obtained at 36th hour. After that point, transglutaminase

activity begins to decrease due to increased proteolytic activity. To conclude, extracellular proteolytic activity of *Pichia pastoris* expression system is a handicap for recombinant protein production that prevents the recombinant MTG to reach higher levels. Actually, the main advantage of *Corynebacterium ammoniagenes,* produced 2500mg L^{-1} MTG and *Corynebacterium glutamicum,* produced 881 g L^{-1} MTG, over *Pichia pastoris* is their low extracellular proteolytic activity (Itaya *et al*., 2008; Date *et al.,* 2004).

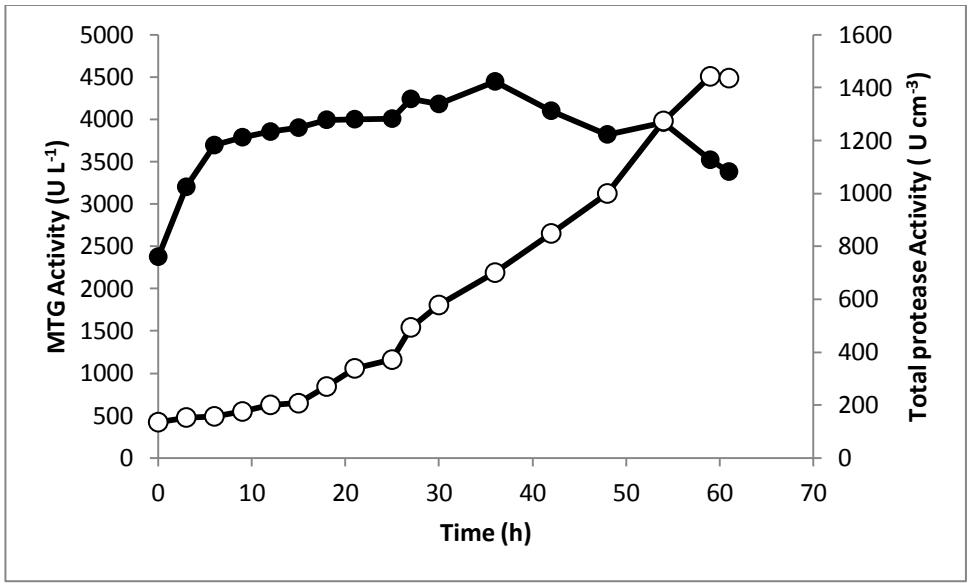


Figure 4.33: Relationship between recombinant microbial transglutaminase activity and total proteolytic activity with respect to time throughout the bioprocess. MTG activity, U L^{-1} (●); total protease activity, U cm^{-3} (○).

4.3.5 Organic Acid Profile

In order to have a better insight for the intracellular reaction network of recombinant *Pichia pastoris* E8, organic acid profile throughout the bioprocess is very useful. For this purpose, organic acid profile of the

fermentation medium was investigated and variations in organic acid contents throughout the bioprocess are given in Table 4.3.

In methanol utilization pathway of *Pichia pastoris*, methanol is converted to hydrogen peroxide and formaldehyde by AOX enzyme and these products are toxic to the *Pichia* cells (Zhang *et al*., 2000). To eliminate toxic formaldehyde, some of the formaldehyde molecules were used as building blocks within the cell while others were oxidized to formic acid. Therefore, formic acid concentration is related with the AOX activity of the cells. As can be seen in the Table 4.3, at the beginning of the bioprocess formic acid concentration was high and then no formic acid was detected in the production medium. This result is compatible with the AOX activity results as the maximum AOX activity was detected at the beginning of the bioprocess. After that, AOX activity didn't reach such high values. The reason why formic acid wasn't detected after 3rd hour of the bioprocess is that, it should has been used in anabolic reactions of *Pichia pastoris* so it wasn't detected.

Acetic acid, pyruvic acid, fumaric acid, succinic acid, oxalic acid and citric acid are involved in TCA cycle reactions. From these organic acids, acetic acid was observed earlier than others and then it disappeared again untill t=36 h. That means, acetic acid incorporation into TCA cycle was failed between 3rd to 12th hours of the bioprocess. Until the 24th hour of the bioprocess these organic acids metabolized in TCA cycle efficiently, but after that point, accumulation of them began. First, fumaric and pyruvic acid were detected at t=24 h, citric acid was detected at t=30 h and then succinic and acetic acid were detected at t=36 h. Finally, oxalic acid was detected at 48th hour of the bioprocess. It should be concluded that, organic acids except acetic acid consumed efficiently until t=24. After 36th hours of the bioprocess all organic acids except oxalic acid was detected and after that point their concentrations increased throughout the fermentation. From all, oxalic acid was utilized efficiently throughout the bioprocess. Lactic acid was observed at

t=18 h and its concentration increased as the bioprocess continued. Lactic acid is the product when fermentative mode of metabolism is occurred. Additionally, TCA cycle require oxygen to perform the reaction efficiently. Depending on the accumulation of organic acids related with TCA cycle and lactic acid it should be concluded that, especially after 36^{th} hour of the bioprocess oxygen was not sufficient for metabolic activities of the recombinant *Pichia* cell.

Table 4.3 Organic Acid Profile of the fermentation medium throughout the bioprocess. Organic acid concentration are given in g L^{-1}

Experiment t(h)	0	3	6	12	18	24	30	36	42	48	54
Oxalic Acid	-	-	-	-	-	-	-	-	-	0,1057	0,1428
Gluconic Acid				2,1313	2,1830	4,0760	3,7247	2,2423	2,0128	2,3688	4,915298
Formic Acid	0,7814	0,7276	-	-	-	-	-	-	-	-	-
Malic Acid	-	-	-	-	0,1763	0,2447	1,4275	1,7864	1,9416	1,9224	2,433251
Lactic Acid	-	-	-	-	0,51802	0,802693	-	0,755482	1,018226	1,42354	2,249221
Acetic Acid	-	-	0,3055	0,3412	-	-	-	0,428098	0,729666	1,192921	2,070269
Maleic Acid	-	-	-	-	-	-	0,00143	0,00344	0,005392	0,006816	0,010893
Citric Acid	-	-	-	-	-	-	0,161653	0,27187	0,364239	0,452961	0,607881
Succinic Acid	-	-	-	-	-	-	-	0,377005	0,770719	1,120018	1,735864
Fumaric Acid	-	-	-	-	-	0,007485	0,01078	0,015893	0,020198	0,023973	0,03682
Pyruvic Acid	-	-	-	-	-	0,006578	0,053618	0,069748	0,085208	0,096643	0,18089

4.4 Summary of the Results

Aim of this M.Sc thesis study is to develop *Pichia pastoris* strains producing extracellular and intracellular microbial protransglutaminase, separately and to produce microbial protransglutaminase by genetically engineered *P. pastoris*. In this context, the thesis study was divided into 2 main research programs. In the first phase of the study, recombinant *Pichia pastoris* strains producing microbial protransglutaminase both extracellular and intracellular were developed with genetic engineering techniques. To accomplish this step, *pro-mtg* gene which encodes pro-MTG was cloned into pPICZα-A expression vector and pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids were constructed and then, pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids were integrated into *Pichia pastoris* genome at the AOX1 locus. In the second part of this program, using Dot blot and SDS-PAGE analysis, the best expression strains were selected and named as E8 and I1, respectively.

The results obtained from this study are explained below:

- Depending on the metabolic engineering design, both for extracellular and intracellular expression systems of recombinant *Pichia pastoris*, a reverse primer and two different forward primers were designed to amplify *pro-mtg* gene. Intracellular forward primer and extracellular forward primer are 29bp oligonucleotides and composed of restriction enzyme recognition site, *Bsp*119I for intracellular primer and *Eco*RI for extracellular primer, start codon and 19 nucleotides from the 5' end of the *pro-mtg* gene sequence. Reverse primer is 30bp oligonucleotide and composed of *Xba*I restriction enzyme recognition site and 22 nucleotides from the 3' end of the *pro-mtg* gene sequence. *pro-mtg*$_{intra}$ and *pro-mtg*$_{extra}$ genes were amplified with the designed primers by PCR. Amplified *pro-mtg* genes were cloned into pPICZα-A expression vectors via restriction and ligation reactions. Constructed plasmids

were named as pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ and transferred into competent *E. coli* TOP 10 cells. True transformants were selected by Zeocin containing selective media, PCR, restriction digestion analyses and gene sequencing. Followed by transformant selection, plasmids were isolated and linearized by *Pme*I restriction enzyme. pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids were integrated into AOX1 locus of *P. pastoris* genome. By Dot blot and SDS-PAGE analyses best microbial protransglutaminase producing strains were selected.

- During genetic engineering experiments, we came across some new findings. First, after construction of pPICZα*A::pro-mt*$_{gextra}$ and pPICZα*A::pro-mt*$_{gintra}$ plasmids, *Xba*I restriction site within the plasmid was verified by gene sequencing. Although its recognition site was found in the plasmid, *Xba*I couldn't digest the recombinant vector. This phenomenon was probably the result of three dimensional conformations of the recombinant plasmids. Secondly, in the transfection of *Pichia pastoris,* plasmid DNA concentration was found to be key point. Although pPICZα A, B and C manual suggests using 5-10 µg of plasmid DNA, its concentration was found to be insufficient for cloning. Cloning efficiency is directly proportional with the DNA concentration and transfection of the *Pichia pastoris* cells with pPICZα*A::pro-mt*$_{gextra}$ and pPICZα*A::pro-mt*$_{gintra}$ plasmids were achieved with 90µg and 70µg of plasmid DNA, respectively.

In the second research program, a pilot scale bioreactor experiment with E8 clone was performed to investigate the cell growth and recombinant transglutaminase activity profile under controlled conditions of pH, temperature and oxygen. The pilot scale bioreactor system has 1 dm^3 working volume and it's including pH, temperature, stirring rate, high foam and dissolved oxygen controls. Pilot scale bioreactor experiment was performed at

pH set to 5.0, temperature set to 30^0C and dissolved oxygen level set to 20%. In the bioprocess, sorbitol was used as co-carbon source with methanol and at the beginning o the production phase it was fed to the system batchwise at an initial sorbitol concentration of 50 g L^{-1}. Methanol feeding profile was calculated due to specific growth rate of 0.03 h^{-1}, and it was fed to the bioreactor system regarding to predetermined feeding profile. The results obtained from pilot scale bioreactor experiment are given below:

- Sorbitol was totally utilized by recombinant *Pichia pastoris* cells within 30 hours.
- The maximum cell concentration was achieved as C_x=74.1 g L^{-1} at t=36 h of the bioprocess. Regarding to the cell growth profile, lag phase did not last long depending on the pulse sorbitol feed.
- The maximum recombinant microbial transglutaminase activity was observed as 4447.6 U L^{-1} at t=36 h of the bioprocess and it began to decrease after that point due to increased proteolytic activity of the media. For the first time, microbial transglutaminase was produced in active form with *Pichia pastoris* expression system.
- The maximum microbial protransglutaminase concentration was observed at t=42 h of the bioprocess with SDS-PAGE analyses. After that, its concentration began to decrease due to the increase in proteolytic activity of the fermentation medium.
- The maximum AOX activity was obtained as 39 U g CDW^{-1} at the beginning of the production phase, after that point it never reached such high levels and shown fluctuations throughout the bioprocess. After 27^{th} hour, due to the methanol accumulation in the fermentation medium, it increased for 15 hours.
- Proteolytic activity of the fermentation media increased throughout the bioprocess and the maximum total protease activity was detected as 1442.19 U cm^{-3} at 59^{th} hours of the bioprocess. A significant increase in

total protease activity and a significant decrease in microbial transglutaminase activity was observed between t=36 h to t=48 h.

- Organic acid profiles showed that, untill the 24th hour of the bioprocess fumaric acid, succinic acid, citric acid, pyruvic acid, acetic acid, and oxalix acid incorparated into TCA cycle reactions effectively, after that time they began to accumulate, sequentially. Among them, oxalic acid was the most effectively utilized organic acid throughout the bioprocess and it was detected at the very late hours of the production, t=48 h. Formic acid is the product of methanol utilization pathway and AOX catalyzes the formic acid formation. The highest formic acid concentration as 0.7814 g L^{-1} was detected at the beginning of the bioprocess when the maximum AOX activity was observed. Formic acid accumulation is the result of high AOX activity and after 3rd hour it was not detected in the fermentation media that means it was utilized in metabolic pathways of *Pichia pastoris*. Lactic acid was first detected at 18th hour of the bioprocess. After 36th hour of the bioprocess most of the organic acids take place in TCA cycle and lactic acid accumulation increased. As TCA cycle reactions require oxygen and lactic acid is the product of the fermentative mode of metabolism, It should be concluded that, after 36th hour, oxygen wasn't sufficient for metabolic activities of *Pichia pastoris*.

CHAPTER 5

CONCLUSION

In this study, for the first time, microbial transglutaminase was produced in active form by *Pichia pastoris* both intracellularly and extracellularly. *pro-mtg* genes which encodes pro-MTG from *Streptomyces mobaraenis* were cloned into pPICZα-A expression vector and pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids and then pPICZαA*::pro-mtg*$_{extra}$ and pPICZαA*::pro-mtg*$_{intra}$ plasmids were integrated into AOX1 locuses of different *Pichia pastoris* cells. After construction of recombinant *Pichia pastoris* cells', the strain having the highest transglutaminase production potential was selected by Dot Blot and SDS-PAGE analyses. Finally, a pilot scale bioreactor experiment was performed with the selected *Pichia pastoris* clone producing extracellular microbial protransglutaminase to investigate the cell growth and recombinant transglutaminase production. During pilot scale bioreactor experiment pH was set to 5.0, temperature was set to 30^0C and dissolved oxygen level was set to 20%. In the bioprocess, sorbitol was used as co-carbon source with methanol and at the beginning of the production phase sorbitol was fed to the system batch-wise at an initial sorbitol concentration of 50 g L^{-1}; whereas, where methanol was fed to the bioreactor with the specific growth rate of μ_0=0.03 h^{-1}. The maximum cell concentration was achieved as C_x=74.1 g L^{-1} at t=36 h of the bioprocess. The maximum recombinant microbial transglutaminase activity was observed as 4447.6 U L^{-1} at t=36 h of the bioprocess and then it decreased. The maximum microbial

protransglutaminase concentration was observed at t=42 h of the bioprocess. The maximum AOX activity was obtained as 39 U g CDW^{-1} at the beginning of the production phase. Proteolytic activity of the fermentation media increased throughout the bioprocess and the maximum total protease activity was detected as 1442.19 U cm^{-3} at t=59 h.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

Genetic Engineering Experiment Solutions

Alkaline Lysis Solution I	50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0). The buffer was prepared from stock solutions in batches of 100 ml. The buffer was autoclaved and stored at 4^{0}C.
Alkaline Lysis Solution II	0.2 N NaOH, 1% (w/v) SDS. The buffer was prepared fresh and used at room temperature.
Alkaline Lysis Solution III	60 ml of 5 M Potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of dH_2O was mixed. The solution was stored at 4^{0}C.
Yeast Lysis Solution for Genomic DNA Isolation	2 g Triton X-100, 1 g SDS, 5.844g NaCl, 10 ml of Tris-Cl pH 8.0, 0.37224 g Na_2EDTA was dissolved in dH_2O and the final volume was made up to 1L. The solution was autoclaved at 121 ^{0}C for 20 minutes and stored at room

	temperature.
0.5 M EDTA	18.61 g Ethylenediaminetatra acetic acid disodium salt dehydrate was dissolved in 80 ml dH_2O. NaOH was added until EDTA was dissolved. The final pH was adjusted to pH 8.0 and the final volume was adjusted to 100 ml with d H_2O. The buffer was autoclaved at 121 ^0C for 20 minutes and stored at room temperature.
1 M Tris-Cl, pH 8.0	12.1 g Tris base was dissolved in 80 ml of dH2O and the pH was adjusted to 8.0 by added concentrated HCl. The volume was made up to 100 ml with dH2O. The buffer was autoclaved at 121 ^oC for 20 minures and stored at room temperature.
3 M Sodium acetate, pH 5.2	24.6 g sodium acetate was dissolved in 80 ml dH2O and the pH was adjusted to 5.2 with 3 M acetic acid. Sodium acetate buffer was filter sterilized and stored at 2-8 ^oC.
5X TBE	54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) was dissolved in dH_2O and the final volume made up to 1 L with sterile dH_2O. Before use the stock solution was diluted 1:5 with dH_2O.
10 X TE Buffer (pH 8.0)	10 ml of 1 M Tris-Cl (pH 8.0) , 2 ml of 0.5 M

EDTA (pH 8.0) was mixed with dH_2O. Final volume was made up to 100ml with dH_2O. The buffer was autoclaved at 121 ^0C for 20 minutes and stored at room temperature. Before use the stock solution was diluted 1:10 with dH_2O.

Dot-Blot Analysis Solutions

10 X TBS Buffer — 12.11 g Tris-base and 87.66 g NaCl was dissolved in 900ml dH_2O. pH was adjusted to 7.6 with glacial acetic acid. The buffer was stored at room temperature.

TBS-T Solution — 0.1% (v/v) Tween 20 was added into 1 X TBS solution. The buffer was prepared on the day of use.

TBS-T Milk Solution — 5 % (w/v) non-fat milk powder was dissolved in TBS-T. The solution was prepared on the day of use.

Transglutaminase Activit Assay Buffers

350 mM Tris-HCl pH 8.0 — 10.5 ml of Tris-HCl pH 8.0 stock solution was diluted with 19.5 ml dH_2O.

1 M Tris Buffer, pH 6.0 — 6.057 g Trizma base dissolved in 50 ml dH_2O. pH was adjusted to 6.0 with glacial acetic acid.

200 mM Hydroxylamine with 20 mM glutathione	0.13898 g hydroxylamine and 0.061464 g reduced glutathione were dissolved in 10 ml dH_2O.
10 mM L-Glutomic Acid γ-Monohydroxamate Solution	0.016214 g L-glutomic acid γ-monohydroxamate was dissolved in 10 ml dH_2O.
12 % TCA Solution	3 ml of TCA Solution (6.1 N) was mixed with 22 ml dH_2O. The solution was stored in dark bottle.
5 % (w/v) $FeCl_3$	5 g $FeCl_3$ was dissolved in 100 ml 0.1 M HCl.
Transglutaminase Reaction Cocktail	101 mg Z-Gln-Gly, 2 ml of Tris Buffer pH 6.0 and 5 ml of hydroxylamine glutathione solution was mixed by inversion. pH was adjusted to 6.0 at 37^0C with 0.1 NaOH. The final volume made up to 10 ml with dH_2O.
Stop Solution	1 volume of 3M HCl, 1 volume of 12% TCA and 1 volume of 5% $FeCl_3$ was mixed. The solution stored in dark bottle.
Dispase Buffer	50mM Tris-HCl Buffer pH 8.0, 300mM NaCl, 2mM $CaCl_2$, 1 mM glutathione. The buffer was

	prepared with dH_2O.

SDS-PAGE Solutions

10% (w/v) APS (Ammonium Persulfate)	0.1 g ammonium persulfate was dissolved in 1 ml of dH_2O. The solution was prepared freshly.
1.5 M Tris-HCl, pH 8.8	36.3 g tris base was dissolved in 150 mL dH_2O and pH was adjusted to 8.8 with 6 N HCl. The buffer was made up to 200 mL with dH_2O. The buffer was autoclaved at 120^0C for 20 min and stored at 2-8°C.
0.5 M Tris-HCl , pH 6.8	12.1 g Tris base was dissolved in 150 mL dH_2O and pH was adjusted to 6.8 with 6N HCl. The buffer was made up to 200 mL with dH_2O. The buffer was autoclaved at 120^0C for 20 min and stored at 2-8°C.
Stacking Solution (5%)	2.8mL dH_2O, 0.85mL 30% Acrylamide-bis, 1.25mL 0.5M Tris-HCl pH6.8, 50µL 10% SDS was mixed. Prior to gel mixture pour into gel cast, 25µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine were added and mixed by swirling.
Resolving Solution (12%)	3.4 mL dH_2O, 4 mL 30% Acrylamide-bis, 2.5 mL

	1.5 M Tris-HCl pH 8.8, 100 µL 10% SDS was mixed. Prior to gel mixture pour into gel cast 50 µL APS and 5µL N,N,N',N'-Tetramethylethylenediamine were added and mixed by swirling.
4 x Sample Loading Buffer for SDS-PAGE	200 mM Tris-HCl pH 6.8, 40% glycerol, 6% SDS, 0.013% Bromophenol blue, 10% 2-mercaptoethanol was mixed. 1 ml of the mixture was distributed into microcentrifuge tubes and stored at -20°C.
5 x SDS-PAGE Running Buffer	15 g Tris Base, 72 g glycine, 5g SDS was dissolved in dH_2O, final volume was made up to 1 L with dH_2O. The buffer was stored at 2-8°C and diluted 1:5 with dH_2O prior to each use.
Fixer Solution	150 mL methanol, 36 mL acetic acid, 150 µL 37% formaldehyde and 114 ml dH_2O was mixed.
Pretreatment Solution	0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) was dissolved in 400 ml distilled water.
Silver Nitrate Solution	0.8 g silver nitrate was dissolved in 400 mL

	distilled water and 300 µL of 37% formaldehyde was added into mixture and mixed.
Developing Solution	9 g potassium carbonate was dissolved in 400 mL distilled water. 8 mL from pretreatment solution and 300 µL 37% formaldehyde were added into solution.
Stop Solution	200 mL methanol, 48 mL acetic acid and 152 ml dH_2O was mixed together.

AOX Activity Assay Solutions

Yeast Lysis Buffer	100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10% glycerol. 1 protease inhibitor coctail tablet was added for every 50 mL of buffer. The buffer was filter sterilized and stored at +4°C for 3 months.
1 M potassium phosphate buffer, pH 7.5	1 M KH_2PO_4, 1M K_2HPO_4 was dissolved in dH_2O and KH_2PO_4 was tittered with K_2HPO_4 while controlling the pH. The buffer was autoclaved and stored at room temperature.

Protease Activity Assay Solutions

0.05 M Sodium Acetate	0.713 ml acetic acid was mixed with 24.287 ml

buffer (for acidic proteases)	dH_2O. 2.052 g sodium acetate was dissolved in 50 ml dH_2O. Sodium acetate solution was adjusted to pH 5.0 with acetic acid solution. Then, the solution was diluted to 500 ml with dH_2O. The buffer was autoclaved and stored at +4°C.
0.05 M Sodium Phosphate Buffer (for neutral proteases)	6.70 g $Na_2HPO_4.7H_2O$ was dissolved in 50 mL dH_2O. 3.90 g $NaH_2PO_4.2H_2O$ was dissolved in 50 mL dH_2O. Titrate till pH 7.0 and final V=50 mL. The dilute to 500 mL. Autoclave and store at room temperature.
Borate Buffer (for alkali proteases)	2.381 g Boraks ($Na_2B_4O_7.10H_2O$) was dissolved in 250 ml dH_2O. pH was adjusted to 10 with 1 M NaOH and the volume made up to 500 ml with dH_2O. The buffer was filtered and store at 4^0C.

<u>Fermentation Medium</u>

1 M potassium phosphate buffer, pH 6.0	56.48 g KH_2PO_4, 14.8 g K_2HPO_4 was dissolved in dH_2O and the volume made up to 500 mL. The pH was adjusted to 6.0 and the buffer was autoclaved and stored at room temperature.
Antifoam	10% (v/v) antifoam solution was prepared with dH_2O. Can be autoclaved once.
Base	25% NH_3OH (Sigma). No need to sterilize.

APPENDIX B

GROWT MEDIA

All growth media were autoclaved at 121°C for 20 minutes for sterilization and appropriate antibiotics were added after cooling to approximately 50°C.

Table B.1 Composition of solid YPD medium

Compound	**Concentration (g L^{-1})**
Yeast extract	10
Peptone	20
Glucose	20
Agar	20
Zeocin	0.15

Table B.2 Composition of solid LSLB medium

Compound	**Composition (g L^{-1})**
Tryptone	10
Yeast extract	5
NaCl	5
Zeocin	0.025
Agar	15

Table B.3 Composition of precultivation medium, BMGY

Compound	**Composition (g L^{-1})**
Yeast extract	10
Peptone	20
Potassium phosphate buffer (pH=6.0)	0.1 M
Yeast Nitrogen Base (YNB) (w/o aminacids)	13.4
Biotin	4 x 10^{-5}
Glycerol	10
Chloramphenicol (34 mg ml^{-1})	1 ml L^{-1}

Table B.4 Production medium of *P. pastoris* for laboratory scale air filtered shake bioreactor experiments

Compound	**Composition (g L^{-1})**
Methanol	1 ml
Sorbitol	30
Ammonium sulphate	4.35
Potassium phosphate buffer (pH=6.0)	0.1 M
$MgSO_4 \cdot 7H_2O$	14.9
$CaSO_4 \cdot 2H_2O$	1.17
Chloramphenicol (34 mg ml^{-1})	1 ml
PTM1	4.35 ml

Table B.5 Trace salt solution (PTM1) composition

Compound	Composition (g L^{-1})
$CuSO_4 \cdot 5H_2O$	6
NaI	0.08
$MnSO_4 \cdot H_2O$	3
$Na_2MoO_4 \cdot 2H_2O$	0.2
H_3BO_3	0.02
$ZnCl_2$	20
$FeSO_4 \cdot 7H_2O$	65
$CoCl_2$	0.5
H_2SO_4	5
Biotin	0.2

Table B.6 BSM medium composition

Compound	**Composition (g L^{-1})**
85% H_3PO_4	26.7 ml
$CaSO_4 \cdot 2H_2O$	1.17
$MgSO_4 \cdot 7H_2O$	14.9
KOH	4.13
K_2SO_4	18.2
Glycerol	40

APPENDIX C

NUCLEOTIDE SEQUENCES AND PLASMIDS

Sequence of Microbial Protansglutaminase Gene (1131 bp)

GACAATGGCGCGGGGGAAGAGACGAAGTCCTACGCCGAAACCTACCGCCTCACGG
CGGATGACGTCGCGAACATCAACGCGCTCAACGAAAGCGCTCCGGCCGCTTCGAGC
GCCGGCCCGTCGTTCCGGGCCCCCGACTCCGACGACAGGGTCACCCCTCCCGCCGA
GCCGCTCGACAGGATGCCCGACCCGTACCGTCCCTCGTACGGCAGGGCCGAGACGG
TCGTCAACAACTACATACGCAAGTGGCAGCAGGTCTACAGCCACCGCGACGGCAGG
AAGCAGCAGATGACCGAGGAGCAGCGGGAGTGGCTGTCCTACGGCTGCGTCGGTG
TCACCTGGGTCAATTCGGGTCAGTACCCGACGAACAGACTGGCCTTCGCGTCCTTCG
ACGAGGACAGGTTCAAGAACGAGCTGAAGAACGGCAGGCCCCGGTCCGGCGAGAC
GCGGGCGGAGTTCGAGGGCCGCGTCGCGAAGGAGAGCTTCGACGAGGAGAAGGG
CTTCCAGCGGGCGCGTGAGGTGGCGTCCGTCATGAACAGGGCCCTGGAGAACGCCC
ACGACGAGAGCGCTTACCTCGACAACCTCAAGAAGGAACTGGCGAACGGCAACGAC
GCCCTGCGCAACGAGGACGCCCGTTCCCCGTTCTACTCGGCGCTGCGGAACACGCC
GTCCTTCAAGGAGCGGAACGGAGGCAATCACGACCCGTCCAGGATGAAGGCCGTCA
TCTACTCGAAGCACTTCTGGAGCGGCCAGGACCGGTCGAGTTCGGCCGACAAGAGG
AAGTACGGCGACCCGGACGCCTTCCGCCCCGCCCCGGGCACCGGCCTGGTCGACAT
GTCGAGGGACAGGAACATTCCGCGCAGCCCCACCAGCCCCGGTGAGGGATTCGTCA
ATTTCGACTACGGCTGGTTCGGCGCCCAGACGGAAGCGGACGCCGACAAGACCGTC
TGGACCCACGGAAATCACTATCACGCGCCCAATGGCAGCCTGGGTGCCATGCATGTC
TACGAGAGCAAGTTCCGCAACTGGTCCGAGGGTTACTCGGACTTCGACCGCGGAGC

CTATGTGATCACCTTCATCCCCAAGAGCTGGAACACCGCCCCCGACAAGGTAAAGCA
GGGCTGGCCGTGA

Sequence of pPICZα-A (3593 bp)

agatctaacatccaaagacgaaaggttgaatgaaacctttttgccatccgaca
tccacaggtccattctcacacataagtgccaaacgcaacaggaggggatacac
tagcagcagaccgttgcaaacgcaggacctccactcctcttctcctcaacacc
cacttttgccatcgaaaaaccagcccagttattgggcttgattggagctcgct
cattccaattccttctattaggctactaacaccatgactttattagcctgtct
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aatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgt
aacctatatagtataggatttttttgtcattttgtttcttctcgtacgagct
tgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggggtttg
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cttcaaaatgtttctactccttttttactcttccagattttctcggactccgc
gcatcgccgtaccacttcaaaacacccaagcacagcatactaaattttccctc
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aaagagaccgcctcgtttctttttcttcgtcgaaaaaggcaataaaaatttt
atcacgtttctttttcttgaaatttttttttttagtttttttctctttcagtg
acctccattgatatttaagttaataaacggtcttcaatttctcaagtttcagt
ttcatttttcttgttctattacaacttttttacttcttgttcattagaaaga
aagcatagcaatctaatctaaggggcggtgttgacaattaatcatcggcatag
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gaccagtgccgttccggtgctcaccgcgcgcgacgtcgccggagcggtcgagt
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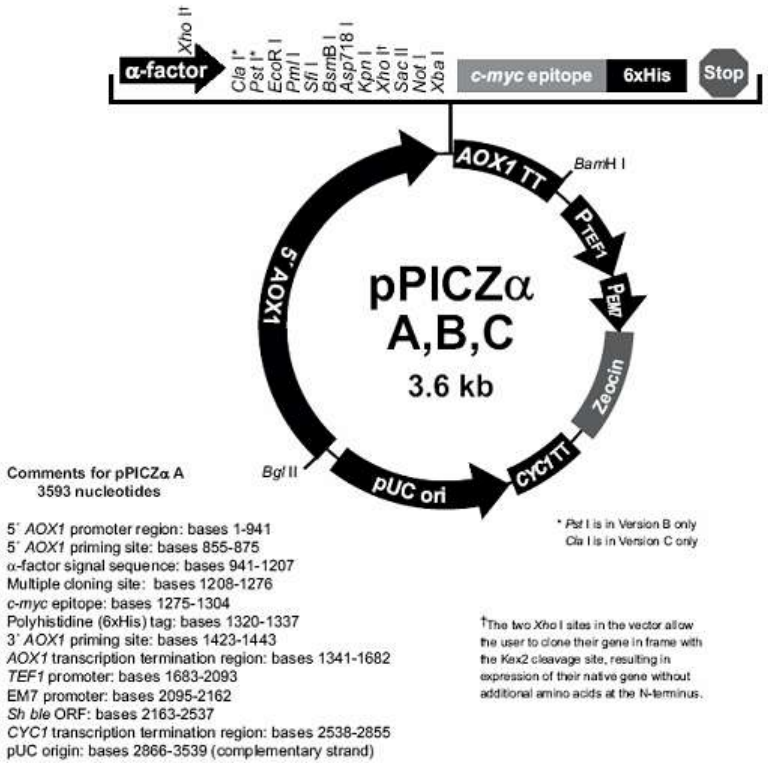

Figure B.1 Schematic representation of pPICZα-A (Invitrogen, 2001)

Multiple Cloning Sites of pPICZα-A Expression Vector

Sequence of pPICZαA*::pro-mtg$_{extra}$* (4664 bp)

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGG
TCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTG
CAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACCAGC
CCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACAC
CATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCC
GAATGCAACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTG
GGGTCAAATAGTTTCATGTTCCCCAAATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGG
AACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAAGTTTGGTTCGTTGAAATG
CTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTTTGG
TATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCT
TCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGAT
TATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGATAGCCTA
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TTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTT
GCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATT
GCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTC**ATGGACA**
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CGGGCCCCCGACTCCGACGACAGGGTCACCCCTCCCGCCGAGCCGCTCGACAGGATGCCCG
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TGGCTGTCCTACGGCTGCGTCGGTGTCACCTGGGTCAATTCGGGTCAGTACCCGACGAACA
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AAGGAGCGGAACGGAGGCAATCACGACCCGTCCAGGATGAAGGCCGTCATCTACTCGAAGC
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CGCCTTCCGCCCCGCCCCGGGCACCGGCCTGGTCGACATGTCGAGGGACAGGAACATTCCG
CGCAGCCCCACCAGTCCCGGTGAGGGATTCGTCAATTTCGACTACGGCTGGTTCGGCGCCC
AGACGGAAGCGGACGCCGACAAGACCGTCTGGACCCACGGAAATCACTATCACGCGCCCAA
TGGCAGCCTGGGTGCCATGCATGTCTACGAGAGCAAGTTCCGCAACTGGTCCGAGGGTTAC
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TCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTT
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CAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACT
AAATTTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAA
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GGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGG
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AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACG
AAAACTCACGTTAAGGGATTTTGGTCATGAGATC

Underlined sequences are restriction enzyme recognition sites: *EcoR*I and *Xba*I, respectively.

Bold sequence is the nucleotide sequence of *pro-mtg*.

Sequence of pPICZαA*::pro-mtg*$_{intra}$ (4391 bp)

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGG
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CCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACAC
CATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCC
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AACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAAGTTTGGTTCGTTGAAATG
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CAGGATGAAGGCCGTCATCTACTCGAAGCACTTCTGGAGCGGCCAGGACCGGTCGAGTTCG
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GCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTC
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TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGA
TC

Underlined sequences are restriction enzyme recognition sites: *Bsp*119I and *Xba*I, respectively.

Bold sequence is the nucleotide sequence of *pro-mtg*.

APPENDIX D

PROPERTIES OF DESIGNED PRIMERS

Reverse Primer

(XbaI RE) (*pro-mtg* gene sequence 22nt)
5′- CC TCT AGA TCA CGG CCA GCC CTG CTT TAC C-3′

Reverse Primer

```
Name           : Reverse Primer
Primer         : 5´-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3´
Reverse        : 3´-CCATTTCGTCCCGACCGGCACTAGATCTCC-5´
Length         : 30 nt

Tm (basic)     : 96,0 °C
Tm (salt)      : 76,2 °C
Tm (NN)        : 75,2 °C

GC %           : 60,0 %
dG             : -67,5 kCal/mol

3'-tail GC %   : 42,9 %
3'-tail dG     : -8,0 kCal/mol

Molecular weight : 9100,8 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 3,49 µM
and contains 31,8 µg ssDNA
```

Reverse Primer self annealing:

```
                    5'-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3'
                         ||||||
3'-CCATTTCGTCCCGACCGGCACTAGATCTCC-5'
 dG: -7,35 kcal/mol

  5'-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3'
                ||||
3'-CCATTTCGTCCCGACCGGCACTAGATCTCC-5'
```

```
 dG: -7,28 kcal/mol

5'-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3'
       :: : :: |||  ::: :: : ::
  3'-CCATTTCGTCCCGACCGGCACTAGATCTCC-5'
 dG: -3,93 kcal/mol

              5'-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3'
                    :  ||||  :
3'-CCATTTCGTCCCGACCGGCACTAGATCTCC-5'
 dG: -3,30 kcal/mol

5'-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3'
                :   |||   :::   :
           3'-CCATTTCGTCCCGACCGGCACTAGATCTCC-5'
 dG: -2,46 kcal/mol

5'-CCTCTAGATCACGGCCAGCCCTGCTTTACC-3'
                  |||  :::
        3'-CCATTTCGTCCCGACCGGCACTAGATCTCC-5'
 dG: -0,88 kcal/mol
```

Reverse Primer loops:

```
5'-CCTCTAGATCACGGCCAGCC
                :   ||| C
           3'-CCATTTCGT
 dG: -2,04 kcal/mol
```

Intracellular Forward Primer

（Bsp119I) (start codon) (*pro-mtg* gene 19nt)

5′- G TTC GAA ATG GAC AAT GGC GCG GGG GAA G-3′

Intracellular Forward Primer

```
Name          : Intracellular Forward Primer
Primer        : 5´-GTTCGAAATGGACAATGGCGCGGGGGAAG-3´
Reverse       : 3´-GAAGGGGGCGCGGTAACAGGTAAAGCTTG-5´
Length        : 29 nt

Tm (basic)    : 92,0 °C
Tm (salt)     : 74,7 °C
Tm (NN)       : 72,7 °C

GC %          : 58,6 %
dG            : -63,3 kCal/mol

3'-tail GC %  : 71,4 %
```

```
3'-tail dG    : -12,5 kCal/mol

Molecular weight : 9158,9 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 2,95 µM
and contains 27,0 µg ssDNA
```

Intracellular Forward Primer self annealing:

```
                      5'-GTTCGAAATGGACAATGGCGCGGGGGAAG-3'
                          ||||||
3'-GAAGGGGGCGCGGTAACAGGTAAAGCTTG-5'
 dG: -6,74 kcal/mol

5'-GTTCGAAATGGACAATGGCGCGGGGGAAG-3'
               :    ||||    :
         3'-GAAGGGGGCGCGGTAACAGGTAAAGCTTG-5'
 dG: -6,66 kcal/mol

5'-GTTCGAAATGGACAATGGCGCGGGGGAAG-3'
                     ||||
           3'-GAAGGGGGCGCGGTAACAGGTAAAGCTTG-5'
 dG: -5,14 kcal/mol

5'-GTTCGAAATGGACAATGGCGCGGGGGAAG-3'
    |||      : :: :: :      :::
3'-GAAGGGGGCGCGGTAACAGGTAAAGCTTG-5'
 dG: -0,62 kcal/mol
```

Intracellular Forward Primer loops:

```
5'-GTTCGAAATGGACA
    |||      : : A
3'-GAAGGGGGCGCGGT
 dG: -0,20 kcal/mol
```

Extracellular Forward Primer

(EcoR1) (start codon) (*pro-mtg* gene 19nt)

5′- G GAA TTC ATG GAC AAT GGC GCG GGG GAA G -3′

Extracellular Forward Primer

```
Name          : Extracellular Forward Primer
Primer        : 5´-GGAATTCATGGACAATGGCGCGGGGGAAG-3´
Reverse       : 3´-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5´
Length        : 29 nt
```

```
Tm (basic)     : 92,0 °C
Tm (salt)      : 74,7 °C
Tm (NN)        : 73,0 °C

GC %           : 58,6 %
dG             : -64,1 kCal/mol

3'-tail GC %   : 71,4 %
3'-tail dG     : -12,5 kCal/mol

Molecular weight : 9158,9 g/mol

1 ml of the primer solution with an
absorbance of 1 at 260 nm is 2,95 µM
and contains 27,0 µg ssDNA
```

Extracellular Forward Primer self annealing:

```
5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
               :    ||||    :
         3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: -6,66 kcal/mol

                     5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
                         ||||||
3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: -5,54 kcal/mol

5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
                     ||||
           3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: -5,14 kcal/mol

             5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
                    : |||| :
3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: -2,29 kcal/mol

5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
       |||     :      :     :::
   3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: -0,62 kcal/mol

      5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
               ||| : : :::
3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: -0,08 kcal/mol

          5'-GGAATTCATGGACAATGGCGCGGGGGAAG-3'
             :  |||       :::  :
3'-GAAGGGGGCGCGGTAACAGGTACTTAAGG-5'
 dG: 1,02 kcal/mol
```

Extracellular Forward Primer loops:

```
5'-GGAATTCATGGACAAT
       |||     :   )
   3'-GAAGGGGGCGCGG
 dG: -0,20 kcal/mol

      5'-GGAATTCATGG
                |||  A
3'-GAAGGGGGCGCGGTAAC
 dG: 0.34 kcal/mol

          5'-GGAATTCAT
             :  |||   G
3'-GAAGGGGGCGCGGTAACAG
 dG: 1,44 kcal/mol
```

APPENDIX E

MOLECULAR WEIGHT MARKERS

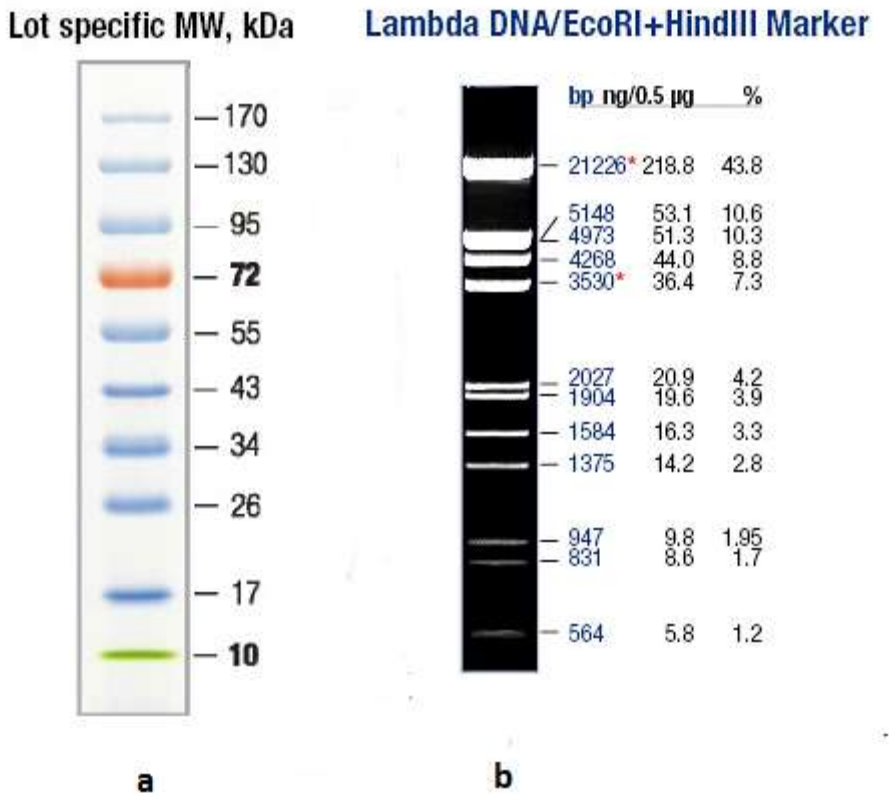

Figure E.1 Molecular weight markers used in this study. **(a)** PageRuler Prestained Protein Ladder used in SDS-PAGE analyses, **(b)** Lambda DNA/*EcoR* I HindIII Marker used in Agarose Gel Electrophoresis.

APPENDIX F

CALIBRATION CURVE FOR SORBITOL CONCENTRATION

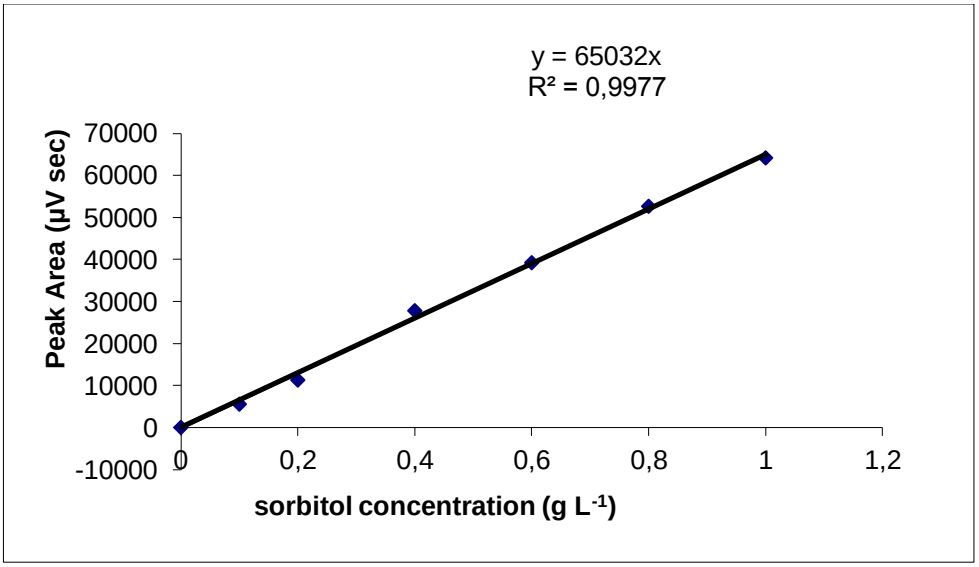

Figure F.1 Calibration curve for sorbitol concentration; analysis was performed by HPLC.

APPENDIX G

CALIBRATION CURVE FOR METHANOL CONCENTRATION

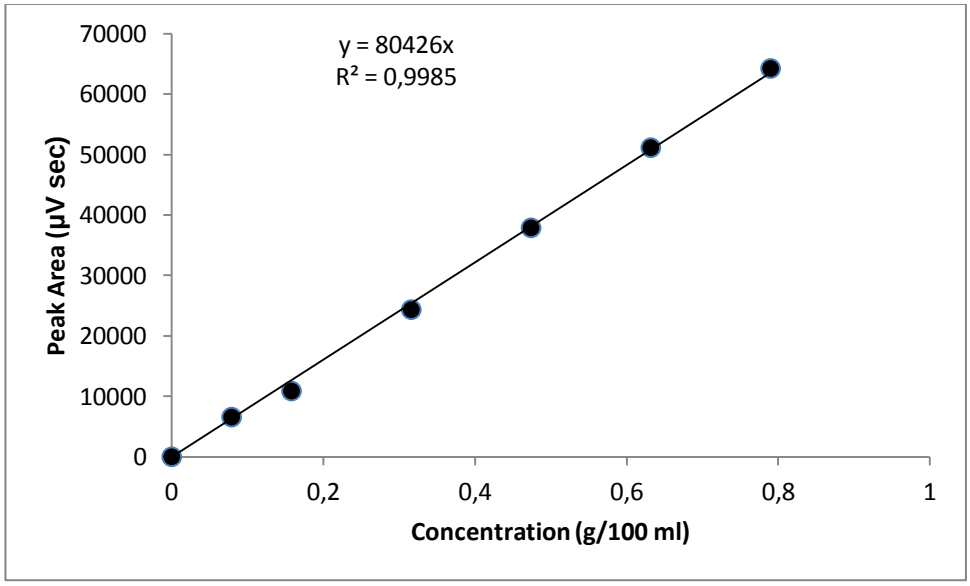

Figure G.1 Calibration curve for methanol concentration; analysis was performed by HPLC.

APPENDIX H

CALIBRATION CURVE FOR ORGANIC ACID CONCENTRATION

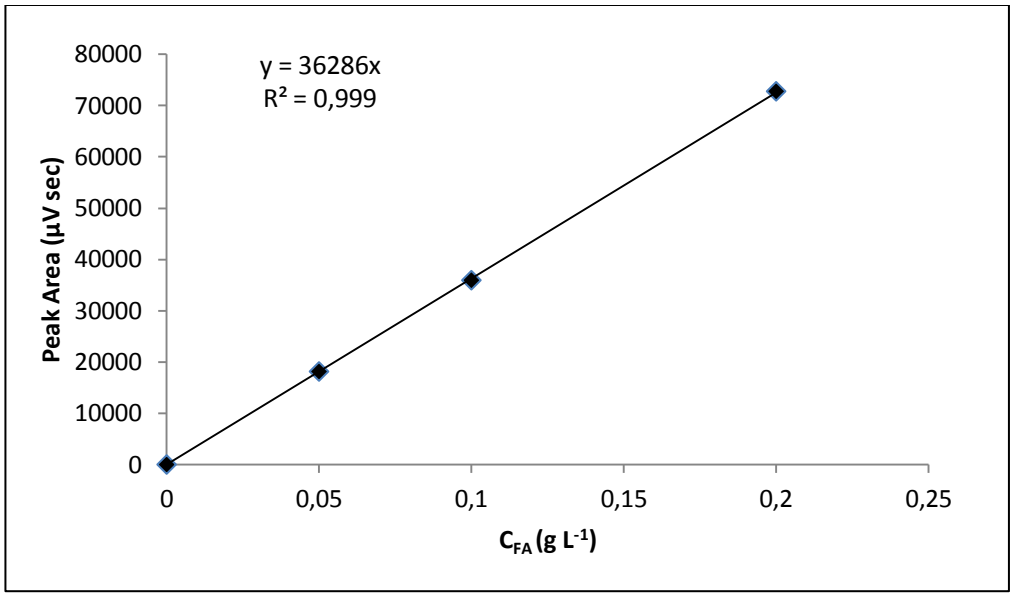

Figure H.1 Calibration curve obtained for formic acid concentration; analysis was performed by HPLC.

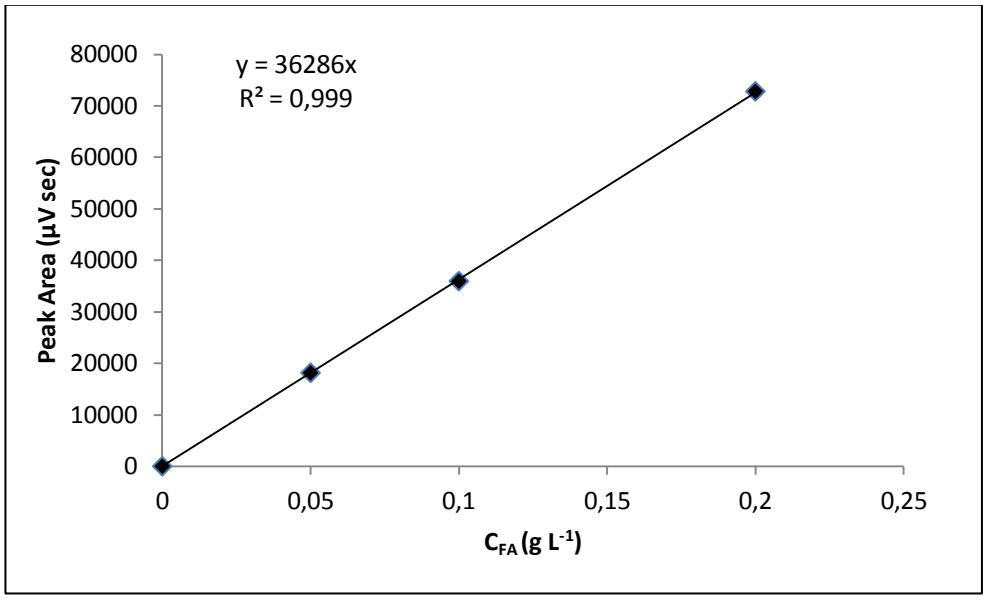

Figure H.2 Calibration curve obtained for fumaric acid concentration; analysis was performed by HPLC.

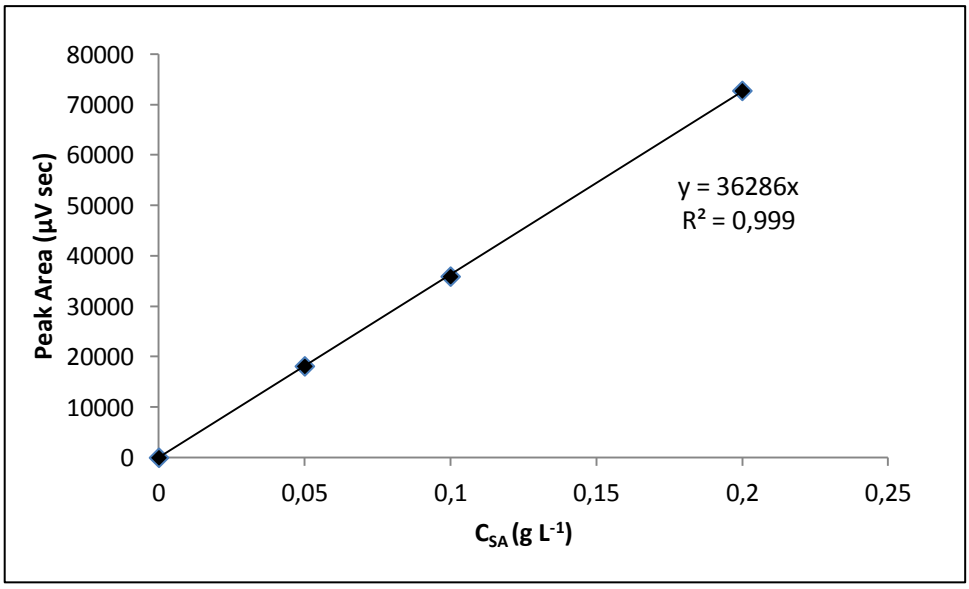

Figure H.3 Calibration curve obtained for succinic acid concentration; analysis was performed by HPLC.

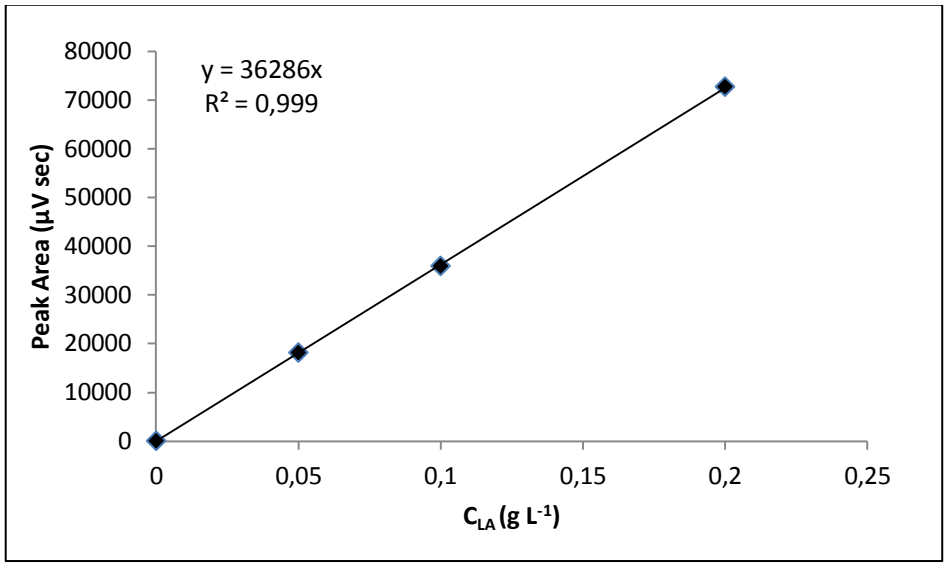

Figure H.4 Calibration curve obtained for lactic acid concentration; analysis was performed by HPLC.

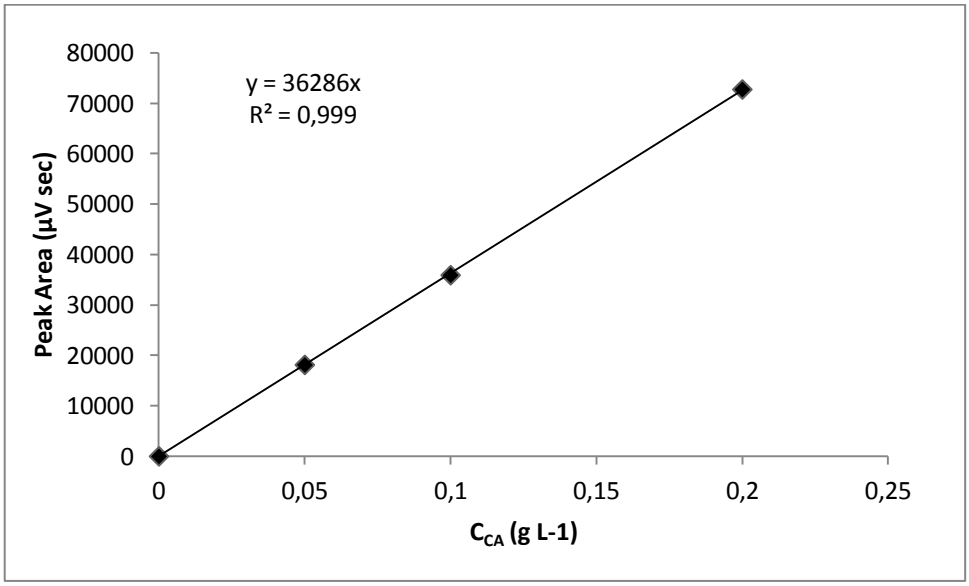

Figure H.5 Calibration curve obtained for citric acid concentration; analysis was performed by HPLC

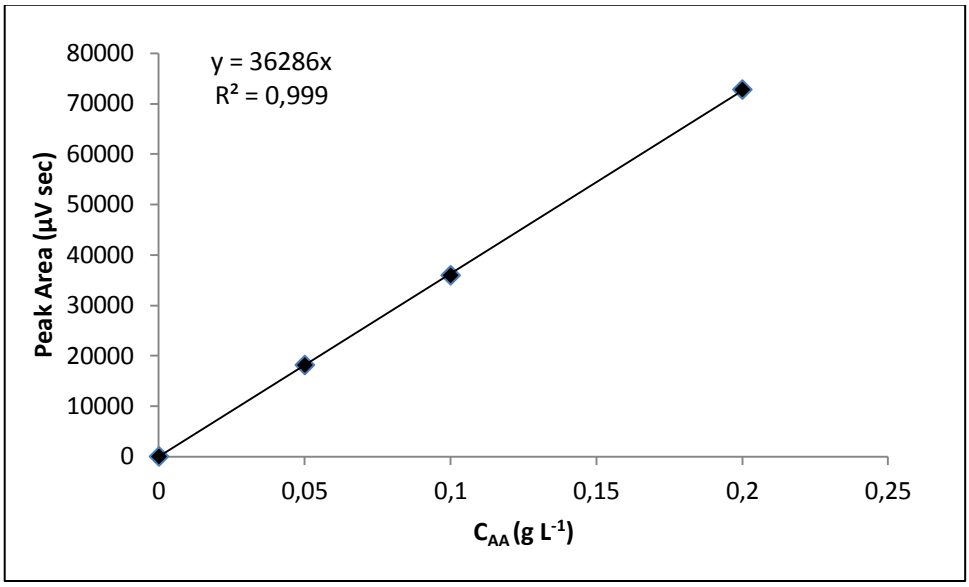

Figure H.6 Calibration curve obtained for acetic acid concentration; analysis was performed by HPLC.

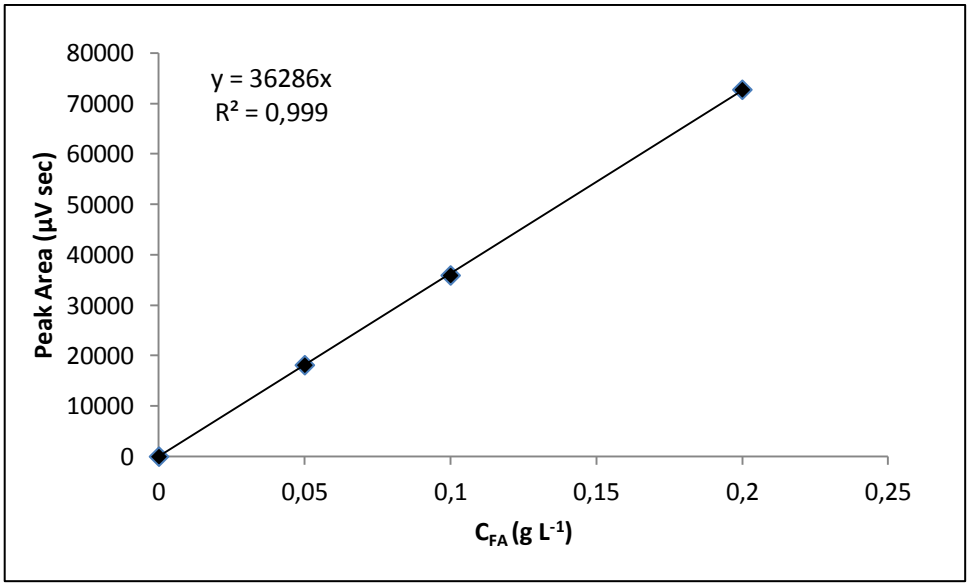

Figure H.7 Calibration curve obtained for oxalic acid concentration; analysis was performed by HPLC.

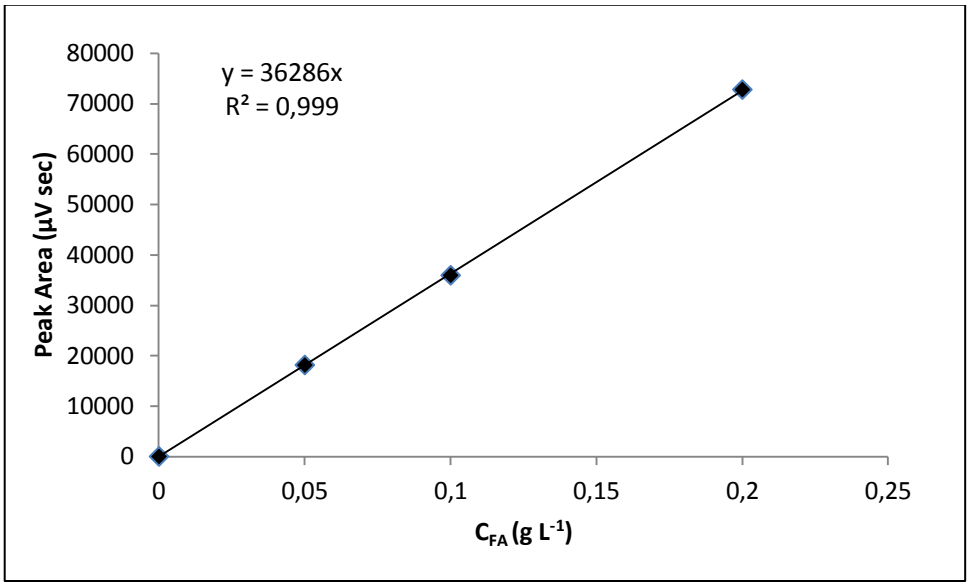

Figure H.8 Calibration curve obtained for pyruvic acid concentration; analysis was performed by HPLC

APPENDIX I

CALIBRATION CURVE FOR AOX ACTIVITY ASSAY

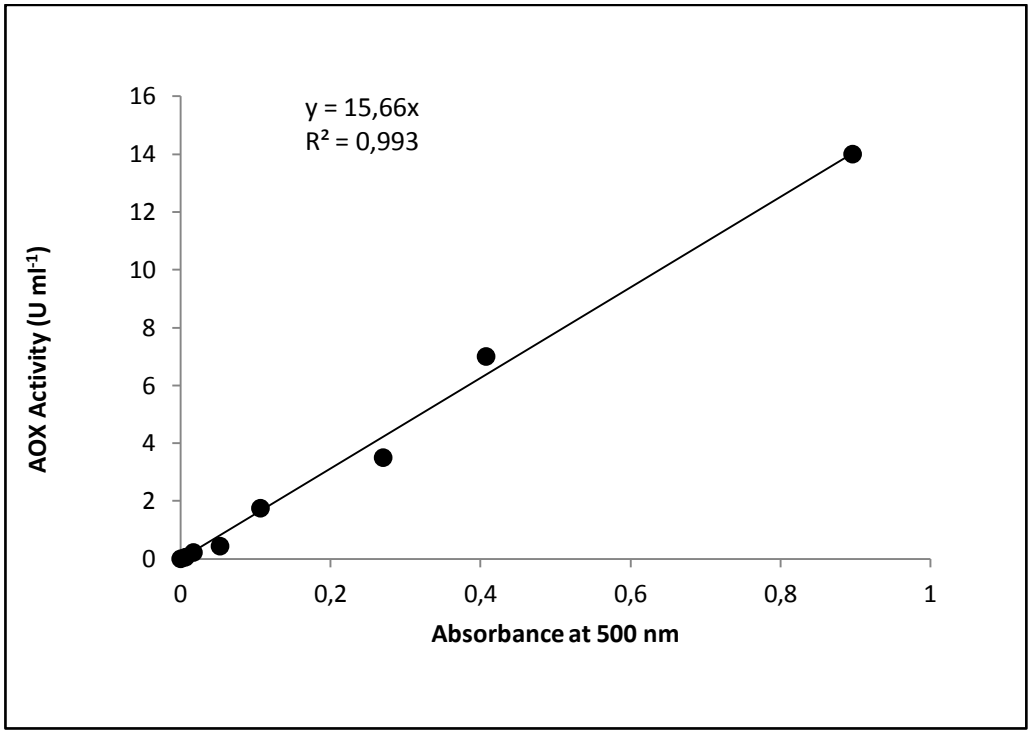

Figure I.1 Calibration curve for AOX activity assay.