

**T.C.
AKDENİZ UNIVERSITY**



**EXTRACELLULAR PRODUCTION OF RECOMBINANT MICROBIAL
TRANSGLUTAMINASE ENZYME in *Pichia pastoris***

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INSTITUTE OF NATURAL AND APPLIED SCIENCES

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This thesis in 20.10.2023 unanimously accepted by the jury.

Assoc. Prof. Dr. Aysun ÖZÇELİK (Supervisor)

Assoc. Prof. Dr. Münevver AKSOY

Assist. Prof. Dr. Tuğba ÖZAKTAŞ

ÖZET
REKOMBİNANT MİKROBİYAL TRANSGLUTAMİNAZ ENZİMİNİN *Pichia pastoris*'te HÜCRE DIŞI ÜRETİMİ

Leyla Nur KORKMAZ
Yüksek Lisans Tezi, Tarımsal Biyoteknoloji Anabilim Dalı
Danışman: Doç. Dr. Aysun ÖZÇELİK
Ekim 2023; 39 sayfa

Biyoteknolojik gelişmeler hızla ilerlerken, enzimler gıda sektöründe ve diğer pek çok alanda üretimin iyileştirilmesi, verimlilik ve kalite artışı için kullanılmaktadır. Transglutaminaz enzimi uzun zamandır biyoteknoloji alanında gıda katkı maddesi olarak kullanılan ve pek çok organizma tarafından sentezlenebilen bir enzimdir. Metilotrofik bir maya olan *Pichia pastoris* (*P. pastoris*) ekspresyon sistemi, son yıllarda birçok rekombinant proteinin üretiminde hem araştırma hem de endüstriyel amaçlarla başarıyla kullanılmaktadır ve en etkili heterolog protein ekspresyon sistemlerinden biridir.

Bu çalışmada, *Streptomyces mobaraensis* mikrobiyal transglutaminaz (MTGaz) enziminin *P. pastoris*'te alkol dehidrojenaz (*ADH*) promotörü kontrolünde hücre dışı ekspresyonu yapılmıştır. Bu amaçla, öncelikle pADH2 α -proMTGase ekspresyon vektörü oluşturulmuş ve *P. pastoris* KM71 suşuna transforme edilmiştir. Zeosin antibiyotikli agar plakalarda seçilen transformant *Pichia* klonları ile çalkalamalı erlenmayer ölçeğinde standart *Pichia* gelişim koşulları altında (28 °C sıcaklık, pH 6.0) protein ekspresyonu çalışması yapılmış ve protein ürettiği SDS-PAGE ile doğrulanan klon ile çalışmaya devam edilmiştir. Düşük sıcaklığın MTGaz proteininin üretiminde uygun olduğu daha önce ekibimiz tarafından yapılan çalışmada belirlenmiştir. Bu yüzden sıcaklık 20°C'de sabit tutularak üç farklı pH (3.0, 5.0 and 7.0) değerinde üretim tekrarlanmıştır. Çalkalamalı erlenmayerde 96 saatlik protein ekspresyonu sonucunda en iyi üretim pH 7.0 değerinde elde edilmiş ve ulaşılan protein miktarı 396 ng/mL olarak hesaplanmıştır. Enzim aktivitesi ise pH 7.0 için 10200 U/mL olarak belirlenmiştir.

Bu tez çalışması kapsamında *Streptomyces mobaraensis* MTGaz enzimi ilk kez *P. pastoris*'te *ADH* promotörü kontrolünde rekombinant olarak üretilmiştir. MTGaz enziminin üretiminde *ADH* promotorunun uygun bir promotor olduğu yapılan çalışma ile gösterilmiştir.

ANAHTAR KELİMELEER: ADH, mikrobiyal transglutaminaz, *Pichia pastoris*, rekombinant protein

JÜRİ: Doç. Dr. Aysun ÖZÇELİK

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ABSTRACT
EXTRACELLULAR PRODUCTION OF RECOMBINANT MICROBIAL
TRANSGLUTAMINASE ENZYME in *Pichia pastoris*

Leyla Nur KORKMAZ

MSc Thesis in Agricultural Biotechnology

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While biotechnological developments are advancing rapidly, enzymes are produced and used in the food industry and many other fields for improving production, increasing efficiency and quality. Transglutaminase enzyme is an enzyme that has been used as a food additive in the field of biotechnology for a long time and can be synthesized by many organisms. *Pichia pastoris* expression system, a methylotrophic yeast, has been successfully used in the production of many recombinant proteins for both research and industrial purposes in recent years and is one of the most effective heterologous protein systems.

In this study, extracellular expression of *Streptomyces mobaraensis* microbial transglutaminase (MTGase) enzyme was performed in *P. pastoris* under the control of alcohol dehydrogenase (*ADH*) promoter. For this purpose, firstly the pADH2 α -proMTGase expression vector was constructed and transformed into *P. pastoris* KM71 strain. Protein expression study was performed with transformant *Pichia* clones selected on zeocin antibiotic agar plates under standard *Pichia* growth conditions (28°C temperature, pH 6.0) in shake flask, and the study was continued with the protein producing clone which was confirmed by SDS-PAGE. It was determined in the previous study by our laboratory that low temperature is suitable for the production of MTGase protein. Therefore, the production was repeated under three different pHs (3.0, 5.0 and 7.0), while keeping the temperature constant at 20°C. As a result of 96 hours of protein expression in shake flask, the best production was obtained at pH 7.0 and the amount of protein reached was calculated as 396 ng/mL. The enzyme activity was determined as 10200 U/mL for pH 7.0.

Within the scope of this thesis, the *S. mobaraensis* MTGase enzyme was produced recombinantly under the control of *ADH* promoter in *P. pastoris* for the first time. It has been shown by the study that the *ADH* is a suitable promoter for the production of the MTGase enzyme.

KEYWORDS: *ADH*, *Pichia pastoris*, microbial transglutaminase, recombinant protein

COMMITTEE: Assoc. Prof. Dr. Aysun ÖZÇELİK

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This thesis is attributed to my friend Gökhan Demirağ.

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[CV](#)

AKADEMİK BEYAN

Yüksek Lisans Tezi olarak sunduğum “**EXTRACELLULAR PRODUCTION OF RECOMBINANT MICROBIAL TRANSGLUTAMINASE ENZYME in *Pichia pastoris***” adlı bu çalışmanın, akademik kurallar ve etik değerlere uygun olarak yazıldığını belirtir, bu tez çalışmasında bana ait olmayan tüm bilgilerin kaynağını gösterdiğimi beyan ederim.

...../...../20....

Leyla Nur KORKMAZ

İmzası

XXXXXX

SYMBOLS AND ABBREVIATIONS

Symbols

h	: Hour
Da	:Dalton
°C	: Celcius degree
U	: One unit of an enzyme
OD	: Optical density
kDa	: Kilo dalton
μ	: Mikro (10 ⁻⁶)
n	: Nano (10 ⁻⁹)
ms	: Millisecond
V	: Voltage
mA	: Milliamper
M	: Molar
L	: Litre
g	: Gram
m	:Minute
×g	: Centrifuge force
rpm	: Revolutions per minute
,	: Decimal separator

Abbrevations

ADH	: alcohol dehydrogenase
GAP	: glyceraldehyde 3-phosphate dehydrogenase
AOX	: alcohol oxydase
TGase	: transglutaminase
MTGase:	microbial transglutaminase
GRAS	: generally recognized as safe
YPD	:yeast extract peptone dextrose
LB	:Luria-Bertani
BMGY:	buffered glycerol- complex medium
BMEY:	buffered ethanol-complex medium
NCBI	:National Center for Biotechnology Information

SDS :sodium dodecyl sulfate
PAGE :sodium dodecyl sulfate
TGS :Tris-Glycine-SDS
TAE :Tris-asetat-EDTA
BSA :Bovine serum albumin
NFW :nuclease free water
DTT :dithiothreitol
PCR :polymerase chain reaction



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1. INTRODUCTION

The using of biotechnology industry also called as 'white biotechnology', predominantly relies on enzymes as biocatalysts and cellular factories, utilizing their innate or engineered capabilities to convert a wide range of renewable substrates into a diverse array of products for the food, feed, textile, pulp, chemistry, and energy sectors. The main objective of industrial biotechnology is to design environmentally friendly manufacturing strategies for chemicals, materials, pharmaceuticals, and bio-energy. The swift and secure technological advancements, genetic manipulation techniques, process improvements, modeling, novel cutting-edge tools, etc., along with the necessity for sustainable development and ongoing globalization and competition, act as the primary catalysts of industrial biotechnology (Olguin et al., 2012). Industrial biotechnology offers benefits by reducing energy consumption and utilization, while also minimizing expenses. Additionally, renewable resources are employed in place of non-renewable ones. In the microbial biotechnology approach, synthetic biology, microbial physiology, metabolic engineering, and pathway engineering are commonly utilized to generate highly complex natural products. Strategies for enhancing strains have reduced costs by increasing the productivity of the desired product and inhibiting the formation of undesired by products (Wolgmuth, 2009).

Enzymes are proteins found in the structure of organisms and they accelerate the reaction rates by entering into chemical reactions. In many fields since ancient times; enzymes secreted by living things are used for production of foodstuffs such as beer, bread, wine and cheese making. Today, developments in civilization have expanded the usage of enzymes in the food sector. These enzymes are used in the food industry, either by being modified or by using their natural strains, such as homogenization and preservatives.

In the contemporary era, there exists an unprecedented demand for products and services, surpassing anything experienced throughout history. To meet this ever-growing demand, diverse sources are being explored while innovative production strategies are continuously developed. Green technology, in particular, has gained significance in industrial processes, striving to protect the environment while ensuring sustainable economic expansion and production. Enzymes assume a crucial role in white biotechnology and bioprocesses, acting as indispensable components. Industrial operations necessitate substantial quantities of enzymes to sustain their operations. With this objective in mind, researchers are devising novel bioprocesses to obtain substantial quantities of enzymes and other protein-based compounds, including antibiotics, hormones, vaccines, and more.

The face of biotechnology has changed rapidly, at least in some respects, as recombinant DNA techniques have become applicable. This technology allows manipulation of DNA in vitro. A particular gene region of an organism can be isolated and transfer of this gene to another organism is possible. Also, nucleotide sequencing techniques allow a gene to be identified at nucleotide level. The amino acid sequence of a polypeptide encoded by a gene whose nucleotide sequence is known can be determined from the genetic code, and the structure of proteins can be changed by directed mutation methods. Furthermore, fully synthetic DNA molecules or genes can be produced. Again,

this technology allows controlled recombinations and addition of mutations with desired characteristics, characteristics that cannot be added naturally to an organism. In addition, microorganisms are also used for the production of animal, especially human proteins, which can be produced very little by classical methods and have a very high economic cost. The most striking example of this is the production of human insulin in *Escherichia coli* cells. Insulin, which was obtained from tons of pig pancreas and was very costly in the pre-recombinant DNA period, also caused allergic reactions in patients given due to antigenic incompatibility. With the help of recombinant DNA techniques, the human insulin gene was cloned and transferred to *E. coli* cells and human insulin was produced more economically. Moreover, allergic reactions caused by porcine insulin have also disappeared.

Transglutaminases were first discovered by Heinrich Waelsch in 1957 (Sarkar et al., 1957). It was initially discovered in guinea pig liver through research and has since been found in various living organisms across different kingdoms. In 1985, Folk and coworkers developed and produced transglutaminase (TGase) under in vivo conditions. TGase finds significant application in the food industry, primarily for improving the functional properties of proteins. The enzyme catalyzes the bonding of primary amines of proteins with the γ -carboxylamide group of reduced glutamine and various other primary amines. In the absence of amines as substrates, TGase catalyzes the deamination of reduced glutamine through hydrolysis. When applied in bread production, it has been observed that transglutaminase has the ability to enhance the structure, texture, and volume of the bread (Moore et al., 2006). Simultaneously, when used in the production of meat products, it contributes to increased firmness of the meat.

Pichia pastoris, also known as *Komagataella phaffii*, is a eukaryotic, single-celled methylotrophic yeast that possesses the ability to perform post-translational modifications required for the production of recombinant proteins. Compared to bacteria, *Pichia* has a higher expression yield and can proliferate rapidly in culture environments containing a carbon source. *P. pastoris* belongs to the Fungi kingdom, Ascomycota clade, Saccharomycetales order, Saccharomycetaceae family, and Komagataella genus. The *P. pastoris* expression system, which utilizes this methylotrophic yeast, has been successfully employed for the production of numerous recombinant proteins in both research and industrial settings in recent years (Higgins and Cregg, 1998). The expression system of *P. pastoris* has gained recognition as one of the most well-established heterologous protein systems, with successful expression reported for over five thousand proteins (Goodrick and Kurtzman, 2009; Spohner et al., 2015).

The *P. pastoris* expression system has gained popularity due to its user-friendly nature, cost-effectiveness, and rapid expression capabilities. Several factors, including oxygen levels, pH value, and temperature, can influence cell growth and impact the quantity of the desired product. Factors such as the choice of *Pichia* species, promoter selection, and gene copy number can also significantly affect the protein production levels (Cregg et al., 1993). These considerations highlight the importance of optimizing various parameters to achieve the desired protein expression and yield in the *P. pastoris* expression system.

Studies have indicated that the optimal temperature for *P. pastoris* cell growth is around 30°C (Cos et al., 2006). However, it has been observed that protein production

can still occur when the induction temperature is reduced to as low as 22 °C (Wang et al., 2009). Furthermore, research has shown that the optimal pH range for *P. pastoris* production lies between 3.0 and 7.0. These findings highlight the importance of temperature and pH control in optimizing protein expression and production in yeast (Cregg et al.,)

The aim of this thesis is to produce the microbial transglutaminase enzyme extracellularly in *P. pastoris* under the control of *ADH* promoter. For this purpose, the pro-MTGase gene was cloned into the pADH2 α -A vector and then plasmids were integrated into the genome of *P. pastoris* yeast, which contained the pADH2 α A-pro-MTGase construct for the protein production. Protein expression studies were carried out at three different pHs (3.0, 5.0 and 7.0) by keeping the temperature constant at 20°C. The protein produced was determined by total protein analysis, SDS-PAGE analysis and enzyme activity determination in the supernatant samples obtained.

2. LITERATURE REVIEW

2.1 Transglutaminase

Escherichia coli glutaminase (EC 3.5.1.2, L-glutaminase, glutamine aminohydrolase, glutaminase I) (GAC; GAM; KGA; GLS1; AAD20; DEE71; GDPAG; CASGID; EIEE71) is an enzyme which generates glutamate from glutamine which is crucial metabolic role for living organisms (Figure 2.1). The enzyme segregated from L-glutamic acid and ammonia (Stumvoll et al., 1999).

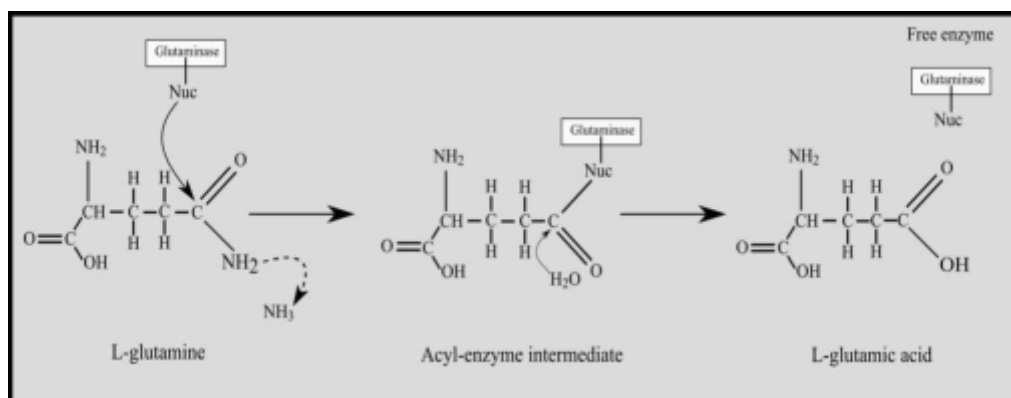


Figure 2.1:Chemical pathway of L-glutamine (Patel et al., 2021)

The transglutaminase enzyme derived from microorganisms is referred to as microbial transglutaminase. TGase from microorganisms is a single-unit protein comprising 331 amino acids, with a molecular weight of 38 kDa. It has three potential N-glycosylation sites. Within the cellular environment, TGase is synthesized in the form of an inactive proenzyme. To activate the pro-TGase enzyme, it is essential to remove the 45 amino acids prosequence (Özçelik et al., 2019)

Transglutaminases are enzymes that play a role in facilitating the interaction between a suitable nucleophile, referred to as the acyl acceptor and the carboxamide group of a glutamyl residue present in a protein or peptide substrate, known as the acyl donor. The acyl acceptor can be various entities, including the ϵ -amino group of a lysine residue in a protein or peptide, an amine, diamine, polyamine, water, or a glutamyl-bound polyamine. In addition to their primary function, transglutaminases can also catalyze certain esterification reactions (Ando et al., 1989).

Overall, transglutaminases contribute to the modification and cross-linking of proteins, playing a significant role in various biological processes (Figure 2.2). Hydrolysis reactions involving water can indeed lead to deamidation and modifications in the charge of proteins (Ando et al., 1989).

The amino group of lysine is classified as a primary amine and serves as a favorable substrate for transglutaminases. In contrast, other amino acids may possess amidated, esterified, or decarboxylated α -carboxyl groups, resulting in variations in their negative charges. In transglutaminase reactions, peptides containing lysine residues act

as acyl acceptors, while proteins function as acyl donors. Conversely, peptides containing glutamine residues serve as acyl donors, with proteins acting as acyl acceptors (Ando et al., 1989).

The three-dimensional structure of substrate proteins also plays a crucial role in enhancing the reactivity of transglutaminase enzymes. Denatured proteins generally exhibit higher substrate potential than native proteins (Beninati et al., 2009).

Cross-links involving ϵ -lysine can be formed both intramolecularly and extramolecularly, resulting in physical changes, particularly in protein-rich foods containing lysine and glutamine (Yokoyama et al., 2004). Transglutaminase can also act on other bonds in proteins, such as the bonds between casein and gluten (Kang et al., 1994). The addition of polyphenoloxidase and lipoxygenase can further contribute to cross-link formation by affecting the sulfhydryl and disulfide bonds of enzymes (Lantto et al., 2005).

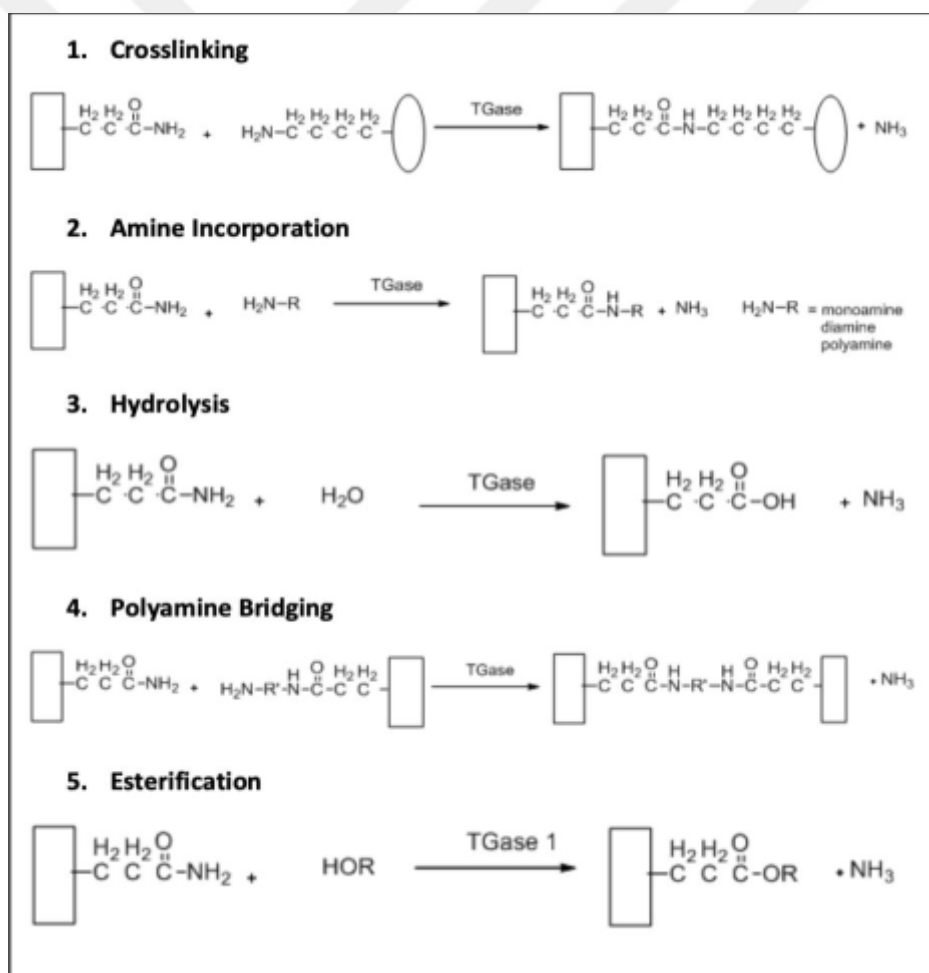


Figure 2.2 :Transglutaminase catalyze reactions (Kim, 2007)

2.2 Transglutaminase using areas

Transglutaminase, also known as "bio glu," was initially introduced in the food industry using the source of *Streptomyces mobaraensis* (Ando et al., 1989; Shimba et al., 2002; Washizu et al., 1994). Its primary applications include cross-linking proteins in meat and fish, as well as supporting gelling processes in yogurt and cheese (Farnsworth et al., 2008). Additionally, it is utilized for the production of heat-resistant gels and as an ion modifier (Caballero et al., 2005). Once activated, transglutaminase serves as a catalyst in various reactions and enables post-translational modifications of proteins (Martin et al., 2013).

Using of the area also concludes medicine, textile and wood are for better applications such as producing of cross-linking collagen (Stachel et.al., 2010), bone grafting (Cavelier et al., 2018), upgrading shrink resistance, wood colour fastness, wool smoothing (Cui et al., 2009).

Transglutaminase causes cross-linking of proteins both within and between each other (Mahmood and Sebo 2009). The effect on the product; sponging, gelation, solubility is observed when used as an additive (Giosafatto et al., 2012). For example, when used in bread production, it has been determined that it has the effect of increasing the texture, structure and volume of bread (Moore et al., 2006). At the same time, when it is used in the production of meat products, the firmness of the meat increases. For dairy products microbial transglutaminase optimize heat resistance and makes firm gel which improves structure. The water holding capacity of yogurt also improved and make positive affect for other beneficial living organisms (Farnsworth et al., 2006)

2.3 Transglutaminase sources

The isolation of high-yield production strains has played a significant role in enhancing the production of transglutaminase. On microbial transglutaminase production using *Streptoverticillium mobaraense*, continuous efforts have been made to select new strains with higher enzyme yield and optimize the production process using various methods (Ando et al., 1989). Numerous studies have focused on increasing the production of microbial transglutaminase. Among them, *Streptomyces* spp. has been found to have significantly higher yield compared to other strains, making it the preferred choice for industrial production of microbial transglutaminase. Additionally, efforts have been made to optimize substrates, metabolic processes, and environmental controls such as pH, dissolved oxygen level, and temperature to improve the yield of microbial transglutaminase. Developed a two-stage environmental control strategy, resulting in an increase in transglutaminase activity from 2.0 U/mL to 3.40 U/mL (Zheng et. al., 2000)

These studies have contributed to the production of transglutaminase from various strains isolated from nature. More recently, solid-state fermentation techniques have been explored for the production of microbial transglutaminase by *Streptomyces* spp., offering a more cost-effective approach (Nagy et al., 2008). Despite the significant progress made in microbial transglutaminase production over the past two decades, the productivity levels are still relatively low. The high cost and price of microbial transglutaminase limit its widespread application in the food industry and also other sectors. Consequently, many

studies have shifted their focus from conventional optimization to genetic engineering, aiming to construct recombinant transglutaminase production strains with higher yields.

2.4 *Pichia pastoris* expression system

Pichia pastoris (formerly known as *K. phaffii*) is a eukaryotic, unicellular methylotrophic yeast capable of performing post-translational modifications necessary for producing recombinant proteins (Higgins et al., 1998).

P. pastoris possesses several important features that make it suitable for expressing proteins from diverse species. Firstly, it can be easily manipulated at the genetic level, allowing for efficient genetic engineering of the expression system. Secondly, *P. pastoris* has the ability to produce high levels of proteins, both intracellularly and extracellularly. Furthermore, it exhibits a wide pH range (3.0 - 7.0) for growth and protein expression (Cregg et al., 1993). Importantly, *P. pastoris* is a higher eukaryotic organism capable of performing post-translational modifications such as glycosylation, disulfide bond formation, and correct protein folding (Cregg et al., 2000). These characteristics enable the successful expression of proteins that may not be functionally active when expressed in bacteria, *Sacharomyces cerevisiae* or baculoviruses (Cereghino et al., 2001). There is a fundamental difference in the role and origin of transglutaminases between baculoviruses and *Streptomyces morabensis*. Baculoviruses are not natural producers of transglutaminases but are used as a host system for the expression of transglutaminases from other sources, while *S. morabensis* naturally produces microbial transglutaminase.

Numerous studies have utilized the *P. pastoris* expression system to produce a wide range of proteins. Examples include the production of erythropoietin (Soyaslan and Çalık, 2011), xylosidase (Dehnavi et al., 2016), and polygalacturonate lyase (Wang et al., 2010). Since the 1970s, *P. pastoris* has been widely used in the market for expanding recombinant protein production, capitalizing on its versatility and reliability. *P. pastoris* expression system has become a very popular system due to its features such as easy to use, low cost and rapid expression ability. The amount of oxygen in the environment, the pH value and temperature factors affect cell growth or change the amount of the product. Some factors, such as species selection (Table 2.1), promoter selection and gene copy number, change the amount of protein produced. (Cregg et al. 1993).

Table 2.1: The pioneer strains, vectors and usage for target product in *P.pastoris*.

Product	Strain of <i>P.pastoris</i>	Vector	Usage	References

Table 2.1 continue

Lycopene and β -carotene	X-33	GAPZA	Feed supplements	Araya-Garay et al. (2012)
Class I chitinase	KM71	PICZ α A	Antifungal peptide	Landim et al. (2017)
Goat chymosin	X-33	PICZ α A	Hydrolysis of κ -casein	Tyagi et al. (2016)
Transglutaminase	GS115	PIC9K	Restructured meat products	Yang and Zhang (2019)

The optimal temperature for the growth of *P. pastoris* cells is 30°C (Coss et al., 2000). Regarding pH, Determined that the best pH range for *P. pastoris* production is between pH 3.0 and 7.0 (Cregg et al., 2000)

The *P. pastoris* expression system utilizes the alcohol oxidase 1 (AOX1) promoter, which is known for its strength and efficiency as an inducible promoter (Cereghino and Cregg, 2000). Another commonly used promoter is the constitutive glyceraldehyde-3-phosphate (GAP) promoter, which serves as a regulatory element in *P. pastoris* expression (Cereghino et al., 2002; Daly et al., 2005). Promoters play a crucial role in controlling gene expression and recombinant protein production in the *P. pastoris*.

More than one promoter was found to be used in this fungi species, in which the promoter region became important. Examples of inducible promoters are methanol-inducible AOX1, inducible methanol or methylamine FLD1 (Shen et al., 1998).

The methanol-inducible *P. pastoris* system utilizes the AOX1 promoter, which is specifically activated in the presence of methanol. Methanol serves as the energy source for the cell, and the initial product formed during methanol utilization is methanol oxidase, which accounts for approximately 35% of the total protein in the cell (Hill et al., 1986). It has been observed that *P. pastoris* cells do not grow when provided with ethanol, glucose, or glycerol as the sole carbon source. However, excessive levels of methanol in the environment can pose a fire risk and act as a toxic substance (Couderc and Baratti, 1980).

On the other hand, the GAP promoter is a constitutive promoter that has been used for expressing heterologous proteins in *P. pastoris* since 1997 (Waterham et al., 1997). The GAP system utilizes glycerol, glucose, or methanol as the energy source for cell growth, depending on the specific requirements of the expression system. The presence of glucose or glycerol is necessary for protein production in the GAP system, and the dosage of the GAP regulatory gene can also influence its functionality (Vassileva et al., 2001). Compared to the methanol-inducible system, the GAP system offers a safer alternative as it does not rely on methanol for induction.

P. pastoris is a Crabtree-negative yeast, which means it does not produce ethanol in environments with high glucose concentrations. However, under aerobic conditions, *P. pastoris* can produce a small amount of ethanol, as noted by Inan and Meagher in 2001. The presence of ethanol in the environment has been found to have an impact on recombinant protein production, specifically by suppressing the AOX1 promoter that is induced by methanol. In contrast, the GAP promoter is not suppressed by ethanol and can even lead to a slight increase in the expression level of recombinant proteins in productions using the GAP promoter (Maurer et al., 2006).

These observations suggest that ethanol can interfere with the AOX1 promoter induction system but does not have the same suppressive effect on the GAP promoter. This finding opens up possibilities for developing inexpensive and non-hazardous alternatives to the currently available promoter induction systems derived from *P. pastoris*. Such alternatives could provide more flexibility and options for optimizing recombinant protein production in *P. pastoris* (Vogl and Glieder, 2013) (Table 2.2).

New inducible promoters that could use inexpensive and safe induction alternatives to currently available promoter induction systems derived from *P. pastoris* were investigated, and two alcohol dehydrogenase ADH genes were identified for the yeast *P. pastoris*. The gene investigated as the promoter region was named the ADH1 promoter. When this gene was searched in the NCBI database, it was named the ADH3 gene because of its close resemblance to the amino acid sequence of *S. cerevisiae* ADH3. The result of recent studies, and in their study, the function of this gene, which is responsible for ethanol metabolism, was investigated and characterized. with protein accession number (XP_002491382) in the NCBI database (Karaoğlu et al., 2016).

As a result, it was stated that the ADH3 gene is the only gene responsible for ethanol consumption. This promoter, called ADH3, investigated in the study, has been reported in the literature. In order to avoid systematic confusion, it was named ADH2 in later studies and within the scope of this thesis (Karaoğlu et al., 2019).

Table 2.2: Used pioneer promoters in *P.pastoris* (Vogl T and Glieder A., 2013)

Gene name	Gene	Inducer	Expression level
Inducible promoters			
AOX1	Alcohol oxidase 1	Methanol	Strong
ADH3	Alcohol dehydrogenase	Ethanol	Strong
DAS	Dihydroxyacetone phosphate	Methanol	Strong
FLDI	Formaldehyde dehydrogenase	Methanol/methylamine	Strong
PEX8	Peroxisomal matrix protein	Methanol/oleate	Weak
ICL1	Isocitrate lyase	Ethanol	-

Table 2.2 continue

LRA3	L-rhamnonate dehydratase	Rhamnose	~75% of GAP
LRA4	L-KDR aldolase	Rhamnose	~22% of GAP
THI1	Thiamine biosynthesis protein	Repressed by thiamine	~70% of GAP
GTH1	High affinity glucose transporter	Limited glucose	-
CUP1	Copper-binding metallothionein protein	Copper	-
PPLCC1	Laccase	Copper	-
Constitutive Promoters			
Gene name	Gene product	Expression level	
GAP	Glyceraldehyde 3-phosphate dehydrogenase	Strong	-
YPT1	GTPase involved in secretion	Weak	-
TEF1	Translation elongation factor-1 alpha	Strong	-
GCW14	Glycosylphosphatidylinositol	Strong	-
PGKI	Phosphoglycerate kinase	~10% of GAP	-

The ADH2 promoter was compared with the AOX1 and GAP promoters in terms of recombinant protein production. Specifically, the xylanase gene was expressed under the control of the GAP, ADH2, and AOX1 promoters, and their expression activities were evaluated. The results showed that expression under the control of the ADH2 promoter in Erlenmeyer experiments resulted in a two-fold lower xylanase enzyme activity compared to expression under the control of the AOX1 promoter. On the other hand, expressions under the control of the GAP promoter and the ADH2 promoter exhibited approximately the same activity. It should be noted that Erlenmeyer conditions are uncontrolled, but the highest enzyme activity was observed in the samples obtained from ADH2-controlled xylanase production. Based on these findings, it was concluded that the ADH2 promoter could be used for large-scale applications and may be more suitable for recombinant protein production. The study suggested that the ADH2 promoter could serve as an alternative to other powerful promoters (Karaoğlu et al. 2016, Özçelik et al 2019).

Along with these advantages, GRAS (Generally Recognized as Safe) status, crab-tree negative and no toxic production segregation are the other reasons for using *P.*

pastoris (FDA, 1993). On the other hand some disadvantages exist such as longer cultivation time according to bacteria, high protease segregation and the hard monitoring of the leaking of methanol.

In this study, *P. pastoris* in ADH production used as the gene source. The DNA fragment encoding the microbial transglutaminase enzyme gene was transferred to the plasmid using molecular biology techniques such as transformation and ligation. Thus, an expression vector encoding the desired protein was obtained. Electroporation transfer of expression vector to *Pichia* strain capable by lithium acetate method was achieved.

However, *P. pastoris* will be proven to be available in the production of recombinant TGase under the new structural ADH promoter. Although TGase enzyme production by using *P.pastoris* is not preferred because its widespread use in industry is dangerous due to its induction with ethanol due to the AOX1 promoter used. As an alternative to this method, *Pichia* with an ADH promoter in its structure was observed that produces good results in the production of recombinant proteins.

The objective of the proposed thesis is to investigate the enzyme microbial transglutaminase and explore the factors that influence its production. In this study, the MTGase gene which is responsible for transglutaminase enzyme were cloned into pADH2 α -A expression vectors individually. These plasmids were then integrated into the genome of *P. pastoris*. The strain KM71, which produced pADH2 α A-pro-MTGase, was selected for further analysis. The pro-MTGase enzyme was produced at a temperature of 20°C, and three different pH values were tested. The purpose of this analysis is to evaluate the effect of pH on the production of recombinant microbial transglutaminase enzyme.

By studying the production and enzyme activity of TGase under different conditions, this research aims to contribute to a better understanding of the enzyme and its potential applications.

3. MATERIAL AND METHOD

3.1 Material

3.1.1 Chemicals and enzymes

The restriction enzymes and their buffer solutions used in the study were obtained from Thermo Fisher Scientific (FastDigest Restriction Enzymes-Thermo Fisher Scientific, USA). The manufacturer's instructions were followed when using enzymes.

The protein marker PageRuler Plus prestained protein ladder (Thermo Fisher Scientific, USA) was used for SDS-PAGE analysis. Dispaase enzyme was used to activate pro-MTGase enzyme with diluted 1 mg/ml from stock. Plasmid isolations, transformation, ligation operations were carried out according to the manufacturer's instructions using Qiagen (Germany) kits.

Molecular techniques used in the study were carried out according to Sambrook and Russell (2001) and kit protocols. The National Biotechnology Information Center Network Service (Bethesda, MD, USA; www.ncbi.nlm.nih.gov) was used for obtaining gene and protein sequences.

3.1.2 Buffer and stock removers

All buffers and stock solutions were prepared with distilled water, sterilized by autoclaving at 121°C for 15-20 minutes and stored at +4°C or room temperature until time of use.

3.1.3. Gene source and microorganisms

S. mobaraensis microbial transglutaminase gene was obtained from pGAPZ α -pro-MTGase clone that was constructed in our laboratory. pADH2 α A vector was used to obtain pADH2 α -pro-MTGase expression vector (Figure 3.1).

Among the microorganisms used in the study, *E. coli* NEB5 α strain was used for the reproduction and storage of plasmids, *P. pastoris* KM71 strain was used as the host for protein expression.

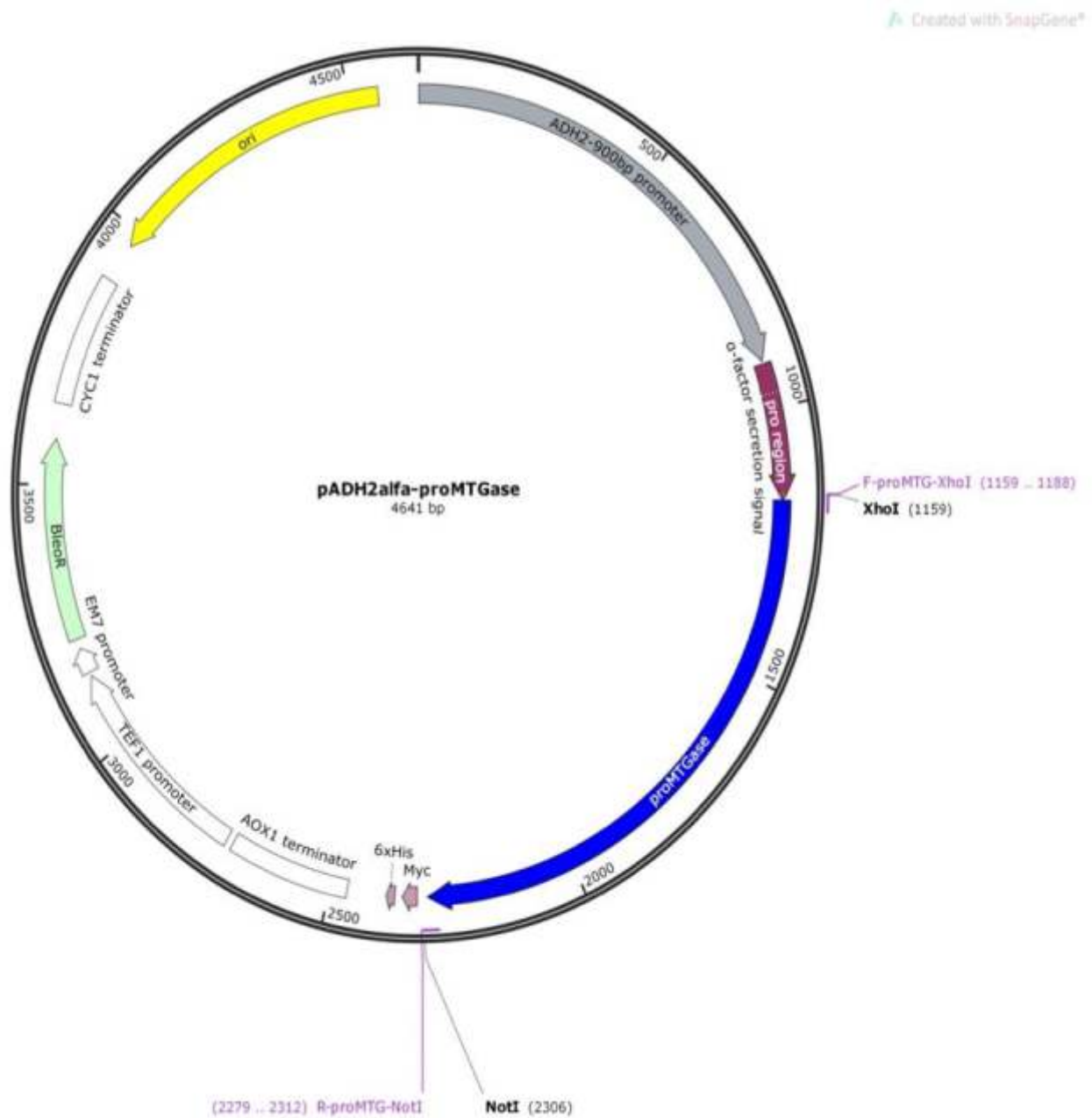


Figure 3.1 : pADH2 α -proMTGase expression vector map

All microorganisms were kept at -80°C in a suitable medium containing 50% glycerol. The strains to be used in the study were developed by drawing a single colony from the main stock onto the agar plates, and keeping them in incubators at the appropriate temperature and time (3 days at 30°C for *P. pastoris*, an overnight at *E. coli* NEB5 α at 37°C).

3.1.4. Media, growth media and antibiotics

LB Lenox (5 g/L yeast extract, 10 g/L Tryptone and 5 g/L NaCl) was used to grow *E. coli* NEB5 α cells. Appropriate antibiotics were added to the broth and solid media used to ensure appropriate selection for the resistance gene on the plasmid.

Stock solutions of zeocin antibiotic were prepared at concentrations of 100 mg/mL, filter sterilized using a sterile 0.22 μ m filter (Corning, AB), aliquoted in 1 mL and stored at -20°C until use. In order to prepare the medium with antibiotic, the medium was sterilized at 121°C for 15 minutes, then cooled to 50°C, and zeocin dissolved on ice were added to the medium at a concentration of 25 μ g/mL for *E. coli* NEB5 α strain. In order to ensure the selection of *P. pastoris* strains, zeocin was added to the media at rates of 100 μ g/mL, 500 μ g/mL and 1000 μ g/mL.

In the general growth of *P. pastoris* strains used in the study; YPD (1% yeast extract, 2% peptone, 2% glucose), buffered glycerol-complex medium (BMGY) (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L YNB, 4×10^{-5} g/L biotin, 10% glycerol and 0.1 M phosphate buffer, pH 6.0) were used. Buffered ethanol-complex medium (BMEY) (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L YNB, 4×10^{-5} g/L biotin, 10% ethanol, and finally prepared at different pHs (3.0, 5.0 and 7.0) was used in protein expression studies.

3.2. Method

3.2.1. Integration of *Xho*I and *Not*I restriction sites to the pro-MTGase gene with Polymerase Chain Reaction (PCR)

The pGAPZ α -proMTGase clone was constructed in our laboratory previously (Özçelik et al., 2019). However, restriction sites of pro-MTGase gene were not suitable for the cloning into the pADH2 α plasmid. Therefore, the restriction sites of *Xho*I and *Not*I enzymes were added to the pro-MTGase gene with the help of the PCR. The PCR was carried out according to KOD Hot Start DNA polymerase (EMD Biosciences, San Diego, CA, USA) kit protocol. PCR mixture were given in Table 3.1. The sequence of primers was given in Appendix section. The PCR conditions were as follows; initial denaturation at 95°C for 120 seconds, denaturation at 95°C for 20 seconds, annealing at 56°C for 10 seconds, extension at 70°C for 100 seconds and final extension at 72°C for 120 seconds. The amplification program consisted of 35 cycles of denaturation, annealing and extension steps. After reaction completed, the PCR product visualized on agarose gel.

Table 3.1 Components of PCR mixture

Constituent	Final concentration	Volume added
NFW		15.5 μ l
10X KOD Buffer	1X	2.5 μ l
2mM dNTP mix	0.2 mM	2.5 μ l
25mM MgSO ₄	1.5 mM	1.5 μ l
10 μ M forward primer	15 pmol	0.75 μ l
10 μ M reverse primer	15 pmol	0.75 μ l

Table 3.1 continue

Template DNA (5 ng/μl)		1 μl
KOD polymerase (1U/μl)	0.02 U/μl	0.5 μl
TOTAL		25 μl

3.2.2. Preparation of competent *E.coli* and *P. pastoris* cells

E. coli NEB5α cells were made competent by the CaCl₂ method. The *E. coli* NEB5α strain, which was developed so that a single colony fell on the LB Miller plate, was cultivated from a single colony in 3 ml of LB Miller broth and left to grow overnight in a 37°C shaking incubator. The next day, the optical density of the growing culture was measured and seeded in 50 ml of LB Miller broth with an initial OD_{600nm} value of 0.01 and grown in a 37°C shaking incubator until the OD_{600nm} value was 0.3 (approximately 3 hours). When the culture reached the expected density, the cells were harvested by centrifugation at 3000xg at 4°C for 5 minutes. The procedures were then carried out on ice. The resulting cell pellet was suspended in 5 ml of 0.1 M CaCl₂ solution and incubated on ice for 30 minutes. The cell pellet, which was re-harvested by centrifugation in the previous conditions, was resuspended in 1.5 ml of 0.1 M CaCl₂ solution containing 15% glycerol and brought to -80°C for later use in 100 μl volumes.

Cells of the *P. pastoris* KM71 strain were potentiated according to the Lithium Acetate (LiAc) Method (Wu and Letchworth 2004). *P. pastoris* KM71 strain desired to be made competent was grown from frozen culture or from single colonies on agar plate stored at +4°C, inoculated into 3 ml of YPD broth in a test tube, in a 250 rpm, 30°C shaking incubator overnight. The next day, culture was grown overnight, and the initial OD_{600nm} value was 0.1, and 80 ml of YPD broth was cultured in 250 ml erlenmeyer flasks. It was developed in a 250 rpm, 30°C shaking incubator until the OD_{600nm} value was 1.3-1.5. After the cells showed sufficient growth, they were harvested by centrifugation at 3000xg at +4°C for 5 minutes, and the supernatant part was removed. The cell pellet was suspended in 8 ml of LiAc mixture (100 mM LiAc, 10 mM DTT, 0.6 M Sorbitol, 10 mM Tris-HCl pH 7.4) and incubated for 30 minutes at room temperature. At the end of the period, the cells were re-harvested by centrifugation at 3000xg for 5 minutes at 4°C, suspended in 1.5 ml of ice-cold 1 M sorbitol and transferred to a 2 ml centrifuge tube. Cells harvested by centrifugation at 3000xg for 5 minutes at 4°C were washed 3 times with 1.5 ml cold 1 M sorbitol, then suspended in ice-cold yeast resuspension buffer (1 M sorbitol, 20% glycerol) to a final concentration of 10¹⁰ cells/mL. It was cooled to -80°C for later use in 80 μl volumes.

3.2.3. Determination of plasmid DNA concentrations

Concentration measurements of the genes encoding the pro-MTGase enzyme were performed using the “Qubit dsDNA BR Assay Kit (Invitrogen, CA, USA)” according to the manufacturer's instructions. The readings of the Qubit dsDNA BR Assay buffer/dye/sample mixture prepared in a total of 200 μl at the ratios specified in the protocol were made and the concentration calculations against the standards were made on the Qubit 2.0 Fluorometer (Invitrogen, CA, USA).

3.2.4. Determination of cell density

Cell densities of cultures grown in broth were measured spectrophotometrically. After the necessary dilutions were made to the cultures, the cell densities were determined by measuring the absorbance at 600 nm wavelength in the LibraS50 (Biochrom, UK) spectrophotometer device, and the term optical density (OD) was used as the expression unit.

3.2.5. Construction of pADH2 α -proMTGase expression vector

The pGAPZ α -pro-MTGase clone had been previously created in our laboratory (Özcelik et al., 2019). However, the restriction sites in the pro-MTGase gene were not compatible with the cloning process into the pADH2 α plasmid. To overcome this issue, we introduced *XhoI* and *NotI* restriction sites into the pro-MTGase gene using PCR amplification, and then we successfully ligated it with the pADH2 α plasmid (Figure 3.2).

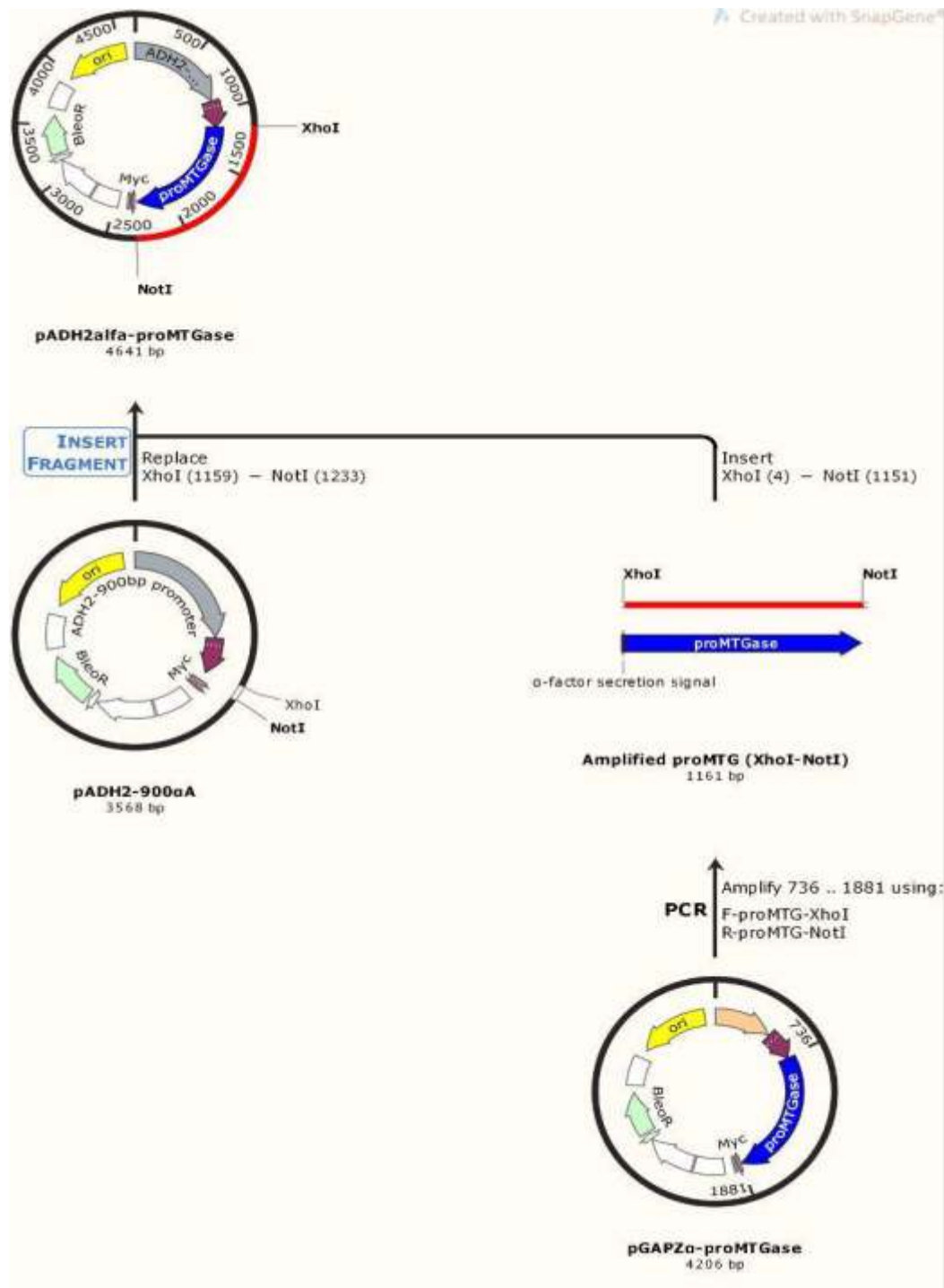


Figure 3.2: The history of construction of the pADH2 α -pro-MTGase expression vector.

3.2.5.1 Ligation of DNA fragments in plasmid

E.coli NEB5 α cells were transformed with vectors by means of the electroporation method. Before the transformation, ligation completed for better results. In order to prevent the vector from closing on its own, dephosphorylation process was applied to the vector. 1:3 vector:insert ratio (M:M) was used in the ligation reaction.

Dephosphorylation was performed to prevent the plasmid from closing on itself. For calculating at least 50 ng insert lenght; 1134 bp insert pro-MTG and 3600 bp vector pADH2 α ligated.

For the ligation process, the amount of insert DNA (pro-MTGase) calculated for 50 ng of vector DNA (pADH2 α) in a total of 10 μ l was mixed, and 5U of T4 DNA ligase was added in the presence of 1X T4 DNA ligation buffer. After 5 minutes of incubation at 22°C, transformation into competent bacterial cells was continued with 1/10 of the ligation mixture, and the remaining amount of the mixture was stored at -20°C.

3.2.5.2. Transformation of plasmid into competent *E.coli* cells after ligation

Transformation of plasmids into *E. coli* NEB5 α cells which were was carried out. After the control of the obtained colonies, the cultures prepared were kept at -80 °C. Confirmation of the presence of vectors in *E. coli* cells is the first step by propagating *E.coli* cells in an antibiotic medium.

5 μ l of ligation mixture was added to 100 μ l of competent *E. coli* NEB5 α cells which were thawed on ice. After incubation on ice for 30 minutes, the cells were heat shocked at 42°C for 60 seconds, cooled by keeping them on ice for 5 minutes. Transformant cells were inoculated on LB Lenox broth and incubated at 37°C for 60 minutes. Cells were then added to media containing the appropriate antibiotic and incubated overnight at 37°C. Then, plasmid isolation was performed in accordance with the protocol using the “QIAprep Spin Miniprep Kit (QIAGEN, Germany)”.

After measuring the concentrations of the plasmids, the restriction digestion was carried out in order to confirm the plasmids. The reaction mixture was prepared in a total of 20 μ l by adding 10X green buffer and NFW, 1 U enzyme and 1 μ g plasmid. After incubation at 37°C for 60 minutes, the evaluation of cut plasmids and uncut plasmids as control was done by agarose gel electrophoresis.

In order to transfer the confirmed plasmids to competent *P. pastoris* cells, different 6 enzymes which are *Bam*HI and *Bgl*II, *Eco*9II and *Bgl*II, *Xho*I and *Not*I that cleaves the vector at different points was used. The plasmids, which were validated with enzymes were carried out on a 1% agarose gel to control. After DNA fragments were validated, their concentrations were measured for transformation.

3.2.6. Transformation of pADH2 α -proMTGase plasmid into compotent *P. pastoris* KM71 cells

After the confirmation analyses, in order to transform the selected expression vectors into competent *P. pastoris* cells, the vector was digested with the restriction enzyme *Bgl*II, which was selected according to the region where the integration was targeted in the *Pichia* genome and cut the cloning vector in a single place and was made linear and confirmed on the gel (Figure 3.3). The linearized DNA was purified using the MinElute PCR Purification Kit (Qiagen) and then its concentration was measured.



Figure 3.3: Agarose gel photo of linearization of expression vector

Transfer of the confirmed plasmids to component *P. pastoris* cells was done by electroporation method. 3-5 μg of linear DNA was added to 80 μl of competent *P. pastoris* cells were thawed on ice, mixed by pipetting and transferred to an ice-cold 2 mm electroporation cuvette. The cuvettes were kept on ice for 5 minutes and subjected to 1500 V electric current for 5 ms in the electroporation device. Immediately after, 1 ml of ice-cold 1 M sorbitol was added and transferred to a 1.5 ml centrifuge tube. Cells incubated for 1-2 hours at 28°C were spread on YPD agar medium plates containing zeocin antibiotic at 100,500, 1000 $\mu\text{g}/\text{ml}$ and incubated at 28°C until colonies formed (approximately 3 days).

3.2.7. pro-MTGase protein expression in shake flask

Colonies that developed on the plates after transformation of *P. pastoris* were then inoculated on YPD agar medium in order to be singled out. After 3 days of incubation, the colonies that fell one by one were inoculated into 3 ml YPD medium and transglutaminase expression was initiated in the erlenmeyer of *P. pastoris* KM71 strain. MTGase clones carrying the expression cassette for expression and *P. pastoris* cells not carrying the expression cassette (negative control) were inoculated into 3 ml YPD broth. Cells that developed in YPD broth at 28°C until the OD_{600nm} absorbance value was between 6-8 absorbance units were inoculated into BMGY (pH:6) with the final OD_{600nm} of the medium being 0.1 and left to grow in a shaking incubator for 18-20 hours.

While the OD_{600nm} value of the cells grown in BMGY medium at 28°C was approximately 10 absorbance units, they were harvested by centrifugation at +4°C, 3000 g for 15 minutes and the supernatants were discarded. The same volume of BMEY different three pHs (3.0, 5.0 and 7.0) was added to the cell pellets, suspended, and then placed in a shaking incubator at 20°C for protein expression and protein expression was maintained for 96 hours. Since the protein expression of *P. pastoris*, which is a methylotrophic yeast, occurs under the control of the ADH promoter, 300 microliters of 100% methanol was added every 12 hours during the expression, with a final concentration of 1% in the medium. At the end of 96 hours, the cells were

harvested. The samples taken during the fermentation were analyzed by SDS-PAGE analysis and the MTGase enzyme activity was measured.

3.2.7.1 Determination of optimum pH at the operating temperature of the enzyme

The transformant cells inoculated into test tubes containing 3 ml YPD media and incubated at 250 rpm for 24 hours at 28°C. These cells were used as inoculum for 30 ml of erlenmeyer containing YPD media with an initial density of OD_{600nm}=0.1. It performed 24 hours at 250 rpm at 20°C. Samples that will be taken from developing cultures every 24 hours will be centrifuged for 5 minutes at 4000×g, and cells were precipitated, supernatants were stored at -80°C. At the end of production, enzyme activity measured and SDS-PAGE analysis were performed to confirm the presence of MTGase protein in the collected supernatant samples to select the most efficient clone and to determine the optimal pH for the production of proMTGase enzyme in *P. pastoris* at 20°C. Therefore pH 3.0, 5.0 and 7.0 media for protein production tested in the shake flask experiments.

3.2.8. SDS-PAGE analysis

Expression of the gene was first obtained by conducting production experiments in shake flask enzyme activity from supernatants will be measured and also SDS-polyacrylamide gel electrophoresis (SDS-PAGE) will confirm that the protein is produced. SDS-polyacrylamide gel electrophoresis in OWL p8d8 (Thermo Scientific, IL, USA) gradient gel (5% polyacrylamide loading gel and 10% polyacrylamide separation gel, 0.8 mm) it will be prepared and implemented.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was applied in Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, USA) device by preparing 5% polyacrylamide loading gel and 10% polyacrylamide separation gel of 1 mm. The supernatant samples for which protein production will be detected, were mixed with a 1:3 ratio of 4X SDS gel loading buffer (200 mM Tris-Cl, pH 6.8, 8% SDS, 0.4% Bromphenol Blue, 40% glycerol, 100 mM DTT) in a total of 30 µl. The mixture was incubate at 70 °C for 10 minutes and to ensure denaturation of the proteins. 20 µl of the prepared sample-loading mixture was taken and loaded into the gel wells. Protein samples were run in 1X TGS (0.025 M Tris base, 0.192 M Glycine, 0.1% SDS, pH 8.3) buffer at 120 V current for 90 minutes during electrophoresis. After electrophoresis, the gel was stained with staining buffer (10% Acetic acid, 50% Methanol, 0.1% Coomassie brilliant blue) for 2 hours on an orbital shaker. The protein bands were washed until the protein bands were visible to remove excess protein-bound dye with dye-free wash buffer (10% Acetic acid, 10% Methanol). Afterwards, the acrylamide gel was imaged with the ChemiDoc MP imaging system, and the target protein was verified by the alignment method with the help of the protein standard loaded into a well in the same gel.

3.2.9. Total protein determination

Total protein determination was performed with the Coomassie plus™ (Bradford) Assay kit (USA) according to the manufacturer's instructions. In order to calculate the protein amount, the albumin standard (BSA) was diluted with buffer solution (0.9 saline 0.05 sodium azide) in accordance with the protocol (Thermo Scientific) (0 µg/ml, 25µg/ml, 125 µg/ml, 250 µg/ ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml, 1500 µg/ml,

2000 µg/ml). Then, the prepared dilutions were mixed with the reaction reagent at a ratio of 1:30 and kept at room temperature for 10 minutes, and the absorbance values were read at 595 nm wavelength. The protein amount of the unknown sample was calculated from the standard graph drawn with the absorbance values obtained from the standard series measured in parallel.

3.2.10 MTGase enzyme activity measurement

In order to measure the enzyme activity, first of all, the pro part of the produced proMTGase enzyme must be cut and converted into the active MTGase enzyme. For this purpose, proMTGase was first converted to active MTGase enzyme by treatment with dispase I enzyme. To cut the pro portion, 0.21 U/mg dispase I enzyme was added for 0.25 mg/mL protein in the supernatant sample and incubated at 37°C for 2 hours. MTGase activity was determined spectrophotometrically according to the colorimetric hydroxymet method. According to this method, Z-glutaminyglycine (Z-Gln-Gly), hydroxylamine and L-glutamic acid-γ-monohydroxymet were used as amine acceptor substrate, amine donor substrate and standard, respectively. After 200 µL of reaction mixture (Z-Gln-Gly, 1M Tris buffer pH 6 and 200 mM hydroxylamine 20 mM reduced glutathione) was kept at 37 °C for a while, 30 µL of supernatant sample was added and mixed upside down and mixed at 37 °C. It was incubated for 10 minutes. After 10 minutes, the reaction catalyzed by the MTGase enzyme was stopped by adding 1 mL of stop solution (1 volume of 3 M HCl, 1 volume of 12% TCA, 1 volume of 5% FeCl₃). After this process was done, the reaction mixture was centrifuged at 4000×g for 5 minutes and the mixture was taken into a cuvette. Absorbance measurement was done at 525 nm. Samples that were measured by applying these procedures were called tests. In addition, to prepare the test blanks, the reaction mixture and the supernatant sample were mixed in the same way, but without incubation at 37°C for 10 minutes, the reaction stop solution was added and immediately measured in the spectrophotometer after centrifugation. After adding 100 µL of L-glutamic acid - γ-monohydroxymet solution at concentrations ranging from 1-10 mM to the standard tubes, 1 mL of stop solution was added without incubation and centrifuged and the measurement was made. To prepare the standard curve, 100 µL of water was substituted for the standard solution. 1 unit of enzyme activity was defined as 1 µmol of hydroxymethane formed per minute under the given conditions, and the enzyme activity was expressed as U/L.

4.RESULTS

4.1.Construction of pADH2 α -proMTGase expression vector

4.1.1. Integration of restriction sites to proMTGase gene

The intact mass spectrometry analysis of purified MTGase unveiled two distinct masses: 5,629 Da and 39,198 Da. These masses correspond to the prodomain and mature domain of MTGase, respectively. It's worth noting that the expected mass for the prodomain is 5,629 Da, and for the mature domain, it is 39,200 Da. This observation suggests that the detected masses accurately correspond to the cleaved MTGase prodomain and the mature domain, respectively. The *Xho*I and *Not*I regions integrated into the proMTGase gene to be transferred were visualized on agarose gel and 1161 kb fragment was obtained (Figure 4.1).

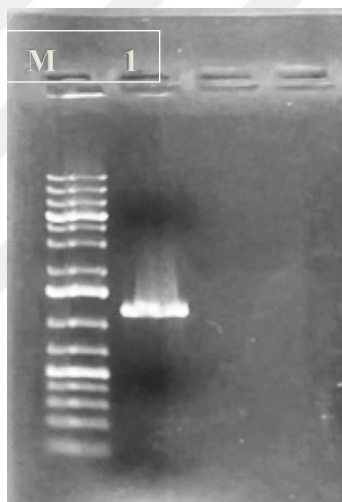


Figure 4.1:Agarose gel photo of integration restriction enzyme sites to the proMTGase gene, *Xho*I and *Not*I restriction enzyme sites to the proMTGase gene by PCR

After the restriction sites were added to proMTGase gene with PCR (Figure 4.1), the expression vector was obtained by ligation with the pADH2 α plasmid. The created expression vector was transformed into *E.coli* NEB5 α cells.

Ligation was performed using a 1:3 vector:insert ratio (M:M) to the pADH2 α plasmid and MTGase genes to generate expression vectors. During ligation, dephosphorylation was performed to prevent the pADH2 α plasmid from closing on itself.

4.1.2 Validation of the pADH2 α -proMTGase expression vector

Validation the pADH2 α -proMTGase expression vector was performed with restriction enzyme digestion. For this aim, seven different plasmids were digested with *Bam*HI and *Bgl*II, *Eco*9II and *Bgl*II, *Xho*I and *Not*I enzymes. The expected band sizes were 2530 and 2111 bp, 3932 and 709 bp, 3494 and 1147 bp, respectively. The

appearance of fragments at the expected locations in the agarose gel proved confirmation (Figure 4.2). According to the results, #1, #2, #6 and #12 numbered plasmids were confirmed and #6 numbered plasmid was selected to continue study.

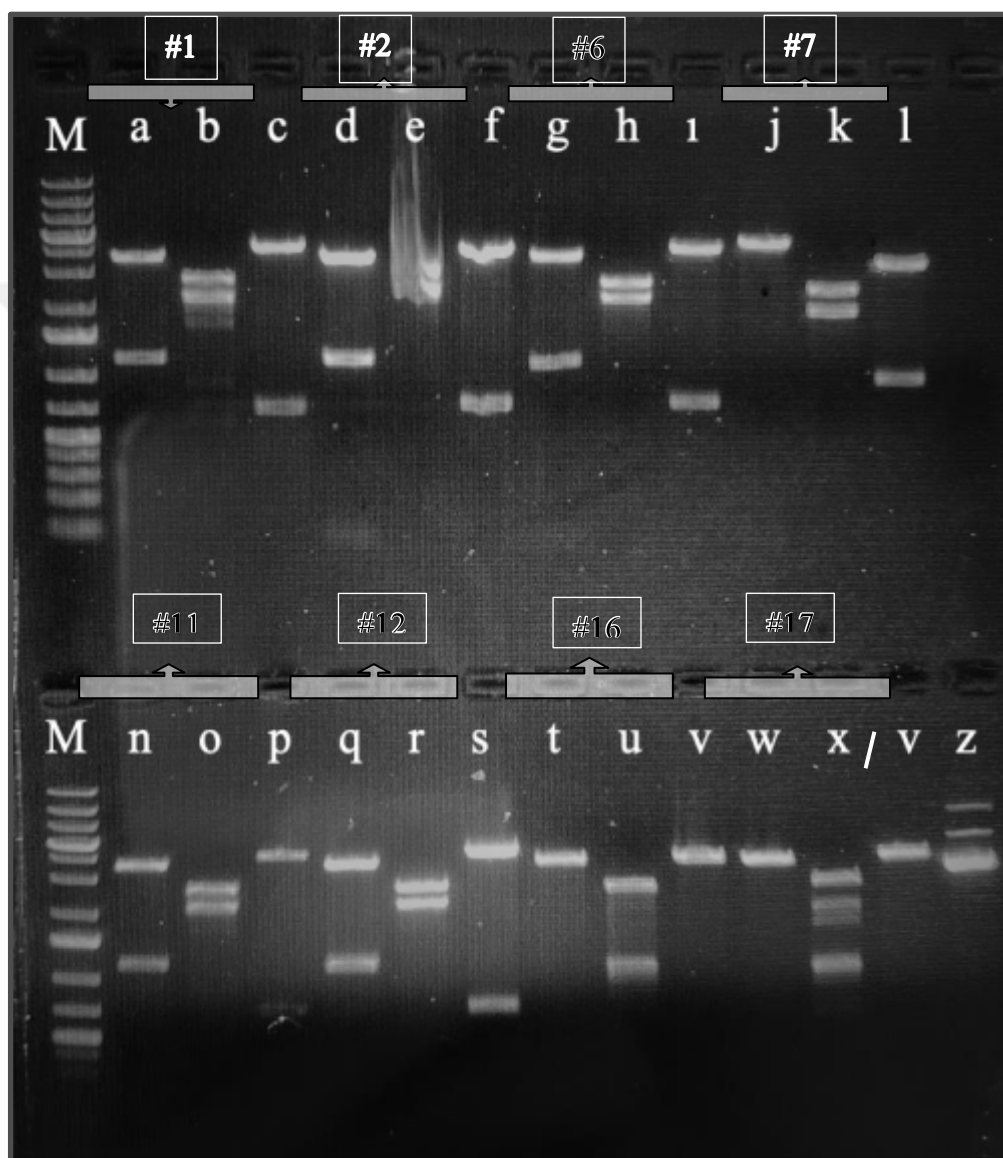


Figure 4.2: The confirmation of pADH2 α -proMTGase plasmids with different restriction enzymes digestion.

4.1.3 Transfer of the verified vector to *P. pastoris*

P. pastoris KM71 strain electro-competent cells prepared according to the method used by Wu and Letchworth (2004). The resulting expression vector pADH2 α -proMTGase was linearized and transformed into *P. pastoris* KM71 strain by electroporation technique.

4.2 Protein expression assays

Transformants named in section 4.1.2 were first developed in YPD and BMGY medium, and then protein expressions were performed in BMEY at pH:6.0 at 28°C. The collected samples were analyzed by SDS-PAGE and the results were given in Figure 4.3. According to SDS-PAGE result, all of the selected clones produce pro-MTGase protein.

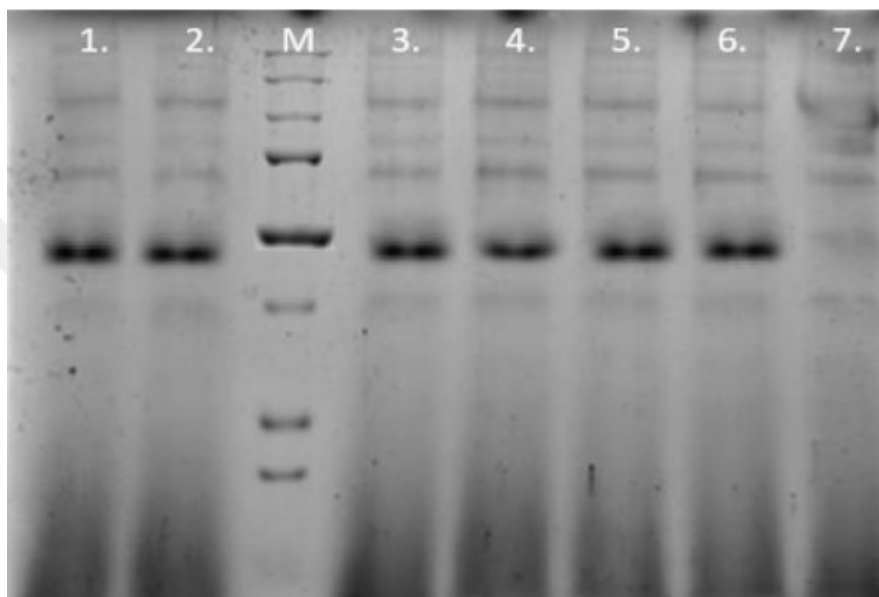


Figure 4.3: SDS-PAGE results of protein expression study. M marker PageRuler Unstained Protein Ladder (Thermo Fisher Scientific, MA, ABD), 1-6 Pichia clones, 7-negative control KM71 host strain

4.2.1. Transglutaminase expression assays at different pH

After validation of protein expression, best developed clear 5 numbered clone was selected for the optimization of protein expression under three different pHs. Transformant was first developed in YPD and BMGY medium, and then protein expressions were performed in shake flask at pH:3, pH:5.0 and pH:7.0 in BMEY medium for 96 hours. Pure ethanol was added to make a total ethanol of 1% every 12 hours during the induction period, since their expression was made under the control of the *P. pastoris* ADH promoter and SDS-PAGE analysis was performed by taking samples every 24 hours. Samples that will be taken from developing cultures every 24 hours will be centrifuged for 5 minutes at 4000×g, and cells will be precipitated, supernatants will be stored at -80°C. At the end of production, enzyme activity measured and SDS-PAGE analysis will be performed to confirm the presence of MTGase in the collected supernatant (Figure 4.4)

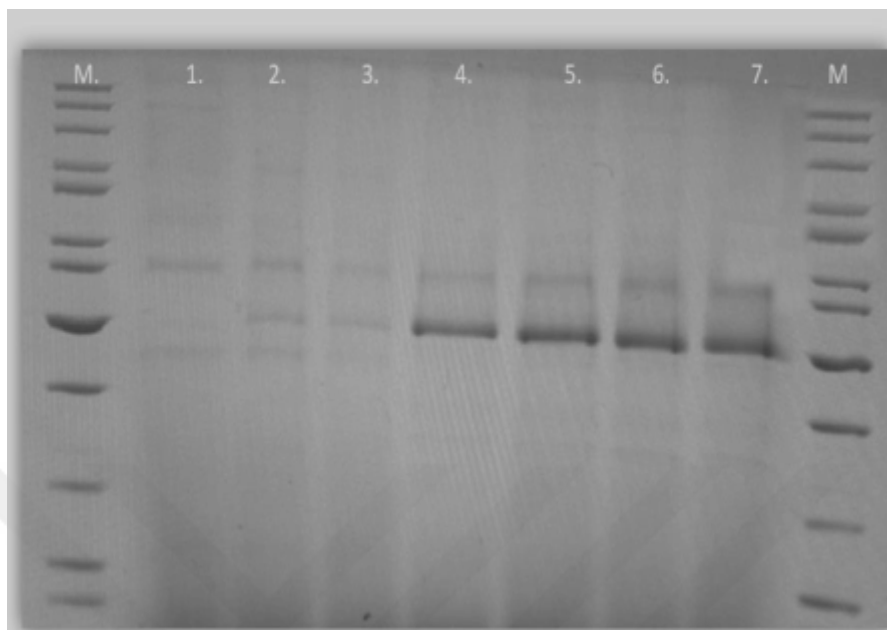


Figure 4.4: SDS-PAGE analysis of pADH2 α -proMTGase clone at different pHs. M; marker PageRuler Unstained Protein Ladder (Thermo Fisher Scientific, MA, ABD), 1: Negative control KM71 host strain, 2-3: pH 3.0 at 96. hour , 4-5: pH:5.0 at 96 hour, 6-7: pH 7.0 at 96 hour

4.2.2 Total protein concentration determination and transglutaminase enzyme activity detection

Detailed activity procedure is that the total protein amount in the supernatant samples was measured with the Coomassie plus Assay kit using the Bradford method. Bovine serum albumin (BSA) was used as the protein standard. Protein concentration was determined by reference to the absorbance values of BSA standard solutions (1-25 μ g/ml) read with the samples. 50 μ l of supernatant sample was placed in 1.5 ml of Bradford solution and incubated for 10 minutes at room temperature, and the absorbance was read in a spectrophotometer at 595 nm wavelength. The protein amount of the unknown sample was calculated from the standard graph drawn with the absorbance values obtained from the standard series measured in parallel. The results are given in Figure 4.6, 4.7 and 4.8.

In order to measure enzyme activity, it is necessary to first cut the pro-MTGase enzyme produced into the active MTGase enzyme by cutting the pro part. For this purpose, pro-MTGase will first be treated with dispase and converted into the active MTGase enzyme. The supernatant sample was incubated at 37°C for 10 minutes with the activation mixture to cut off the pro part.(Figure 4.5)

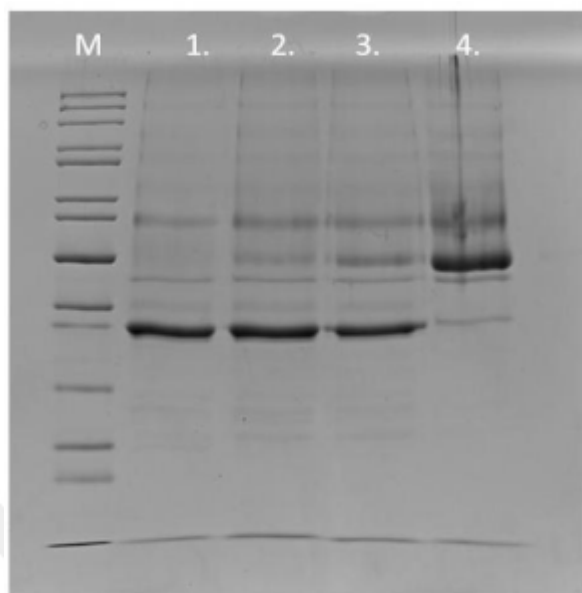


Figure 4.5 : Activation of pro-MTGase with the dispase enzyme. M: marker, 1-3: Activated 38kDa MTGase enzyme, 4:pro-MTGase enzyme.

Although the amount of protein reached as a result of 96 hours of transglutaminase expression performed at the same temperature and conditions with different pH ranges varies between 125-396 ng/mL, the highest protein production level was observed as 396 ng/mL at pH:7.0 at 96th hour (Figure 4.6,4.7,4.8)

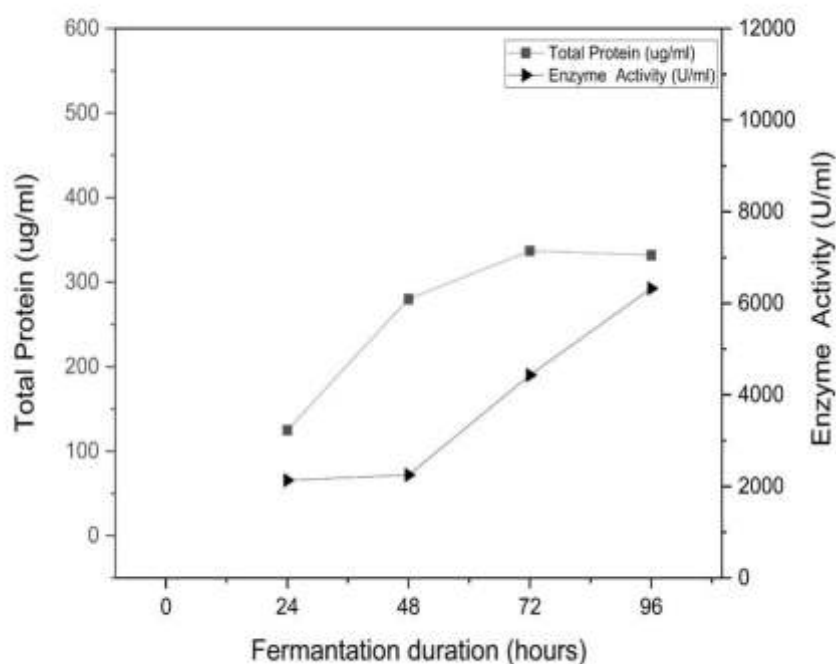


Figure 4.6 : Enzyme activity and total protein amount of MTGase protein expression at pH 3.0

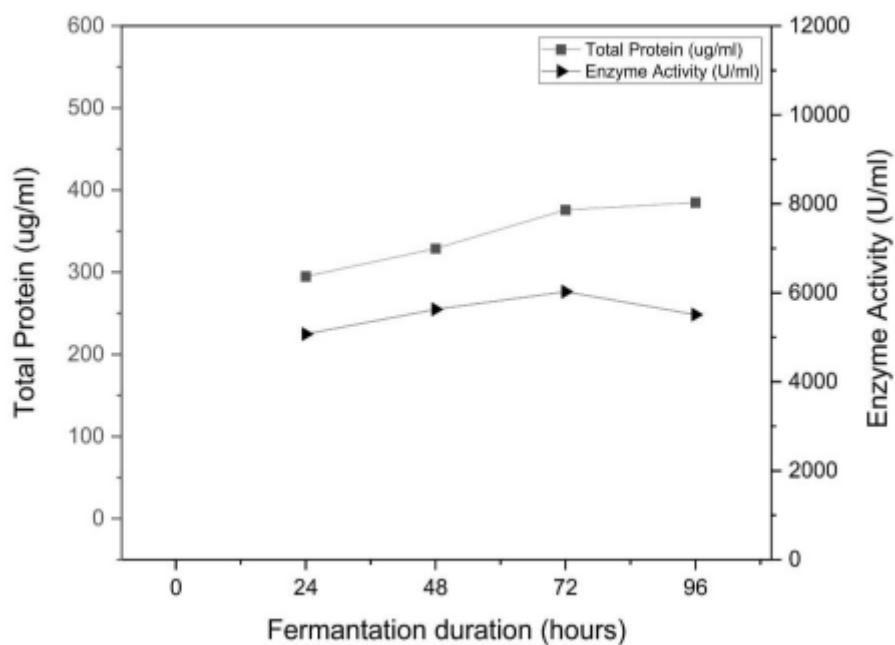


Figure 4.7 : Enzyme activity and total protein amount of MTGase protein expression at pH 5.0

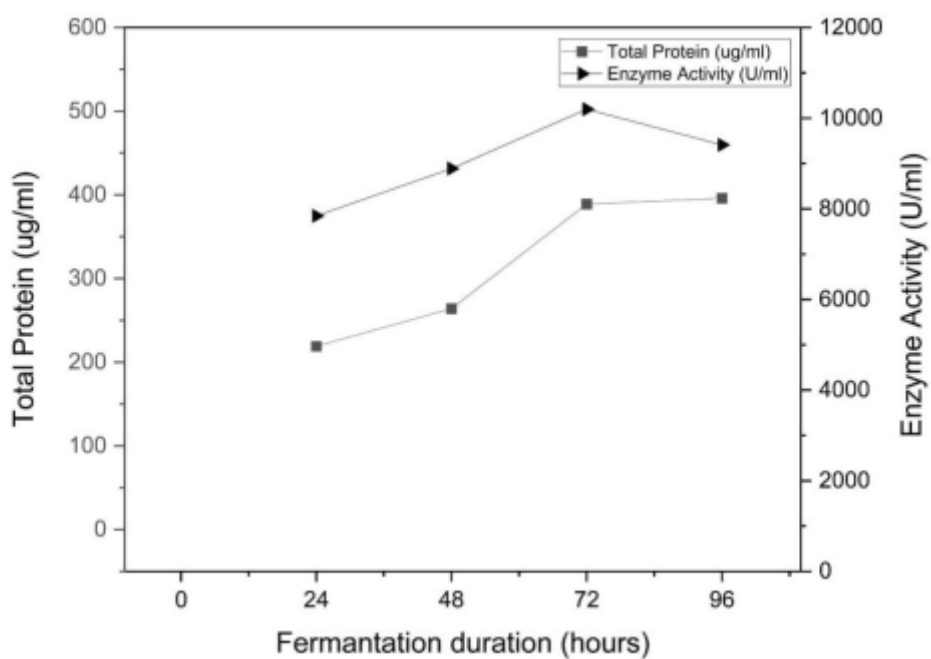


Figure 4.8 : Enzyme activity and total protein amount of MTGase protein expression at pH 7.0

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, this colored complex was detected at 525nm.

$$A = \frac{(\text{Abs. Test} - \text{Abs. Test Blank})}{(\text{y value at calibration curve})} \cdot \frac{1,23 (\text{total V})}{0,03 (\text{enzyme V})} \cdot \frac{1}{10 (\text{minutes})}$$

Equation 1: Formulation for detection of protein activity at 525 nm

As a result, the values were calculated according to the specific formula and the values are in U/mL, supernatant samples taken every twenty-four hours at pH:3.0 of KM 71 strain were 2130 U/mL, 2025 U/mL, 4430 U/mL and 6320 U/mL for 96th hour, respectively (Figure 4.6).

Supernatant samples taken every twenty-four hours at pH:5.0 of KM 71 strain were 5671 U/mL, 5079 U/mL, 6025 U/mL and 5500 U/mL for 96th hour, respectively (Figure 4.7)

Supernatant samples taken every twenty-four hours at pH:5.0 of KM 71 strain were 7841 U/mL, 8887 U/mL, 10200U/mL and 9400 U/mL for 96th hour, respectively (Figure 4.8)

4. DISCUSSIONS

Numerous studies have focused on increasing microbial transglutaminase production. Isolation of high-yielding production strains has played an important role in increasing transglutaminase production. Since the first report by Ando et al., about microbial transglutaminase production using *S. mobaraense* (Ando et al., 1989), continuous efforts have been made to select new strains with higher enzyme yields and to optimize the production process using various methods. In studies by Ando et al., TGase *Streptoverticillium* sp. The enzyme activity was found to be 2102 U/L. In another study, in 2007, Itaya et al. produced the transglutaminase enzyme produced by using *Corynebacterium glutamicum*, and the protein activity was measured as 23 U/L.

Pichia pastoris, a methylotropic yeast, has been used for many years in the production of recombinant protein. *P. pastoris* is a eukaryotic organism and is found in small amounts in nature, it can be used as a biofactory in the production of proteins that are found of industrial importance. *P. pastoris* yeast makes the post-translational modifications in the proteins correctly, allows the proteins to be folded in their active forms, and has the ability to produce proteins at g/L levels. Methods that allow their successful transformation are available and secrete very little native protein out of the cell, therefore they have been widely used in the production of heterologous proteins in recent years. In addition, efforts have been made to optimize substrates, metabolic processes, and environmental controls such as pH, dissolved oxygen level, and temperature to improve microbial transglutaminase yield.

Recombinant protein production is usually carried out under the control of the promoter of alcohol oxidase gene I (AOX1) in *P. pastoris*. The AOX1 promoter is induced by methanol and repressed by glucose and ethanol. The regulatory mechanisms of the AOX1 promoter have been studied in recent years. Another promoter used in recombinant protein production is derived from glyceraldehyde 3-phosphate dehydrogenase (GAP). It is a structural supporter. In another study, xylanase enzyme was performed indirectly as a promoter in *P. pastoris*. Isolation of high-yielding production strains has played an important role in increasing transglutaminase production. Since the first report by Ando et al. On microbial transglutaminase production using *S. mobaraense* (Ando et al., 1989), continuous efforts have been made to select new strains with higher enzyme yields and to optimize the production process using various methods. Numerous studies have focused on increasing microbial transglutaminase production. In addition, efforts have been made to optimize substrates, metabolic processes, and environmental controls such as pH, dissolved oxygen level, and temperature to improve microbial transglutaminase yield. Zheng et al. developed a two-stage environmental control strategy that resulted in an increase in transglutaminase activity from 2.0 U/mL to 3.40 U/mL (Zheng et al., 2002).

As a result of the work of another enzyme xylanase activity measured in 72-hour samples was ~228 U/mL, 416 U/mL, and ~219 U/mL for ADH3, AOX1 and GAP-induced clones, respectively. Interestingly, in contrast to the shake flask results, enzyme production at the fermentor scale with ADH3 was approximately 1.8 times higher than that of AOX1. In addition, the highest enzyme production was obtained with AOX1 cultures at the shaker level, while ADH3 cultures at the fermenter scale were more efficient (Karaoğlu et al., 2016). Recent literature has shown that newly identified promoters of *P. pastoris* in addition to AOX1 and GAP are also promising.

In the observation made in three different pH ranges at 20 °C in this study, it was observed that the most active protein ratio reached the highest value at an average of 72 hours, and this was at pH:7.0. When *P. pastoris* yeast is considered, previous studies show that 20 °C is the most efficient temperature on a small scale when higher and lower values are compared for this yeast. According to Cos et al. (2006), the optimal temperature for the growth of *P. pastoris* cells is 30 °C. However, protein production can still occur when the induction temperature is reduced to 15 °C, as observed by Wu et al.,. Regarding pH detection study, Cregg et al. (2000) determined that the best pH range for *P. pastoris* production is between 3.0-7.0.

In another study, by Türkanoglu et al., the result of transglutaminase enzyme produced by using the GAP promoter in *P. pastoris* was found in the bioreactor highest enzyme activity was obtained as 9120U/L at pH 7.5 and 20°C for transglutaminase enzyme as a result of 40 hour samples. On the other hand, 8425 U/L enzyme activity was measured at pH 7 and 20°C after bioreactor production. In this paper, the enzyme activity reached its highest level for pH 7 and was measured as 10200 U/ml considering the results of enzyme producing.

Consequently, research must continue on a large scale. As a result of different enzymes and different modifications, it seems that the ADH promoter seems promising in terms of efficiency for the production of extracellular transglutaminase using *P. pastoris* host KM71 strain at 20 °C.

5. CONCLUSION

This study covers the expression of the extracellular transglutaminase enzyme gene in the *P.pastoris* host using recombinant cloning techniques and the effect of promoter change on the production of recombinant transglutaminase at different pH values at the same temperature. Recombinant vector carrying the transglutaminase gene was created, and its extracellular production was carried out in *P. pastoris* yeast under the ADH promoter.

The main purpose of the study is to examine the recombinant production of the transglutaminase enzyme under the ADH promoter. In order to produce the enzyme recombinantly, the genes encoding the transglutaminase enzyme were cloned into *P. pastoris* yeast by cloning techniques. In order to find the clones with the best production as a result of cloning, clone screening productions were carried out in Erlenmeyer flask, and then production and protein activity calculations were made on the selected clones. Partial characterization (temperature and pH characterization) of the obtained supernatant was performed. In the SDS-PAGE analysis of the supernatants, the protein bands of the transglutaminase enzyme were found to be approximately 50 kDa in size. Although the amount of protein reached as a result of 96 hours of transglutaminase expression performed at the same temperature and conditions with different pH ranges varies between 125-396 ng/mL, the highest protein production level was observed as 396 ng/mL at pH:7 at 96th hour. Research shows that while low pH value affects production negatively, production increases around pH:7.

As a result of the study, it was concluded that the change in the promoter region significantly affects the production of the transglutaminase enzyme. In studies that can be done as a continuation of this study, using large-scale production systems and testing other environmental factors can make the enzyme more effective. In addition, the stabilization and other effects of the enzyme produced under this promoter can be examined.

If we look at its contributions to Turkey, it is thought that it will contribute to the techniques in the field of recombinant DNA technology, which we lack scientifically. If we look at the industrial-scale contributions, it is thought that a local source of transglutaminase enzyme can be obtained with the support of further projects in which the transglutaminase enzyme can be produced, and our country's dependence on foreign sources can be reduced in this context.

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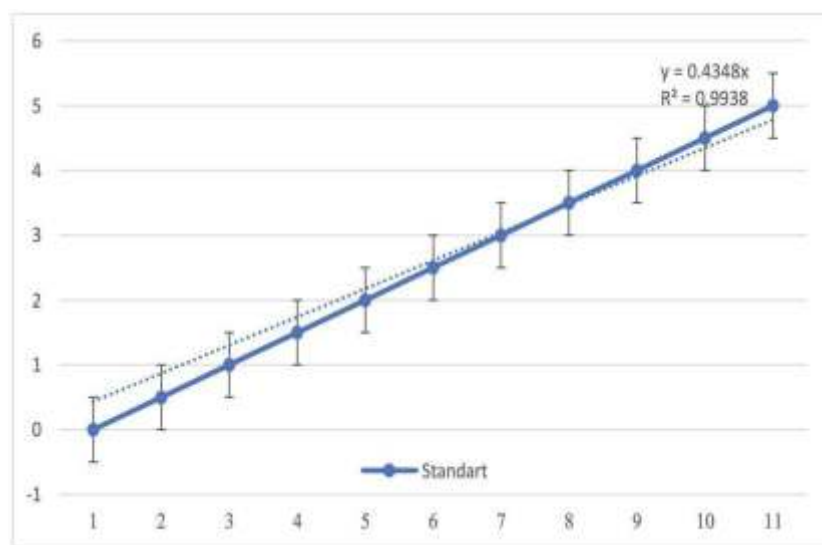
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Standard graph drawn with 595 nm absorbance values of BSA standard at different concentrations for total protein determination

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