

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**CLONING, EXPRESSION AND CHARACTERIZATION OF
THE ASPERGILLUS NIGER XYNB GENE**

KÜBRA BAYRAK
A THESIS SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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KARAKTERİZASYONU

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YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

Aspergillus niger secretes xylanases, and degrade xylans of plant origin, outside the cell. Xylanases used in various industrial applications can be produced mostly by microorganisms and also by using strains from which microorganism-derived xylanase genes are transmitted. Strains and enzymes obtained by recombinant methods are currently preferred.

In this study, *xynB* gene (UNEN*xynB2*) encoding functional xylanase B enzyme was transferred from *A.niger* MRC200803 strain belonging to TUBITAK MRC fungal collection to homologous and heterologous host microorganisms.

The UNEN*xynB2* gene is homologously integrated into the *A.niger* N593 chromosome under its own signal and control of the *glaA* promoter. 15 out of total of 17 transformants selected in uridine-free medium were found to be PCR positive. UNEN*xynB2* was heterologously transferred to *P.pastoris* with the *P.pastoris* PHO1 and *S.cerevisiae* α -factor signal peptides under the control of the AOX1 promoter. 84 of His⁺ candidate transformants were controlled by PCR. 10 candidate clones were identified as *xynB2* positive. UNEN*xynB2* expression was examined by flask culture with 0.5% methanol induction. At the end of the study, xylanase B enzyme was released extracellularly by PHO1 secretion signal in *P.pastoris* host. Expression-dependent activity in clone GH10S-6 was measured at 2U/ml at 48h when wheat arabinoxylan was used as substrate. Limited amount of enzyme was released in flask culture and it was observed that measured activity decreased rapidly. Crude xylanase expressed in *P.pastoris* was found to have optimum activity at pH 5 and 50°C. After 30 minutes of incubation at 50°C, the enzyme retains only 6% of its activity.

It was found that the recombinant xylanase enzyme obtained from the expression of UNEN*xynB2* gene expressed in *P.pastoris* GS115 was not efficient enough to start production studies in fermenter size.

Keywords: Xylanase, Recombinant protein expression, *Aspergillus niger*, *Pichia pastoris*.

ÖZET

Aspergillus niger bitkisel kaynaklı ksilanları degrades etmeye yarayan ksilanazları hücre dışına salgılamaktadır. Çeşitli endüstriyel uygulamalarda kullanılan ksilanazlar, en çok mikroorganizmalar tarafından ve yine mikroorganizma kaynaklı ksilanaz genlerinin aktarıldığı suşlar kullanılarak üretilmektedir. Rekombinant yöntemlerle elde edilmiş suşlar ve enzimleri günümüzde tercih edilmektedir.

Bu çalışmada, TÜBİTAK MAM kültür koleksiyonuna ait *Aspergillus niger* MRC200803 kodlu suştan, fonksiyonel ksilanaz B enzimini kodlayan *xynB* geni (UNEN*xynB2*) homolog ve heterolog konakçı mikroorganizmalara aktarılmıştır.

UNEN*xynB2* geni, kendi sinyali ile ve *glaA* promotörü kontrolünde, homolog olarak *Aspergillus niger* N593 suşu kromozomuna entegre edilmiştir. Üridin eklenmemiş ortamda seçilmiş toplam 17 transformantın 15'inin PCR pozitif olduğu tespit edilmiştir.

UNEN*xynB2*, AOX1 promotörü kontrolünde *Pichia pastoris*'e ait PHO1 ve *S. cerevisiae*'ye ait α -faktör sinyal peptitleri ile heterolog olarak *P. pastoris*'e aktarılmıştır. His⁺ aday transformantlarının 84 tanesi PCR ile kontrol edilmiştir. 10 tane aday klonun *xynB2* pozitif olduğu tespit edilmiştir. UNEN*xynB2* ekspresyonu, %0,5 metanol indüklemesi ile erlen kültürü yapılarak incelenmiştir. Çalışma sonucunda UNEN*xynB2* geninin ürünü olan ksilanaz B enzimi, *P. pastoris* konakçısında PHO1 sekresyon sinyali ile hücre dışına salınmıştır. GH10S-6 klonunda ekspresyona bağlı aktivite, buğday arabinoksilani substrat olarak kullanıldığında 48. saatte 2 U/ml olarak ölçülmüştür. Erlen kültüründe sınırlı miktarda enzim salınmış ve ölçülen aktivitenin hızla düştüğü görülmüştür. *P. pastoris*'te ifadesi sağlanmış ham ksilanazın pH 5 ve 50 °C sıcaklıkta optimum aktivite gösterdiği belirlenmiştir. Enzimin 50 °C'de 30 dakika inkübasyon sonunda aktivitesinin yalnızca %6'sı kalmaktadır. *P. pastoris* GS115'te ifade edilmiş olan UNEN*xynB2* geninin anlatımından elde edilen rekombinant ksilanaz enziminin fermentör boyutunda üretim çalışmalarına başlanması için yeterli verimlilikte olmadığı anlaşılmıştır.

Anahtar kelimeler: Ksilanaz, Rekombinant protein ekspresyonu, *Aspergillus niger*, *Pichia pastoris*.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
E.C.	: Enzyme Commission
ng	: Nanogram
µg	: Microgram
mg	: Milligram
µl	: Microliter
ml	: Milliliter
mM	: Millimolar
OD	: Optical density
amp	: Ampicillin
kan	: Kanamycin
α	: Alpha
β	: Beta
°C	: Centigrade degree
REase	: Restriction endonuclease
PCR	: Polymerase chain reaction
DNA	: Deoxyribonucleic acid
RNA	: Ribonucleic acid
gDNA	: Genomic DNA
cDNA	: Complementary DNA
sscDNA	: Single-stranded complementary DNA
dscDNA	: Double-stranded complementary DNA
dNTP	: Deoxynucleotide triphosphate
bp	: base pair
LB	: Luria-Bertani
rpm	: Revolutions per minute
min	: minute
dH ₂ O	: Distilled water
UNENxynB2	: Xylanase B cDNA from <i>A. niger</i> MRC200803 strain

pMxynBc2	: UNENxynB2 cDNA ligated with pMiniT vector
pAExynB2	: UNENxynB2 cDNA ligated with pGWf vector
SxynB2	: Codon-optimized UNENxynB2 cDNA without signal sequence
pBCK9	: SxynB2 sequence ligated with pPIC9 vector
pBKC-S1	: SxynB2 sequence ligated with pHIL-S1 vector
kDa	: Kilodalton
BSA	: Bovine serum albumin
APS	: Ammonium persulfate
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
AZCL	: Azurine-dye crosslinked
U	: Enzyme unit

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1. INTRODUCTION

Enzymes, the catalytic cornerstones of metabolism, are highly specific catalyst proteins that govern all metabolic events in living cells. Enzymes regulate the specificity and speed of many studies in the cell, as well as catalyze many chemical reactions *in vitro* [Kirk et al., 2002]. Enzymes having important metabolic functions in living structure; as a part of daily life, it makes important contributions in many fields such as economy, food, agriculture and industry [Wiseman, 1983]. The production and use of enzymes are increased and they are mainly from microorganism and some also from plant and animal origins [Sharma et al., 2018]. In recent years, the interest in industrial enzymes in biotechnological fields has increased due to the developing and growing enzyme technology, the usage areas of the products produced and their high economic value, and the studies and researches in this field have gained great importance. Again, enzyme production studies have gained great importance with recombinant DNA technology [Hols et al., 1994]. Generally, wild type microorganisms produce a lower level of enzyme in comparison with recombinant microorganisms. Enzyme production cost is an important topic at the industrial level. Purposes of the recombinant enzyme productions are; natural sources can't produce in sufficient amount of enzyme or designed protein is not found in nature [Damasceno et al., 2012].

Among the applications of recombinant DNA technology are the homologous and/or heterologous expressions of enzymes. With bioengineering applications, the enzyme can be produced on a large scale using suitable microorganisms [Gao et al., 2014]. To date, in general, a xylanase gene has been integrated into the chromosome as single or multiple copies. Extracellular release of the enzyme was achieved by different signal sequences. However, there is still need to well produced recombinant enzymes by obtaining new organisms by the help of the molecular strategies.

2. LITERATURE REVIEW

2.1. Enzymes

Enzymes are organic biomolecules that catalyze biochemical reactions in cells. Enzymes that can show activity in natural environment can be isolated from various living groups by biotechnological methods and can be used in different industrial fields. Although enzymes originate from animal, plant and microorganisms, the enzymes used in the industrial field are generally obtained from microorganisms. The main reasons for this situation: The enzymes obtained from microorganisms have higher catalytic activity than animal- and plant-based enzymes; they are cheaper and more stable, they do not form unwanted by-products; they can be produced in high purity and in large quantities [Kiran and Çömlekçioğlu, 2003; Kiran et al., 2006]. Enzymes obtained from living cells with the development and progress of biotechnology; beer production, dairy, processing meats, fruit syrups, clarification of food, food industry, for the breakdown of oil waste in the detergent industry, leather and weaving industry, diagnosis in medicine, medical treatment methods, detergents and other chemical cleaners also contribute to the bleaching process [Voget et al., 2006].

2.2. Xylan

Hemicellulose is the second most abundant polysaccharides in nature and xylan is one of the constituents of hemicellulose. Hemicelluloses are composed of arabinan, galactan, and mannan or combining of those polysaccharides such as arabinoxylan, galactoglucuronoarabinoxylan. In the plant cell wall, xylans are localized between cellulose fiber and lignin [Scheller et al., 2010]. Xylans are one of the main polysaccharides that are widely found in nature and form the structure of hemicelluloses. They are located on the cell walls of terrestrial plants, on the interface of lignin and cellulose, that is, on the secondary cell wall of plant cells [Wong et al., 1988]. Xylan, cellulose and lignin are connected to each other by

covalent or non-covalent bonds and together form the main structure of the plant cell wall [Beg et al., 2001]. Xylans are broken down into D-xylose units by hydrolysis of β -1,4 bonds by xylanases [Biely, 1985], it is a complex polysaccharide and different enzymes have different properties such as substrate specificity and mode of actions to degrade heterogenous xylans [Dey et al., 2018]. It consists of β -1,4-linked xylopyranosyl residues and has other substituted groups such as arabinose, acetyl, glucuronic acids, and ferulic acids [Moreira et al., 2016]. Primary cell walls of grasses and monocot plant cereals such as wheat, rye, barley, oat, rice, corn, and sorghum are contained arabinoxylan as a major part of the hemicellulosic component [Saha, 2000]. Arabinoxylan is the primary constituent in wheat grain and two forms of arabinoxylans are present; water-soluble form and water-insoluble form [Sharma et al., 2018]. Conversions of water-insoluble arabinoxylan to water-soluble arabinoxylan by xylanases provide the highest bread volume, uniform, and finer crumbs and decrease dough firmness [Butt et al., 2008]. The hydrolysis mechanism of the endoxylanases are shown in Figure 2.1 [Butt et al., 2008].

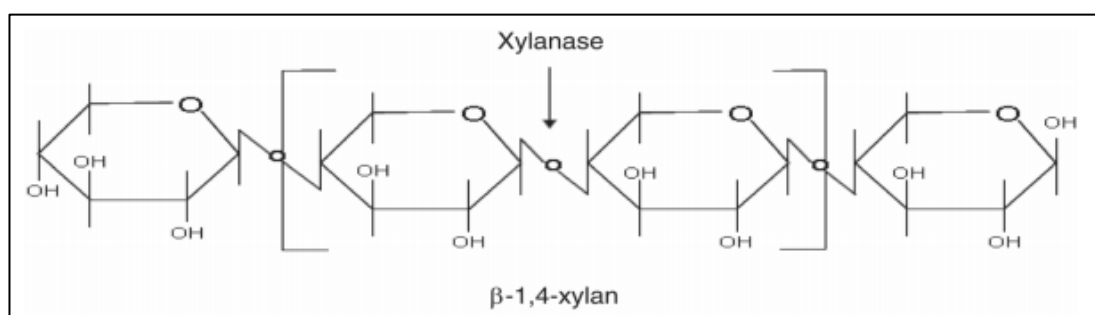


Figure 2.1: Hydrolysis of the xylan polymer by xylanase.

Arabinoxylans have been identified in barley, wheat, rye, rice, oats [Izydorczyk et al., 1995]. Although these polysaccharides are minor components of all grains, they form an important part of the plant cell walls. Glucuronoxylans and glucuronoarabinoxylans act as adhesives by forming covalent and non-covalent bonds with lignin, cellulose and other polymers that are essential to the integrity of the cell wall. Xylans are the most important class of hemicelluloses. They constitute 15-30% of the total dry weight in angiosperms, but are less common in

gymnosperms (7-12% xylan) [Haltrich et al., 1996]. Glucuronoxylans are linear polymers of β -D-xylopyranosyl units bound by xylose.

2.3. Xylanases

Xylanases are mainly produced by microorganisms. Many microorganisms are secreted different carbohydrate-degrading enzymes to obtain energy from plant biomass and evolved their cellular machinery. These enzymes are capable of degrading of complex mixtures. Xylan is one of these complex mixtures and heterogeneity of xylan needs different types of enzymes for complete degradation such as endo- β -1,4-xylanase (E.C. 3.2.1.8), β -1,4-xylosidase (E.C. 3.2.1.37), α -L-arabinofuranosidase (E.C. 3.2.1.55), α -glucuronidase (E.C. 3.2.1.139), acetyl xylan esterase (E.C. 3.1.1.72) and phenolic acid esterase (E.C.3.1.1.73) [Dhiman et al., 2008]. Among those enzymes, endo- β -1,4-xylanases are the most important one because they degrade the main chain of xylans and liberate xylooligosaccharides.

Endo- β -1,4-xylanases are the key enzymes for the degradation of xylan backbone [Walia et al., 2017]. According to the CAZy database, endo- β -1,4-xylanases are belong to the GH10 and GH11 families. Major researches are focused on the GH10 and GH11 family xylanases action mechanism for xylan degradation. GH10 family xylanases hydrolyze the xylan backbone and xylan specificity is less. GH11 family xylanases are known as true xylanases and specific for xylan. GH10 and GH11 family of xylanases have family-specific structure, $(\alpha/\beta)_8$ barrel fold and β -jelly roll fold, respectively [Moreira et al., 2016]. Since 1988, xylanases have been subdivided into many types. Firstly, xylanases are classified based on the molecular weight and isoelectric point (pI) of the enzyme [Wong et al., 1988]. They are also classified in the CAZy database by comparison with the catalytic sites in the primary structure of the enzyme. According to this database, xylanases are divided into 14 different GH families. Endo- β -1,4-xylanases are subdivided in two glycoside hydrolase family. Family G xylanases or GH11 xylanases have low molecular weight (<30 kDa) and basic pI. In spite of the GH11 xylanases, Family F or GH10 xylanases have high molecular weight (>30 kDa) and acidic pI [Paës et al., 2012]. Fungal

xylanases are not categorized in this way because some fungal GH11 xylanases have low molecular weight and acidic pI [Dey et al., 2018]. Later on, different classification systems are came up based on xylanase properties such as size, shape, physicochemical properties, structural properties, kinetic parameters, etc. [Hmida-Sayari et al., 2012].

Xylanases have also been categorized depending on the optimum pH values: Acidophilic xylanases active at low pH, neutral xylanases active at neutral pH and alkaliphilic xylanases active at high pH [Fushinobu et al., 2011]. Acidophilic xylanases are valuable for many industries such as biobleaching, fruit juice clarification, baking, and xylooligosaccharide production.

Endo- β -1,4-xylanases also hydrolyze β -1,4-D-xylosidic bonds and produce xylooligosaccharides [Dhiman et al., 2008]. Xylooligosaccharides are used as prebiotics and food supplements. It is produced by auto-hydrolysis, chemical or enzymatic treatments. Auto-hydrolysis necessitates specialized equipment for high temperature and chemical methods require corrosive acids [Sharma et al., 2018]. Enzymatic treatment for xylooligosaccharide production is an alternative method that is safe, provides easy downstream processing and does not require special equipment [Samanta et al., 2017]. In enzymatic treatment, xylooligosaccharides are produced random hydrolysis of heteroxylan by xylanases [Sharma et al., 2018].

Xylanases are widely used alone or in combination with other enzymes for different industries [Liu et al., 2017]. Xylanases are used in bread making [Dornez et al., 2011], biobleaching [Satyanarayana, 2013], animal feed additive [Gao et al., 2014] and biofuel production [Gray et al., 2006].

Xylanases are produced by different organisms such as bacteria, archaea, plants, fungus, animals, and nematodes [Sharma et al., 2018]. Bacteria and fungi produce a large amount of xylanases. Due to both high xylanase production and advantages over plants and animals such as fast growth, cheap medium, and controlled production, industrially valuable xylanases are mainly produced by microorganisms [Mitreva-Dautova et al., 2006].

Fungi produce biomass-degrading enzymes including different types of xylanases and these are important organisms for the heterologous protein production. Fungal xylanase production yield is much higher than other

microorganisms but in contrary articles are present [Li et al., 2015]. While *Aspergillus*, *Trichoderma* hosts are preferred in commercial xylanase production, mold-derived genes are recombinantly cloned and expressed to produce xylanase more and to improve its properties industrially. Recombinant xylanases obtained from the expression of fungal xylanase genes in homologous and heterologous systems are reported to be promising for industrial production [Ahmed et al., 2009; Karaoglan et al., 2014].

Gao et al. (2014) reported that *Aspergillus niger* IA-001 xylanase B (*XynB*) (JX560731.1) was expressed in *Pichia pastoris*. In this study, the amino acid sequence has been optimized according to the codon usage of *Pichia*, and the gene region has been designed to produce two repetitive proteins. Thus, the recombinant enzyme was obtained 5.3 times more than the natural *xynB* and the enzyme that can be used in the feed sector was obtained.

Xu et al., (2016) reported that *xynA* gene from *Streptomyces* sp. FA1 expressed in *Pichia pastoris* KM71 host and obtained xylanase used in making for Chinese steam bread.

In Chang et al., (2017), the alkali-tolerant xylanase from *Bacillus subtilis* Lucky9 was expressed in *E. coli* and obtained xylanase used for prebiotic production.

2.4. Industrial Use of Xylanases

Xylanases have found application in a variety of industries, particularly food, due to their ability to hydrolyse plant-derived xylans into simple sugars. The feed and food industry use xylanases alone or in combination with other enzymes when lignocellulosic raw materials are used. In the food industry, enzymes are used as the main auxiliary components, especially for bakery. Hydrolysis of peptides is possible by proteases, hydrolysis of esters by lipases and esterases, oxidation of phenol groups by laccases, oxidation of hydroxyl groups by sulfhydryl oxidases. The arabinoxylans in the flour are hydrolyzed with endoxylanases. In the bakery sector, xylanase is usually combined with amylase and other enzymes to participate in bread making [Melim Miguel et al., 2013]. The complete hydrolysis of arabinoxylan

from wheat flour occurs with the joint activity of α -L-arabinofuranosidase and B-xylosidase [McCleary et al., 2015]. The use of xylanase reduces the internal hardness of the bread, slows down the staling and increases the specific volume of the bread, facilitates the processability of the dough and improves the bread quality positively [Güner et al., 2008].

2.5. Microbial Recombinant Protein Expression Systems

E. coli has been used as a host for recombinant protein expression. This microorganism can't process post-translational modification because lacks of intracellular machinery. So, eukaryotic protein production in *E. coli* expression system becomes not favorable. Therefore, *E. coli* is not suitable for the production of protein containing disulphide bridges or requiring PTMs such as glycosylation [Spohner et al., 2015].

S. cerevisiae is another host system for recombinant protein production. The difference between *E. coli* and *S. cerevisiae*, *S. cerevisiae* can produce active eukaryotic protein. *S. cerevisiae* expression system can process the post-translational modifications of the protein. However, this system hyperglycosylate the protein so, it can be undesirable for production of some proteins.

2.6. *Aspergillus niger* Expression System

Filamentous fungi secrete many proteins mainly hydrolytic enzymes and this feature is attractive for the production of recombinant protein. *Aspergillus niger* is a filamentous fungi and used as both homologous and heterologous recombinant protein expression system. *A. niger* is a GRAS status microorganism [Schuster et al., 2002] and different industrial products produced in this organism. There are some advantages for the production of proteins in this system: *A. niger* can secrete proteins to culture medium, *A. niger* is an eukaryotic microorganism so it can post-translationally modify eukaryotic proteins. *A. niger* has most efficiently secreted protein glucoamylase (glaA), and glucoamylase gene promoter and its secretion

signal are widely used for recombinant protein expression [Ganzlin and Rinas, 2008]. *glaA* promoter is an inducible promoter to express target gene and is required the presence of starch or its hydrolytic products in the culture media [Gladden et al., 2019, US 2019/0169584 A1] and xylose repress the *glaA* promoter-controlled gene expression [Ganzlin and Rinas, 2008].

2.7. *Pichia pastoris* Expression System

In recent years, methylotrophic yeast *Pichia pastoris* (*Komagataella pastoris*) from industrial yeasts has been used as a host system for protein and enzyme production [Macauley-Patrick et al., 2005]. *Pichia pastoris* (*P. pastoris*) is classified by the USA FDA as GRAS (Generally Recognized as Safe) microorganism [Ahmad et al., 2014]. It was developed in the early 1980s by Philips Petroleum Company and Salk Institute Biotechnology/Industrial Associates (SIBIA, La Jolla, CA, USA). *P. pastoris*-based system for recombinant protein expression was developed to produce a single-cell protein (SCP) [Juturu and Wu, 2018].

P. pastoris is one of the most popular eukaryotic microorganisms as a host for recombinant protein production and it is released out of the cell and is being used to produce properly folded and functional proteins. It uses methanol as a sole carbon source. *P. pastoris* expression system has some advantages over other recombinant protein producers; it capable of high cell densities, ease of genetic manipulation, both intracellular and extracellular protein can be produced, allows post-translational modifications such as glycosylation and disulfide bond formation, can grow in cheap media. Also, recombinant products do not contain endotoxins, oncogenic or viral DNA [Damasceno et al., 2012].

The expression system of *P. pastoris* offers strong and inducible promoter name as alcohol oxidase 1 (AOX1) promoter. Alcohol oxidase is the first enzyme in *P. pastoris* methanol utilization pathway. *AOX1* and *AOX2* genes produce alcohol oxidase enzyme in peroxisome which catalyzes methanol to formaldehyde conversion. *AOX1* gene is synthesizing 90% of the alcohol oxidase enzyme. It strongly induced by methanol [Duan et al., 2009]. There are vectors and strains

containing the promoter which enable the integration of the gene that expresses the target recombinant protein into the genome and is effective in protein production. Integration into the *P. pastoris* genome is possible by gene insertion and gene replacement. The insertion or replacement of the *AOX1* locus may occur as well as insertion into the *his4* locus.

When methylotrophic yeast *P. pastoris* is induced by AOX1 promoter, it uses only methanol as the carbon source [Daly and Hearn, 2005]. AOX oxidize the methanol and formed formaldehyde in the peroxisomes [Hartner and Glieder, 2006] (Figure 2.2). AOX suppresses other carbon sources in case of carbon starvation and methanol induction. In methanol metabolism, the AOX enzyme needs oxygen to methanol oxidization, during which H_2O_2 and formaldehyde are formed.

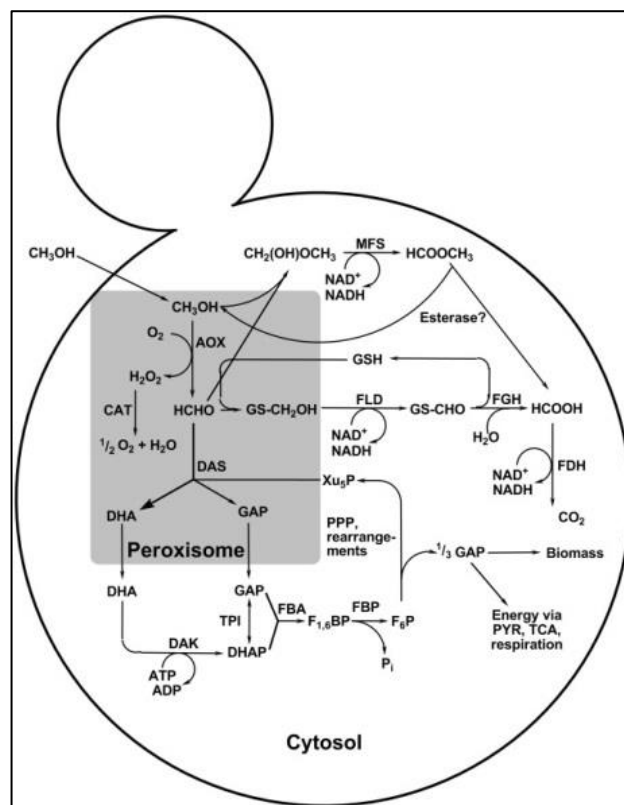


Figure 2.2: Methanol utilization pathway.

Regardless of the genetic structure, the physiological effects that occur during the culture of yeast affect the efficiency of industrial enzymes or other important heterologous proteins produced in these systems. Therefore, optimization is

needed. Cultivation conditions are based on the culture protocols provided by Invitrogen (USA). Alcohol oxidase genes can be produced up to gr/L levels for recombinant enzyme or protein, using recombinant strains in methanol oxidation by Mut⁺ and Mut^s phenotype. In the development of recombinant protein production in *P. pastoris*, it can appear unpredictable results specific to the strain. Therefore, the experimental characteristics of each strain should be determined.

There are several examples of recombinant proteins produced by *Pichia pastoris*. The first *P. pastoris*-produced therapeutic protein KALBITOR® approved by FDA in 2009 [Walsh, 2009].

Codon optimization, multi-copy gene transfer, optimization of signal peptide, strengthening of promoter and optimization of fermentation conditions are among the reported strategies to increase the expression of heterologous protein in *P. pastoris* system [Li et al., 2010].

2.8. Aim of The Thesis

The objectives of the thesis are: Firstly, to clone *A. niger xynB* gene. Secondly, transformation/integration of this gene into both *A. niger* and *P. pastoris*. Finally, to screen and characterize *xynB* expression in *P. pastoris*.

This thesis has been carried out in collaboration with TUBITAK MRC. It is part of ARDEB 1003 project no 115O052 and Sub-project no 115O053. It is related to two work packages of the project.

3. MATERIALS & METHODS

3.1. Materials

3.1.1. Microorganisms

Recombinant *P. pastoris* GH10S-6 strain was obtained using pBKC-S1 plasmid via homologous recombination in this study. Other microorganisms and plasmids are given in Table 3.1 and Table 3.2.

Table 3.1: Microorganisms used in this work.

Microorganisms	Genotype	Source/Reference
<i>Escherichia coli</i>		
DH5 α	F ⁻ , Φ 80d <i>lacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk ⁻ , mk ⁺), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	TUBITAK MRC (Turkey)
DH5 α (pMxynBc2)		This work
DH5 α (pAExynB2)		This work
TOP10F'	F' { <i>proAB</i> , <i>lacIq</i> , <i>lacZ</i> Δ M15, Tn10 (Tet ^R)} <i>mcrA</i> , Δ (<i>mrr-hsdRMS-mcrBC</i>), ϕ 80 <i>lacZ</i> Δ M15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , λ^- <i>araD139</i> , Δ (<i>ara-leu</i>) 7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Str ^R), <i>endA1</i> , <i>nupG</i>	Invitrogen Life Technologies (USA)
TOP10F' (pBCK9)		This work
TOP10F' (pBKC-S1)		This work
<i>Aspergillus niger</i>		
MRC200803	Wild type	TUBITAK MRC (Turkey)
N593	<i>cspA pyrA6</i>	Karasugen Biotechnology (Turkey)
N593 (pAExynB2)		This work
<i>Pichia pastoris</i>		
KM71	<i>arg4 his4 aox1::ARG4</i>	Invitrogen Life Technologies (USA)

Table 3.1: Table of continued.

GS115	<i>his4</i>	Invitrogen Life Technologies (USA)
GH10S-6		This work

Table 3.2: Plasmids used in this work.

Plasmid	Construct	Source/Reference
pMiniT		New England Biolabs (USA)
pGWf		Karasugen Biotechnology (Turkey)
pHIL-S1		Invitrogen Life Technologies (USA)
pPIC9		Invitrogen Life Technologies (USA)
pMxynBc2	pMiniT::UNENxynB2*	This work
pAExynB2	pGWf::UNENxynB2	This work
pBKC-S1	pHIL-S1::SxynB2**	This work
pBCK9	pPIC9::SxynB2	This work

*UNENxynB2: *A. niger* MRC200803 cDNA sequence encoding xylanase B.

**SxynB2: Synthetic UNENxynB2 cDNA sequence.

3.1.2. Primers

Table 3.3: List of primers.

Primer name	Primer sequence	Purpose of use
MiniTf	ACCTGCCAACCAAAGCGAGAAC	sscDNA preparation
MiniTr	TCAGGGTTATTGTCTCATGAGCG	sscDNA preparation
XBPf	GGTTAATTAAATGCTCACCAAGAACCTTCTCC	Cloning and PacI site addition to UNENxynB2 cDNA
XBNr	GCGGCCGCTTACTGAACAGTGATGGA	Cloning and NotI site addition to UNENxynB2 cDNA
GWf	CTTCAGCTTCCCCTCGTGCA	For PCR control and DNA sequencing
GWR	GCGAGGTCCAATGCATTAATGC	For PCR control and DNA sequencing

Table 3.3: Table of continued.

AOX1F	GACTGGTTCCAATTGACAAGC	For PCR control and DNA sequencing
AOX1R	GCAAATGGCATTCTGACATCC	For PCR control and DNA sequencing
AOX1pF3	TACACCCGAACATCACTCCA	For DNA sequencing
optF	CTGCACAAGACATCACCT	For PCR control
optR	CACGCTTATTCTGACGGA	For PCR control

3.1.3. Equipments

Table 3.4: List of the equipments.

Name of Equipment	Brand, Country
Thermocycler	TC-512, Techne, UK
Heater	Techne Dri-Block, DB-2A, UK
Electrophoresis System	PowerPac HC, Bio-Rad, USA
Power Supply	Bio-Rad, USA
NanoDrop	ND-1000, Thermo Fisher Scientific, USA
Electroporation System	GenePulser Xcell, Bio-Rad, USA
Biological Safety Cabinet	Esco Class II, Singapore
PCR Cabinet	Biosan, Latvia
Vortex	Dragon Lab, China
Ice Machine	F125 Compact, Icematic, Italy
15 ml, 50 ml Falcon Tubes	Axygen, USA and Falcon, USA
Petri	Greiner, Germany
Water Bath	Lauda LCB 0726, Germany
Gel Doc Imaging Systems	Azure Biosystem C600, USA
UV Transilluminator	Vilber Lourmat, France
pH Meter	Metrohm, Switzerland
Autoclave	Sistek, Turkey
Hotplate Stirrer	Wisestir, Korea
Lyophilizator	Alpha 1-2 LDplus, Christ, Germany
Shaker	KS 260, IKA, Germany
37° C Incubator	Nüve, Turkey
28° C Incubator	Innova 4230, New Brunswick Scientific, USA Innova 43, New Brunswick Scientific, USA
Spectrophotometer	SmartSpec™ Plus, Bio-Rad, USA
Centrifuges	5417R, Eppendorf, USA SL 16R, Thermo Scientific, USA Avanti J-26 XPI, Beckman Coulter, USA
Eppendorf Research^(R) plus	Eppendorf, Germany
0.5 ml, 1.5 ml Microcentrifuge Tubes	Axygen, USA

3.1.4. General kits and Reagents

Table 3.5: List of kits, enzymes and chemicals.

Name of Kits and Reagents	Code, Country
Bead	FGD0150, Karasugen Biotechnology, Turkey
NucleoSpin RNA Isolation Kit	740955.50, Macherey-Nagel, Germany
ProtoScript First Strand cDNA Synthesis Kit	E6300S, New England Biolabs (NEB), USA
PCR Cloning Kit	E1202S, New England Biolabs (NEB), USA
Qiaprep Spin Miniprep Kit	27106, Qiagen, Germany
ZymoClean Gel DNA Recovery Kit	D4007, Zymo Research, USA
Genomic DNA Clean&Concentrator Kit	D4011, Zymo Research, USA
PacI, Restriction Enzyme	R0547L, New England Biolabs (NEB), USA
NotI, Restriction Enzyme	R3189L, New England Biolabs (NEB), USA
Quick Stick Ligase	BIO-27027, Biotline, UK
ZymoPURE™ Midiprep Kit	D4200S, Zymo Research, USA
Aspergillus Transformation Kit	ATK593, Karasugen Biotechnology, Turkey
Quick-DNA™ Fungal/Bacterial Miniprep Kit	D6005, Zymo Research, USA
Pichia Expression Kit	K1710-01, Invitrogen, USA
PureLink™ Genomic DNA Mini Kit	K1820-02, Invitrogen, USA
EcoRI-HF	R3101S, New England Biolabs (NEB), USA
XhoI	R0146S, New England Biolabs (NEB), USA
Sall-HF	R3138S, New England Biolabs (NEB), USA
Xylazyme AX Tablets	T-XAX-1000T, Megazyme, USA
Arabinoxylan (Wheat Flour; Medium Viscosity ~ 30 cSt)	P-WAXYM, Megazyme, USA
Amicon® Ultra-15 Centrifugal Filter Unit	UFC901024, Millipore, USA
GeneRuler DNA Ladder Mix	SM0331, Fermentas, USA
PageRuler™ Plus Prestained Protein Ladder	26619, Thermo Scientific, USA
Kanamycin	K-4378, Sigma-Aldrich, USA
Ampicilline	10835269001, Roche, Switzerland
EZ-Vision Three Gel Loading Dye	N313-KIT, VWR, USA
DreamTaq DNA Polymerase	EP0702, Thermo Scientific, USA
Q5® High-Fidelity DNA Polymerase	M0491S, New England Biolabs (NEB), USA
Standart Taq DNA Polymerase	M0320S, New England Biolabs (NEB), USA
Agarose	A5093, Sigma, USA
Acrylamide/Bis-acrylamide, 30% solution	A3574, Sigma, USA
Ammonium persulfate	A3678, Sigma, USA
Acetic acid	27225, Sigma, USA

Table 3.5: Table of continued.

Bacto agar	214010, BD
Bacto tryptone	211705, BD
Bovine serum albumin (BSA)	A7030, Sigma, USA
Calcium chloride dihydrate	208290, Merck, Germany
Coomassie brilliant blue R-250	A1092.0025, Applichem
Ethanol	100983.2500, Merck
Ethylenediaminetetracetic acid (EDTA)	ED4S, Sigma, USA
Glycerol	G2025, Sigma, USA
Glycine	G8898, Sigma, USA
Methanol	24229, Sigma, USA
2-Propanol	100995, Merck, Germany
Sodium chloride	13423, Sigma, USA
Sodium dodecyl sulfate (SDS)	L3771, Sigma, USA
TEMED (N, N, N', N'-tetramethylethylenediamine)	T7024, Sigma, USA
Trizma base	T1503, Sigma, USA
Coomassie brilliant blue G-250	1.15444.0025, Merck, Germany
Perchloric acid	311421, Sigma, USA
Magnesium chloride	M8266, Sigma, USA
Magnesium sulfate heptahydrate	M7506, Sigma, USA
D-(+)-Glucose	G5400, Sigma, USA
BBL Yeast extract	21929, BD
Sodium hydroxide	S8045, Sigma, USA
Peptone	MC004, LabM
Yeast nitrogen base without amino acids	Y0626, Sigma, USA
d-Biotin	B4501, Sigma, USA
Antifoam C emulsion	A8011, Sigma, USA
Tryptic soy broth	1.05459.0500, Merck, Germany
Sodium carbonate	13418, Sigma, USA
Silver nitrate	85228, Sigma, USA
Potassium sodium tartarate tetrahydrate	218255, Sigma, USA
Sodium sulfate	13464, Sigma, USA
Copper (II) sulfate penta-hydrate	209198, Sigma, USA
Sulfuric acid 95-97%	1.00731.2500, Merck, Germany
Ammonium molybdate tetrahydrate	09878, Sigma, USA
Sodium arsenate dibasic heptahydrate	S9663, Fluka, USA
Citric acid	251275, Sigma, USA
D-(+)-Xylose	95729, Sigma, USA
PEG 6000	528877, Calbiochem, Germany

Table 3.5: Table of continued.

Bromophenol blue	B0126, Sigma, USA
2-Mercaptoethanol electrophoresis reagent	194834, MP
Formaldehyde solution (37%)	252549, Sigma, USA
Sodium thiosulfate pentahydrate	1.06516.1000, Merck, Germany
Sodium citrate tribasic dihydrate	S4641, Sigma, USA
Potassium phosphate monobasic	P5655, Sigma, USA
Potassium phosphate dibasic	P2222, Sigma, USA
Sodium citrate monobasic	71497, Sigma, USA

3.1.5. Buffers and Solutions

Table 3.6: List of buffers and solutions.

Buffer / Solution	Content / Preparation
LB Broth Medium (for 1L)	10 g bacto tryptone, 5 g yeast extract, 10 g NaCl, pH 7.5 using 1 M NaOH, adjust the volume to 1 liter with dH ₂ O.
LB Agar Medium (for 1L)	LB Broth Medium, 15 g agar
100X ampicillin	10 mg ampicillin, 10 ml dH ₂ O
100X kanamycin	5 mg kanamycin, 10 ml dH ₂ O
50X TAE Buffer (for 1 L)	242 g Tris base, 57,1 ml glacial acetic acid, 100 ml 0.5 M EDTA, adjust the volume to 1 liter with dH ₂ O.
1X TAE Buffer (for 1 L)	20 ml 50X TAE buffer, 980 ml dH ₂ O.
1% Agarose gel (For 50ml)	0,5 g Agarose, 50 ml 1X TAE buffer
SOC medium	0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ . After autoclaving, 20 mM glucose is added.
Cryo solution	80 g/L 100% glycerol, 2.4 g agar, and 720 ml dH ₂ O
YPD broth medium	1% yeast extract, 2% peptone, 2% dextrose
BMGY/BMMY broth medium	1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10 ⁻⁵ % biotin, 1% glycerol or 0.5% methanol
MD/MM agar plate	1,34% YNB, 4×10 ⁻⁵ % biotin, 2% dextrose or 0.5% methanol, 15 g agar

Table 3.6: Table of continued.

YPTG/YPTM broth medium (for 250 ml)	182 ml dH ₂ O, 5 ml %2 TSB, 25 ml 10% glycerol or 25 ml 5% methanol, 25 ml 20% peptone, 12,5 ml 10% yeast extract, 0.5 ml 0,02% Biotin.
Reagent A	25 g anhydrous sodium carbonate, 25 g sodium potassium tartrate, 200 g anhydrous sodium sulfate are dissolved in 800 ml dH ₂ O. The volume is adjusted to 1 L with dH ₂ O.
Reagent B	30 g copper sulfate pentahydrate in 200 mL of demineralised water containing 4 drops concentrated sulfuric acid.
Reagent C	Dissolve 50 g of ammonium molybdate in 900 mL of demineralised water and carefully add 42 mL of concentrated sulfuric acid. Separately, dissolve 6 g of sodium arsenate heptahydrate in 50 mL of water. Then mix the solutions and adjust the volume of the solution to 1 L with dH ₂ O.
Reagent D	Add 1 mL of reagent B to 25 mL of reagent A.
Reagent E	Dilute Reagent C 5-fold with demineralised water just before use.
Sodium citrate buffer (1 M, pH 6)	294.1 g sodium citrate tribasic dihydrate is dissolved in 800 ml dH ₂ O. 1 M citric acid is added to the buffer until pH 6. Volume is adjusted to 1 L with dH ₂ O.
Potassium phosphate buffer (1 M, pH 6)	132 mL of 1 M K ₂ HPO ₄ , 868 mL of 1 M KH ₂ PO ₄ are mixed and pH = 6.0 is confirmed (phosphoric acid or KOH used for pH adjustment).
1% arabinoxylan	1 g substrate, 6 mL of 95% v/v ethanol and 80 mL of cold water are dissolved using heat stirrer. 10 mL of sodium citrate buffer (1 M, pH 6) is added to the substrate solution. The volume is adjusted to 100 mL.
Stock xylose solution	0.05 g xylose is dissolved in 100 ml, 100 mM pH 6 sodium citrate buffer.

Table 3.6: Table of continued.

Dye-binding solution (for 1 liter):	0.6 g Coomassie brilliant blue G-250 and 2% perchloric acid is dissolved in water. The solution is placed in a dark bottle by filtration through a filter paper. A spectrophotometer is used to read at 465 nm from the filtrate to a glass cuvette. The reading range of the solution should be between 1.3 and 1.5.
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3.1.6. Kit Protocols

The RNA isolation protocol (Nucleospin RNA kit) from *A. niger* is given below.

- 250 mg myceliums disrupt with beads, 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol using homogenizer at high speed for 2.5 min.
- Centrifuge lysate to a collection tube using NucleoSpin® Filter for 1 min at 11,000 x g.
- Filter discard and 350 µl ethanol (70%) add to lysate and mix by pipetting.
- Lysate load to NucleoSpin® RNA Column then centrifuge for 30 s at 11,000 x g.
- 350 µL MDB add and centrifuge at 11,000 x g for 1 min.
- DNase reaction mixture preparation: Mix 10 µL reconstituted rDNase and 90 µL Reaction Buffer for. Add 95 µL DNase reaction mixtures to the column then incubate at room temperature for 25 min.
- 200 µL Buffer RAW2 add to the column and centrifuge for 30 s at 11,000 x g.
- 600 µL Buffer RA3 add to the column and centrifuge for 30 s at 11,000 x g. Discard filtrate.
- 250 µL Buffer RA3 adds to column and centrifuge for 2 min at 11,000 x g. The column placed into a nuclease-free eppendorf.
- RNA elutes using 40 µL RNase-free H₂O and centrifuge at 11,000 x g for 1 min.

Quick-DNA™ Fungal/Bacterial Miniprep Kit used for plasmid isolation and the protocol is given below.

- 50 – 100 mg (wet weight) fungal or bacterial cells resuspended in 200 µl of water or isotonic buffer (e.g., PBS) to a ZR BashingBead™ Lysis Tube. 750 µl BashingBead™ Buffer added to the tube.
- Sample was homogenized using vortex (>20 min) or homogenizer.
- ZR BashingBead™ Lysis Tube centrifuged at 10,000 x g for 1 minute.
- 400 µl supernatant transferred to a Zymo-Spin™ III-F Filter and centrifuged at 8,000 x g for 1 minute.
- 1,200 µl of Genomic Lysis Buffer added to the filtrate from Step 4.
- 800 µl of the mixture from Step 5 transferred to a Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute.
- Discard the flow through from the Collection Tube and repeated Step 6.
- 200 µl DNA Pre-Wash Buffer added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute.
- 500 µl g-DNA Wash Buffer added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute.
- Zymo-Spin™ IICR Column transferred to a clean 1.5 ml microcentrifuge tube and 35 µl water or DNA Elution Buffer directly added to the column matrix and centrifuged at 10,000 x g for 30 seconds.

Zymoclean™ Gel DNA Recovery Kit used for cleaning of the DNA from agarose gels and the protocol is given below.

- DNA fragment from the agarose gel was excised using a scalpel and transferred it into a 1.5 ml microcentrifuge tube.
- 3 volumes of ADB added to each volume of agarose excised from the gel
- Incubation was occurred at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved.
- Melted agarose solution transferred to a Zymo-Spin™ Column.
- Centrifuge for 30-60 seconds. Discard the flow-through.

- 200 µl of DNA Wash Buffer added to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
- ≥ 6 µl DNA Elution Buffer or water directly added to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

ZymoPURE™ II Plasmid Midiprep Kit used for high volume of plasmid isolation and the protocol is given below.

- 8 ml ZymoPURE™ P1 added to the bacterial cell pellet and resuspended completely by vortexing or pipetting.
- 8 ml ZymoPURE™ P2 added and immediately mixed by inverting the tube 6 times. Cells lysed when the solution appears clear, purple, and viscous.
- 8 ml ZymoPURE™ P3 added to the mixture and mixed gently. The sample turned yellow when the neutralization complete and a yellowish precipitate will form.
- The syringe filter placed upright in a tube rack and the lysate loaded into the ZymoPURE™ Syringe Filter2. 5-8 minutes waited for the precipitate to float to the top.
- Luer Lock plug removed from the bottom of the syringe and placed into a clean 50 ml conical tube. Plunger placed in the syringe and the solution pushed through the ZymoPURE™ Syringe Filter in one continuous motion until approximately 20 ml of cleared lysate is recovered.
- 8 ml ZymoPURE™ Binding Buffer added to the cleared lysate from step 5 and mixed by inverting the capped tube 8 times.
- Ensure the connections of the Zymo-Spin™ III-P Column Assembly are finger-tight and place onto a vacuum manifold.
- With the vacuum off, the entire mixture added from step 6 into the Zymo-Spin™ III-P Column Assembly, and then turned on the vacuum1 until all of the liquid has passed completely through the column.
- The 50 ml Reservoir removed and discarded from the top of the Zymo-Spin™ III-P Column Assembly.

- With the vacuum off, 2 ml ZymoPURE™ Wash 1 added to the Zymo-Spin™ III-P Column. The vacuum turned on until all of the liquid has passed completely through the column.
- With the vacuum off, 2 ml ZymoPURE™ Wash 2 added to the Zymo-Spin™ III-P Column. The vacuum turned on until all of the liquid has passed completely through the column. Wash step repeated.
- Purple Luer Lock cap unscrewed from the top of the Zymo-Spin™ III-P Column and the 15 ml Conical Reservoir discarded. Zymo-Spin™ III-P Column placed in a Collection Tube and centrifuged at $\geq 10,000 \times g$ for 1 minute.
- The column transferred to a clean 1.5 ml microcentrifuge tube and 200 μ l ZymoPURE™ Elution Buffer^{2,3,4} directly added to the column matrix. After 2 minutes, column centrifuged at $\geq 10,000 \times g$ for 1 minute. Eluted plasmid DNA stored at $\leq -20^{\circ}\text{C}$.

Genomic DNA Clean and Concentrator Kit used for cleaning of the extracted genomic DNA and the protocol is given below.

- In a 1.5 ml microcentrifuge tube, add 2-5 volumes of DNA Binding Buffer to each volume of DNA sample. Mix thoroughly.
- Transfer mixture to a provided Column in a Collection Tube.
- Centrifuge for 30 seconds. Discard the flow-through.
- Add 200 μ l DNA Wash Buffer to the column. Centrifuge for 1 minute. Repeat the wash step.
- Add $\geq 10 \mu$ l DNA Elution Buffer or water directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge at for 30 seconds to elute the DNA.

3.2. Methods

3.2.1. Cloning of The UNENxynB2 Gene into *A. niger* N593

3.2.1.1. RNA Isolation

A. niger MRC200803 spores were used for RNA isolation. For this purpose strain was grown in VT3 culture media (Vogel salts ($C_6H_5Na_3O_7 \cdot 2H_2O$ 2,5 g/L, KH_2PO_4 5 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, NH_4NO_3 2 g/L, $CaCl_2 \cdot 2H_2O$ 0,1 g/L), medium viscosity wheat arabinoxylan 0.1 g/50 ml, xylose 0.2 g/L) at 30 °C, at 200 rpm, for 96 hours. Myceliums were collected in eppendorf tubes approximately 250 mg and quickly frozen in liquid nitrogen then stocked in -80 °C. For the isolation of RNA, first beads, Nucleospin RNA kit buffer, and beta-mercaptoethanol were added to the homogenizer for 2.5 minutes at high speed. RNA isolation from the obtained lysate was continued. The DNase step was performed for 25 min instead of 15 min for obtaining cleaner total RNA. Elution was done using 40 µL RNase-free H_2O and isolated RNA was quantified using Nanodrop spectrophotometer. Care was taken to ensure that no RNase contamination was observed during the study with RNA.

3.2.1.2. cDNA Preparation

To obtain UNENxynB2 cDNAs, single-strand cDNAs were first obtained from the isolated total RNAs using ProtoScript First Strand cDNA Synthesis kit according to the manufacturer's protocol. 950 ng total RNA was used as a template. The UNENxynB2 primers (XBPf and XBNr) were designed for cloning into the expression vector. In order to select the UNENxynB2 cDNA from many synthesized cDNAs, PCR reaction with UNENxynB2 cDNA specific XBPf and XBNr primers from single-stranded cDNAs (sscDNA) was established and double-stranded UNENxynB2 cDNA (dscDNA) was obtained by *in vitro* PCR amplification using 150 ng sscDNA as a template. The content and conditions of the reaction are given in Table 3.7. and Table 3.8., respectively.

Table 3.7: PCR reaction conditions.

PCR ingredients	Stock concentration	Final amount	Volume (50 µl)
sscDNA	30 ng/µl	150 ng	5 µl
Dream <i>Taq</i> buffer	10X	1X	5 µl
XBPf	10 µM	0,5 µM	2,5 µl
XBNr	10 µM	0,5 µM	2,5 µl
dNTP mix	10 mM	200 µM	1 µl
Dream <i>Taq</i> DNA polymerase	5 U/µl	2,5 U	0.5 µl
dH ₂ O	-	-	33.5 µl

Table 3.8: PCR cycle set up.

Step		Temperature	Time
Initial denaturation		95 °C	3 min
40 cycles	Denaturation	95 °C	30 sec
	Annealing	56 °C	30 sec
	Extension	72 °C	1 min
Final extension		72 °C	15 min
Final hold		4 °C	∞

3.2.1.3. Ligation and Transformation

40 µl dscDNA was loaded on the 0.1% DEPC-treated 1% agarose gel then 686 bp band was isolated from agarose gel using ZymoClean Gel DNA Recovery Kit. Gel extracted dscDNA was quantified using Nanodrop spectrometer.

Preparation of CaCl₂ competent *E. coli* cells: Single colony was inoculated into 50 ml LB medium and grown until OD₆₀₀ 0.4-0.5 at 37 °C. Cells are aliquoted into 20 ml sterile cooled tubes and incubated on ice for 10 min. Cells were centrifuged at 4000 rpm at 4 °C for 10 min and supernatant was removed. Pellet was dissolved in 10 ml ice-cold 100 mM CaCl₂ (pH 7) and incubated on ice for 30 min then centrifuged at 4000 rpm at 4 °C for 10 min and supernatant was removed. Pellet was dissolved in 1 ml ice-cold 100 mM CaCl₂ (pH 7) and cells are aliquoted into 100 µl eppendorfs. 11 µl 100% glycerol was added to the eppendorfs before -80 °C storage.

1 µl gel-extracted dscDNA (67 ng) was used for ligation using PCR cloning kit. pMiniT vector (supplied with the kit) and dscDNA were ligated with 3:1 insert-to-vector ratio. 2 µl ligation products were mixed with 100 µl CaCl₂ competent *E. coli* DH5α cells. Cells were incubated on ice for 45 min then the tube was heated at 37 °C for 5 min (heat shock). 900 µl LB medium was added to the tube and incubated for 1 hour at 37°C. Cells were centrifuged at room temperature at 6800 g for 1 min. Pellet was dissolved in 100 µl LB and spread on 100 µg/ml ampicillin containing LB agar plate. The plate was incubated overnight at 37 °C. After incubation, colonies appeared. 24 colonies were spread on new LB (100 µg/ml amp) agar plate for obtaining pure colonies and were incubated for overnight at 37 °C. These colonies are randomly selected for screening and sequencing. After one day, pure colonies were obtained and 3 colonies were selected for colony PCR. Selected colonies were dissolved in 40 µl nuclease-free water and used as a template. PCR reaction was carried out using vector primer (MiniTf and MiniTr, supplied with the PCR cloning kit). Colony PCR components and PCR conditions are given in Table 3.9. and Table 3.10., respectively. Two selected clones were identified as transformants including the UNENxynB2 cDNA. The plasmid was isolated and confirmed by PCR and DNA sequencing of the 678 bp UNENxynB2 insert from the target cDNA.

Table 3.9: Colony PCR reaction conditions.

PCR ingredients	Stock concentration	Final amount	Volume (50 µl)
Single colony in 40 µl ddH ₂ O	-	-	38.5 µl
Taq (Mg-free) buffer	10X	1X	5 µl
MgCl ₂	25 mM	1,5 mM	3 µl
MiniTf	10 µM	0,2 µM	1 µl
MiniTr	10 µM	0,2 µM	1 µl
dNTP mix	10 mM	200 µM	1 µl
Taq DNA polymerase	5 U/µl	2,5 U	0.5 µl

Table 3.10: Colony PCR cycle set up.

Step		Temperature	Time
Initial denaturation		95 °C	3 min
30 cycles	Denaturation	95 °C	30 sec
	Annealing*	51-54 °C	1 min
	Extension	68 °C	1 min
Final extension		68 °C	5 min
Final hold		4 °C	∞

* Annealing temperature varies according to the primers.

pMiniT vector was used for cloning of the dscDNA and vector map was shown in Figure 3.1.

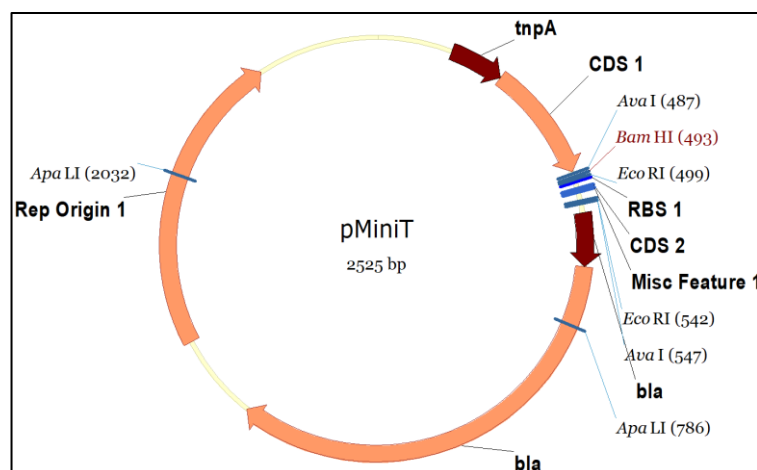


Figure 3.1: pMiniT vector map (NEB, USA).

For gene sequencing, one colony was grown overnight in 5 ml LB (100 µg/ml amp) at 37 °C. The culture was aliquoted into 2 eppendorfs and centrifuged at 4500 g, at 4 °C for 10 min. Pellet was used for plasmid isolation using the Qiaprep Spin Miniprep Kit. Elution was done in 50 µl nuclease-free water. Selected candidates were purified using DNA Clean&Concentrator Kit. Isolated plasmid named as pMxynBc2 was measured with NanoDrop spectrometer. Plasmids and pMiniTf and pMiniTr primers sent to the company (MedSanTek, Turkey) for sequencing.

3.2.1.4. Preparation of UNENxynB2 cDNA and Linear Vector for Obtaining Expression Plasmid

Restriction sites added UNENxynB2 cDNA was confirmed with nucleotide sequencing and pMxynB2 plasmid isolation was done. Firstly, 500 ng purified pMxynBc2 plasmid was cut with 0.5 μ l *PacI* restriction enzyme for 37 °C at 10 min, then the enzyme was inactivated at 65 °C for 20 min. After *PacI* digestion, the pMxynBc2 plasmid was cut with 0.5 μ l *NotI* restriction enzyme at 37 °C for 10 min and enzyme was inactivated at 65 °C for 20 min. About 500 ng digested pMxynBc2 plasmid was loaded in 1% agarose gel then linear cDNA was recovered from the gel using ZymoClean Gel DNA Recovery Kit. Similarly, pGW635 derived [Tamayo Ramos et al., 2011] fungal expression vector pGWf (Figure 3.2.) was cut with both *PacI* and *NotI* enzymes. About 500 ng pGWf vector was loaded in 1% agarose gel then linear vector was recovered from the gel using ZymoClean Gel DNA Recovery Kit.

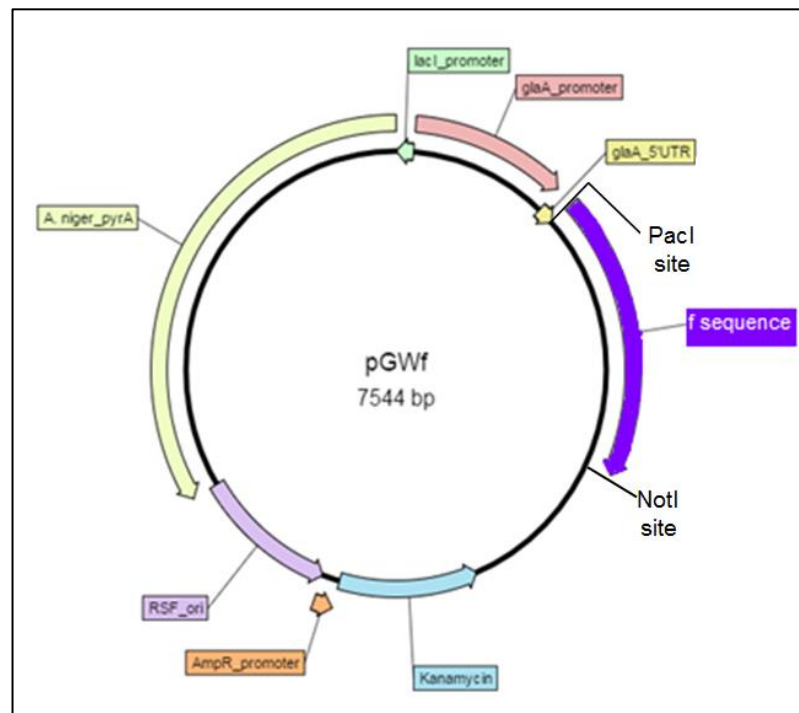


Figure 3.2: pGWf plasmid map (Karasugen Biotechnology, Turkey).

3.2.1.5. Ligation and Subcloning into The *E. coli* DH5α Cells

Linear pGWf vector (6130 bp) was ligated with UNENxynB2 cDNA 5:1 insert-to-vector ratio. Ligation was established in 20 µl reaction volume and 1 µl Quick-Stick (QS) Ligase was used for ligation. After ligation, 3-5 µl ligation mixture (10-20 ng) was transformed to 50 µl CaCl₂ competent *E. coli* DH5α cells. Competent cells were incubated in 1 ml SOC medium at 37 °C for 1 hour. After incubation cells were spread on LB (with 100 µg/ml kanamycin) and incubated at 37 °C for overnight. 4 colonies were selected for colony PCR after incubation. Colony PCR was made same as described at Table 3.9. and 3.10. GWF and GWR primers were used in PCR reaction and annealing temperature was 54.1 °C.

After colony PCR, obtained plasmids were also confirmed using restriction enzyme analysis. For this purpose, plasmids were isolated from *E. coli* DH5α cells. Plasmids were cut with both PaeI and NotI restriction enzymes and obtained bands were confirmed in 1% agarose gel. Plasmids are also used for sequencing using GWF and GWR primers. Plasmid sequencing was done by MedSantek (Turkey). The obtained expression plasmid was named as pAExynB2.

3.2.1.6. Preparation of pAExynB2 Plasmid for Cloning and Transformation of The Expression Host *A. niger* N593

pAExynB2/*E. coli* DH5α cells were grown in 50 ml LB-kanamycin medium at 37 °C for overnight for the transformation of pAExynB2 plasmid. After 16-18 hours, cells were collected by centrifugation at 6800 g, for 3 min. The obtained cell pellet was used for plasmid isolation using Midiprep Kit. Plasmids were precipitated with 17,5 µl sodium acetate (pH: 5.2) and 440 µl 100% ethanol at -80 °C for 1 hour. Precipitated plasmids were collected with centrifugation at 13.000 rpm, at 4 °C for 30 min. The supernatant was removed; 1 ml 70% ethanol was added to the pellet and centrifuged at 13.000 rpm, at 4 °C for 10 min. This step was repeated twice. Pellet was dried and solved in 35 µl nuclease-free water.

Transformation to the *A. niger* N593: 2.5 gr PEG 6000 was dissolved in 7.5 ml TC solution (supplied with the ATK593 kit) using the microwave and cool out at

room temperature. Minimal medium with top agar (0.6% agar) was warmed to 48 °C until use. 200 µl protoplast (10^8 spores/ml) and 10 µg pAExynB2 circular plasmid were mixed, 50 µl PEG was added to the mixture. The mixture was incubated for 20 min at room temperature then 2 ml PEG was added. After 5 min incubation at room temperature, 4 ml STC solution (supplied with the kit) was added. pAExynB2 containing protoplasts were mixed with 40 ml top agar then were poured onto the fungal minimal agar plate in 145 mm Petri dish. The plate was incubated at 30 °C for 5 days for the development of transformants.

3.2.1.7. PCR Analysis of UNENxynB2 Transformants

Pure colonies were obtained from the transformants with the streak plate technique. Colonies were grown on minimal plate then spores were solved in 100 µl ST solutions (supplied with the ATK593 kit). 50 µl spores were spread on to complex medium agar plates (supplied with the kit). Stock culture for preservation was done using cryo solution. Transformants were grown on the minimal agar plate for 7 days then spores were collected with cryo solution and stocked at -80 °C. Transformants were also used for genomic DNA isolation. For genomic DNA isolation, transformants were collected with 5 ml sterile 0.1% Tween 80 and 150 µl spore solution was used as a starting material. Genomic DNA isolation was done using Quick-DNA Fungal/Bacterial Miniprep Kit. Elution of the genomic DNA was done with 25 µl elution buffer. Transformants were confirmed by PCR. For PCR reaction; 150 ng genomic DNA was used as a template. Vector primers (GWF and GWR) were used for PCR amplification. PCR ingredients and reaction cycles are same as given in Table 3.9. and Table 3.10.

3.2.2. Heterologous *SxynB2* Expression in *P. pastoris*

3.2.2.1. Preparation of *SxynB2* Gene and Vector Constructs

UNEN*xynB2* gene (MF170635.1) was used as an optimized gene template for xylanase B heterologous expression. In the project, the wild type UNEN*xynB2* gene without the secretion signal (624 bp) was prepared using the IDT Codon Optimization Tool (<https://eu.idtdna.com/CodonOpt>). pHIL-S1 and pPIC9 vectors were supplied with the kit (K1710-01, Invitrogen). Optimized gene sequence named as *SxynB2* was modified according to the pHIL-S1 and pPIC9 vectors. Firstly, EcoRI restriction endonuclease site was added to 5' end and XhoI restriction endonuclease site was added to 3' end. These restriction sites are suitable for both pHIL-S1 and pPIC9 vectors. The KEX2 and STE13 protease cleavage sites of the *S. cerevisiae* α factor signal sequence at pPIC9 is located at the beginning of the *SxynB2*. UNEN*xynB2* sequences compatible with *Pichia pastoris* was synthesized (GeneArt) for both pHIL-S1 and pPIC9 vectors named as *SxynB2*-1 (636 bp) and *SxynB2*-2 (654 bp), respectively. Lyophilized pHIL-S1 and pPIC9 vectors were dissolved in nuclease-free water (0.5 $\mu\text{g}/\mu\text{l}$ concentration). 200 ng pHIL-S1 and pPIC9 vectors were transformed into electrocompetent *E. coli* TOP10F' cells for vector stock. 200 ng vector and 40 μl electrocompetent *E. coli* TOP10F' cells were mixed and incubated for 5 min in ice. Then vector and cell mixture put on the 0.2 cm electroporation cuvette (165-2086, Bio-Rad) and pulse using GenePulser Xcell. After electroporation, cells were incubated with 1 ml SOC medium at 37 °C for 1 hour then centrifuged at 10,000 rpm for 1 min. The cell pellet was dissolved in 100 μl LB medium, and 30 or 70 μl of it was spread on ampicillin-containing LB plates (100 $\mu\text{g}/\text{ml}$).

3.2.2.2. Preparation of *SxynB2-1* and *SxynB2-2* DNA Fragments and Expression Vectors for Ligation

In order to prepare *SxynB2-1* and *SxynB2-2* fragments for ligation, 120 ng DNA fragments were cut at 37 °C for 15 minutes using 0.5 µl EcoRI-HF and 0.5 µl XhoI restriction enzymes at 25 µl reaction volume. Digested products were purified with DNA Clean&Concentrator kit and eluted in 25 µl nuclease-free water.

Plasmid DNA was isolated from the pPIC9/TOP10F' and pHIL-S1/TOP10F'. 500 ng plasmids were used for each of 50 µl reaction volumes and 1 µl XhoI and EcoRI-HF enzymes were used for digestion at 37 °C, for 15 min. Vector pieces with sticky ends compatible with inserts were obtained. Prior to ligation, they were purified with the kit and eluted in 50 µl nuclease-free dH₂O.

3.2.2.3. Ligation of *SxynB2* with pPIC9 and pHIL-S1

Linearized vector and synthetic DNA fragments were ligated using QS ligase enzyme. A total ligation of 20 µl was established with a pHIL-S1 vector at vector to insert ratio of 1:7 for *SxynB2-1* and 1:3 ratios for the pPIC9 vector and *SxynB2-2*. Ligation was carried out at 22 °C for 15 min using 1 µl QS Ligase. Prior to transformation, the ligation mix was purified using DNA Clean&Concentrator kit and reduced to 8 µl.

3.2.2.4. Transformation into The Intermediate Host

Preparation of electrocompetent *E. coli* TOP10F' cells: Single colony was inoculated into 5 ml LB medium and grown for overnight at 37 °C. The overnight culture was inoculated into 500 ml LB medium and incubated at 37 °C, at 200 rpm until OD₆₀₀ 0.5-0.7 (approximately 2.5-3 hours). Cells were cooled on ice for 20 min then centrifuged at 4 °C, at 4000 g for 15 min. The supernatant was removed and the pellet was dissolved in 250 ml sterile cold water then centrifuged at 4 °C, at 5000 rpm for 15 min. The supernatant was removed and the pellet was dissolved in 20 ml sterile cold 10% glycerol then centrifuged at 4 °C, at 5000 rpm for 15 min. The

supernatant was removed and the pellet was dissolved in 2 ml sterile cold 10% glycerol then 40 µl aliquotes were prepared in cold eppendorfs. Electrocompetent cells were stored at -80 °C until use.

2 µl (15 ng) of the ligation product were added to electrocompetent *E. coli* TOP10F' cells, transferred to 0.2 mm electroporation cuvette and incubated on ice for 1 min. The cuvette was put into the electroporator and bacterial transformation protocol was selected (2500 V, 200 Ω, 25 µF) then pressed the 'PULSE' button. After discharging 1 ml SOC medium was quickly added to cells, mixed well then cells were taken to a sterile tube and incubated for 1 hour at 37 °C. After incubation, cells were centrifuged at 10000 rpm for 1 min. Pellet was dissolved in 100 µl LB medium and spread onto LB plate containing 50 µg/ml Amp. The plate was incubated for overnight at 37 °C, next day colonies were appeared.

3.2.2.5. Selection of Transformants in The Intermediate Host

The colonies in the selective medium for both pHIL-S1-*SxynB2*-1 and pPIC9-*SxynB2*-2 were grown on LB plates containing 50 µg/ml Amp for obtaining pure colony. Then, one colony was taken into 5 ml of LB medium containing 50 µg/ml Amp and the plasmid was isolated with Qiaprep Spin Miniprep Kit. The resulting plasmids were examined for the presence of the insert by restriction endonuclease digestion using EcoRI and XhoI enzymes and PCR with the AOX1F and AOX1R primers (Table 3.9. and Table 3.10.), only annealing temperature was changed to 51 °C. One of the positive clones was selected for DNA sequencing confirmation. The recombinant plasmid which was amplified and sequenced was named pBKC-S1 for pHIL-S1-*SxynB2*-1 and pBCK9 for pPIC9-*SxynB2*-2.

3.2.2.6. Transformation of pBKC-S1 and pBCK9 Vectors into *Pichia pastoris* GS115 and KM71

Preparation of plasmids: Plasmid isolation was performed from 10 ml overnight cultures of the pBCK9/TOP10F' and pBKC-S1/TOP10F' and 5 µg plasmids

were obtained. 5 µg plasmids were digested with Sall-HF enzyme for gene insertion from the HIS4 ORF region of the pBCK9 and pBKC-S1 vectors. After the restriction enzyme digestion, digested products were purified using DNA Clean&Concentrator kit. ~5 µg linear vectors were transformed into electrocompetent *P. pastoris* GS115 and KM71 strains via electroporation.

Preparation of electrocompetent *Pichia pastoris* GS115 and KM71 cells: *P. pastoris* GS115 and KM71 strains were grown in 2.5 ml YPD culture for about 8 hours at 30 °C at 200 rpm. 250 µl pre-culture was inoculated into 250 ml fresh YPD medium and grown for overnight at 30 °C, at 200 rpm. Cells were harvested at 4 °C, at 1500 g, for 5 min when OD₆₀₀ value was between 1.3-1.5. After centrifugation, supernatant was removed and the cell pellet was washed with 250 ml sterile cold water then centrifuged. The cell pellet was dissolved with 125 ml sterile cold water then centrifuged. The cell pellet was dissolved with 10 ml of ice-cold 1 M sorbitol and centrifuged again. The supernatant was removed and the cell pellet was dissolved in 500 µl of ice-cold 1 M sorbitol and aliquoted into 80 µl. Electrocompetent cells were freshly used for high transformation efficiency.

Transformation to electrocompetent *P. pastoris* GS115 and KM71: 80 µl of the electrocompetent cell and ~5 µg of linearized DNA (both for pBCK9 and pBKC-S1 vectors) were mixed and transferred to an ice-cold 0.2 cm electroporation cuvette. The cuvette with the cells was incubated on ice for 5 minutes. The cells were pulsed according to the electroporation parameters (2000V, 25 µF, 200 Ω). 1 ml of ice-cold 1 M sorbitol was added to the cuvette. The cuvette was transferred to a sterile microcentrifuge tube. 100 µl, 300 µl and 600 µl of aliquots were spread on MD or RDB plates. The plates were incubated at 30 °C until colonies appeared.

3.2.2.7. Determination of Methanol Utilization Phenotype and PCR Analysis of *SxynB2* in *Pichia pastoris* Transformants

Transformant colonies were first spread onto MD plate for obtaining pure colony. Plates were incubated at 28 °C for 3 days then pure colonies were patched onto the plates according to the generated phenotypes. In KM71 transformants, phenotype is Mut^s and it is not necessary for phenotype screening. Transformants

patched onto the MD plates. In GS115 transformants phenotype is Mut⁺ and Mut⁺ phenotype is controlled in both MM and MD plates. Colonies were inoculated in 1 ml YPD medium for stocking of the pure colonies for further analysis. Cultures were grown for 8 hours at 28 °C then stocked in 15% glycerol containing YPD medium. Cultures were stocked at -80 °C.

Genomic DNA of the candidate transformants were isolated using kit (Quick-DNA Fungal/Bacterial Miniprep Kit) for the control of the *SxynB2* gene at the DNA level. 150 ng genomic DNA was used as a template with vector primers (AOX1F and AOX1R) for PCR reaction. PRC reaction conditions and cycle set up were used same as Table 3.9. and Table 3.10., only annealing temperature was changed to 51 °C.

3.2.2.8. Flask Cultures of Recombinant *P. pastoris* Transformants

Preparation of media for expression: Media were prepared in accordance with the kit protocol (K1710-01, Invitrogen). Buffered Glycerol-Complex Medium (BMGY) was used for growth and Buffered Methanol-Complex Medium (BMMY) was used for expression.

Considering the phenotype of *Pichia pastoris* recipient, the expression volumes of flasks were changed and baffled flasks were used due to oxygen demand. The medium volumes vary depending on the phenotype for the release of the enzyme. In the liquid culture of Mut⁺ phenotype, one candidate colony from MD plate was taken and transferred into 5 ml BMGY medium and incubated at 28 °C at 150-220 rpm for overnight. When OD₆₀₀ was between 2-6, the cells were collected by centrifugation at 3000 g, at room temperature, for 5 min. The BMGY medium was removed and the pellet was washed with BMMY medium to remove residual glycerol from the pellet. 10-30 ml BMMY medium was added to the pellet to adjust OD₆₀₀ 1. Expression was continued for 4 days in 250 ml baffled flask. After switching to the expression medium, 1 ml of sample was taken every 24 hours and 100% methanol was added to the culture and methanol concentration was adjusted to 0.5%.

For the liquid culture of the Mut^s phenotype, one candidate colony from the MD plate was transferred to 100 ml BMGY medium and incubated at 28 °C at 150-

220 rpm for overnight in 1 L baffled flask. When OD₆₀₀ was between 2-6, the cells were collected by centrifugation at 3000 g, at room temperature, for 5 min. The BMGY medium was removed and the pellet was washed with BMMY medium to remove residual glycerol from the pellet. Pellet was dissolved in 10-20 ml BMMY medium (according to the 1/5 to 1/10 of the original culture volume) in 100 ml baffled flask. The culture was continued for 7 days, 1 ml of sample was taken for analysis every 24 hours and 100% methanol was added at 0.5% daily. In order to prevent foams formed in flasks, 1:1000 diluted (with water) 1% Antifoam C was added daily.

Expression of clone GH10S-6 was assayed with 0.5%, 1% and 3% methanol induction and 25 °C and 28 °C incubation temperatures. GS115 host was used as negative control. Cells were grown on BMGY medium and expressed on 30 ml BMMY medium. Expression was continued for 144 hours. 100% methanol was added at a final concentration of 0.5% for every 24 hours and 1 ml of the samples were taken from the cultures.

GH10S-6 and GH10S-19 clones were expressed with BMMY and YPTM medium mentioned in Ramchuran et al., (2005). Cultures were induced with 0.5% methanol and expression was monitored for 4 days.

Enzyme activity in crude supernatants was monitored by qualitative xylanase activity. Protein determination and SDS-PAGE analysis were performed with the supernatants obtained from the cultures.

3.2.2.9. Preparation of Master and Working Cell Bank

To make -80 °C stocks, a colony grown in MD plate was inoculated into 3 ml BMGY medium and grown at 28 °C for 8 hours and then inoculated into 50 ml BMGY medium. Cells were grown overnight at 28 °C and 120 rpm and were mixed with 1:1 ratio of BMGY medium containing 50% glycerol when OD₆₀₀ was equal to 11 and -80 °C stocks were divided into 1 ml cryo tubes. These stocks are named as master cell bank. 1 ml of the remaining cells were inoculated in 100 ml BMGY medium and cells were grown overnight at 28 °C and 120 rpm. When OD₆₀₀ was

about 10, -80 °C stocks were prepared by mixing 1:1 with BMGY medium containing 50% glycerol. These stocks were named as working cell bank.

3.2.2.10. Investigation of Different Methanol Concentrations on GH10S-6 Expression

1 ml GH10S-6 transformant from working cell bank stock was used for pre-culture in 25 ml BMGY medium. Cells were grown for approximately 8 hours at 28 °C with shaking at 220 rpm. 1.5 ml pre-culture was inoculated into 600 ml BMGY medium and incubated for 16 hours at 28 °C and 120 rpm. After a day, cells were collected by centrifuging at 5000 rpm at room temperature for 15 min when their OD₆₀₀ values were approximately 10.5. After the washing steps using BMMY medium for removing residual glycerol from cells, cell pellets were dissolved in BMMY medium and cell density (OD₆₀₀) was 1. Cells were aliquoted 3 liters baffled flasks in 600 ml expression culture. 3 flask were induced with 0,5% methanol and 3 flask were induced with 1% methanol. Flasks were shaken at 28 °C and 220 rpm. 100% methanol was used every 24 hours for 0.5% methanol-induced flasks and every 12 hours for 1% methanol-induced flasks [Ersöz and Inan, 2019]. The sample was collected every 24 hours from methanol-induced culture. The sample was used for enzyme activity, protein determination, and OD₆₀₀ values.

3.2.2.11. Xylanase Activity Measurement

3.2.2.11.1. Qualitative Xylanase Activity

In order to determine the secretion of the enzyme qualitatively, the enzyme reaction was carried out with the dyed substrate. 100 µl crude supernatants from the cultures were mixed with 100 µl 0.2% azurine cross-linked wheat arabinoxylan in 100 mM sodium citrate pH 5 and incubated for 2 hours at 40 °C on a 96-well plate or 1.5 ml tubes.

3.2.2.11.2. Quantitative Xylanase Activity

Quantitative enzyme activity was followed by the protocol for the determination of activity modified by Megazyme [https://secure.megazyme.com/files/Booklet/T-XAX_DATA.pdf] based on Nelson-Somogyi [Somogyi, 1952] reducing sugar analysis. 500 µl 1% medium viscosity wheat arabinoxylan and 200 µl suitably diluted enzyme with buffer were incubated at 40 °C for 5 min. After incubation, 200 µl enzyme was added to substrate and mixed by vortex then incubated at 40 °C for 5 min. The reaction was stopped by adding to 500 µl Reagent D and mixed well by vortex then boiled for 20 min. The mixture was cooled at 4 °C for 5 min. Color development was observed by adding 3 ml Reagent C after 15 min incubation at room temperature. Samples were read at OD₅₂₀ against blank (just arabinoxylan and buffer).

3.2.2.12. Protein Determination

Preparation of standard curve: 10 mg BSA was dissolved in 10 ml dH₂O. 1 ml solution was added to 9 ml dH₂O and dilutions were prepared (Table 3.11).

Table 3.11: BSA dilutions for the standard curve.

Reagent	BSA concentration (µg/ml)					
	5	10	15	20	25	30
0.1 mg/ml BSA	0	0.1	0.2	0.3	0.4	0.5
dH ₂ O (ml)	2	1.9	1.8	1.7	1.6	1.5

1 ml of these tubes were taken, 1 ml of dye-binding solution [Sedmak and Grossberg, 1977] was added. OD₅₉₅ values were read and plotted against the BSA solution concentration. The slope of the graph was used in the following formula to calculate protein concentration.

$$\text{Amount of protein (mg / ml)} = ((\text{OD}_{595} / \text{Slope}) * \text{Dilution factor}) / 1000$$

3.2.2.13. SDS-PAGE Analysis

12% SDS-PAGE gel [Ausubel et al, 1987] was used in all studies. The gel content is given in Table 3.12, sample loading buffer is given in Table 3.13 and electrophoresis buffer is given in Table 3.14.

Table 3.12: Preparation of SDS-PAGE gel.

	Separation gel (12%)	Stacking gel (5%)
dH ₂ O	1.675 ml	2.25 ml
1.5 M Tris-HCl, pH 8.8	1.25 ml	-
0.5 M Tris-HCl, pH 6.8	-	1 ml
30% Acrylamide/Bisacrylamide	2 ml	0.66 ml
10% SDS	50 μ l	40 μ l
10% Ammonium persulfate (APS)	50 μ l	40 μ l
TEMED	5 μ l	10 μ l

Table 3.13: Preparation of 4X sample loading buffer (10 ml).

	Amount
ddH ₂ O	3.1 ml
1 M Tris-HCl, pH 6.8	2.4 ml
100% Glycerol	4 ml
SDS	0.8 gr
Bromophenol blue	4 mg
β -mercaptoethanol	0.5 ml

Table 3.14: 5X SDS electrophoresis buffer (600 ml).

	Amount (gr)
Tris base	9
Glycine	43.2
SDS	3

Protein samples were mixed with the sample loading dye and loaded into the gel. After 1X SDS electrophoresis buffer was placed in the tank, the current was set to 40 V for the protein samples to run on the stacking gel. After 40 minutes the current was increased to 120 V. After completion of the run, the gel was stained with silver nitrate. The steps to be followed after the gel is executed are given in Table 3.15.

Table 3.15: Silver nitrate staining for SDS gel.

Solutions	Time
Fixation (50% methanol, 12% acetic acid, 0.5 ml/L 37% formaldehyde)	>1 hour
Wash (50% ethanol)	3*20 min
Sensitize (0.2 gr/L $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$)	1 min
Wash (dH_2O)	3*20 sec
Silver stain (2 gr/L AgNO_3 , 0.75 ml/L 37% formaldehyde)	20 min
Wash (dH_2O)	2*20 sec
Developer (60 gr/L Na_2CO_3 , 0.5 ml/L formaldehyde, 4 mg/L $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$)	<10 min
Wash (dH_2O)	2*2 min
Stop solution (50% methanol, 12% acetic acid)	10 min
Wash (50% methanol)	+4 °C for 1 month

3.2.2.14. The Concentration of Crude Enzyme

Samples were concentrated using Amicon Ultra-15 10 kDa regenerated cellulose membrane centrifugal filters. The activity of the enzyme and the amount of the proteins obtained after the concentration were calculated and some properties of the concentrated crude enzyme were determined using these samples.

3.2.2.15. Determination of Optimum pH, Temperature and Thermal Stability of Crude Enzyme

In order to determine the optimum pH value, the enzyme solution diluted with 200 μl 100 mM buffer at different pH's in the range of pH 3-10 was mixed with 500 μl of 1% substrate solution was prepared in the same range of pH buffers and quantitative xylanase activity assay was carried out following the steps in the activity assay.

To determine the optimum temperature value of the concentrated crude enzyme, the enzyme solution diluted with 200 μl 100 mM sodium citrate pH 6 was

mixed with 500 µl of the 1% substrate solution prepared in the same buffer and incubated in the range of 30-70 °C. Quantitative xylanase activity assay was carried out following the steps in the activity assay.

The enzyme preparation (diluted 1:10 with 0,1 M sodium citrate pH:6) was incubated alone at a temperature range of 40-60 °C for 30 minutes, and xylanase activity was measured to determine thermal stability following the steps in the activity determination method.

3.2.2.16. Control of Crude Enzyme Samples

For control of the stability of integrated gene, -80°C stocks were controlled using genomic DNA from the yeast cell pellet and culture supernatant. After separation of the pellet and supernatant by centrifugation, gDNA was isolated from the supernatant and the presence of DNA residuals of *SxynB2* was checked with appropriate primers. gDNAs were measured in NanoDrop and PCR was performed using 150 ng template and AOX1 primers (Table 2.8 and Table 2.9, only annealing temperature was changed to 51 °C.). The DNA sequencing was done using PCR products with designed primer (AOX1pF3) and AOX1 primers.

4. RESULTS

4.1. Cloning of the *A. niger* MRC200803 UNENxynB2 cDNA

4.1.1. Preparation of UNENxynB2 cDNA

RNA was isolated for *in vitro* synthesis of UNENxynB2 cDNA. Mycelia from *A. niger* MRC200803 strain was grown in liquid culture medium containing Vogel salt, medium viscosity wheat arabinoxylan and xylose for 96 hours. The resulting RNAs were checked on agarose gel (Figure 4.1). Total RNA and Oligo (dT) primer were used for cDNA synthesis by RT-PCR. UNENxynB2 dscDNA amplification from obtained single-stranded cDNA was performed with primers (XBPf and XBNr) designed according to the nucleotide sequence of UNENxynB2. Both sscDNA and dscDNA were controlled in 1% DEPC-treated agarose gel (Figure 4.1).

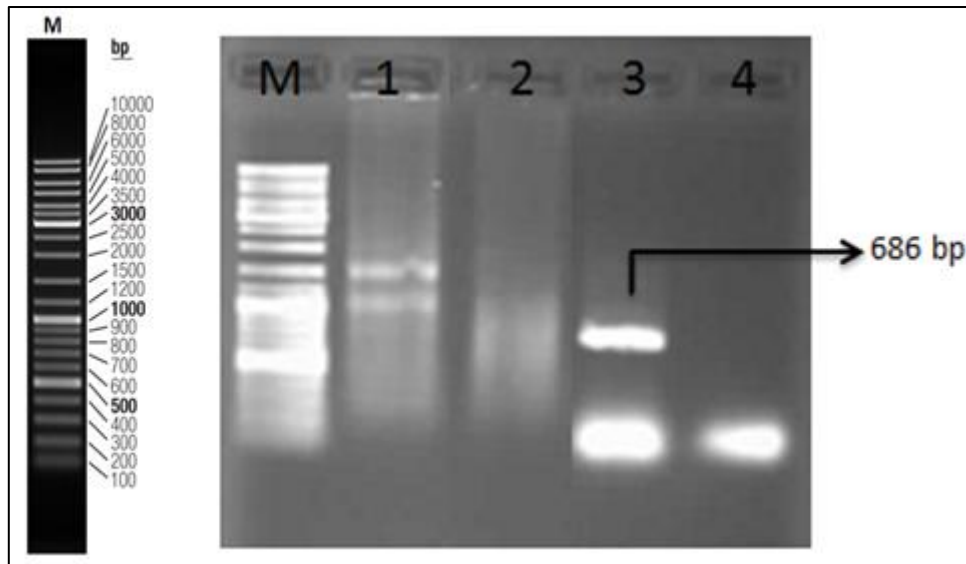


Figure 4.1: RNA and cDNA from *A. niger* MRC200803 isolate. (M) GeneRuler DNA ladder mix, (1) total RNA, (2) sscDNA, (3) dscDNA, (4) control (w/o template).

Thus; first, single-stranded cDNAs were obtained by RT-PCR followed by PCR amplification of 686 bp length double-stranded UNENxynB2 cDNAs using gene-

specific and expression-appropriate XBPf and XBNr primers and low-error Dream *Taq* DNA polymerase.

4.1.2. Obtaining and Analysis of Plasmids Integrated with UNENxynB2 cDNA

The cDNA was loaded onto the gel and extracted from the gel. The cDNA was fused with the intermediate vector (pMiniT) to check and stock the sequence before fusing the cDNA with the expression vector (pGWf). First, the pMiniT plasmid was ligated with UNENxynB2 cDNA following the kit protocol and the ligation product was transferred to *E. coli* DH5 α cells. Selection of transformants was performed in ampicillin-containing LB medium. The presence of inserts in candidate colonies growing in ampicillin was examined by colony PCR. 3 colonies were selected and colony PCR was performed to confirm the presence of insert. PCR products were run on 1% agarose gel (Figure 4.2). The two selected candidates after restriction endonuclease (REase) cleavage were identified as transformants carrying the UNENxynB2 cDNA. Two of the plasmids were isolated and the 924 bp fragment including the UNENxynB2 cDNA insert of the target region was confirmed by DNA sequencing. The 12-3 coded transformant was found to carry the expected UNENxynB2 cDNA with correct orientation and was named as pMxynBc2 (Figure 4.3).

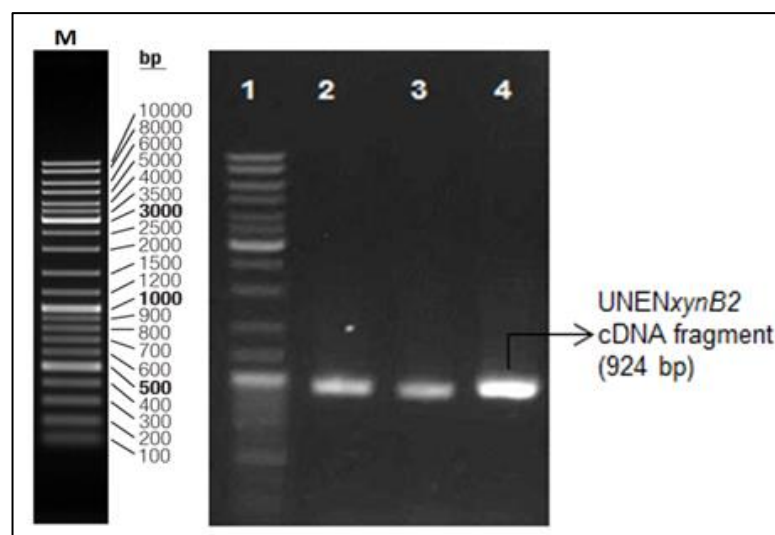


Figure 4.2: Colony PCR amplification. (1) GeneRuler DNA ladder mix, (2) 12-1, (3) 12-2 and (4) 12-3 coded candidates in *E. coli* DH5α.

Thus, 678 bp length UNEN xylanase (UNENxynB2) cDNA sequence (including signal sequence) was registered in NCBI (GenBank no. MF170635).

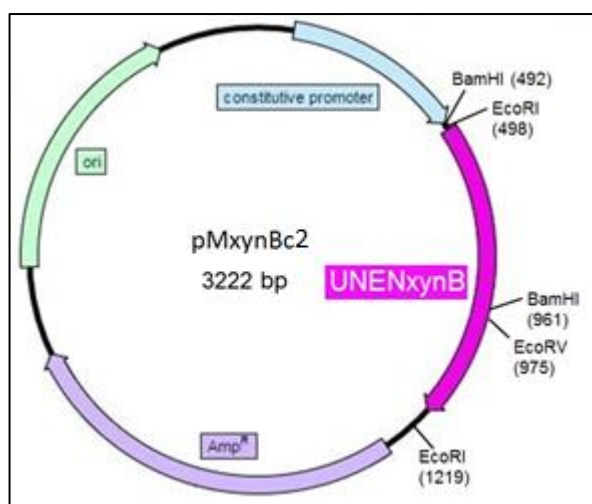


Figure 4.3: pMxynBc2 plasmid map.

The amino acid sequence of the cDNA clone was determined using the EMBOSS Transeq program. The cDNA sequence contains an 18 amino acid-long signal peptide. The Science Gateway Protein Molecular Weight Calculator was used to calculate the molecular weight of the protein (24.10 kDa). Comparisons with

other published xynB amino acid sequences from *A. niger* were made for UNEN xylanase B using EMBL Clustal Omega program (Figure 4.4).

AAM95167.1	MLTKNLLLCFAAAKAVLAVPHDSVVERSDALHKLSESTPSSTGENNGFYYSFWDGGGD	60
AGG38091.1	MLTKNLLLCFAAAKAVLAVPHDSVVERSDALHKLSESTPSSTGENNGFYYSFWDGGGD	60
AEC13626.1	MLTKNLLLCFAAAKAVLAVPHDSVVERSDALHKLSESTPSSTGENNGFYYSFWDGGGD	60
UNENxynB2	MLTKNLLLCFAAAKAVLAVPHDSVVERSDALHKLSESTPSSTGENNGFYYSFWDGGGD	60

AAM95167.1	VTYTNGDAGSYTVEWSNVGNFVGGKGWNPQSAQDITYSGFTPSGNGYLSVYGWTTDPLI	120
AGG38091.1	VTYTNGDAGSYTVEWSNVGNFVGGKGWNPQSAQDITYSGFTPSGNGYLSVYGWTTDPLI	120
AEC13626.1	VTYTNGDAGSYTVEWSNVGNFVGGKGWNPQSAQDITYSGFTPSGNGYLSVYGWTTDPLI	120
UNENxynB2	VTYTNGDAGSYTVEWSNVGNFVGGKGWNPQSAQDITYSGFTPSGNGYLSVYGWTTDPLI	120

AAM95167.1	EYYIVESYGDYNPGSGGTYKGTVTS DGSVYDIYTATRTNAASIQTATFTQYWSVRQNK	180
AGG38091.1	EYYIVESYGDYNPGSGGTYKGTVTS DGSVYDIYTATRTNAASIQTATFTQYWSVRQNK	180
AEC13626.1	EYYIVESYGDYNPGSGGTYKGTVTS DGSVYDIYTATRTNAASIQTATFTQYWSVRQNK	180
UNENxynB2	EYYIVESYGDYNPGSGGTYKGTVTS DGSVYDIYTATRTNAASIQTATFTQYWSVRQNK	180

AAM95167.1	VGGTVTTSNHFNAWAKLGMNLGTHNYQIVATEGYQSSGSSSITVQ	225
AGG38091.1	VGGTVTTSNHFNAWAKLGMNLGTHNYQIVATEGYQSSGSSSITVQ	225
AEC13626.1	VGGTVTTSNHFNAWAKLGMNLGTHNYQIVATEGYQSSGSSSITVQ	225
UNENxynB2	VGGTVTTSNHFNAWAKLGMNLGTHNYQIVATEGYQSSGSSSITVQ	225

Figure 4.4: Comparison of amino acid sequence of the UNENxynB2 cDNA clone with other published *A. niger* xylanase B amino acid sequences.

UNENxynB2 is highly similar to *A. niger* xynB's as expected (Figure 4.4). Also, UNENxynB2 xylanase was picturized using EzMol tool (Figure 4.5). As a result, cDNA-derived UNENxynB2 fragment was isolated from the genome and subcloned to a plasmid for its further analysis.

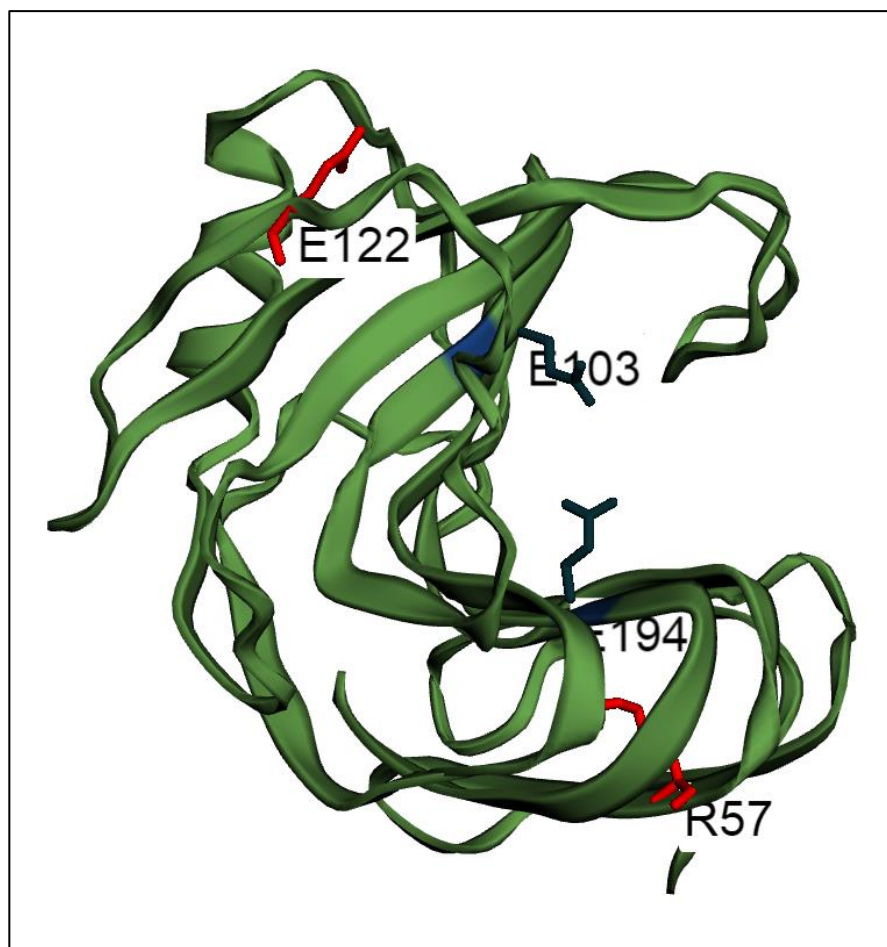


Figure 4.5: 3D structure of mature UNENxynB2 xylanase.

4.1.3. Integrative Plasmid Construction for UNENxynB2 and Transferring to the *Aspergillus niger* N593 Chromosome

The shuttle vector pGWf plasmid was provided for the expression of UNENxynB2 gene. This plasmid proliferates in *E. coli* and contains the *PacI* and *NotI* REase cleavage sites suitable for cloning. The non-complementary cohesive ends can be obtained after cutting so that the cloned sequence can be placed in the correct orientation. Fungal pGWf also contains the *A. niger pyrA* gene, so that the integration of transformed DNA into the chromosome is possible from this sequence.

UNENxynB2 will be inserted by directional cloning using the *PacI* and *NotI* region into the fungal vector (pGWf). *PacI* and *NotI* digested pGWf vector is shown

in Figure 4.6. The UNENxynB2 cDNA region from the pMxynBc2 plasmid digested with PacI and NotI was shown in Figure 4.7.

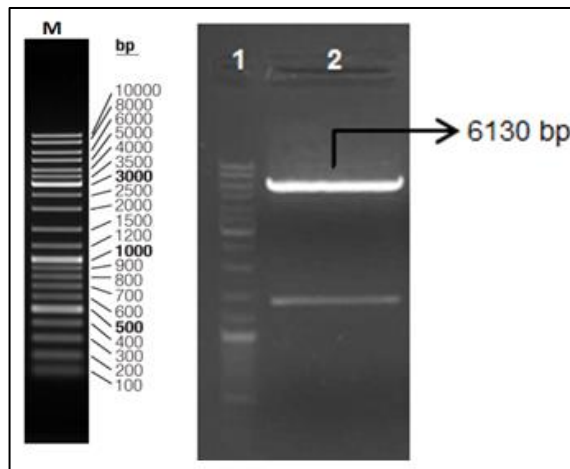


Figure 4.6: pGWf plasmid cut with PacI and NotI enzymes.
(1) GeneRulerDNA Ladder mix, (2) REase digested pGWf vector (6130 bp).

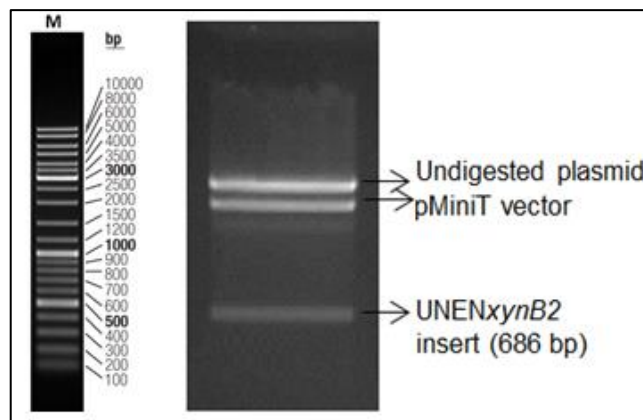


Figure 4.7: Restriction enzyme digested UNENxynB2.
(M) GeneRuler DNA Ladder mix and PacI and NotI digested product of plasmid pMxynBc2.

Ligation products of UNENxynB2 were transferred to *E. coli* DH5 α cells. The plasmids received the UNENxynB2 fragment selected in kanamycin medium and controlled by PCR. Four colonies were selected from transformants grown in selective medium. Colony PCR was performed for confirmation of the presence of UNENxynB2 (Figure 4.8). PCR products run on agarose gel yielded 918 bp of the expected size. From these transformants, the plasmid was then isolated. Obtained plasmid DNA also confirmed with REase digestion (Figure 4.9). The plasmid DNA

carrying the selected UNENxynB2 was sequenced using the GWF and GWR primers designed from vector region sequences, and the UNENxynB2 insertion was confirmed by DNA sequencing. The generated expression plasmid was named pAExynB2. The plasmid pAExynB2 obtained is 6813 bp in size and the plasmid map is given in Figure 4.10.

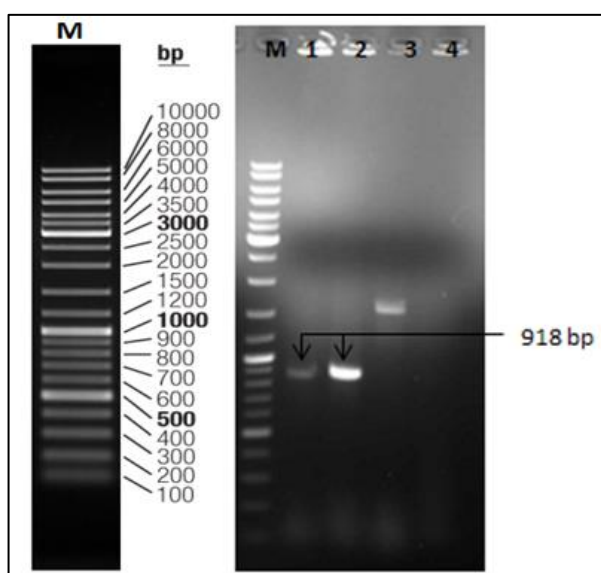


Figure 4.8: Colony PCR amplification of pAExynB2 candidates.
(M) GeneRuler DNA ladder mix, (1,2,3,4) PCR products.

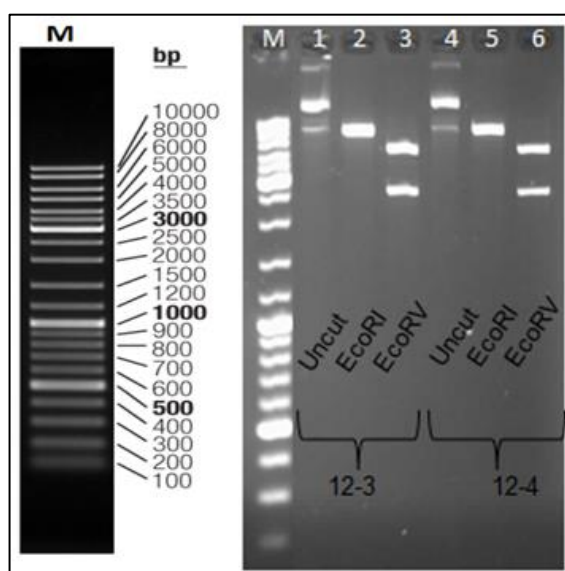


Figure 4.9: Confirmation of selected clones by REase treatment.
(M) GeneRuler DNA ladder mix (1-6) Uncut, EcoRI digest and EcoRV digest for plasmids 12-3 (1-3) and 12-4 (4-6) respectively.

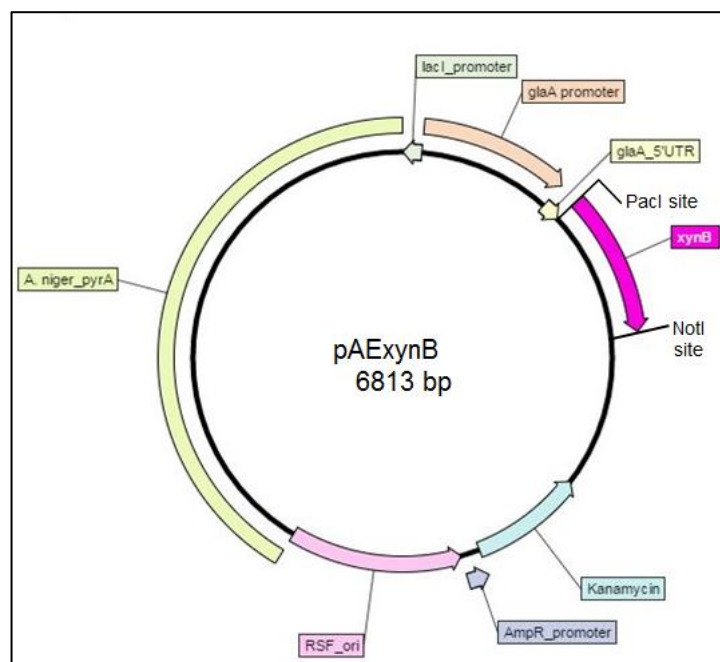


Figure 4.10: pAExynB2 plasmid map (pGWf-derivative).

Thus, the UNENxynB2 protein coding regions are under the control of the fungal *glaA* (glucoamylase) promoter and suitable for selection in *A. niger*. The pAExynB2 expression plasmid is transferred to the recipient organism *A. niger* N593, resulting in the recombination of the *pyrA* gene.

The expression of the protein encoded by the transferred UNENxynB2 is under the control of the *glaA* promoter region, therefore expression should be occurred in the presence of maltose. At the same time, because of *A. niger pyrA* complementation, transformants have to be selected in minimal medium without uridine that synthesized by the the intact *pyrA* gene.

UNENxynB2 was then transferred from plasmid DNA by homologous recombination to recipient *A. niger* N593 strain by the protoplast-mediated transformation. So 17 transformants (pAExynB2/*A. niger* N593) were taken into the minimal medium. The obtained transformants were grown in minimal medium to give a single colony (Figure 4.11).

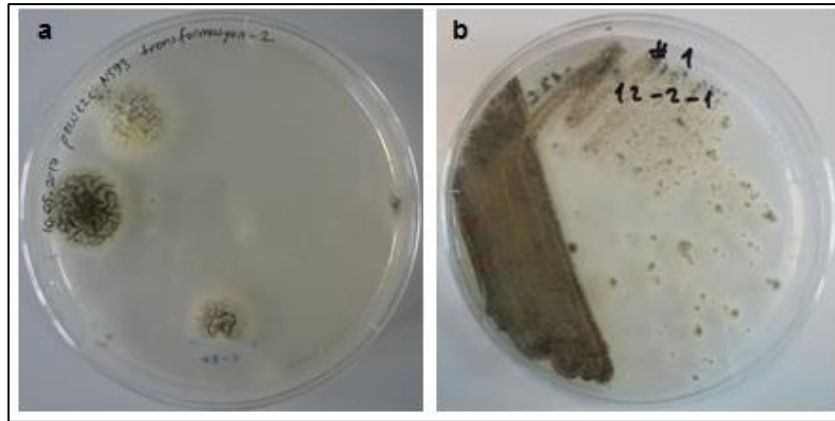


Figure 4.11: *A. niger* transformants on minimal plate.
 (a) pAExynB2/*A. niger* N593 transformants, (b) Pure colony from pAExynB2/*A. niger* N593 transformant.

4.1.4. Analysis of the UNENxynB2 Transformants by PCR

Genomic DNA was isolated from candidate colonies and PCR was performed with vector primers (GWF and GWR) to question of the presence UNENxynB2 gene. PCR products were analyzed on agarose gel (Figure 4.12). As a result of PCR, 918 bp product was obtained including vector sequences and 678 bp UNENxynB2. *A. niger* N593 strain was used as a negative control in terms of GWF and GWR primers.

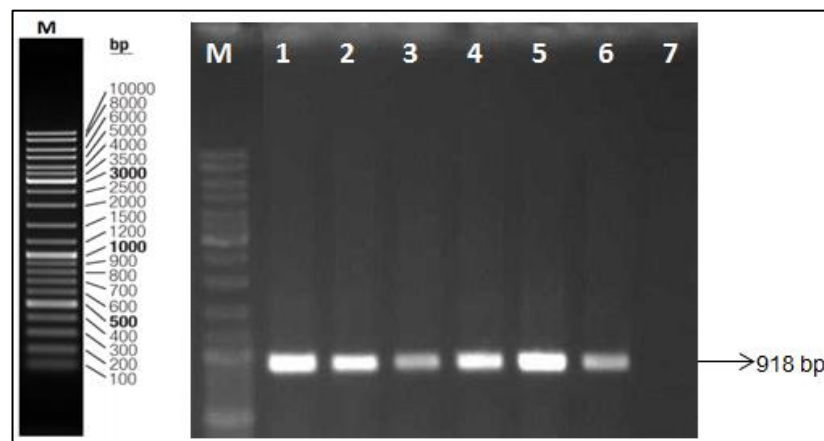


Figure 4.12: PCR analysis of *A. niger* UNENxynB2 fungal transformants.
 (1-6) *A. niger* UNENxynB2 candidate transformants, (7) *A. niger* N593 host (negative control).

Transformation of the gene construct depicted on Figure 4.13 was resulted in 17 transformants by homologous recombination. UNENxynB2 integrated transformants were analyzed by PCR in the frame of this thesis. But they were also analyzed to determine their xylanase activity in the frame of UN-EN Project and studied in detail for its expression, so xynB expression optimized in flask culture. High expression of the xynB in *A. niger* were observed (MSc thesis by Akdeniz, C., 2019).

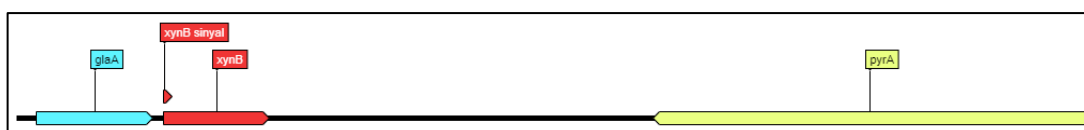


Figure 4.13: Schematic representation of the UNENxynB2 gene with its own signal sequence and *pyrA* region of the pGWf vector in the control of *glaA* promoter.

In this thesis study, selected transformant 12-1-7 chromosomal DNA was sequenced (See, Appendix) and its cDNA sequence is used to subclone *Pichia* vectors and *Pichia* transformants were analyzed.

4.2. Cloning of The Codon Optimized UNENxynB2 cDNA and Expression in *Pichia pastoris*

As an alternative extracellular xylanase secreting expression system, *Pichia pastoris* was used since it is suitable for pilot scale enzyme production.

4.2.1. Codon Optimization of UNENxynB2 Gene and Transfer to *Pichia pastoris*

Two *SxynB2* sequence originated from UNENxynB2 cDNA sequence were synthesized by GeneArt (Germany). *SxynB2-1* was designed to be cloned into pHIL-S1 and *SxynB2-2* was designed to be cloned into pPIC9. Synthesized *SxynB2* sequences are given in Figure 4.14.

SxynB2-1 sequence

CTCGAGGTTCCCATGATTCTGTTGTAGAAAGATCCGACGCACTTCATAAATTGTCAGAAAGGTCTACACCTTCTCTACTGG
CGAGAATAACGGCTTTTACTACTCATTCTGGACCGACGGTGGCGGAGATGTAACCTATACCAATGGAGATGCAGGAAGTTATA
CCGTCGAACGTAGTAACGTGCTAATTTCGTCGGCGGAAAAGGTTGGAACCCCGGCTCTGCACAAGACATCACCTATTGAGGA
ACCTTCACCCCATCAGGAATGGTTATTTGTCGGTGTATGGCTGGACTACCGATCCCTAATCGAATATTACATAGTAGAGAG
TTATGGAGATTACAACCCCGGCTCCGGAGGTACTTACGAGGTACAGTTACCTCTGATGGTAGTGTGTACGACATCTATACGG
CCACAAGAACAATGCGCTTCTATTGAGGGCACCCTACGTTTACACAATACTGGTCTGTCCGTCAGAATAAGCGTGTGGGC
GGAACCGTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAGGTATGAATTGGGTACACATAATTATCAGATAGTCGC
AACAGAAGGTTACAGTCTAGTGGCTCATCTTCAATAACCGTGCAATAAGAATTC - 636 bp

SxynB2-2 sequence

CTCGAGAAAAGAGAGGCTGAAGCTGTTCCTCATGATTCTGTTGTAGAAAGATCCGACGCACTTCATAAATTGTCAGAAAGGTC
TACACCTTCTCTACTGGCGAGAATAACGGCTTTTACTACTCATTCTGGACCGACGGTGGCGGAGATGTAACCTATACCAATG
GAGATGCAGGAAGTTATACCGTCGAACGTAGTAACGTGCGTAATTTGTCGGCGGAAAAGGTTGGAACCCCGGCTCTGCACAA
GACATCACCTATTGAGGAACCTTCACCCCATCAGGAATGGTTATTTGTCGGTGTATGGCTGGATACCGATCCCTAATCGA
ATATTACATAGTAGAGAGTTATGGAGATTACAACCCCGGCTCCGGAGGTACTTACGAGGGTACAGTTACCTCTGATGGTAGTG
TGTACGACATCTATACGGCCACAAGAACAAATGCCGCTTCTATTACGGGCACCGCTACGTTTACACAATACTGGTCTGTCCGT
CAGAATAAGCGTGTGGCGGAACCGTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAGGTATGAATTGGGTACACA
TAATTATCAGATAGTCGCAACAGAAGGTTACAGTCTAGTGGCTCATCTTCAATAACCGTGCAATAAGAATTC - 654 bp

Figure 4.14: Codon optimized *SxynB2-1* and *SxynB2-2* sequences.

UNENxynB2 <i>SxynB2</i>	GTTCOCACGACTCTGTCGTGGAGCGTTGGATGCCTTCACAAAGCTCTCTGAGCGTTCG GTTCOCATGATTCTGTTGTAGAAAGATCCGACGCACTTCATAAATTGTCAGAAAGGTC *****
UNENxynB2 <i>SxynB2</i>	ACCCGAGCTCGAOCGGCGAGAACAACGGCTTCTACTACTCCTTCTGGACCGAOCGGT ACACCTTCTCTACTGGCGAGAATAACGGCTTCTACTACTCCTTCTGGACCGAOCGGTGGC **
UNENxynB2 <i>SxynB2</i>	GGTGTGTGACCTACACCAACGGTGAAGCTGGCTCGTACACCGTTCGACGGCTTAACGT GGAGATGTAACCTATACCAATGGAGATGCGAGGAGTTATACCGTCGAACTAGTAACGTC **
UNENxynB2 <i>SxynB2</i>	GGCAACTTTGTGGTGGAAAGGGCTGGAACCTGGAACTGGCAGGACATTACCTACAGC GGTAATTTCTGTCGGCGGAAAAGGTTGGAACCCGGCTCTGCACAAGACATCACCTATCCA **
UNENxynB2 <i>SxynB2</i>	GGCACCTTCACCCCTAGCGGCAACGGCTACCTCTCCGTCTATGGCTGGACCACTGACCCC GGAACTTCACCCCTACGAGGAATGGTTATTTGTCGGTGTATGGCTGGACTACCGATCCC **
UNENxynB2 <i>SxynB2</i>	CTGATCGAGTACTACATCTGTCGAGTCCCTACGGCGACTACAACCCCGGCACTGGAGGCACC CTAATCGAATATTACATAGTAGAGAGTTATGGAGATTACAACCCCGGCTCCGGAGGTACT **
UNENxynB2 <i>SxynB2</i>	TACGAGGGCACCGTCACTTCGATGGATCCGTCTACGATATCTACACGGCTACCCGCACC TACGAGGGTACAGTTACCTCTGATGGTAGTGTGTACGACATCTATACGGCCACAAAGACA *****
UNENxynB2 <i>SxynB2</i>	AACGCGCTTCATCCAGGAACCGCTACCTTCACCACTATGGTCCGTTCGCCAGAAC AATGCGCTTCTATTGAGGCAACCGCTACGTTTACCAATACGGTCTGTCGGTCAGAAAT **
UNENxynB2 <i>SxynB2</i>	AAGAGGTCGGAGGAAGTTTACCACTTCAACCACTTCAACGCTTGGGCCTAAGCTGGC AAGCTGTGGCGGAACGTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAGGT **
UNENxynB2 <i>SxynB2</i>	ATGAACCTGGGTACTCACAACTACGAGATCGTGGCTACCGAGGGCTACGAGGAGCGGA ATGAATTTGGGTACACATAATATCAGATAGTGGCAACGAGGTTACAGTCTAGTGGC *****
UNENxynB2 <i>SxynB2</i>	TCCTCTCCATCACTGTTCACTAA TCATCTCAATACCGTGCATTA *stop **

Figure 4.15: Comparison of the changed bases of UNENxynB2 and *SxynB2*.

UNENxynB2 and optimized SxynB2 bases were compared using NCBI BLAST (Figure 4.15). The optimized SxynB2 gene and the UNENxynB2 nucleotide sequences was only 72.92% identical. However, amino acid sequences did not changed. It has *Pichia pastoris* codon preference.

pHIL-S1 and pPIC9 vectors were transferred to *E. coli* TOP10F' for vector stock. Thus, vectors were propagated from pPIC9/TOP10F' and pHIL-S1/TOP10F'. Plasmid DNA was isolated and cut using the same XhoI and EcoRI enzymes to produce vector fragments having insert-compatible adhesive ends. The ligations of SxynB2 with digested vectors were performed. Ligation products were transferred to electrocompetent *E. coli* TOP10F' cells and selected in LB-ampicillin agar plates. Plasmid was isolated for control of the SxynB2 gene from *E. coli* TOP10F' transformants. Obtained plasmids were examined for the presence of the insert by PCR using the AOX1F and AOX1R primers complementary to the vector sequence, and also controlled with this restriction endonucleases. The plasmid confirmed by DNA sequencing was named as pBKC-S1 for containing the SxynB2-1 sequence and pBCK9 for containing the SxynB2-2 sequence.

The presence of SxynB2-1 was investigated by PCR from the colonies in selective media. PCR products were run on 1% agarose gel (Figure 4.16). Obtained plasmid was examined for the presence of SxynB2-1 by the REase treatment (Figure 4.17).

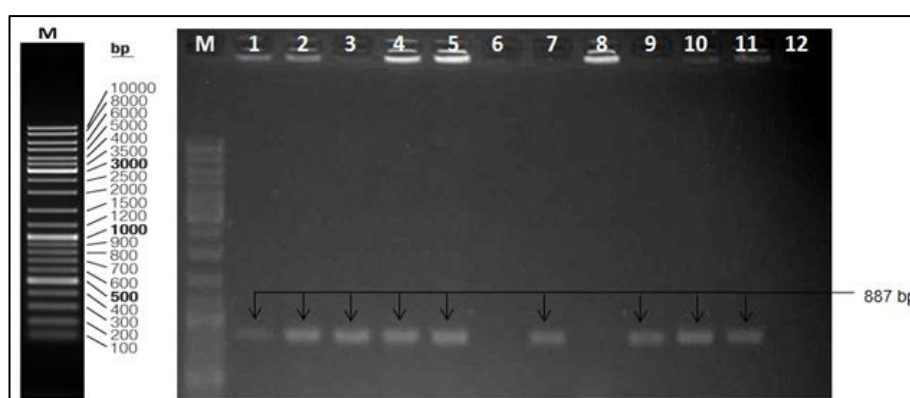


Figure 4.16: PCR analysis of candidate pBKC-S1/*E. coli* TOP10F' using AOX1F and AOX1R primers. 887 bp bands belongs to SxynB2 sequence and part of pHIL-S1 vector.

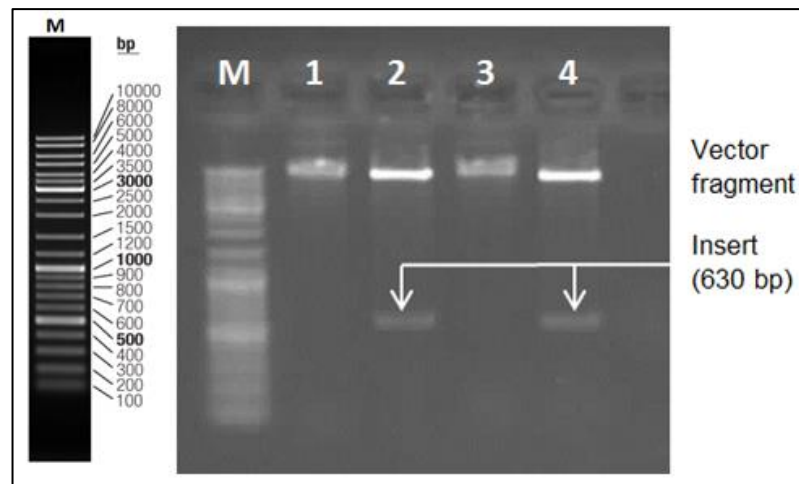


Figure 4.17: XhoI and EcoRI enzyme digestions of pBKC-S1/*E. coli* TOP10F' transformants. (1 and 3) uncut plasmids, (2 and 4) XhoI and EcoRI digested plasmids.

The candidate clone was sent for sequencing (Figure 4.18). Obtained plasmid map is given in Figure 4.22 (a).

pBKC-S1:	
F	-----
RC	TTGACAGCAATATATAAACAGAGGGAAGCTGCCCTGTCTTAAACCTTTTATCAT
F	-----
RC	CATTATTAGCTTACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTAAC
F	-----CAAAAAACAACCTAATTATTCGAAACGATGTTCTC
RC	GACTTTTAAACGACAACCTTGAGAAGATCAAAAAACAACCTAATTATTCGAAACGATGTTCTC *****
F	TCCAATTTTGCTCTTGAAATATTTTAGCTTTGGCTACTTTGCAATCTGTCTTCGCTCG
RC	TCCAATTTTGCTCTTGAAATATTTTAGCTTTGGCTACTTTGCAATCTGTCTTCGCTCG *****
F	AGGTTCCCATGATTCTGTTGTAGAAAGATCGACGCCTTCATAAATGTCAGAAAGGT
RC	AGGTTCCCATGATTCTGTTGTAGAAAGATCGACGCCTTCATAAATGTCAGAAAGGT *****
F	CTACACCTTCTCTACTGCGGAGAATAACGGCTTTTACTACTCATTCTGGACCGGTG
RC	CTACACCTTCTCTACTGCGGAGAATAACGGCTTTTACTACTCATTCTGGACCGGTG *****
F	GCGGAGATGTAACCTTATACCAATGGAGATGCAGGAAGTTATACCGTCGAACGTAGTAACG
RC	GCGGAGATGTAACCTTATACCAATGGAGATGCAGGAAGTTATACCGTCGAACGTAGTAACG *****
F	TCGGTAATTTTCGTGCGGGAAGGTTGGAAACCCGGCTCTGCACAAGACATCACCTATT
RC	TCGGTAATTTTCGTGCGGGAAGGTTGGAAACCCGGCTCTGCACAAGACATCACCTATT *****
F	CAGGAACCTTCACCCCATCAGGAAATGGTTATTGTCCGTGTATGGCTGGACTACCGATC
RC	CAGGAACCTTCACCCCATCAGGAAATGGTTATTGTCCGTGTATGGCTGGACTACCGATC *****
F	CCCTAATCGAATATTACATAGTAGAGAGTTATGGAGATTACAACCCCGCTCCGGAGGTA
RC	CCCTAATCGAATATTACATAGTAGAGAGTTATGGAGATTACAACCCCGCTCCGGAGGTA *****
F	CTTACGAGGTACAGTTACCTCTGATGGTAGTGTGTAACGACATCTATACGGCCACAAGAA
RC	CTTACGAGGTACAGTTACCTCTGATGGTAGTGTGTAACGACATCTATACGGCCACAAGAA *****
F	CAAATGCGCTTCTATTACAGGGCACCGCTACGTTTACACAATACTGGTCTGTCGTCAGA
RC	CAAATGCGCTTCTATTACAGGGCACCGCTACGTTTACACAATACTGGTCTGTCGTCAGA *****
F	ATAAGCGTGTGGGCGGAACCGTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAG
RC	ATAAGCGTGTGGGCGGAACCGTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAG *****
F	GTATGAATTTGGGTACACATAATTATCAGATAGTCGCAACAGAAGGTTACCAGTCTAGTG
RC	GTATGAATTTGGGTACACATAATTATCAGATAGTCGCAACAGAAGGTTACCAGTCTAGTG *****
F	GCTCATCTTCAATAACCGTGCAATAAGAATTCCCCGGATCCTTAGACATGACTGTTCTT
RC	GCTCATCTTCAATAACCGTGCAATAAGAATTCCCCGGATCCTTAGACATGACTGTTCTT *****
F	CAGTTCAAGTTGGGCAC
RC	CAGTTCAAGTTGGGCAC *****

Figure 4.18: *SxynB2-1* sequence from the pBKC-S1 vector using AOX1F and AOX1R primers. The gray-marked arrays belong to the *SxynB2* sequence.

The presence of *SxynB2-2* was investigated by PCR (Figure 4.19) and REase treatment (Figure 4.20). One of the *SxynB2-2* positive clones ligated with the pPIC9

vector was selected for sequencing to check the accuracy of the DNA sequence (Figure 4.21). Obtained plasmid map is given in Figure 4.22 (b).

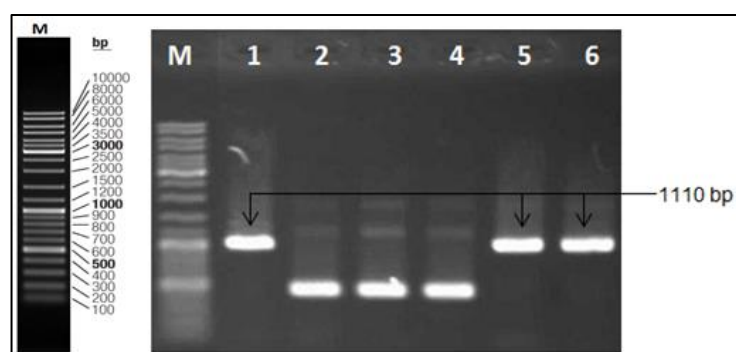


Figure 4.19: PCR analysis of candidate pBCK9/*E. coli* TOP10F'.

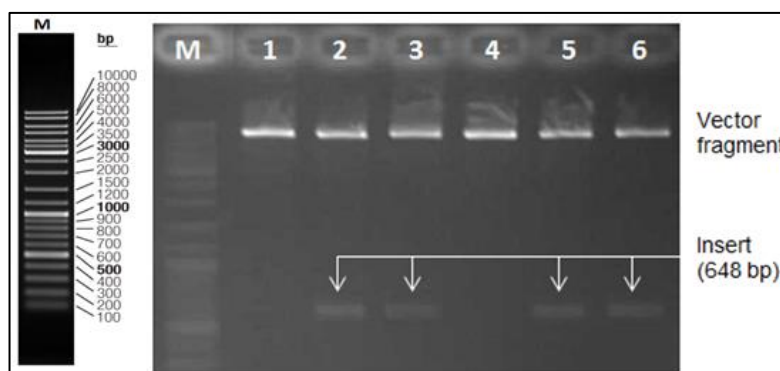


Figure 4.20: XhoI and EcoRI enzyme digestion of candidate pBCK9/*E. coli* TOP10F' transformants. (1-6) XhoI and EcoRI digested plasmids.

pBCK9:

```

F      CT TGAGAAGATCAAAAAACAATAATATTTCGAAGGATCCAAACGATGAGATTTCCTTCA
R      -----CTTCA
          *****

F      ATTTTACTGCAGTTTATTTCGAGCATCCTCCGCATAGCTGCTCCAGTCAACACTACA
R      ATTTTACTGCAGTTTATTTCGAGCATCCTCCGCATAGCTGCTCCAGTCAACACTACA
          *****

F      ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTAGAA
R      ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTAGAA
          *****

F      GGGGATTTCGATGTTGCTGTTTTCGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTT
R      GGGGATTTCGATGTTGCTGTTTTCGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTT
          *****

F      ATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGA
R      ATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGA
          *****

F      GAGGCTGAAGCTGTTCCCATGATTCGTGTTGTAGAAAGATCCGACGCACTTCATAAATTG
R      GAGGCTGAAGCTGTTCCCATGATTCGTGTTGTAGAAAGATCCGACGCACTTCATAAATTG
          *****

F      TCAGAAAGGTCTACACCTTCCTCTACTGGCGAGAATAACGGCTTTTACTACTCATTCTGG
R      TCAGAAAGGTCTACACCTTCCTCTACTGGCGAGAATAACGGCTTTTACTACTCATTCTGG
          *****

F      ACCGACGGTGGCGGAGATGTAACCTATACCAATGGAGATGCAGGAAGTTATACCGTCGAA
R      ACCGACGGTGGCGGAGATGTAACCTATACCAATGGAGATGCAGGAAGTTATACCGTCGAA
          *****

F      CGTAGTAACGTCGGTAATTTTCGTCGGCGGAAAAGGTTGGAACCCCGGCTCTGCACAAGAC
R      CGTAGTAACGTCGGTAATTTTCGTCGGCGGAAAAGGTTGGAACCCCGGCTCTGCACAAGAC
          *****

F      ATCAOCTATTACAGGAACCTTCACCCCATCAGGAAATGGTTATTGTCGGTGTATGGCTGG
R      ATCAOCTATTACAGGAACCTTCACCCCATCAGGAAATGGTTATTGTCGGTGTATGGCTGG
          *****

F      ACTACCGATCCOCTAATCGAATATTACATAGTAGAGAGTTATGGAGATTACAACCCCGGC
R      ACTACCGATCCOCTAATCGAATATTACATAGTAGAGAGTTATGGAGATTACAACCCCGGC
          *****

F      TCCGGAGGTACTTACGAGGGTACAGTTACCTCTGATGGTAGTGTGTACGACATCTATACG
R      TCCGGAGGTACTTACGAGGGTACAGTTACCTCTGATGGTAGTGTGTACGACATCTATACG
          *****

F      GCCACAAGAACAAATGCCGCTTCTATTACGGGCACCGCTACGTTTACACAATACTGGTCT
R      GCCACAAGAACAAATGCCGCTTCTATTACGGGCACCGCTACGTTTACACAATACTGGTCT
          *****

F      GTCCGTCAGAAATAAGCGTGTGGCGGAAACCGTTACGACATCTAACCCTTTAATGCTTGG
R      GTCCGTCAGAAATAAGCGTGTGGCGGAAACCGTTACGACATCTAACCCTTTAATGCTTGG
          *****

F      GCCAAATTAGGTATGAATTTGGGTACACATAATTATCAGATAGTCGCAACAGAAGGTAC
R      GCCAAATTAGGTATGAATTTGGGTACACATAATTATCAGATAGTCGCAACAGAAGGTAC
          *****

F      CAGTCTAGTGGCTCATCTTCAATA-----
R      CAGTCTAGTGGCTCATCTTCAATAACCGTGCAATAAGAATTCCTAGGGCGGCGCGAAT
          *****

F      -----
R      TAATTGOCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACCTTACGAGAAGACCGG

```

Figure 4.21: *SxynB2*-2 sequence from the pBCK9 vector using AOX1F and AOX1R primers. The gray-marked arrays belong to the *SxynB2* sequence.

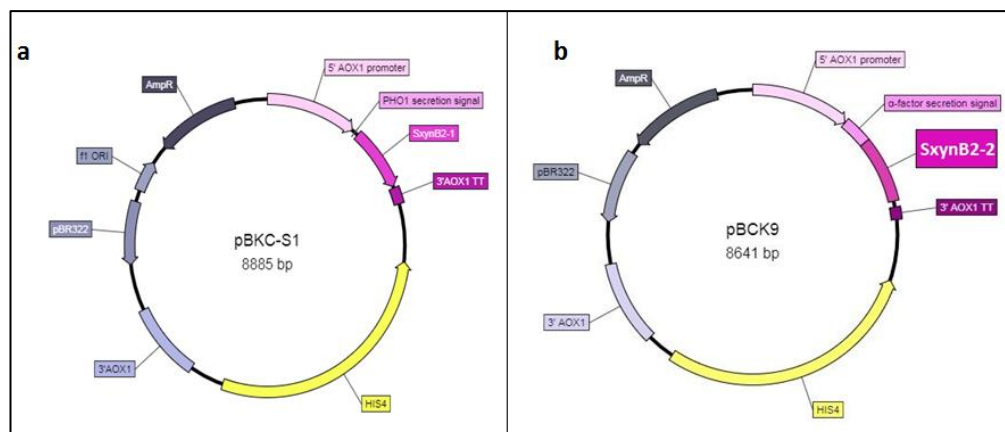


Figure 4.22: Maps of pBKC-S1 and pBCK9 Pichia expression plasmids containing *SkynB2* gene. (The recombinant plasmids were scaled with the tool of <http://www.molbiotools.com/WebDSV/>)

4.2.2. The Production of Fungal Xylanase B in *Pichia pastoris* Strains

5 μ g linearized pBKC-S1 obtained from Sall digestion was transferred to *P. pastoris* GS115 host. The cells were spread onto MD medium and after 3 days incubation at 28 °C, transformants were obtained (Figure 4.23).

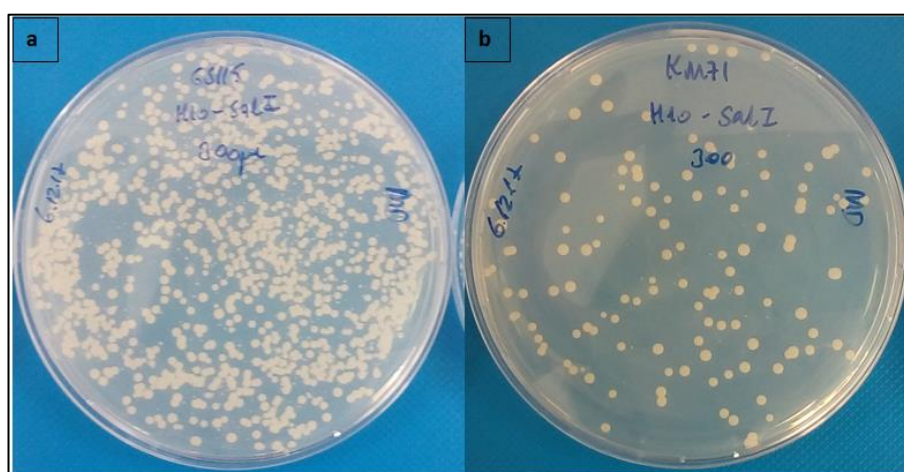


Figure 4.23: pBKC-S1 transformants obtained from *P. pastoris* GS115 a) and KM71 b) hosts.

Similar studies have been carried out for the transformation of pBCK9 containing *Saccharomyces cerevisiae* alpha-factor signal sequence to see the effect of xylanase B on secretion. 5 μ g of pBCK9 plasmid was digested with Sall enzyme for

transformation. The resulting linear DNA fragments were checked on 1% agarose gel (Figure 4.24). Thus, fragments suitable for homologous recombination were obtained. 4,5 µg Sall-digested linear pBCK9 plasmid was transferred to freshly prepared electrocompetent *P. pastoris* KM71 and GS115 cells by electroporation. Transformants appeared after incubation at 28 °C for 5 days (Figure 4.25).

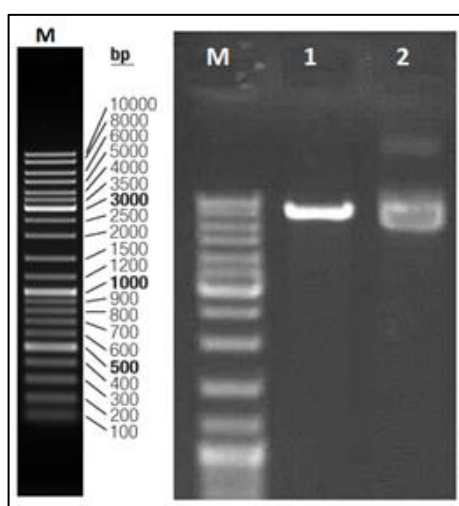


Figure 4.24: Restriction enzyme digestion of pBCK9 plasmid. (1) Sall-digested pBCK9 plasmid, (2) Uncut plasmid.

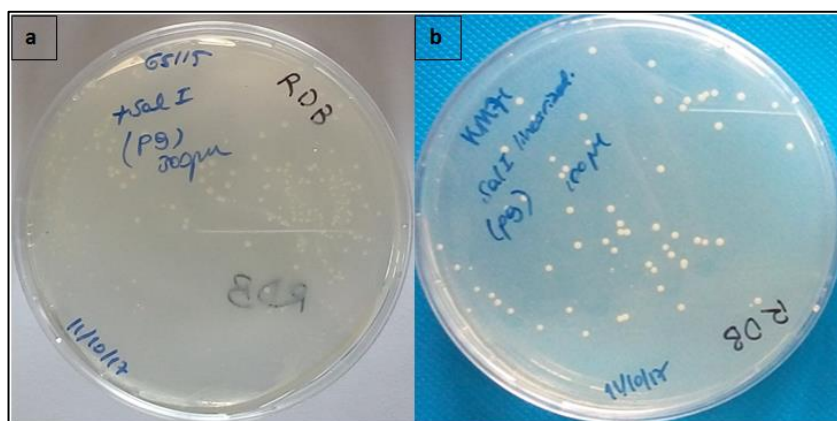


Figure 4.25: pBCK9 transformants obtained from *P. pastoris* GS115 a) and KM71 b) hosts.

Genetic constructs encoding fungal xylanase B sequence (Figure 4.26) with different signal sequences were transferred to histidine auxotroph *Pichia* strains by electroporation.

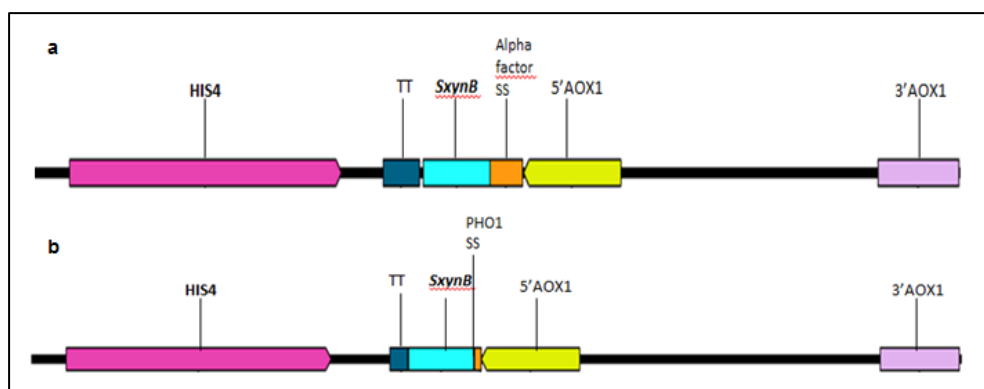


Figure 4.26: Schematic representation of the *SxynB2* gene transferred to *Pichia pastoris*. *Sall*-restricted vectors pBCK9 (a) and pBKC-S1 (b) based 8,6 kb and 8,8 kb genetic fragment, respectively. HIS4: selection marker, TT: transcription termination, SS: signal sequence, 5'AOX1: promoter, 3'AOX1: complementary fragment for AOX1 gene.

Sall enzyme cuts the plasmid within the HIS4 ORF region. As a result of gene insertion in HIS4 region, His⁺ Mut⁺ phenotype is found in *P. pastoris* GS115 cells and His⁺ Mut^s phenotype in *P. pastoris* KM71 cells. The colonies to be screened were grown in YPD medium and stocked with glycerol for further analysis. The characteristics of the recipient strain and signal of *Pichia pastoris* transformants to be screened are given in Table 4.1.

Table 4.1: Nomenclature of *P. pastoris* candidate clones.

Recipient strain	Type of signal peptide	
	Alpha factor	PHO1
KM71	KP9S	KH10S
	(50 clones)	(50 clones)
GS115	GP9S	GH10S
	(50 clones)	(50 clones)

Thus, *SxynB2* was integrated into the yeast chromosome by two separate secretion signals. A total of 200 recombinants from *P. pastoris* KM71 and GS115 yeast transformant were obtained.

In the resulting recombinant clones, the integrated regions of the DNA were checked by PCR and DNA sequencing. The AOX1 primers were used for the control of *SxynB2* and AOX1 promoter sequences in *P. pastoris*.

For *SxynB2* control at the DNA level, yeast genomic DNA was isolated from the transformants (Figure 4.27). PCR reaction was established using obtained genomic DNAs and the vector primers (AOX1F and AOX1R). PCR products were examined on 1% agarose gel and *SxynB2* integrated transformants were determined (Figure 4.28). In GP9S coded transformants, transformation occurred with gene insertion at *his4* locus.

Depending on the vectors and the length of the signal sequences used during gene transfer, *SxynB2* and the portion from the vector obtained with the AOX1 primers vary. In GP9S coded transformants the *AOX1* gene is intact, both *SxynB2* and *AOX1* gene (2.2 kb) amplified at the end of PCR amplification with AOX1 primers (2 bands). The *SxynB2* band is larger than 624 bp because it also includes the vector fragment. In the KP9S coded transformants, amplification of *AOX1* gene is not performed because of KM71 host. This transformants have *aox1::arg4* insertion and give 3,6 kb band with AOX1 primers.

In GH10S coded transformants, on the other hand, 2 bands are obtained by *AOX1* and *SxynB2* amplification and this band size is 0.8 kb due to *SxynB2* and a portion of the plasmid sequence.

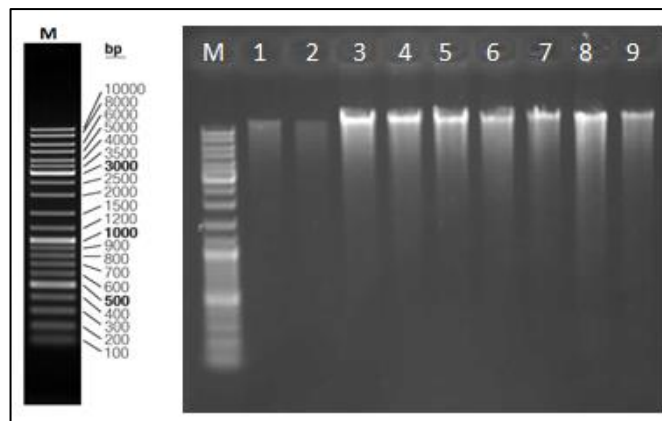


Figure 4.27: Genomic DNAs from *Pichia pastoris* candidate transformants. (M) GeneRuler DNA Ladder mix, (1-10) gDNA obtained from *P. pastoris* candidate transformants.

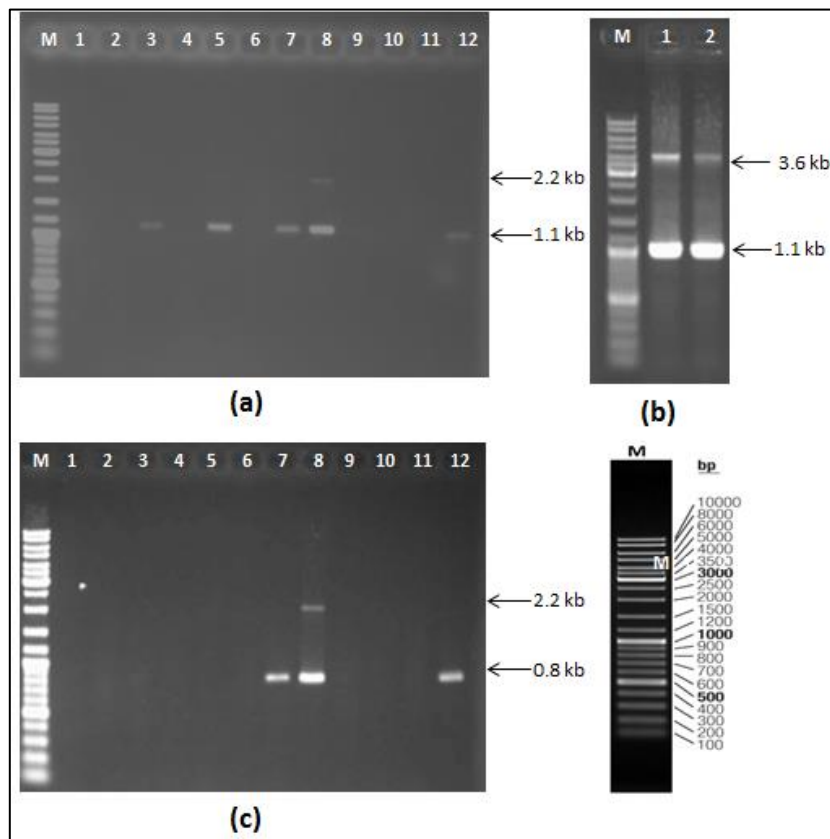


Figure 4.28: PCR analysis of recombinant *P. pastoris* clones. (M) GeneRuler DNA Ladder mix, (a) (1-12) GP9S coded *P. pastoris* GS115 transformants, (b) (1-2) KP9S coded *P. pastoris* KM71 transformants, (c) (1-12) GH10S coded *P. pastoris* GS115 transformants.

As a result of PCR screenings, 10 of 84 candidate clones were found to be positive for AOX1 and *SxynB2* region. 7 Mut⁺ and 3 Mut^s *Pichia* transformants were used to select from candidate recombinant yeasts in which integration of *SxynB2* was determined.

Flask cultures were performed to investigate the expression and release of the UNEN xylanase B enzyme under the control of the alcohol oxidase 1 (AOX1) promoter. The α -factor and PHO1 signal carrying candidate recombinant yeasts to induce the release of xylanase from transformants with Mut⁺ or Mut^s phenotypes based on their presence in *P. pastoris* KM71 and GS115 and to the chromosome were investigated by being induced with methanol in the BMMY medium.

4.2.3. Screening of The Transformants in The Flask Culture

Selected transformants by PCR were used to determine *SxynB2* expression. For this, 7 Mut⁺ and 3 Mut^s *Pichia* transformants were tested in flask culture and xylanase secretion was screened using AZCL-arabinoxylan as a substrate. Mut⁺ transformants were grown in 5 ml BMGY medium then expression was established in 10-20 ml BMMY medium (Figure 4.29). Mut^s transformants were grown in 100 ml BMGY medium then expression was established in 10 ml BMMY medium. 1% methanol was added every 24 hours to culture and 1 ml sample was taken for the determination of xylanase secretion. Xylanase secretion was observed during the culture (Figure 4.30).

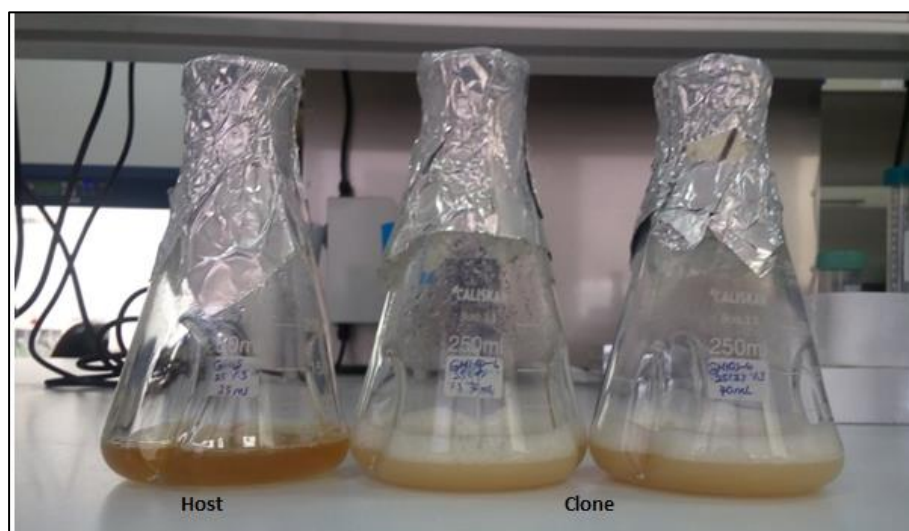


Figure 4.29: Flask cultures of *P. pastoris* host and clone.

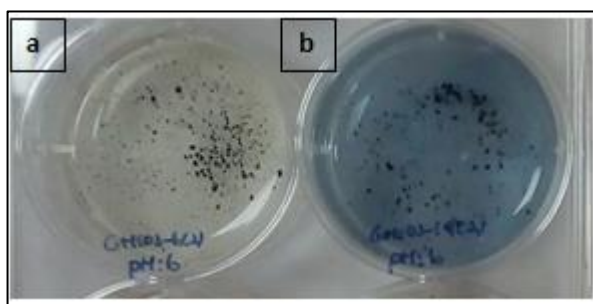


Figure 4.30: Screening of the xylanase secretion from flask culture using AZCL-arabinoxylan as a substrate. (a) Clone GH10S-6, (b) clone GH10S-19.

As shown in Figure 4.30, xylanase secretion is qualitatively observed in GH10S-19 transformant. No significant measurable xylanase activity with GH10S-19 transformant using modified Nelson-Somogyi reducing sugar method. Expression volumes were changed for observing measurable xylanase activity. Mut⁺ transformants were grown in 25 ml BMGY medium and expressed in 100-200 ml BMMY medium. Mut^s transformants were grown on 50 ml BMGY medium and expressed in 20 ml BMMY medium using 0.5% methanol as an inducer. In this case, GH10S-6 coded *Pichia pastoris* GS115 transformant and KP9S-4 coded *Pichia pastoris* KM71 transformant were shown qualitative xylanase activity (Figure 4.31 and Figure 4.32).

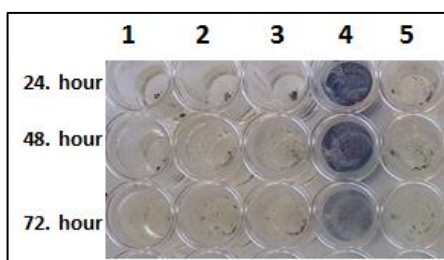


Figure 4.31: Qualitative xylanase activity in candidate transformants. (1) *P. pastoris* GS115, (2) KP9S-1, (3) GP9S-1, (4) GH10S-6 and (5) GH10S-9 coded *Pichia pastoris* transformants.



Figure 4.32: Day-dependent (up to 168 hours) qualitative xylanase activity in KP9S-4 transformant.

There are 3 *SxynB2* expressed transformants, one of them has Mut^s phenotype (KP9S-4), and two of them have Mut⁺ phenotype (GH10S-6 and GH10S-19). In the literature, Mut^s and Mut⁺ *Pichia* clones have some advantages over each

other [Macauley-Patrick et al., 2005]. Some researchers obtained more activity in Mut^s clones and others in Mut⁺ clones. In the Mut^s clones, culture is required to continue for 7 days (K1710-01, Invitrogen). Expression studies were continued with Mut⁺ candidates because of the short duration of culture.

4.2.4. Investigation of The Effect of Methanol Concentration on Xylanase B Enzyme Expression

In the literature, protein expression in *P. pastoris* was tested with different methanol concentrations under the control of AOX1 promoter [Damaso et al., 2003; Korona et al., 2006; Ruanglek et al., 2007]. Methanol concentrations published in previous studies were tested in this study. Flask cultures were supplemented with 0,5%, 1% and 3% methanol in the GH10S-6 clone where activity was observed. These cultures separately incubated at both 25 °C and 28 °C. The samples were collected every 24 hours from each culture. Obtained supernatants were used for protein determination and SDS-PAGE analysis (Figure 4.33).

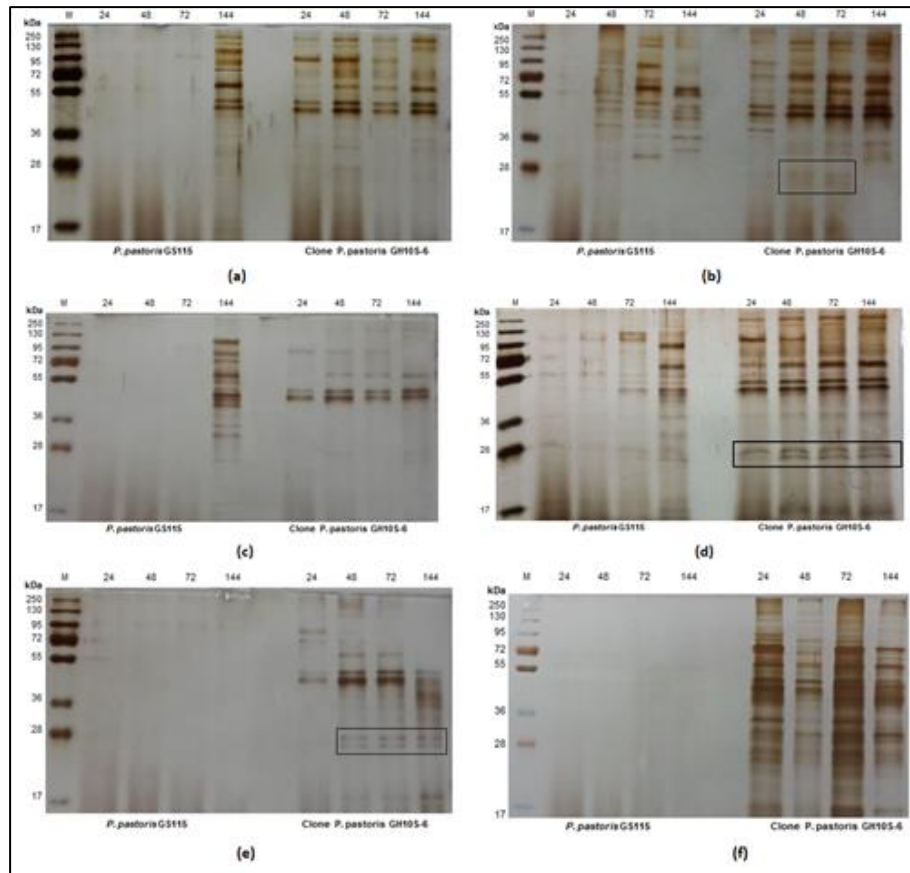


Figure 4.33: Protein profiles of host GS115 (used as a control) and clone GH10S-6 at different temperatures and methanol concentrations. Supernatants obtained at 24, 48, 72, and 144 hours in the presence of 0,5%, 1% and 3% methanol at 25 °C (a), (c) and (e), respectively. Supernatants obtained at 24, 48, 72, and 144 hours in the presence of 0,5%, 1% and 3% methanol at 28 °C (b), (d) and (f), respectively.

When the protein profiles were examined, it is observed that it did not release the protein of the expected size even though 0.5% and 1% methanol were used at 25 °C (Figure 4.33 a,c). However, very weak protein band is observed when induced with 3% methanol (Figure 4.33 e). Two protein bands of the expected size are obtained in the case of inducing with low methanol concentrations at 28 °C (Figure 4.33 b,d). Both glycosylated xylanase and non-glycosylated form have been considered to be present in the expression condition with 28 °C and 0,5% or 1% methanol (Figure 4.33 b,d). From 24 hours to 144 hours, a protein was observed which is better expressed (Figure 4.33 d). At 28 °C and 3% methanol induction, various proteins were synthesized, but the expected protein between 17-28 kDa was not observed (Figure 4.33 f). Better protein profiles were observed to be 0.5%

or 1% methanol concentrations at 28 °C. Qualitative enzyme activity was not measured in this expression conditions.

4.2.5. Xylanase Expression in BMMY and YPTM Medium

GH10S-6 and GH10S-19 were expressed in 100-200 ml volume using BMMY and YPTM medium. When the initial OD₆₀₀ values were between 4-6, 0.5% methanol was added. Qualitative xylanase activity was observed in BMMY medium for GH10S-6 and in YPTM medium for GH10S-19 clones (Figure 4.34). The protein was monitored for 4 days (Figure 4.35). Time-dependent protein profiles are given in Figure 4.36.

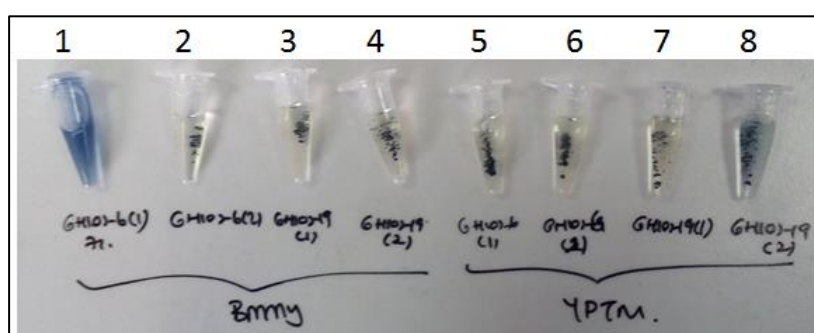


Figure 4.34: Activities of the supernatants (72. hour) obtained from the hydrolysis of AZCL-arabinoxylan after 1 hour incubation at 40°C.

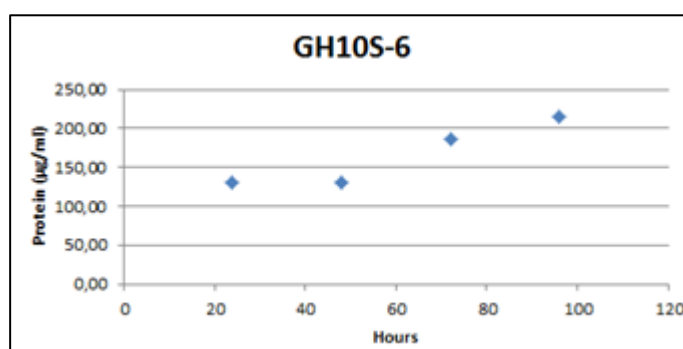


Figure 4.35: Time-dependent change in protein amount of the clone GH10S-6 in BMMY medium.

As shown in Figure 4.34, the GH10S-6 clone expressed in the BMMY medium and GH10S-19 clone expressed in the YPTM medium showed apparent activity

when examined with azurine-dyed substrate. Proteins from GH10S-6 supernatants were increased during the expression (Figure 4.35). The activity of the crude supernatant obtained from GH10S-6 expression of BMMY gave better hydrolysis and the amount of enzyme could be measured, albeit low (Table 4.2).

Table 4.2: Time-dependent xylanase activity in crude supernatants of clone GH10S-6 obtained from BMMY medium.

Clone	Activity (U/ml)			
	24th hour	48th hour	72nd hour	96th hour
GH10S-6				
BMMY	0,15	0,42	0,42	0,41

When we examine the Figure 4.36 (a), time-dependent increases of the proteins in the range of 17-28 kDa are observed. It is seen that two proteins close to each other by weight increase in the enclosed area. These two proteins suggest that xylanase B may have glycosylated and non-glycosylated forms in the crude enzyme supernatant. Figure 4.36 (b) shows no extracellular protein was released between 17-28 kDa in the culture supernatant.

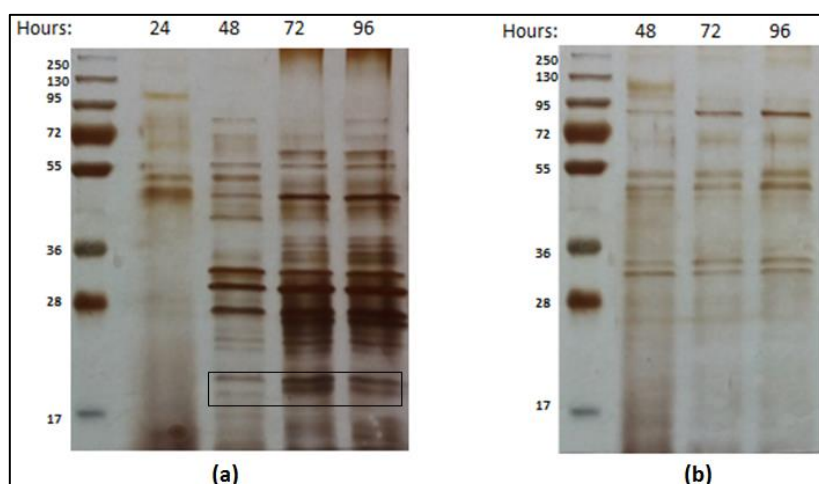


Figure 4.36: Time-dependent protein profiles. (a) Clone GH10S-6 in BMMY medium, (b) Clone GH10S-19 in YPTM medium. 0.5% methanol induction was applied for both case; 2.5 µg protein was loaded into the wells.

4.2.6. Contaminating *SxynB2* DNA and Microorganism Analysis in The Enzyme Preparation

During xylanase expression, the cell pellet and supernatants were checked for residual microorganism and DNA. For this; gDNA was isolated using culture pellet and supernatant and run on 1% agarose gel (Figure 4.37, (a) 6-10). The xylanase expression locus was checked by PCR with AOX1 primers (Figure 4.37, (a) 1-5 and (b) 1).

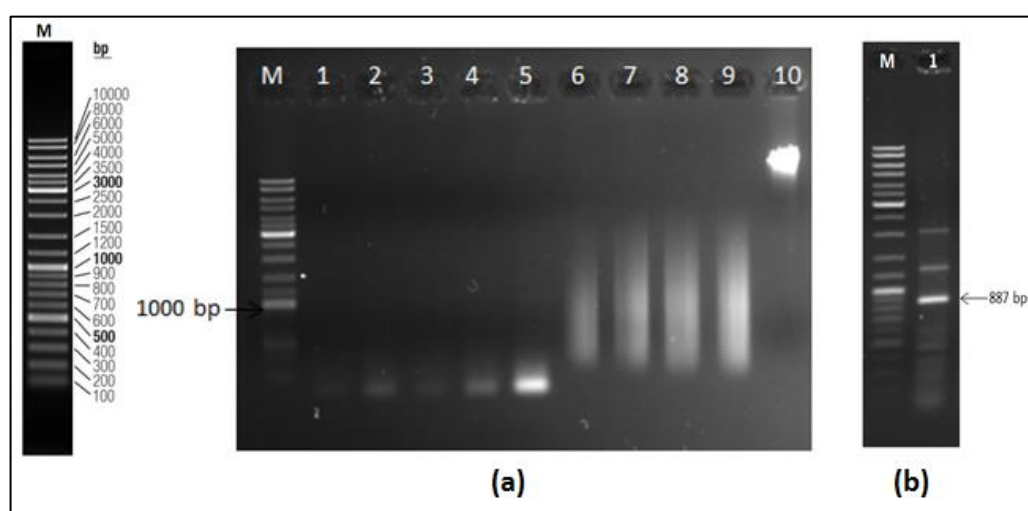


Figure 4.37: gDNA's from crude enzyme preparation and its PCR amplifications. (M) GeneRuler DNA Ladder mix, (a) (1-4) PCR control of the integrated *SxynB2* in the supernatant, (5) Negative control (w/o template), (6-9) Genomic DNA obtained from supernatant, (10) Genomic DNA obtained from culture pellet. (b) (1) PCR made with gDNA from culture pellet.

The crude enzyme preparations obtained from 0,5% methanol induced at 28 °C culture condition in which xylanase expression was observed were centrifuged at different speeds or ultrafiltrated for concentration. These preparations are understood to be organism-free, but only possible by filtrating the supernatants (Figure 4.38).

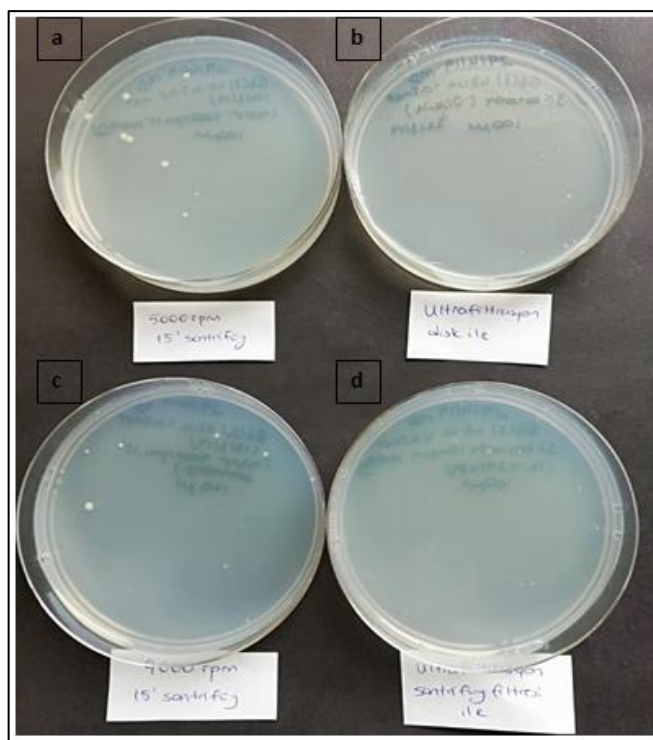


Figure 4.38: Growth profile of GH10S-6 crude enzyme samples in MD medium. After centrifugation at 5000 rpm (a) or 9000 g (b) and after ultrafiltration using ultrafiltration discs (c) or centrifugal filter unit (d).

In the AOX1 promoter region, which is activated by being induced in the heterologous system, integrated *SxynB2* sequence was controlled for a change (mutation) that will be negatively affect the gene expression and/or the DNA sequences in the *in vitro* gene transfer processes in the target protein. The DNA sequence of the PCR product obtained by the genomic DNA of the clone GH10S-6 was the same as the transferred *SxynB2* sequence. When the analysis result was checked, there was no difference in the promoter region, PHO1 signal sequence and *SxynB2* sequence. The resulting DNA sequence is given in Figure 4.39.

```

ATAGTTTCATGTTCCCAAATGGCCCAAACCTGACAGTTTAAACGCTGTCTTGGAACCTA
ATAGTTTCATGTTCCCAAATGGCCCAAACCTGACAGTTTAAACGCTGTCTTGGAACCTA
*****

ATATGACAAAAGCGTGATCTCATCCAAGATGAACTAAGTTTGGTTGTTGAAATGCTAAC
ATATGACAAAAGCGTGATCTCATCCAAGATGAACTAAGTTTGGTTGTTGAAATGCTAAC
*****

GGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGGCATAACCGTTTGTCTTGTTTGGTATT
GGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGGCATAACCGTTTGTCTTGTTTGGTATT
*****

GATTGACGAATGCTCAAAAATAATCTCATTAAATGCTTAGCGCAGTCTCTCTATCGCTTCT
GATTGACGAATGCTCAAAAATAATCTCATTAAATGCTTAGCGCAGTCTCTCTATCGCTTCT
*****

GAACCCCGGTGCACCTGTGTCGAAACGCAAATGGGGAACACCCGCTTTTGGATGATTA
GAACCCCGGTGCACCTGTGTCGAAACGCAAATGGGGAACACCCGCTTTTGGATGATTA
*****

TGCAATGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGATAGCTTAA
TGCAATGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGATAGCTTAA
*****

CGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACAGAA
CGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACAGAA
*****

GAAGCTGCCCTGTCTTAAACCTTTT TTTTATCATCATTATTAGCTTACTTTCATAATTG
GAAGCTGCCCTGTCTTAAACCTTTT TTTTATCATCATTATTAGCTTACTTTCATAATTG
*****

CGACTGGTTC CAATTGACAAAGCTTTTGATT TTAACGACTT TTAACGACAACTTGAGAAGA
CGACTGGTTC CAATTGACAAAGCTTTTGATT TTAACGACTT TTAACGACAACTTGAGAAGA
*****

TCAAAAAACAATAATTATT CGAAACGATGTTCTCTCCAATTTTGTCTTGGAATTATT
TCAAAAAACAATAATTATT CGAAACGATGTTCTCTCCAATTTTGTCTTGGAATTATT
*****

TTAGCTTTGGCTACTTTGCAATCTGTCTTCGCTCGAGGTTCCCCATGATCTGTGTGTAGA
TTAGCTTTGGCTACTTTGCAATCTGTCTTCGCTCGAGGTTCCCCATGATCTGTGTGTAGA
*****

AAGATCCGACGCACTTCATAAATTGTGAGAAAGGTCTACACCTTCTCTACTGGCGAGAA
AAGATCCGACGCACTTCATAAATTGTGAGAAAGGTCTACACCTTCTCTACTGGCGAGAA
*****

TAACGGCTTTTACTACTCATTCTGGACCGACGGTGCGGAGATGTAACCTATACCAATGG
TAACGGCTTTTACTACTCATTCTGGACCGACGGTGCGGAGATGTAACCTATACCAATGG
*****

AGATGCAGGAAGTTATACCGTCGAACGTAGTAACGTCGGTAATTTGTCGGCGGAAAAGG
AGATGCAGGAAGTTATACCGTCGAACGTAGTAACGTCGGTAATTTGTCGGCGGAAAAGG
*****

TTGGAACCCCGGCTCTGCACAAGACATCACCTATT CAGGAACCTT CACCCCATCA
TTGGAACCCCGGCTCTGCACAAGACATCACCTATT CAGGAACCTT CACCCCATCA
*****

```

Figure 4.39: DNA sequence of GH10S-6 clone.

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GGAAATGGTTATTTGTCCGTGTATGGCTGGACTACCGATCCCTAATCGAATATTACATA
GGAAATGGTTATTTGTCCGTGTATGGCTGGACTACCGATCCCTAATCGAATATTACATA
*****

GTAGAGAGTTATGGAGATTACAACCCGGCTCCGGAGGTACTTACGAGGGTACAGTTACC
GTAGAGAGTTATGGAGATTACAACCCGGCTCCGGAGGTACTTACGAGGGTACAGTTACC
*****

TCTGATGGTAGTGTGTACGACATCTATACGGCCACAAGAACAAATGCCGCTTCTATTGAG
TCTGATGGTAGTGTGTACGACATCTATACGGCCACAAGAACAAATGCCGCTTCTATTGAG
*****

GGCACCGCTACGTTTACACAATACTGGTCTGTCCGTCAGAATAAGCGTGTGGGCGGAACC
GGCACCGCTACGTTTACACAATACTGGTCTGTCCGTCAGAATAAGCGTGTGGGCGGAACC
*****

GTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAGGTATGAATTGGGTACACAT
GTTACGACATCTAACCACTTTAATGCTTGGGCCAAATTAGGTATGAATTGGGTACACAT
*****

AATTATCAGATAGTCGCAACAGAAGGTTACAGTCTAGTGGCTCATCTTCAATAACCGTG
AATTATCAGATAGTCGCAACAGAAGGTTACAGTCTAGTGGCTCATCTTCAATAACCGTG
*****

CAATAAGAATTCCCCGGGATCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACTTA
CAATAAGAATTCCCCGGGATCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACTTA
*****

CGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGT
CGAGAAGACCGGTCT-----
*****

```

Figure 4.39: continued.

4.2.7. Investigation of Expression in Recombinant Yeast by Scale-up in The Flask Culture

Expression volume effects the produced recombinant protein level in *Pichia pastoris* strains [Sun et al., 2016]. In this thesis study, activity was observed in the BMMY medium by using the GH10S-6 clone in 600 ml volume with 0.5% methanol. Recombinant xylanase B production was also investigated in 200 ml expression volume by starting with the cells having the OD₆₀₀ of 4.8 and 14. Growth in 600 ml flask culture was initiated with OD₆₀₀ of 12 and induced with 0.5% methanol. Protein determination (Figure 4.40), quantitative activity (Table 4.3) and protein profiles (Figure 4.41) were determined by taking a sample every 24 hours.

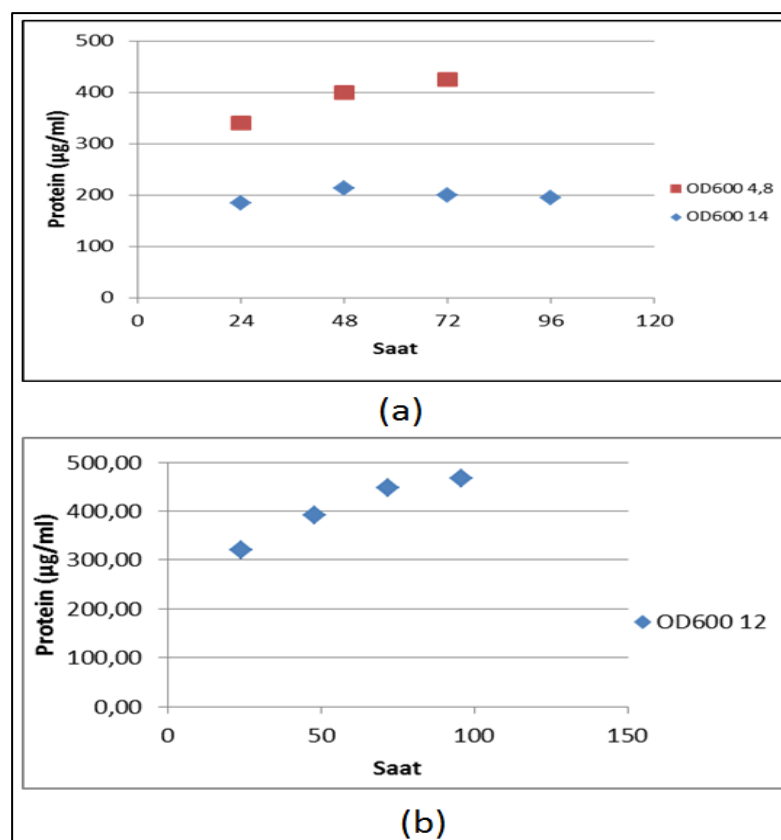


Figure 4.40: Time-dependent protein amounts obtained from clone GH10S-6 with varying inoculations (OD_{600}) in an expression volume of (a) 200 ml (b) 600 ml.

Referring to Figure 4.40, protein amounts differ in the expression volume of 200 ml depending on the starting inoculation amount (4.8 or 14). However, protein amounts obtained with the culture volume of 600 ml having an initial inoculation density of 12 is similar to the amounts of protein obtained with the culture volume of 200 ml having an initial inoculation density of 4.8. When protein amounts and obtained activities are compared, although there are similarities in protein amounts, there are differences between the obtained activities.

Table 4.3: Time-dependent activity of clone GH10S-6 in different culture volumes.

Clone GH10S-6	Activity (U/ml)			
	24th hour	48th hour	72nd hour	96th hour
200 ml culture	0,15	0,42	0,42	0,41
600 ml culture	1,14	1,71	1,26	0,75

Protein profiles and units of crude preparations obtained from 200 and 600 ml cultures are summarized in Table 4.3 and Figure 4.41. The xylanase activity of the crude enzyme sample collected at the 48th hour of the 600 ml culture was obtained as 1.71 U/ml (Table 4.3).

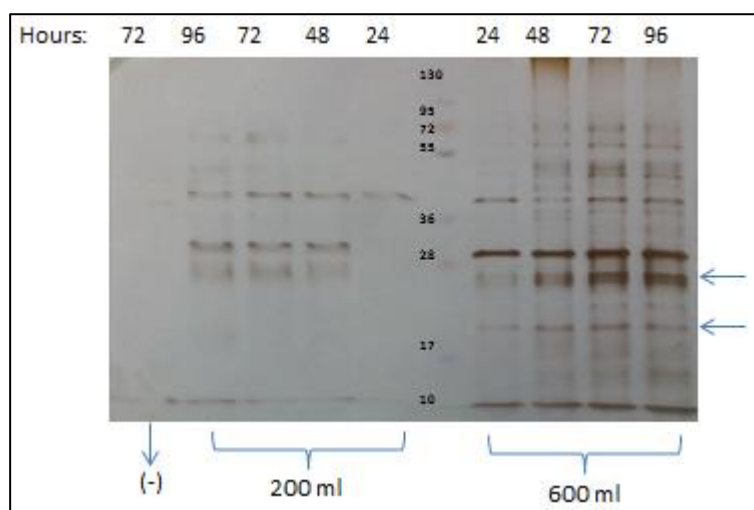


Figure 4.41: Protein profiles of the GH10S-6 clone in BMMY medium with 0.5% methanol induction at different expression volumes.

Figure 4.41 shows a time-dependent increase in proteins between 17-28 kDa in the expression volume of 600 ml. In the expression volume of 200 ml, a protein near 28 kDa is increased. This protein appears in both 200 ml and 600 ml expression volumes, suggesting that there is another protein of higher molecular weight released in the cell in terms of the expected protein size.

The expression was repeated with the GH10S-6 clone by increasing the scale as xylanase activity was found to be measurable in 600 ml flask expression. When the cell density (OD_{600}) was 10.5, the cells were centrifuged and expression with 0.5% and 1% methanol was initiated. The cultures were monitored for 96 hours by withdrawal of the samples at every 24 hours for 0.5% methanol and at every 12 hours for 1% methanol [Ersöz and Inan, 2019]. 3 ml samples were collected at every 24 or 12 hours and used for the spectrophotometric determination of the cell density, protein amount (Figure 4.42), enzyme activity (Table 4.4) and time-dependent expression profile (Figure 4.43).

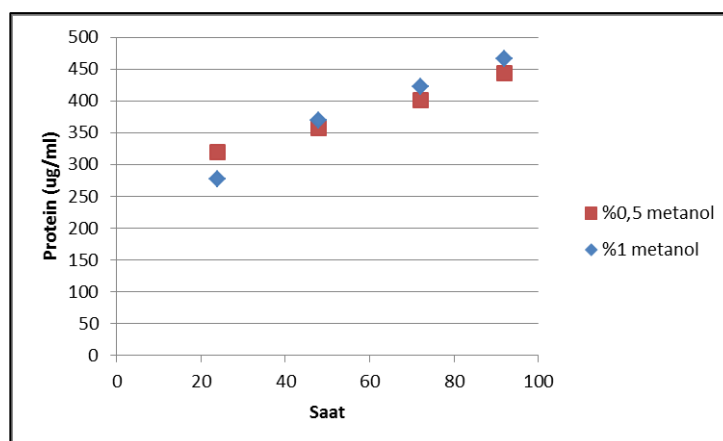


Figure 4.42: Time-dependent protein amounts of the clone GH10S-6 in an expression volume of 600 ml with the induction of 0.5% or 1% methanol.

Table 4.4: Xylanase activities that vary due to the induction of clone GH10S-6 with different methanol concentrations.

Methanol concentration	Activity (U/ml)			
	24th hour	48th hour	72nd hour	96th hour
0.5%	1,55	2,03	1,57	0,82
1%	1,79	1,92	1,74	0,90

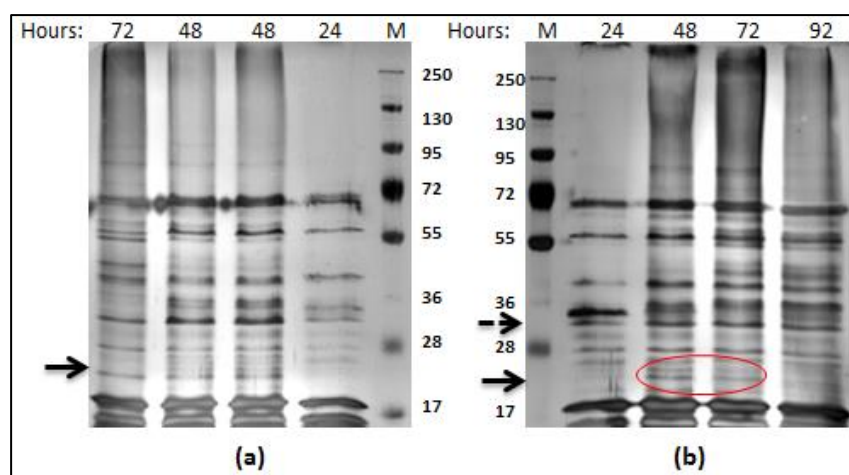


Figure 4.43: Time-dependent protein profiles of the clone GH10S-6.
(a) Induction with 0.5% and (b) 1% methanol.

Protein secretion was observed and xylanase B enzyme activity was determined as 2 U/ml at 48th hour. The extracellular protein profiles were found to

be similar in both methanol percentages. The target protein is expected to be around 22 kDa (non-glycosylated) or released as a larger protein if glycosylated.

In order to increase protein and enzyme content in crude supernatant and to determine basic biochemical properties, the concentrated enzyme was needed. After the induction with 0.5% methanol, the enzyme liquid obtained at 48th hour was concentrated. Then, optimum pH, temperature and thermal stability of the crude enzyme were determined as a priority.

4.2.8. Concentration of The Crude Enzyme and Activity Measurement

In order to determine the optimum pH and temperature values in the concentrated crude preparations, the volume of the crude preparation obtained from the flask culture of the clone GH10S-6 was concentrated three times by ultrafiltration. The obtained activity and protein values are given in the Table 4.5.

Table 4.5: Concentration and purification of the GH10S-6 clone by ultrafiltration.

Clone GH10S-6	Volume (ml)	Activity (U/ml)	Total Activity (U)	Activity yield (%)	Protein (µg/ml)	Total protein (mg)	Protein yield (%)	Specific activity (U/mg)	Purification (fold)
Crude extract	60	1,46	87,71	100	366,67	22,00	100	3,99	1
Ultrafiltration (3X)	19,9	4,20	83,53	95,24	869,96	17,31	78,69	4,82	1,21

Optimum pH, optimum temperature and thermal stability tests were performed with concentrated samples to clarify the identity of the enzyme.

4.2.9. Determination of Optimum pH, Temperature and Thermal Stability with Concentrated Crude Enzyme Preparations

To determine the effect of pH on the activity of the concentrated crude enzyme, the activity was determined at varying pH values between 3.0 and 10.0, and the optimum pH of the concentrated crude enzyme obtained from clone GH10S-6 was determined as 5.0 (Figure 5.44).

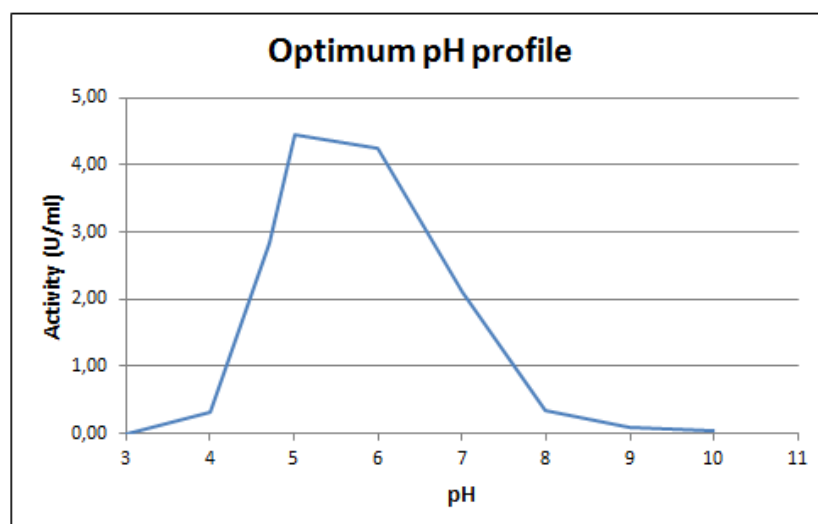


Figure 4.44: Optimum pH profile of concentrated crude xylanase enzyme produced from clone GH10S-6.

To determine the effect of temperature on the activity of the concentrated crude enzyme, the activity was determined at varying temperature values from 30 °C to 80 °C and the optimum temperature of the concentrated crude enzyme obtained from clone GH10S-6 was determined as 50 °C (Figure 5.45).

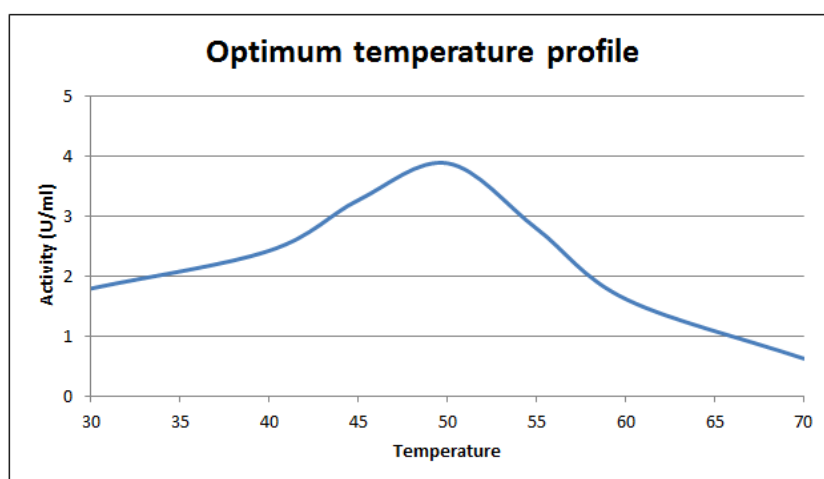


Figure 4.45: Optimum temperature profile of concentrated crude xylanase enzyme produced from clone GH10S-6.

The remaining activity values of the concentrated crude enzyme is calculated and given in the Table 4.6. At temperatures of 50 °C and above, the concentrated crude xylanase enzyme loses its nearly entire activity.

Table 4.6: Stability of concentrated crude xylanase at different temperatures.

Sample	Temperature, °C	Remaining activity, %
Concentrated crude xylanase	40	100
	50	6,03

As a result of the expression studies of Mut⁺ GH10S-6 clone in lab scale flask cultures, extracellular secreted xylanase B activity was found to be insufficient to scale up the tested parameters. The signal of the SxynB2 protein is the acid phosphatase 1 signal of the *Pichia pastoris*, although it partially provides secretion of the protein, but the possible enzyme is rapidly degraded.

5. DISCUSSION

The objective of this study is to clone recombinant GH11 xylanase enzyme and investigate if it could be used to scale up the xylanase production. The xylanase gene, named UNENxynB2, was cloned from *A. niger* MRC200803 strain of the TUBITAK MRC fungal collection and transferred to homologous host *A. niger* N593 and heterologous host *P. pastoris* GS115 and KM71 strains. The UNENxynB2 gene (GenBank no: MF170635) was integrated into the *A. niger* N593 which is considered as GRAS. Furthermore, the *SxynB2* gene after codon optimization of UNENxynB2 cDNA sequence was integrated into *P. pastoris*. The *SxynB2* sequence was ligated with two different vectors carrying the *P. pastoris* PHO1 and *S. cerevisiae* alpha factor signal sequences. When the extracellular secretion of the signal peptides were compared, it was found that the recombinant strain obtained with the PHO1 signal was expressed extracellularly. After 48 hours of expression with 0.5% methanol, 2 U/ml enzyme was obtained from the flask culture.

The length of mature UNENxynB2 xylanase enzyme was 207 amino acids. UNENxynB2 xylanase has differences at 2 positions at the amino acid level compared to other published xylanase B enzymes (AAM95167.1, AGG38091.1, AEC13626.1). The amino acid tryptophan (W) at position 57 was arginine (R) in this xylanase. In position 122, the amino acid lysine (K) was glutamic acid (E). It has been reported in the literature that the catalytic residues are two glutamic acids for the GH10 and GH11 families (Vandermarliere et al., 2008). The prediction of the active sites indicated that two amino acid residues, E103 and E194 of UNENxynB2, were located at the active center. When looking at the R57 and E122, it is seen that these amino acids are not close to the E103 and E194 involved in the catalytic region (Figure 4.5). Therefore, low activity cannot be directly associated with amino acids that differ from the literature. In addition, further studies should be performed experimentally and bioinformatically. For example, the proximity of these amino acids should be calculated using bioinformatics programs, and the substrate binding region of the enzyme must be found by molecular modelling.

Some characteristics of the xylanase enzyme from clone *P. pastoris* GH10S-6 have been described. When wheat arabinoxylan was used as a substrate, the recombinant enzyme reached its optimum values at pH 5 and 50 °C. When the thermal stability of the obtained recombinant enzyme was examined, it was observed that the remaining activity after incubation at 50°C for 30 minutes was 6%. Recombinant xylanase expression observed in the flask culture could be increased by optimization studies in the fermenter and controlled induction conditions. Thus, xylanase activity can be achieved in an efficient unit as reported in other xylanases.

In general, there is a need for detailed study and optimization for the characterization and commercialization of each enzyme using alternative enzyme production strategies and expression systems. Optimizations require working on fermenter size, lab and pilot scale. It is also important to delete the genes of their proteases from the genome so that the microorganism in which chromosomal gene integration of the target enzyme is ensured will not degrade the secreted enzyme. In recent years, studies with alternative expression vectors and strains have been reported. Among the protease deleted strains, the gene expressing protease B enzyme in the SMD1165 (*his4 prb1*) strain, the gene encoding protease A in the strain SMD1168 (*his4 pep4*), and the gene encoding both proteases in the strain SMD1163 (*his4 pep4 prb1*) were deleted.

High levels of expression can be achieved when *xynB* is induced. The *xynB* gene of *A. niger* IA-001 with α factor secretion signal was expressed under control of the AOX1 promoter in *Pichia pastoris* X-33. After 10 hours of methanol induction in 10 L of fermenter, 10.035 U/ml xylanase was produced. It was reported that the purified enzyme was 24 kDa in size and specific to beechwood xylan, and its activity was positively affected by Mg^{2+} ions (Fang et al., 2014).

Another xylanase B gene (from *A. niger* IBT-90) was transferred to *Pichia pastoris* GS115 strain and expressed with its own signal peptide and yeast alpha factor signal under control of the methanol-inducible AOX1 promoter. Both signals were recognized and processed in yeast. Molecular weight of the xylanase B was found as 17.5 kDa. Xylanase secretion with its own signal was found to be more efficient than alpha factor secretion signal. This was also studied for *Xyn6*, another

xylanase, with similar results. These studies showed that the importance of the signal sequence for the secretion of the enzyme. It is known that the signal is important for the release of the enzyme from the cell, in some cases more enzymes are released into the medium if the enzyme has its own signal sequence (Korona et al., 2006).

A. niger xylanase with different secretion signals was investigated in *P. pastoris* KM71H strain. Signal with repeated sequence named *P. pastoris* PIR1 (protein with internal repeats) under the control of the AOX1 promoter was studied and this signal peptide was more effectively secreted than other signal peptides. In this study conducted by Karaoglan et al. (2014), xylanase with a specific activity of 6863 U/mg was reported when using signal with repeated sequence.

Another xylanase cloned metagenome from saline alkaline soil was transferred to *Pichia pastoris* GS115 and produced by induction with methanol. The enzyme was obtained with better activity in wheat arabinoxylan and it was reported to be a high pH, salt and ethanol tolerant enzyme (Wang et al., 2017). Recombinant xylanase derived from *SxynB2* is similar in terms of its optimum temperature. Since the xylanase obtained from *P. pastoris* GS115 is in low amount so it could not be purified, the other properties of the xylanase B cannot be compared with the reported pure xylanases.

Objectives accomplished

- Xylanase B was cloned from *Aspergillus niger* MRC200803 isolated from chickpea in Turkey.
- Bioinformatics analysis revealed that the UNENxynB2 gene was highly homologous to the published *A. niger* xylanases, with only 2 amino acid differences.
- Expression-secretion plasmid was constructed for xylanase B homologous expression.
- *A. niger* transformants containing the UNENxynB2 gene were obtained.
- The UNENxynB2 gene was subcloned into *P. pastoris* vectors.
- *SxynB2* integration was confirmed by analysis of chromosomal DNA from *P. pastoris*.
- Xylanase B production in *P. pastoris* was performed in flask culture.
- Xylanase B secretion using AOX1 promoter was accomplished by using *P. pastoris* acid phosphatase 1 secretion signals.
- PHO1 signal was processed and xylanase B was secreted. However, obtained xylanase B activity was very low.

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APPENDIX

Appendix A: UNENxynB2 DNA sequence in chromosome of *A. niger* transformant rAB2 (12-1-7). Obtained sequence of GWF (forward sequence) and GWR (reverse sequence) were aligned with UNENxynB2.

GWF	-----GTGGATTGGTCGGGGT
GWR	CTTCAGCTTTCCCTCGTGCAGATGAGGTTTGGCTATAAATTGAAGTGGTTGGTCGGGGT
UNENxynB2	-----
GWF	TCCGTGAGGGGCTGAAGTGCTTCCTCCCTTTAGACGCAACTGAGAGCCTGAGCTTCATC
GWR	TCCGTGAGGGGCTGAAGTGCTTCCTCCCTTTAGACGCAACTGAGAGCCTGAGCTTCATC
UNENxynB2	-----
GWF	CCCAGCATCATTACACTTAATTAAATGCTCAACCAAGAACCTTCTCCTCTGCTTGGCCGCA
GWR	CCCAGCATCATTACACTTAATTAAATGCTCAACCAAGAACCTTCTCCTCTGCTTGGCCGCA
UNENxynB2	-----ATGCTCAACCAAGAACCTTCTCCTCTGCTTGGCCGCA *****
GWF	GCTAAGGCTGTCTGGCCGTTCGCCACGACTCTGTGTCGAGCGTTGGATGCCTTGCAC
GWR	GCTAAGGCTGTCTGGCCGTTCGCCACGACTCTGTGTCGAGCGTTGGATGCCTTGCAC
UNENxynB2	GCTAAGGCTGTCTGGCCGTTCGCCACGACTCTGTGTCGAGCGTTGGATGCCTTGCAC *****
GWF	AAGCTCTCTGAGCGTTCGACCCGAGCTCGAACGGCGAGAACAACGGCTTCTACTACTCC
GWR	AAGCTCTCTGAGCGTTCGACCCGAGCTCGAACGGCGAGAACAACGGCTTCTACTACTCC
UNENxynB2	AAGCTCTCTGAGCGTTCGACCCGAGCTCGAACGGCGAGAACAACGGCTTCTACTACTCC *****
GWF	TTCTGGACCGACGGCGGTGGTGATGTGACCTACACCAACGGTGAAGCTGGCTCGTACACC
GWR	TTCTGGACCGACGGCGGTGGTGATGTGACCTACACCAACGGTGAAGCTGGCTCGTACACC
UNENxynB2	TTCTGGACCGACGGCGGTGGTGATGTGACCTACACCAACGGTGAAGCTGGCTCGTACACC *****
GWF	GTGAGCGGTCTAACGTTGGCAACTTTGTTGGTGGAAAGGGCTGGAACCTGGAAGTGCG
GWR	GTGAGCGGTCTAACGTTGGCAACTTTGTTGGTGGAAAGGGCTGGAACCTGGAAGTGCG
UNENxynB2	GTGAGCGGTCTAACGTTGGCAACTTTGTTGGTGGAAAGGGCTGGAACCTGGAAGTGCG *****
GWF	CAGGACATTACCTACAGCGGCACTTCACCCCTAGCGGCAACGGCTACCTCTCGTCTAT
GWR	CAGGACATTACCTACAGCGGCACTTCACCCCTAGCGGCAACGGCTACCTCTCGTCTAT
UNENxynB2	CAGGACATTACCTACAGCGGCACTTCACCCCTAGCGGCAACGGCTACCTCTCGTCTAT *****

Figure A1.1: UNENxynB2 DNA sequence in chromosome of *A. niger* transformant rAB2 (12-1-7).

GWF	GGCTGGACCACTGACCCCCTGATCGAGTACTACATCGTCGAGTCTACGGCGACTACAAC
GWR	GGCTGGACCACTGACCCCCTGATCGAGTACTACATCGTCGAGTCTACGGCGACTACAAC
UNENx ynB2	GGCTGGACCACTGACCCCCTGATCGAGTACTACATCGTCGAGTCTACGGCGACTACAAC *****
GWF	CCCGGCAGTGGAGGCACCTACGAGGGCACCGTCACCTCCGATGGATCCGTCTACGATATC
GWR	CCCGGCAGTGGAGGCACCTACGAGGGCACCGTCACCTCCGATGGATCCGTCTACGATATC
UNENx ynB2	CCCGGCAGTGGAGGCACCTACGAGGGCACCGTCACCTCCGATGGATCCGTCTACGATATC *****
GWF	TACACGGCTACCCGCAACCAAGCCGCTTCCATCCAAGGAACCGCTACCTTCACCCAGTAT
GWR	TACACGGCTACCCGCAACCAAGCCGCTTCCATCCAAGGAACCGCTACCTTCACCCAGTAT
UNENx ynB2	TACACGGCTACCCGCAACCAAGCCGCTTCCATCCAAGGAACCGCTACCTTCACCCAGTAT *****
GWF	TGGTCCGTTGCGCAGAACAAGAGAGTGGAGGAACTGTTACCACCTTCCAAACACTTCAAC
GWR	TGGTCCGTTGCGCAGAACAAGAGAGTGGAGGAACTGTTACCACCTTCCAAACACTTCAAC
UNENx ynB2	TGGTCCGTTGCGCAGAACAAGAGAGTGGAGGAACTGTTACCACCTTCCAAACACTTCAAC *****
GWF	GCTTGGGCTAAGCTGGGCATGAACCTGGGTACTCACAACCTACCAGATCGTGGCTACCGAG
GWR	GCTTGGGCTAAGCTGGGCATGAACCTGGGTACTCACAACCTACCAGATCGTGGCTACCGAG
UNENx ynB2	GCTTGGGCTAAGCTGGGCATGAACCTGGGTACTCACAACCTACCAGATCGTGGCTACCGAG *****
GWF	GGCTACCAGAGCAGCGGATCTTCTCCATCACTGTTTCAGTAAGCGGCGCTGTACAGTTT
GWR	GGCTACCAGAGCAGCGGATCTTCTCCATCACTGTTTCAGTAAGCGGCGCTGTACAGTTT
UNENx ynB2	GGCTACCAGAGCAGCGGATCTTCTCCATCACTGTTTCAGTAA----- *****
GWF	AAACAAGAATAAAACGCGTTTCGGGTTTACCTCTTCCAGATACAGCTCATCTGCAATGCA
GWR	AAACAAGAATAAAACGCGTTTCGGGTTTACCTCTTCCAGATACAGCTCATCTGCAATGCA
UNENx ynB2	AAACAAGAATAAAACGCGTTTCGGGTTTACCTCTTCCAGATACAGCTCATCTGCAATGCA -----
GWF	TTAATGCATGGG
GWR	-----
UNENx ynB2	-----

Figure A1.1: Continued.

BIOGRAPHY

Kübra BAYRAK was born in 1th July, 1992 in İstanbul. In 2016, she was graduated from Gebze Technical University, Department of Molecular Biology and Genetics. She started her MSc. education in Gebze Technical University, Graduate School of Natural and Applied Sciences, Department of Molecular Biology and Genetics. She performed her studies under the supervision of Assist. Prof. Dr. Saliha İŞSEVER ÖZTÜRK and co-supervisor Assoc. Prof. Dr. Sevnur MANDACI. She participated in ARDEB 1003 project in December 2016 during her master studies. She took part in the project for 27 months. She completed her studies at the Enzyme and Microorganism Molecular Genetics Laboratory at TUBITAK MRC GEBİ.