ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

BIOCHEMICAL CHARACTERISATION OF TRUNCATED AMYLOPULLULANASE FROM *Thermoanaerobacter brockii brockii*

M.Sc. THESIS

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

Thermoanaerobacter brockii brockii KAYNAKLI AMİLOPULLULANAZ ENZİMİNİN KESİLMİŞ VARYANTLARININ BİYOKİMYASAL KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

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HAZİRAN 2022

Aycan KAYRAV, a M.Sc. student of İTÜ Graduate School student ID 521201105, successfully defended the thesis/dissertation entitled "BIOCHEMICAL CHARACTERISATION OF TRUNCATED AMYLOPULLULANASE FROM *Thermoanaerobacter brockii brockii*", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and best friends,

FOREWORD

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Aycan KAYRAV
(Molecular Biologist)

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ABBREVIATIONS

AMFED : Association of Manufacturers and Formulators of Enzyme Products
AamyC : α-amylase C-terminal all-β domain
BC : Before Christ
CBM20 : Carbohydrate binding module 20
CD : Cyclodextrin
CTAB : Cetrimonium bromide
DNA : Deoxyribonucleic acid
dNTP : Deoxynucleotide triphosphate
DMF : Dimethylformamide
DMSO : Dimethylsulfoxide
DNSA : Dinitrosalicyclic acid
DTT : Dithiothreitol
EDTA : Ethylene diamine tetra acetic acid
EGTA : Egtazic acid
FADH : Flavin adenine dinucleotide
FnIII : Fibronectin type III domain
IEX : Ion exchange chromatography
IMAC : Immobilized metal affinity chromatography
IPTG : Isopropyl-ß-D thiogalactopyranoside
LB : Luria-Bertani
NADH : Nicotinamide adenine dinucleotide
PCR : Polymerase chain reaction
PEG : Polyethylene glycol
PRR : Pullulan reactive red
RACHITT : Random chimeragenesis
rRNA : Ribosomal RNA
SBD : Starch binding domain
SDS PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS : Sodium dodecyl sulfate
StEP : Staggered extension process

TAE **:** Tris-Acetate-EDTA buffer

TLC **:** Thin layer chromatography

tRNA **:** Transfer RNA

USD **:** United States dollar

UV : Ultraviolet

SYMBOLS

%	**:** Percent
bp	**:** Base pair
° C	**:** Celcius
G	**:** Gram
kb	**:** Kilo base
k_{cat}	**:** Catalytic turnover number
kDa	**:** Kilo Dalton
K_m	**:** Michealis-Menten constant
M	**:** Molar
m	**:** meter
mg	**:** Milligram
min	**:** Minute
mM	**:** millimolar
ml	**:** Milliliter
rpm	**:** Revolutions per minute
W	**:** Watt
x g	**:** Accelaration of gravity
U	**:** Enzyme unit
V	**:** Volt
Vmax	**:** Maximum velocity
µg	**:** microgram
µl	**:** microliter
µM	**:** micromolar

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BIOCHEMICAL CHARACTERISATION OF TRUNCATED AMYLOPULLULANASE FROM *Thermoanaerobacter brockii brockii*

SUMMARY

The last point reached as a result of the chemical transformation carried out by nature is life. Enzymes are responsible biomolecules for the chemical transformation that makes life possible. People had benefited from the reactions carried out by enzymes to obtain various products in many fields throughout history, from the times when they did not know the existence of these catalysts. Over time, people had understood how great potential these biocatalysts have with the structure and working principles. Since then the characterisation of novel enzymes and the usage area of these enzymes have been expanded day by day. Today, more than 3.000 enzymes are used in biotechnological and industrial fields and it is reported that the size of this enzyme market has reached 6 billion USD in 2021. The enzymes that dominate this market with a share of 30 % are those used in the food and beverage industry and the ones that have the upper hand in this group are the enzymes used in starch processing applications.

Starch, the main component of many agricultural products and the primary source of carbohydrates for most people, is the main source of carbon in the world. In addition to starch, oligosaccharides, disaccharides and monosaccharides obtained as a result of hydrolysis are also used in various fields such as food, pharmaceuticals and biofuels. To obtain these hydrolysis products, starch is subjected to a three-step process: gelatinisation, liquefaction and saccharification. The viscous solution which is obtained by hydrolysis of α-1,4- and α-1,6- glycosidic bonds in the starch structure and gelatinisation is achieved by using α-amylase, β-amylase, glucoamylase and pullulanase enzymes during the liquefaction. During all these processes to remain the enzymes stable for a long time and maintain their activity, pH adjustment and the addition of Ca^{2+} ionsare required . Remove both the formed salt as a result of pH adjustments and excess Ca^{2+} ions causes additional steps and all of these lead to an increase in the cost of production. To eliminate these steps and perform a one-step liquefaction-saccharification process can be possible with an alternative enzyme. Amylopullulanases (E.C. 3.2.1.1/41) are enzymes capable of hydrolysing both α-1,4- and α-1,6- glycosidic bonds, while those obtained from extremophilic organisms can maintain their activities under harsh industrial conditions.

In this study, the amylopullulanase (TbbApu) enzyme belonging to the *Thermoanaerobium brockii brockii* organism, is one of the strong candidates for starch hydrolysis processes, is examined. Previously, for the recombinant production of TbbApu, the whole apu gene had been obtained by using the primary walking method by our group and the optimisation of expression studies was in progress. As a result of the studies, pET-28 a (+) vector and *E. coli* BL21 (DE3) were chosen as the appropriate expression vector and and *E. coli* BL21 (DE3) the appropriate host, respectively. Additionally, the variants TbbApuΔSH3 without SH3 domain and TbbApuΔCBM20

without CBM20 domain were constructed to investigate the effect of SH3 and CBM20 domains.
In the scope of this thesis, apart from TbbApuΔSH3 and TbbApuΔCBM20 variants, four additional truncated constructs namely TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1 and TbbApuΔX25-2-CBM20 were also obtained to disclose the effects of X25 domain. The biochemical characterisation, raw starch binding ability and kinetic studies of TbbApu and its six variants have been accomplished. The function of the X25 domain on substrate binding, activity and stability of the enzyme has been revealed for the first time with this study.
The genes belonging to each construct were amplified by PCR with specific forward and reverse primers that have *Sac*I and *Not*I restriction sites at their 5' ends and using the whole apu gene as a template. As a result of the amplification, *tbbApuΔX25-1-SH3*, *tbbApuΔX25-2-SH3*, *tbbApuΔX25-1-CBM20* and *tbbApuΔX25-2-CBM20* genes with a length of 4065, 3750, 3375 and 3060 base pairs, respectively, were obtained. Then, the obtained genes and the pET-28 a (+) expression vector were cut with *SacI* and *NotI* enzymes to form sticky ends and the ligation reactions were set up for the insertion of the genes into the pET-28 a (+) expression vector. Then, transformation was performed using competent *E. coli* BL21 (DE3) host cells. For the determination of positive colonies from the colonies obtained as a result of the transformation, half of the colonies were inoculated on agar plates containing red pullulan. 1 µM IPTG and 40 µg/mL kanamycin were also included in the agar plate for induction and selection respectively. For the control of detected positive colonies from the PRR plate, the other half of the colonies were inoculated into Luria-Bertani (LB) medium and plasmid isolation was performed from these cells. Then, the isolated plasmids were cut with FastDigest *SacI* enzyme and linearised to determine the length of the plasmids. By linearisation, *tbbApuΔX25-1-SH3* - pET-28 a (+) vector with a length of 9434 base pairs; 9119 base pairs long *tbbApuΔX25-2-SH3* -pET-28 a (+) vector, 8744 base pairs *tbbApuΔX25-1-CBM20*- pET-28 a (+) vector and 8429 base pairs *tbbApuΔX25-2-CBM20*- pET-28 a (+) vector were obtained from all selected colonies.
After the cloning, TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were over-expressed using magic media. To purify these recombinant enzymes, purification was achieved in three steps by using metal affinity chromatography, ion exchange chromatography and heat purification respectively. Then, the pullulanase and α-amylase activities of TbbApu and its six variants were checked by the red pullulan and starch-containing polyacrylamide gel. The biochemical characterisation of the enzymes was completed. As a result of the studies, the optimum reaction temperature for pullulanase activities was determined as 70 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 75 °C for TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3 and TbbApuΔX25-1-CBM20 and also 80 °C for TbbApuΔX25-2-CBM20 domain. The optimum reaction temperature for α-amylase activities was specified as 75 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, and 80 °C for TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20. The pure variants were incubated for 24 hours at variable temperatures to examine the effect of temperature on the stability of enzymes. It was observed that the most stable temperature of 60 °C for TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3 and TbbApuΔX25-2-SH3, 50 °C for TbbApuΔX25-1-CBM20 and 40 °C for TbbApuΔX25-2-CBM20. Optimum pH values for both activities were found to be 6.5 for TbbApu, TbbApuΔX25-1-SH3 and TbbApuΔX25-1-CBM20, 6 for TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-2-SH3, and 7 for TbbApuΔX25-

2-CBM20. It has been determined that the enzymes reached 80 % of their α-amylase activities with pH 5 and protected it up to pH 8. When the effects of different pH values on the stability of the enzymes were examined, the most stable pH according to pullulanase activities was found as pH 4 for TbbApu, TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-2-CBM20, pH 6 for TbbApuΔX25-2-SH3, pH 6.5 for TbbApuΔX25-1-SH3 and TbbApuΔX25-1-CBM20 after 24 hours of incubation. According to their α-amylase activities, the most stable pH was found as pH 3 for TbbApu, TbbApuΔSH3, TbbApuΔCBM20, pH 6 for TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20, pH 7 for TbbApuΔX25-1-SH3. In addition, it is represented that TbbApu and its variants can maintain their stability between pH 3 and 8 and TbbApu can be used in starch hydrolysis processes without pH adjustment. When the effect of metal ions was examined, while Mn^{2+} and Co^{2+} ions increased both pullulanase and α-amylase activities of TbbApu and its variants whereas, Mg^{2+}, Zn^{2+} and Al^{3+} ions decreased both activities of all enzymes except pullulanase activity of TbbApuΔCBM20 variant. Although α-amylase enzymes used in starch processing are Ca^{2+} ion-dependent, TbbApu and its variants are not dependent on Ca^{2+} ion for activity and stability, making the enzyme and its variants a strong candidate for starch hydrolsis process. Also, in the presence of 20 % and 50 % hexane and acetone, with some exceptions, both activities of TbbApu and its variants were increased and 20 % and 50 % butanol, DMF and DMSO presence strongly inhibited both activities of the enzymes. When the effects of organic solvents on the stability of enzymes were examined, it was observed that, with some exceptions, the stability of the enzymes increased in the presence of 20 % and 50 % acetone and hexane, compared to both pullulanase and α-amylase activities, after 24 hours of incubation. In the case of inhibitors and detergents, it was observed that inhibitors moderately inhibited pullulanase and α-amylase activities of TbbApu and its variants with some exceptions, whereas all inhibitors increased the pullulanase activity of TbbApuΔCBM20 and nonionic and anionic enzymes inhibited both activities moderately with. However, the activities of all enzymes were strongly inhibited in the presence of CTAB, which is a cationic detergent. Then, the kinetic parameters of the enzymes were elucidated. The results imply that, truncation SH3 domain improves both α-amylase and pullanase activities of the enzyme. However, truncation of CBM20 and X25 domains from TbbApu caused to loss of affinity and specificity of the enzyme to soluble starch and to shift in the specificity of the enzyme to pullulan. The removal of the SH3 domain and also CBM20 domain, the carbohydrate-binding module in the enzymes did not have any effect on the raw starch binding capacity of TbbApu. The removal of the SH3 domain and also CBM20 domain, the carbohydrate binding module in the enzymes did not have any effect on the raw starch binding capacity of TbbApu. However, sequential removal of X25 and SH3 domains resulted in 27.3 % and 58.4 % reduction in raw starch binding ability, while removal of CBM20 and X25 domains resulted in a 90 % decrease in raw starch binding ability. Thus, it was revealed that the X25 domain is involved in binding raw starch. The results of TLC analysis represented that TbbApu and its variants released maltotriose and maltose as a result of pullulan hydrolysis and maltotriose starch hydrolysis, respectively. The results of TLC analysis represented that TbbApu and its variants released maltotriose and maltose in pullulan hydrolysis and maltotriose in starch hydrolysis, respectively.

Thermoanaerobacter brockii brockii KAYNAKLI AMİLOPULLULANAZ ENZİMİNİN KESİLMİŞ VARYANTLARININ BİYOKİMYASAL KARAKTERİZASYONU

ÖZET

Tüm zamanların en iyi kimyageri olan doğanın gerçekleştirdiği kimyasal dönüşüm sonucunda en son gelinen nokta yaşamdır. Yaşamı mümkün kılan kimyasal dönüşümden ise evrimsel süreçle meydana gelen biyolojik katalizörler olan enzimler sorumludur. İnsanlar enzimlerin gerçekleştirdiği reaksiyonlardan bu katalizörlerin varlığını bilmediği dönemlerden tarih boyunca birçok alanda çeşitli ürünlerin eldesi için yararlanmışlardır. Zamanla bu reaksiyonları gerçekleştiren biyomoleküllerin yani enzimlerin yapısı, çalışma prensibinin açığa çıkarılması ve karakterizasyonlarının yapılması ile bu biyokatalizörlerin ne kadar büyük bir potansiyele sahip olduğu anlaşılmış bununla birlikte ekstemofilik organizmaların ve bu organizmalara ait enzimlerin keşfi ayrıca yürütülen protein mühendisliği çalışmaları ile de zorlu endüstriyel koşullara dayanıklı enzimler elde edilerek enzimlerin her geçen gün kullanım alanı genişletilmiştir. Günümüzde 3.000'den fazla enzim biyoteknolojik ve endüstriyel alanda kullanılmakta ve 2021 yılında bu enzim pazarının büyüklüğü 6 milyar ABD dolarına ulaştığı raporlanmaktadır. % 30'luk payla bu pazarı domine eden enzimler ise yiyecek ve içecek endüstrisinde kullanılan enzimler olup bu grup içerisinde de üstünlüğü ele alanlar nişasta işleme uygulamalarında kullanılan enzimlerdir.

Birçok tarım ürününün ana bileşeni olan ve çoğu insan için birincil karbonhidrat kaynağını oluşturan nişasta, dünyada bulunan en temel karbon kaynağıdır. Nişastanın yanı sıra hidrolizi sonucu elde edilen oligosakkarit, disakkarit ve monosakkaritler de gıda, farmasötik ve biyoyakıt gibi çok çeşitli alanlarda kullanılmaktadır. Hidroliz ürünlerinin eldesi için nişasta, jelatinleştirme, sıvılaştırma ve sakkarifikasyon olmak üzere üç basamaktan oluşan bir prosese tabi tutulur. Jelatinleştirme işlemi ile viskoz çözeltisi elde edilen nişastanın, sıvılaştırma ve sakkarifikasyon işlemleri sırasında da α-amilaz, β-amilaz, glikoamilaz ve pullulanaz enzimleri kullanılarak nişastanın yapısında bulunan α-1,4- ve α-1,6- glikozidik bağlarının hidrolizi sağlanıp istenilen hidroliz ürünün eldesi sağlanır. Tüm bu işlemler sırasında enzimlerin uzun süre stabil kalıp aktivitesini sürdürmesi için pH ayarlamaları ve aktivite gösterebilmeleri için Ca^{2+} iyonunun eklenmesi gerekmektedir. Yapılan pH ayarlamaları ve Ca^{2+} iyonunun eklenmesi yeni bir işlem basamağı oluşturmakta ve pH ayarlamaları, oluşan tuz çökeltileri ve fazla Ca^{2+} iyonlarının işlem sonucunda uzaklaştırılması için ek basamak eklenmesi gerekmektedir. Tüm bunlar ise üretim maliyetinin artmasına sebep olmaktadır. Hem bu basamakları elimine etmek hem de tek basamaklı sıvılaştırma-sakkarifikasyon işlemini gerçekleştirmek alernatif bir enzim ile mümkünolmaktadır. Amilopullulanazlar (E.C. 3.2.1.1/41) hem α-1,4- hem de α-1,6- glikozidik bağlarını hidrolize edebilen enzimler olup ekstremofilik organizmalardan elde edilenler ise yüksek sıcaklıklarda ve asidik pH'larda aktivitelerini sürdürebilmektedirler. Bu çalışma kapsamında nişasta hidroliz işlemlerinin güçlü adaylarından biri olan *Thermoanaerobium brockii brockii* organizmasına ait amilopullulanaz (TbbApu)

enzimi incelenmektedir. Yapılan çalışmalar sonucunda, uygun ekspresyon vektörü olarak pET-28 a (+) vektörü, uygun konakçı olarak da *E. coli* BL21 (DE3) konakçısı seçilmiştir. Tüm bunların yanı sıra C-terminalinde bulunan SH3 ve CBM20 bölgelerinin (domain) ekspresyon üzerine olan etkisini incelemek için i) SH3 domaini çıkarılmış olan TbbApuΔSH3 ve ii) SH3 domaini ile birlikte CBM20 bölgesi çıkarılmış olan TbbApuΔCBM20 varyantları elde edilmiştir.

Tamamlanan bu tez kapsamında ise TbbApuΔSH3 ve TbbApuΔCBM20'ye ek olarak SH3 ve CBM20 bölgeleri ile birlikte enzimin N-terminalinde yer alan iki adet X25 bölgelerinin deçıkarılması ile elde edilen dört varyantının- TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 ve TbbApuΔX25-2-CBM20- oluşturulması ve böylece literatürde henüz görevi tanımlanmamış olan X25 bölgelerinin enzimin substrat bağlanmasına, aktivitesine ve stabilitesine olan etkilerinin açığa çıkarılması hedeflenmiştir. Ayrıca TbbApu ve bugüne kadar elde edilen kesilmiş altı varyantının tümünün biyokimyasal karakterizasyonu, ham nişasta bağlama yeteneği ve kinetik çalışmaları tamamlanmıştır.

Öncelikle 5' uçlarında sırasıyla *SacI* ve *NotI* restriksiyon enzimleri kesim noktası bulunan ileri ve geri primerler yardımıyla tüm apu geni kalıp olarak kullanılarak TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 ve TbbApuΔX25-2-CBM20'e ait genler PZR metodu ile çoğaltılmıştır. Çoğaltma sonucunda sırasıyla 4065, 3750, 3375 ve 3060 baz çifti uzunluğunda *tbbApuΔX25-1-SH3, tbbApuΔX25-2-SH3, tbbApuΔX25-1-CBM20* ve *tbbApuΔX25-2-CBM20* genleri elde edilmiştir. Elde edilen genler ve pET-28 a (+) ekspresyon vektörü *SacI* ve *NotI* enzimleri ile kesilerek yapışkan uçlar oluşturulmuş böylece genler pET-28 a (+) ekspresyon vektörüne yapıştırılmaya uygun hale getirilmiştir. Uçları kesilen her bir gen ile pET-28 a (+) ekspresyon vektörü için T4 DNA ligaz enzimi yardımıyla ligasyon reaksiyonu kurulmuş ve kurulan reaksiyonla oluşturulmuş vektörlerin kompetent *E. coli* BL21 (DE3) konakçı hücrelerine transformasyonu gerçekleştirilmiştir. Elde edilen kolonilerden pozitif kolonilerin tayini için kolonilerin yarısı red pullulan içeren agar plakalara ekilmiştir. Ayrıca indükleme için 1 µM IPTG ve seleksiyon için 40 µg/mL kanamisin agara dahil edilmiştir. 16 saat inkübasyon sonrası hem hücrelerin patlaması hem de enzimlerin aktivite gösterebilmesi için 60 °C`de 4 saat boyunca konulan plakalardan *tbbApuΔX25-1-SH3* - pET-28 a (+) vektörü içeren 5 koloni, *tbbApuΔX25-2-SH3* - pET-28 a (+) vektörü içeren 2 koloni, *tbbApuΔX25-1-CBM20*- pET-28a (+) içeren 3 koloni ve *tbbApuΔX25-2-CBM20*- pET-28 a (+) vektörü içeren 3 koloni tespit edilmiştir. Tespit edilen pozitif kolonilerin kontrolü için kolonilerin diğer yarısı Luria-Bertani (LB) sıvı ortamına ekilmiş ve bu hücrelerden plazmit izolasyonu gerçekleştirilmiştir. İzole edilen plazmitler FastDigest *SacI* enzimi ile kesilerek lineer hale getirilmiş ve plazmitlerin uzunluğu tespit edilmiştir. Yapılan linerizasyon ile 9434 baz çifti uzunluğunda *tbbApuΔX25-1-SH3* - pET-28 a (+) vektörü; 9119 baz çifti uzunluğunda *tbbApuΔX25-2-SH3* - pET-28 a (+) vektörü, 8744 baz çifti uzunluğunda *tbbApuΔX25-1-CBM20*- pET-28 a (+) vektörü ve 8429 baz çifti uzunluğunda *tbbApuΔX25-2-CBM20*- pET-28 a (+) vektörü seçilen tüm kolonilerden elde edilmiştir.

Klonlama işleminden elimizde bulunan TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 ve TbbApuΔX25-2-CBM20 varyantlarının ekspresyonu magic media kullanılarak gerçekleştirilmiştir. Ekspresyonu gerçekleştirilen tbbApu enziminin 6 varyantı sırasıyla metal afinite kromatografisi, iyon değiştirme kromatografisi ve ısı ile pürifikasyon yöntemleri kullanılarak üç basamakta saflaştırılabilmiştir. Ardından TbbApu enzimi ve altı varyantının pullulanaz ve α-amilaz aktivitesinin kontrolü için

red pullulan ve nişasta içeren poliakrilamit jel ile analizi yapılmıştır. Enzimlerin biyokimyasal karakterizasyonu kapsamında öncelikle sıcaklık, pH, metal iyonları, organik solvent, deterjan ve inhibitörlerin enzimlerin pullulanaz ve α-amilaz aktivitesine etkileri incelenmiş, ayrıca sıcaklık, pH ve organik solventlerin enzim stabilitesine olan etkileri tespit edilmiştir. Biyokimyasal karakterizasyon sonucunda pullulanaz aktiviteleri için optimum reaksiyon sıcaklık değerlerinin TbbApu, TbbApuΔSH3 ve TbbApuΔCBM20 için 70 °C, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 için 75 °C, TbbApuΔX25-2-CBM20 için 80 °C derece, α-amilaz aktiviteleri için optimum reaksiyon sıcaklık değerleriTbbApu, TbbApuΔSH3 ve TbbApuΔCBM20 için 75 °C, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 ve TbbApuΔX25-2-CBM20 için 80 °Colduğu gösterilmiştir. Sıcaklığın enzimlerin stabilitelerine olan etkisi incelendiğinde ise 24 saat inkübasyon sonrasında en stabil kaldıkları sıcaklık değerleri TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3 ve TbbApuΔX25-2-SH3 için 60 °C, TbbApuΔX25-1-CBM20 için50 °C ve TbbApuΔX25-2-CBM20 için 40 °C olarak tespit edilmiştir. Pullulanaz ve amilaz aktivitelerine göre optimum pH değerleri ise TbbApu, TbbApuΔX25-1-SH3 ve TbbApuΔX25-1-CBM20 için 6.5, TbbApuΔSH3, TbbApuΔCBM20 ve TbbApuΔX25-2-SH3 için 6, TbbApuΔX25-2-CBM20 için ise 7 olarak bulunmuş ve enzimlerin hem pullulanaz hem de amilaz aktivitelerinin % 80'ine pH 5 ile eriştiği ve pH 8'e kadar koruduğu tespit edilmiştir. Farklı pH değerlerinin enzimlerin stabilitelerine olan etkileri incelendiğinde ise 24 saat inkübasyon sonucu pullulanaz aktivitelerine göre TbbApu, TbbApuΔSH3, TbbApuΔCBM20 ve TbbApuΔX25-2-CBM20'nin pH 4, TbbApuΔX25-2-SH3'ün pH 6, TbbApuΔX25-1-SH3 ve TbbApuΔX25-1-CBM20'nin pH 6.5'ta; α-amilaz aktivitelerine göre ise TbbApu, TbbApuΔSH3, TbbApuΔCBM20'nin pH 3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 ve TbbApuΔX25-2-CBM20'nin pH 6, TbbApuΔX25-1-SH3'ün pH 7'de en stabil kaldığı gösterilmiştir. Ayrıca TbbApu ve varyantlarının geniş bir pH aralığı olan pH 3 ve 8'de stabilitelerini koruyabildikleri tespit edilmiştir ve asidik pH'larda stabil kalması ise TbbApu'nun nişasta hidroliz işlemlerinde pH ayarlamasına gerek görülmeksizin kullanılabileceğini böylece bu prosesin işlem basamakları ve dolayısıyla maliyet azaltmasını sağlayabileceği göstermektedir. Metal iyonların etkisi incelendiğinde ise Mn^{2+} ve Co^{2+} iyonlarının TbbApu ve varyantlarının hem pullulanaz hem de α-amilaz aktivitesini arttırdığı; Mg^{2+}, Zn^{2+} ve Al^{3+} iyonlarının ise TbbApuΔCBM20 varyantının pullulanaz aktivitesi hariç bütün enzimlerin her iki aktivitesini de düşürdüğü tespit edilmiştir. Böylece Mn^{2+} ve Co^{2+} iyonlarının aktivatör Mg^{2+}, Zn^{2+} ve Al^{3+} iyonlarının ise inhibitör etkiye sahip olduğu görülmüştür. Nişasta işleminde kullanılan α-amilaz enzimlerinin Ca^{2+} metaline bağımlı olduğu halde TbbApu ve varyantlarının aktivite ve stabilite için Ca^{2+} metaline bağımlı olmaması enzimi bu tür işlemler için kullanılmak üzere güçlü bir aday haline getirmektedir. Organik solventlerden ise % 20 ve % 50 hekzan ve aseton varlığında bazı istisnalar hariç TbbApu ve varyantlarının hem pullulanaz hem amilaz aktivitelerini de arttırdığı; % 20 ve % 50 konsantrasyonlarda butanol, DMF ve DMSO'nun ise enzimlerin her iki aktivitesini güçlü bir şekilde inhibe ettiği tespit edilmiştir. Organik solventlerin enzimlerin stabilitesine olan etkileri incelendiğinde 24 saat inkübasyon sonucu bazı istisnalar hariç enzimlerin hem pullulanaz hem de α-amilaz aktivitelerine göre % 20 ve % 50 aseton ve hekzan varlığında stabilitelerinin arttığı görülmüştür. İnhibitörlerin ise TbbApu ve varyantlarının bazı istisnalar hariç enzimlerinin pullulanaz ve α-amilaz aktivitelerini orta derecede inhibe ettiği buna karşın bütün inhibitörlerin TbbApuΔCBM20'nin pullulanaz aktivitesini arttırdığı görülmüştür. Aynı şekilde kullanılan noniyonik ve aniyonik enzimlerin bazı istisnalar

hariç her iki aktivitesini de orta derecede inhibe ettiği buna karşın katyonik deterjan olan CTAB'ın varlığında bütün enzimlerin aktivitelerinin güçlü bir şekilde inhibe olduğu görülmüştür. Daha sonra hem TbbApu hem de kesilmiş varyantlarının kinetik parametreleri belirlenmiştir. Yapılan çalışmlar sonucu TbbApu'dan X25 domainin çıkarılması enzimin hem pullulanaz hem de α-amilaz aktivitesini iyileştirmiştir. Bununla birlikte CBM20 ve X25 bölgelerinin çıkarılması enzimin çözünür nişastaya karşı hem afinitesi hem de spesifitesinin düşmesine sebep olmuş; enzimin pullulana karşı spesifitesini arttırmıştır. Bunların yanı sıra ham nişasta bağlama kapasiteleri ölçülen enzimlerde SH3 ve karbonhidrat bağlama modülü olan CBM20 bölgesinin çıkarılması herhangi bir etki yaratmazken; SH3 ile X25 bölgelerinin sırayla çıkarılması % 27.3 ve % 58.4, CBM20 ile X25 bölgelerrinin çıkarılması ise % 90 ham nişasta bağlanma yeteneğinde azalışa sebep olmuştur. Böylece X25 bölgesinin ham nişasta bağlamada görevli olduğu ortaya çıkarılmıştır. TLC analiz sonucu ise TbbApu ve varyantlarının pullulan hidrolizi ile maltotrioz, nişasta hidrolizi ile ise maltotrioz ve maltoz bileşenlerinin açığa çıktığını göstermektedir.

1. INTRODUCTION

1.1 Catalysts Created by Evolution: Enzymes

Nature creates products with excellent specificity and diversity from simple, abundant, and renewable starting materials by its amazing chemistry and engineering ability. The ultimate point of this chemical transformation is the life that has been developing for billions of years. The molecules that are specifically responsible for this chemical transformation, which no human being can reach or master, are enzymes and Nature creates enzymes and all biomolecules that makeup life using a single process: evolution (Arnold, 2019).

Evolution reveals the functional protein sequences from the possible protein universe billions of years of work by mutation and natural selection. On the evolutionary path, functional proteins must form a continuous network that can be crossed by unit mutation steps without passing through non-functional intermediates. Although a protein sequence is surrounded by a large number of variants resulting from mutation, only a few of them are functional. However, evolution allows proteins to evolve by following this network despite this complexity and it continues to create new ones from these rare functional sequences that it discovers every day (Maynard Smith, 1970).

People have been benefiting from the reactions of enzymes since they did not know about their existence. Examples of the first usage of the reactions performed by enzymes are ensuring the production of alcoholic beverages from barley by fermentation carried out by yeast by the Babylonians and Sumerians 6000 BC and the realization of cheese production in 400 BC, which Homer mentioned in the epic of the Iliad (Singh et al., 2016). Over time, people have focused on the catalysts that carry out these chemical transformation and conducted several studies to identify these biomolecules. As a result of these studies, these catalysts were named ‘enzymes’ by Wilhelm Friedrich Kühne in 1877; the first enzyme, diastasein, was discovered by Anselme Payen in 1883; Eduard Buchner showed that enzymes do not need cells to work in 1897; James B. Sumner obtained pure urease and crystallized it in 1947. Also,

following these studies, NOVO® produced protease which was expressed by *Bacillus licheniformis* on a commercial scale in 1960. Thus, with these studies, steps were taken for the commercial use of enzymes. Then, with the emerging recombinant DNA technology, the genes belonging to the enzymes were cloned and produced in high quantities, and the steps of commercial enzyme production were established (Gurung et al., 2013).

1.2 Enzymes in Industry

With the increasing human population, to supply growing human population needs have forced the industrial production stages to be shortened and reduced the production cost; the decrease in the number of raw materials in Nature against the increased production has led to production with high efficiency; in order to solve the increasing environmental problems caused by industrial production, the demand for the transition from chemical production to environmentally friendly production has increased. One of the most important steps in ensuring these transformations is the usage of enzymes in industrial applications. The ability to increase the reaction rates up to 10^{18} times of the enzymes provided to shortened processing time. Working under relatively mild reaction conditions allows to give low energy to the systems and reusability together with immobilization of enzymes make available the production cost lower. Also, regioselectivity and stereospecificity of the enzymes both their substrates and products allow the production of chirally pure products without giving by-product that allows reactions to take place with high efficiency. Together with these, obtaining from renewable resources, biodegradable features and reducing the formation of toxic wastes as a result of reacting without the need for organic solvents and heavy metals supplied the environmentally friendly industrial production (Singh et al., 2016; Ordu & Karagüler, 2012; Chapman et al., 2018).

Today, more than 3.000 enzymes are used the biotechnological and industrial area and hydrolases are the dominant enzyme class which are used for these purposes (Dumorné et al., 2017). Proteases, amylases, lipases and cellulases constitute more than 75 % of commercial enzymes and these enzymes are mainly used in applications in food and beverages, animal feed, healthcare, textiles, leather processing, biofuels detergents and cleaners. (Prakash et al., 2013; Grand Viev Research U.S., 2022). In Figure 1, the distribution of industrial market revenue (%) according to application area can be seen.

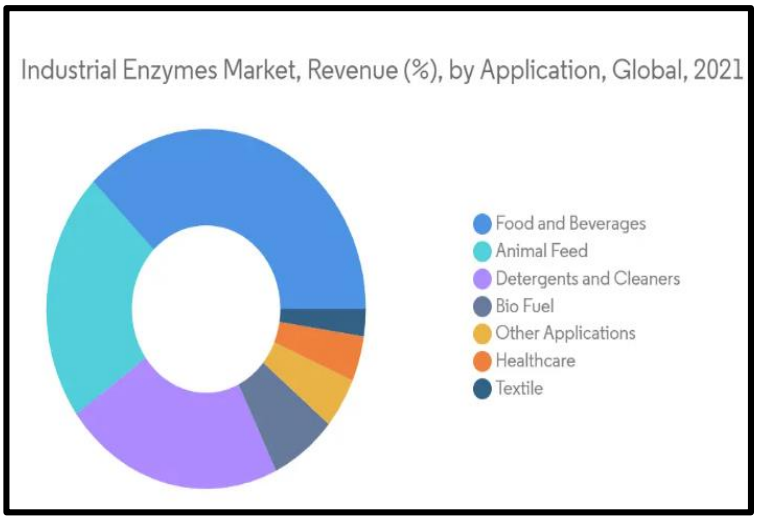

Figure 1.1: Revenue distribution of industrial enzymes market according to application (Grand Viev Research U.S., 2022).

Enzymes are used in food processing since ancient times such as beer brewing, cheese and bread making and today, they are used in a wide range applications in the food industry such as dairy product production, starch processing applications, baking and brewing processes, juice production stages (Zhu et al., 2011). According to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) report (2015), 261 enzymes are used in the food industry and at least 57 of these enzymes are produced from genetically modified organisms such as *Aspergillus niger, Saccharomyces cerevisiae, Escherichia coli, Bacillus subtilis*, and *B. licheniformis*. Aminopeptidase, amylase, catalase, cellulase, glucanase, glucoamylase, glucose oxidase, glucosidase, laccase, hemicellulose, lipase triacylglycerol, pectin lyase, pectinesterase, protease, pullulanase, xylanase and phospholipase A2 are the mainly used enzymes which are responsible for the improvement of flavour, texture, appearance and nutritional value.

Enzymes used in detergents and cleaners, textile and leather, cosmetic, biofuel, healthcare, pulp and paper industries are called technical enzymes. Among these technical industries, the detergent industry has the highest enzyme utilisation rate and the application with the highest revenue. Generally, proteases but also lipases, amylases and cellulases are used in this area to remove hard stains at low temperatures and enzymes (Jemli et al., 2016).

In the textile industry, the enzymes are used both to enhance the look and texture of garments made from cellulosic materials and fibre processing mainly cellulases but

also α-amylases, laccases, peroxidases, lipases, and pectate lyases are used for this purpose in the area. In the leather industry, proteases are used to remove proteins from skins and hides (Kalantzi et al., 2008; Khan, 2013). In the pulp and paper industry, enzymes are used in pulp production, paper manufacturing, fibre de-lignification and pulp bleaching purposes. Cellulanases, xylanases, laccases and lipases are the enzymes used in these areas (Jemli et al., 2016). Also, another developing area which is renewable energy production from natural materials benefits from enzymes to obtain biofuels and lignocellulosic biomass that is composed of lignin, hemicellulose and cellulose are mainly used in this area to obtain bio-alcohol and biogases and some enzymes have been used to perform this conversion. Cellulases and hemicellulases are the mainly used enzymes for these purposes but amylases, glucoamylases, laccases, proteases and pectinases are also used for the degradation of the biomass (Srivastava et al., 2020 Jemli et al., 2016).

In 2021, the value of the industrial market was USD 6.000 million and the market value is expected to grow 6 % by 2027 (Grand Viev Research U.S., 2022). As can be seen that the usage of enzymes in the industrial area is expanding and enzymes are becoming an important component of the production stages. However, despite the advantages of using biocatalysts instead of chemical catalysts in industrial processes, some disadvantages that limit this usage. First of all, many industrial processes are carried out under harsh conditions such as high pressure and temperature, extreme pH, oxidative conditions and nonaqueous solution and these conditions cause denaturation and loss of activity of the enzymes. In addition , some enzymes require a cofactor for their activity such as NADH and FADH and the addition of the cofactor in production steps causes to increase production cost. Also, enzymes can be highly expensive according to chemical catalysis because of the time-consuming, complex and costly expression and purification steps and the usage of enzymes in industrial production processes needs special process design and development. However, some solutions to these problems have been found together with the advancement of science, technology and the accumulation of knowledge. Bioreaction engineering and interdisciplinary team studies provide process the development in a short period of time. Also, development of large-scale fermentation techniques and recombinant DNA technology allowed overexpression of the recombinant protein in suitable hosts and together with the immobilization of enzymes let re-usage of enzymes in the processes.

Thus, the production of enzymes became economically feasible and production costs decreased. Also, cofactor requirements can be eliminated by protein engineering studies and the most critical problem is the instability of the enzymes during harsh industrial production conditions were solved in two ways: using extremozymes where isolated extremophiles in industrial processes and applying protein engineering studies to the existed enzymes. (Wu et al., 2021; Jemli et al., 2016).

1.3 Extremophiles

The first life forms managed to survive and evolve by adapting to the harsh conditions on Earth in which they were found – anoxic atmosphere, high UV radiation, periodically changing temperature, ionizing radiation - and also helped to change the conditions of Earth. Thus, they created conditions that exist today (Singh et al., 2020). When we look at the current limits of life considering the main parameters that determine life such as water activity, temperature, pH, salinity, pressure and radiation, we encounter interesting results. Based on these parameters, two hyperacidophilic Archaea, *Picrophilus oshimae* and *P. torridus* (pH_{opt} 0.7), living at the most acidic pH value -0.06 detected today, were isolated from a solfataric hot spring in Noboribetsu (Hokkaido, Japan) (Schleper et al., 1996). On the other hand, *Serpentinomonas* sp. B1 (pH_{opt} 11), an alkaliphilic, aerobic, mesophilic bacterium that lives at the highest basic pH value of 12.5 was isolated from the terrestrial serpentination system, The Cedars (CA, United States) (Suzuki et al., 2014). When the temperature is examined, the lowest temperature at which vitality and metabolic activity continue was determined as -20 °C as a result of the study with a natural population of bacteria from Siberian permafrost (permanently frozen soil), and *Planococcus halocryophilus* Or1 isolated from the permafrost active layered soil collected from the high Arctic region of Canada, which can survive as a pure culture at -15 °C in the presence of 18 % salt concentration (Mykytczuk et al., 2012; Mykytczuk et al., 2013). Going in the opposite direction, the highest temperature at which life is challenged is 122 °C, where *Methanopyrus kandleri* 116 isolated from the deep-sea hydrothermal habitat in the Kairei hydrothermal area on the Central Indian Ridge (CIR Kairei field) (Takai et al., 2008). Also, if we take into account that most of the Earth consists of salty environments of various proportions, the organism that lives at the highest rate of salinity is *Halarsenatibacter silvermanii* SLAS-1^T strain isolated from alkaline

hypersaline Searles Lake (CA, United States), which survives at 35 % salinity (Blum et al., 2009). Among the other parameters, Haloarchaeal GN-2 and GN-5 strains isolated from Pondicherry solar lakes can live at the lowest water activity (0.635 aw); *Thermococcus piezophilus*, which is isolated from a deep-sea hydrothermal vent in the Cayman Trough at 4964 m water depth, lives at the highest pressure (125 MPa) (Verma et al., 2020; Dalmosso et al., 2016). The organisms (Table 1.1) that live on the edge of life show that it is not possible to draw the real boundaries of life and the factors that determine the existence of life link life to three basic principles: a liquid solvent, an energy source, and building blocks (Schwieterman et al., 2018).

Table 1.1: Organisms that live extreme boundary of life (Merino et al., 2019).

Organism	Domain	Extromophile Type	Isolated Ecosytem	T (°C)	pH	Pressure (MPa)	Salinity (%)	Water Activity (a_w)
Picrophilus oshimae KAW 2/2	Archaea	Hypercidophile	Solfataric hot spring (Hokkaido, Japan)	47–65	-0.06–1.8	not defined	0–20	not defined
Serpentinomonas sp. B1	Bacteria	Alkaliphile	Terrestrial serpentination system, The Cedars (CA, United States)	18–37	9–12.5	not defined	0–0.5	not defined
Methanopyrus kandleri 116	Archaea	Hyperthermophile	Kairei hydrothermal area on the Central Indian Ridge	90–122	6.3–6.6	0.4–40	0.5–4.5	not defined
Planococcus halocryophilus Or1	Bacteria	Halopsychrophile	Permafrost active layered soil collected from the high Arctic region of Canada	-15–37	6–11	not defined	0–19	not defined
Halarsenatibacter silvermanii SLAS-1	Bacteria	Haloalkaliphile	Alkaline hypersaline Searles Lake (CA, United States)	28–55	8.7–9.8	not defined	20–35	not defined
Thermococcus piezophilus CDGS	Archaea	Piezothermophile	Hydrothermal vent in the Cayman Trough (Caribbean Sea)	60–95	5.5–9	0.1–125	2–6	0.635

When looking at the distribution of extremophile organisms, these organisms found three domains of life. However, the great majority of these organisms are found in Archaea and Bacteria domains and researchers have created a classification of extremophile microorganisms according to their physical and biochemical growing condition in order to define these organisms (Dalmaso et al., 2015). In Table 1.2, classification of the extremophiles can be seen.

Table 1.2: Extreme organism classes and their optimum growth conditions (Dalmaso et al., 2015).

Organism Class	Optimum Growth Conditions
Acidophile	pH< 3
Alkaliphile	pH> 10
Halophile	≥ 1 M salt
Hyperthermophile	> 80 °C
Thermophile	60 °C > and < 80 °C
Psychrotolerant	< 15 °C and > 25 °C
Psychrophile	< 20 °C
Piezophile	> 400 atm
Endolithic	inside rock
Hipolith	on rock and cold deserts
Oligotroph	environment with scarce nutrients
Radioresistant	tolerance to high level of radiation
Metallotolerant	tolerance to high level of heavy metals
Toxitolerant	tolerance to high level of toxic agents
Xerophile	low water availability

Extremophiles which have high optimum growth temperature are classified into two groups: thermophiles and hyperthermophiles. While thermophiles have optimum growth temperature between 60 and 80 °C, hyperthermophiles can grow only in temperatures higher than 80 °C. These organisms gained some features to adapt to these high-temperature conditions. First of all, membrane lipids are chemically stable at high temperatures and the composition of their membrane lipids is changed according to changing temperature (Dalmaso et al., 2015). According to studies on *Methanocaldococcus jannaschii*, it was found that increasing the temperature causes

a decrease of diether lipids that is also known as archaeol-based lipids and increase of standard caldarchaeol-based and cyclic archaeol-based lipids (Sprott et al., 1991). Thus, they provide membrane stability against rising temperatures. Also, their nucleic acids such as tRNA, and rRNA have high G+C content, so they provide genomic stability with a high number of hydrogen bonds together with stabilized proteins. Besides all these, their proteins have some differences according to proteins that originated from mesophiles. To give an example, the surface of these proteins contains more polar amino acid residues to make more intramolecular salt bridges (Mehta et al., 2016). Against thermophiles and hyperthermophiles, pyschrophiles can grow only less than 20 °C and psychrotolerants that can survive less than 15 °C and grow optimally at 20–25 °C. These organisms adapted to these environmental conditions by having some polyunsatured fatty acids and cold shock proteins to provide fluidity of the membrane, nutrient transportation and cell integrity (Dalmaso et al., 2015; Feller, 2013).

In the case of halophiles, they can grow only existence of a salt concentration of more than 1 M. Like other extremophiles, these organisms gained some adaptation strategies to survive a salty environment. The organisms use two main strategies to provide osmotic balance. One strategy is the accumulation of K^+ in their cytoplasm or the extraction of salts by the accumulation of compatible osmolytes such as glycerol, ectoine, some amino acid derivatives, and sugars in their cytoplasm. Also, their proteins preserve their stability by having a high ratio of acidic amino acid residues that provide negatively charged surface (Singh et al., 2020; DasSarma & Arora 1997).

Acidophiles which have optimum growth pH of less than 3 and basophiles which have optimum growth pH of more than 10 and maintained their cytoplasmic pH at ~ 6 and ~7-8 respectively through using some transporters such as ion-utilizing ATP synthase to transport protons and ions. Also, these organisms can secrete some organic metabolites to control surrounding pH (Singh et al., 2020; Dalmaso et al., 2015).

Like the explained extremophiles, other extremophiles have some adaptation strategies to survive and grow in their habitat. Piezophiles secrete some molecules such as polyunsaturated fatty acids and eicosapentaenoic acid and pressure-regulated promoters to adapt to high-pressure conditions (Nogi et al., 1998; Jaenicke et al., 1988); radioresistants have some special enzymes and proteins to repair radiation-associated DNA damages and increase metal concentration in the cytoplasm to protect

cellular proteins from oxidative damages (Dalmaso et al., 2015; Daly, 2009); metallotolerants that can survive under high heavy metal contaminated areas can tolerate the presence of heavy metals through their megaplasmids including genes for efflux mechanisms (Gomes & Steiner, 2004); xerophiles that can able to survive in low water availability secrete some compatible solutes such as trehalose and upregulate and downregulate some genes to handle desiccation and low water (De Philippis et al., 1998; Katoh et al., 2004).

On the other hand, some extremophiles adapted to survive under several extreme conditions at the same time that is linked to their habitats and they are called polyextremophiles. Today, more than 200 species that are called psychroalkaliphile, thermoalkaliphile, psychroacidophile and thermoacidophile are defined as adapted to both extreme temperature and extreme pH. As well to pH and temperature parameter, some organisms can survive under other extreme parameter combinations such as temperature and salinity, temperature and pressure, temperature and radiation, pH and salinity, pH and pressure, pH and radiation, salinity and pressure, desiccation, pH and salinity and desiccation and radiation (Capece et al., 2013). In Table 1.3, some polyextremophiles are listed.

Table 1.3: Polyextremophiles and growth conditions.

Organisms	Adapted Conditions	References
Acidianus infernus	65 to 96 °C pH 1.0−5.5	(Segerer et al., 1986)
Thermococcus acidaminovorans	56 to 93 °C pH 5.0−9.5	(Dirmeier et al., 1998)
Spirochaeta americana	10 to 44 °C pH 8.0−10.5	(Hoover et al., 2003)
Psychromonas ingrahamii sp.	−12 to 10 °C 1–10% NaCl	(Auman et al., 2006)
Thermococcus waiotapuensis sp.	60 to 90 °C <13.9 % NaCl	(González et al., 1999)
Methanopyrus kandleri strain 116	90 to 122 °C 20 to 40 MPa	(Takai et al., 2008)
Colwellia piezophila sp.	2-14 °C 0.1-60 MPa	(Nogi et al., 2004)
Thermococcus gammatolerans sp.	55 and 95 ˚C < 30 kGy	(Jolivet et al., 2003)
Natranaerobius thermophiles sp.	35 and 56 °C pH 8.3–10.6 3.1–4.9 M NaCl	(Mesbah et al., 2007)

As can be seen that organisms can evolve and develop survival strategies against the challenging conditions and what makes it possible to live the organisms in the presence of these states is fitting all cellular components and processes as a result of evolution and adaptation. Although living things have gone through this process to survive, today human beings can use these living things and their components to make their lives easier, for example, enzymes of these creatures that can work in extreme conditions: extremozymes.

1.4 Extremozymes and Their Industrial Usage Potentials

As previously explained, the enzymes which are used in industrial processes are faced with a different environment other than their own working conditions such as extreme pH, temperature and pressure, organic solvents, high substrate concentrations and non-natural substrates and all these factors can cause both losses of activity and stability of the enzymes and carrying outside reactions of them. However, these extreme conditions can be the natural habitat states for some enzymes which are isolated from extremophile organisms. As can be seen, all cellular components of these extremophile organisms evolved to help adaptation of the organisms the extreme conditions.As can be seen, all cellular components of these extremophile organisms evolved to help adaptation of the organisms the extreme conditions. Between these cellular components, proteins that are expressed by extremophiles are adapted to stay functional in the growth conditions of originated organisms. These properties make these proteins the solution to an important problem: limited usage of the enzymes in industrial processes because of instability in harsh industrial process conditions (Sarmiento et al, 2015).

Firstly, when looking at cold-absorbed extremozymes, two main properties of these enzymes are utilized in the industry: high activity at low temperatures and thermolability. Thanks to its high activity at low temperatures, reactions can be catalyzed without giving any energy to the system, which saves energy and production cost. In addition, due to its activity at moderate temperatures, it has caused the transition from mesophilic enzymes to psychrophilic enzymes in many applications. Also, as a result of the thermolability feature, the reactions can be carried out at certain time intervals and the reactions can be stopped only by enzyme denaturation with increasing the temperature. This significantly reduces the formation of unwanted by-

products. Today, these properties of psychrophilic enzymes are used in many areas from biotechnology to detergent, textile food and beverage industry (Sarmiento et al., 2015).

However, some industrial processes must keep going under high temperatures and the enzymes which are used in the processes must keep stability and activity at these temperatures. At this point, extremozymes which is originated from thermophilic and hyperthermophilic organisms can take on the task. These extremozymes can preserve their activity and stability under high temperatures. And also, they can work to high pH values, high substrate concentrations, and high pressure and keep their stability in the presence of denaturing agents and organic solvents. Due to these features, they become the target of many industrial areas such as food and beverages, pulp and paper, leather, feed and biofuel (Dumorné et. al, 2017).

Another type of extremozyme that resistant to multiple extreme conditions like hot extremozymes is halophilic extremozymes. Halophilic extremozymes are stable, active and soluble at high salt concentrations; however, they also keep their activity under extreme pH and temperatures. Also, they proceed with their reactions in the low water level and the presence of organic solvents. Thanks to these features, it has paved the way for its use in many industrial processes and today these extremozymes are used in food, pulp and paper, textile and leather industries (Delgado et al.,2012).

Also, other types of extremozymes are very crucial for industrial applications because of their activity under highly acidophilic and alkaliphilic conditions. For these purposes, they are used in food, pulp and paper, detergent and polymer-hydrolysis related processes. Finally, another target extremozymes which are piezophilic extremozymes get into the focal point of processes that carried out under high pressure. Although not much research has been done yet, today, they have been used in the food, detergent and pharmaceutical industries. In Table 1.4, some extremozymes and their industrial usage area can be seen (Dumorné et. al, 2017).

Table 1.4: Extremozymes and their industrial applications (Sarmiento et al., 2015; Dumorné et. al, 2017; Delgado et al.,2012).

Type	Enzyme	Industrial area	Application
Thermophilic	Amylases	Food and beverage	Starch hydrolysis to form syrups Production cakes and fruit juices
Thermophilic	Laccase	Pulp and paper	Bio-bleaching
Thermophilic	Lipases and proteases	Detergent	Detergent additive to clean difficult stains
Thermophilic	Chitanases	Biofuel	Conversion cellulose to ethanol
Psychrophilic	Lipases, proteases and amylases	Detergent	Breaking down lipid, protein and starch based stains at low temperatures
Psychrophilic	Amylases and cellulases	Textile	Desizing and biofinishing of fabrics
Psychrophilic	Pectinases	Food and beverage	Fermentation of beer and wine and clarification of fruit juices
Halophilic	Amylases	Food	Starch hydrolsis
Halophilic	Xylanase	Pulp and paper	Xylan biodegradation
Halophilic	Lipases, Amylases	Detergent	Usage in detergent formulation for breaking down lipid and starch based stains
Acidophilic	Proteases	Animal Feed	Improvement of animal feed
Acidophilic	Oxidases	Fuel	Coal desulfurization
Alkaliphilic	Cellulases	Food	Fermentation of beer and wine and clarification of fruit juices
Alkaliphilic	Proteases	Detergent	Usage in detergent formulation for breaking down protein based stains
Piezophilic	-	Food	Food preservation and sterilization
Piezophilic	-	Pharmaceutical	Antibiotic production

When we look at the general picture, it is seen that the use of enzymes is quite intense in many industrial areas as a result of the extremozymes coming from extremophilic and polyextremophilic organisms. However, the value of these enzymes is still not fully appreciated and cannot be fully integrated into the industry. Besides all this, other extremozymes such as toxitolerant and metallotolerant enzymes that work in the presence of highly toxic substances; xerophilic enzymes that can operate in the presence of low water; radioresistant enzymes that are highly resistant to radiation have not taken their place in the industry (Dalmaso et al., 2015). Day by day, more and more enzyme integration will be achieved in industrial applications by noticing and studying these enzymes, as well as the discovery of new extremozymes as a result of developing metagenomic studies.

1.5 Redesign of Enzymes via Protein Engineering

Although the biocatalysts perform incredible chemical transformations, they cannot meet the needs of some large-scale applications (Adamczak & Krishna, 2004). At this point, protein engineering studies on existing enzymes can provide the solution.

Protein engineering allows the design and construction of novel proteins generally with manipulation of the genes of proteins; thus, it allows to create enzymes with novel characteristics. Three main strategies can be used to create novel enzymes in protein engineering: rational design, directed evolution and semi-rational design.

To design proteins with desired properties in rational design, some prior knowledge is needed. These are 3-dimensional structure of the protein or the estimation of the structure information by homology modeling or molecular dynamics, the amino acid sequence of the protein, the functional information and the amino acids that responsible for the function. Based on all this information, the required amino acid change(s) are determined to gain the desired function and controllable amino acid changes such as insertion, deletion or displacement are carried out by the PCR method with the help of primers containing the required nucleotide sequence changes (Ordu et al., 2012).

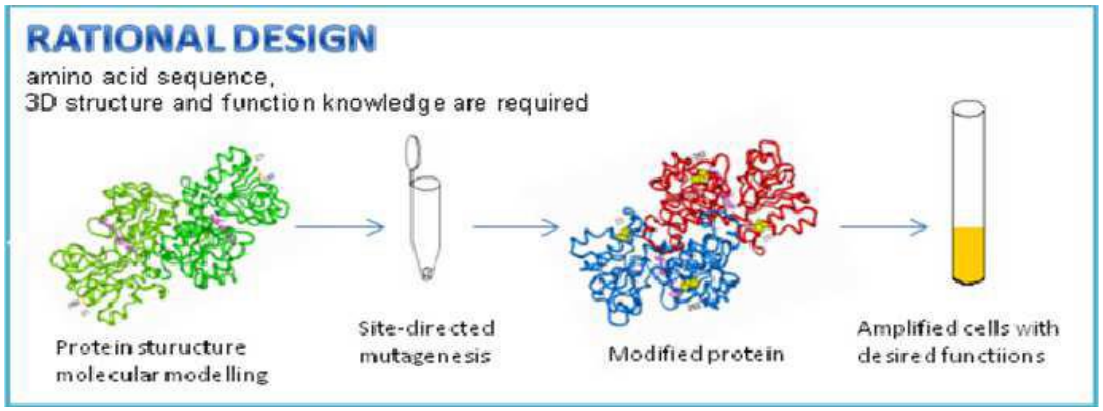

Figure 1.2: Rational design strategy (Ordu et al., 2012).

In case of the directed evolution strategy, proteins evolve in a short time scale in the laboratories by mimicking the evolution mechanism to obtain mutants with the desired properties. The main foundations of this strategy conducted with the studies of Frances Arnold that succeeded increasing subtisilin E activity 256 times in the presence of 60 % DMF by the random mutagenesis and Pim Stemmer that succeed increased the minimum inhibitory concentration of *E. coli* against the antibiotic cefotaxime by DNA shuffling studies on the TEM-1 β-lactamase gene of *E. coli* in the 1990s (Ordu et al., 2012; Chen & Arnold, 1993; Stemmer, 1994).

In this strategy, a gene library containing random mutations is created by recombinative such as DNA shuffling, StEP, RACHITT methods and non-recombinative methods such as site saturation mutagenesis, RID and error prone PCR method without knowing the three-dimensional structure of the relevant protein. Then, mutants with the desired trait are selected from this created library using an appropriate selection method. This process is repeated cyclically until the desired property is reached (Bornscheuer & Pohl, 2001; Williams et al., 2004).

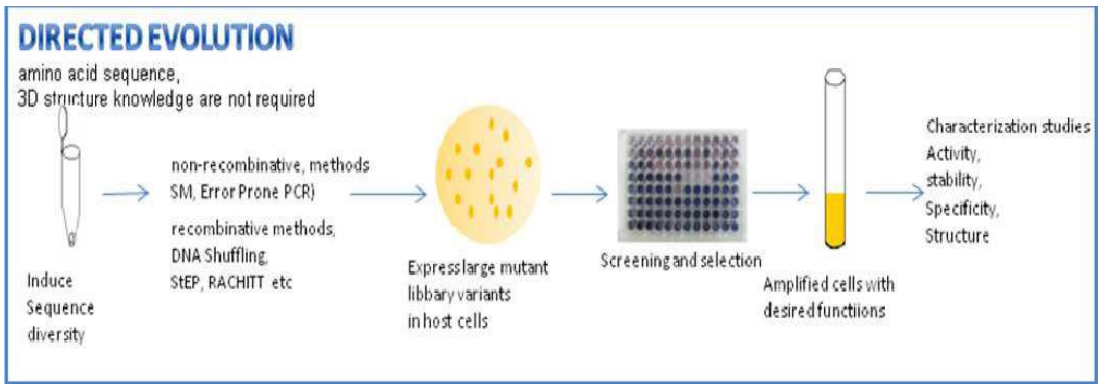

Figure 1.3: Directed evolution strategy (Ordu et al., 2012).

In semi-rational design which is a combination of rational design and directed evolution strategy, small sized gene libraries are obtained by creating mutations on the gene encoding the relevant protein with the site saturation mutagenesis method only the knowledge of the region and amino acids associated with the feature of interest. Then, mutants that have the desired properties are selected from the obtained library by a suitable screening and selection method (Ordu et al., 2012).

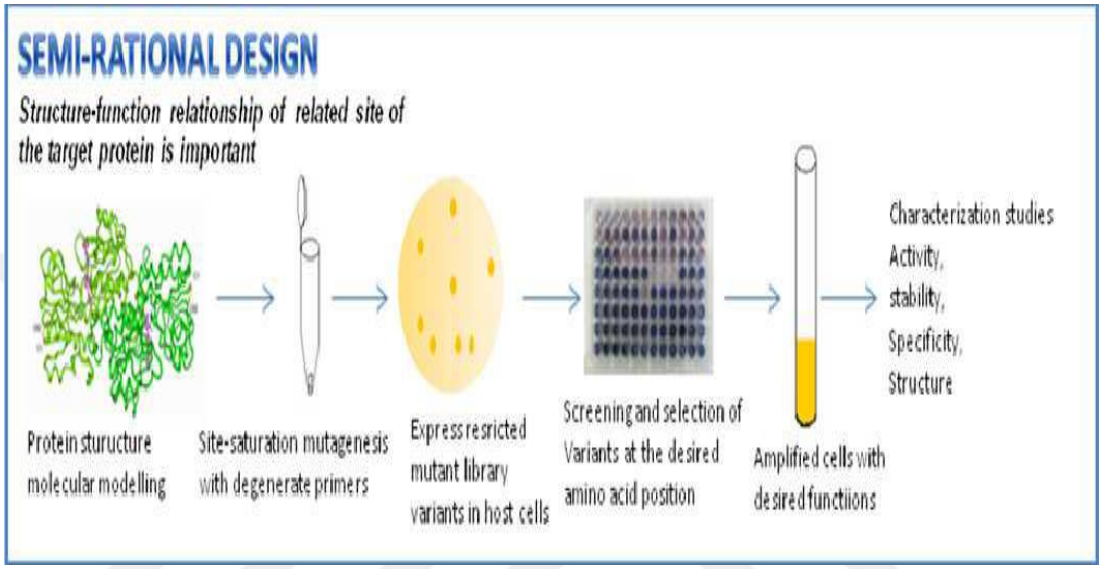

Figure 1.4: Semi-rational design strategy (Ordu et al., 2012)

The semi-rational design has some advantages over rational and directed evolution. In this strategy, the three-dimensional structure information of the proteins that are in rational design is not required, and all possible combinations that are obtained by replacing the relevant amino acid with 19 different amino acids are get with the usage of the saturation mutagenesis method in a single library. In addition, saving time and effort is provided as a result of the creation of smart libraries against large libraries in directed evolution (Bommarius et al., 2006; Lehmann & Wyss, 2001).

Researchers used all three strategies to fit enzymes in industrial processes and usage. The rational design strategy allowed the design of enzymes with high stability at high temperatures and alkali pH and oxidation. Together with stability studies, a rational design strategy let the design of process-specific biocatalysts by changing the substrate and product specificity of the enzymes. Today constructed enzymes by rational design are used in a wide range of industrial areas such as food, detergent, textile, pulp and paper etc. Table 1.5 lists some enzymes used in the industrial field, on which rational design studies have been carried out.

Table 1.5: Industrial enzymes engineered by rational design strategy (adapted from Sing et al., 2013).

Enzyme	Organism	Improved Property	Method	Application
Cyclodextrin glucanotransferase	*Bacillus stearothermophilus* ET1	Modulation of cyclizing activity and thermostability	Site-directed mutagenesis	Bread industry
Protease BYA	*Bacillus* sp. Y	Specific activity1.5-fold higher	Site-directed mutagenesis	Detergents products
Endo-1,4-β-xylanase II	*Trichoderma reesei*	Increased alkali stability	Site-directed mutagenesis	Sulfate pulp bleaching
1,3-1,4-β-D-glucanase	Fibrobacter succinogenes	Thermostability and specific activity	Rational mutagenesis	feed additive
Alkaline amylase	*Alkalimonas amylolytica*	Oxidative stability	Site-directed mutagenesis	Detergent and textile industries
Endoglucanase	*Thermoascus aurantiacus*	4-fold increase in kcat and 2.5-fold improvement in hydrolytic activity on cellulosic substrates	Site-directed mutagenesis	Bioethanol production
D-glucose 1-dehydrogenase isozymes	*Bacillus megaterium*	Substrate specificity	Site-directed mutagenesis	Measurements of blood glucose level
Superoxide dismutase	Potentilla atrosanguinea	Thermostability	Site-directed mutagenesis	Scavenging of $O2^-$

On the other hand, some researchers used to directed evolution strategy to the design industrial enzymes with high stability to high temperatures, organic solvents and acidic pH. Also, together with increasing the activity of the enzymes, activity shifted to the desired reactions using this strategy. Some industrial enzymes that are obtained by directed evolution studies can be seen in Table 1.6.

Table 1.6: Industrial enzymes engineered by directed evolution strategy (adapted from Sing et al., 2013).

Enzyme	Organism	Improved Property	Method	Application
Lipase B	*Candida antarctica*	20-fold increase in half-life at 70 °C	epPCR	Resolution and desymmetrization of compound
Tagatose-1,6-Bisphosphate aldolase	*E. coli*	80-fold improvement in kcat/Km and 100-fold change in stereospecificity	DNA shuffling and screening	Efficient syntheses of complex stereoisomeric products
Xylose isomerase	Thermotoga neapolitana	High activity on glucose at low temperature and low pH	Random Mutagenesis	Used in preparation of high fructose syrup
Amylosucrase	Neisseria polysaccharea	5-fold increased activity	Random mutagenesis and gene shuffling	Synthesis or the modification of polysaccharides
Galactose oxidase	*F. graminearum*	3.4–4.4 fold greater Vmax/Km and increased specificity	epPCR	Derivatization of guar gum
Fructose bisphosphate aldolase	E. coli	Increased thermostablity and stability to treatment with organic solvent	DNA shuffling	Use in organic synthesis
Lipase	*P. aeruginosa*	2-fold increase in amidase activity	Random mutagenesis	Understanding lipase inability to hydrolyze amides
Xylose isomerase	Thermotoga neapolitana	2.3-fold increases in catalytic efficiency	Random mutagenesis	Production of high fructose corn syrup
α-Amylase	Bacillus sp. TS-25	10 °C enhancement in thermal stability	Directed evolution	Baking industry
CotA laccase	B. subtilis	120-fold more specific for ABTS	Directed evolution	Catalyze oxidation of polyphenols
Prolidase	Pyrococcus horikoshii	Thermostability	Random mutagenesis	Detoxification of organophosphorus nerve agents

With the semi-rational designs carried out on industrial enzymes, the enzymes have been stabilized at high temperatures. Also, their substrate specificity has been shifted to the desired direction and their activation has been increased. Some designed industrial enzymes and their applications can be seen in Table 1.7.

Table 1.7: Industrial enzymes engineered by semi-rational design strategy (adapted from Sing et al., 2013).

Enzyme	Organism	Improved Property	Method	Application
Hydantoinase	*Arthrobacter sp.*	Enantioselective hydantoinase and 5 fold more productivity	Saturation mutagenesis	Production of L-Met (L-amino acids)
Xylanase		Tm improved by 25 °C	Saturation mutagenesis	Degradation of hemicellulose
Pyranose 2-oxidase	*Trametes multicolor*	Altered substrate selectivity for D-galactose, D-glucose	Semi-rational design	Food industry
Endoglucanase CelA	*Clostridium thermocellum*	10-fold increase in half-life of inactivation at 86 °C	Saturation mutagenesis	Bioconversion of cellulosic biomass
Phospholipase D	*Streptomyces*	Improved thermal stability and activity	Site-specific saturation mutagenesis	Phosphatidylinositol synthesis

Obtaining proteins and enzymes with the desired properties and activities are possible by changing a few amino acids in the sequence through protein engineering studies; however, Nature also reveals the proteins with novel functions by making larger changes to the genes of existing proteins such as tandem duplications and circular permutations in the evolutionary process. Thus, mimicking the way of Nature, novel functionality and characteristics can be gained fromthe enzymes by swapping elements of the existed enzymes such as secondary structural elements, domains and whole proteins using combinatorial and non-combinatorial methods. In protein engineering, imitating Nature with the domain switching method enables to obtain enzymes these properties: gaining allosteric regulation, activators and inhibitors, substrate specificity, improvement catalytic activity, stability against high temperature, denaturants, inhibitors and expression through aggregation prevention and increasing solubility (Ostermeier & Benkovic, 2001).

At this point understanding the functions and characteristics of the domains and the effect of the domains on the function and biochemical character of enzymes are very important to gain new features to the enzymes through protein engineering studies. In this way adaptation of the enzymes that work in moderate conditions in the industrial processes can be provided with the help of the extremozymes domains.

Today, many characteristics of the enzymes can be changed by protein engineering studies such as remaining stable and functional in extreme conditions, keeping activity in the presence of high substrate and product concentration, eliminating cofactor

requirement and changing substrate and product specificity. Also, designing the enzymes that react with non-natural substrates or catalyze reactions that are not yet discovered in nature make possible with the fourth wave of evolution of biocatalysts. Thus, the limitations of natural enzymes can be overcome and process-specific biocatalysts can be designed. Together with protein engineering studies, recent advances in recombinant DNA technology, metagenomics and proteomic enable both discoveries of new enzymes and the joining of more enzymes in industrial applications (Singh et al., 2013; Ordu et al., 2012; Arnold, 2019; Cobb et al., 2013).

1.6 Promising Enzyme in Industrial Usage and Protein Engineering Studies: Thermostable Amylopullulanases

The global enzyme market is dominated by enzymes which constitute 30 % of the total enzyme market and are used in food and beverage industry. Starch processing applications are the most dominant area in the food industry as enzyme usage and the enzymes which are used in starch processing constitute 15-20 % of the global enzyme market (Grand Viev Research U.S., 2022).

Starch which is the main constituent of food products is a heterogeneous carbohydrate polymer and is stored in plants as insoluble granules. It composes of amylose and amylopectin. While glucose unit is linked by α-1,4-glycosidic bonds in amylose, 5 % of glucose unit in amylopectin is linked by α-1,6-glycosidic bonds (Figure 1.5). The ratio of amylose and amylopectin and size, shape and structure of the granules change according to plant type (Robyt, 2008).

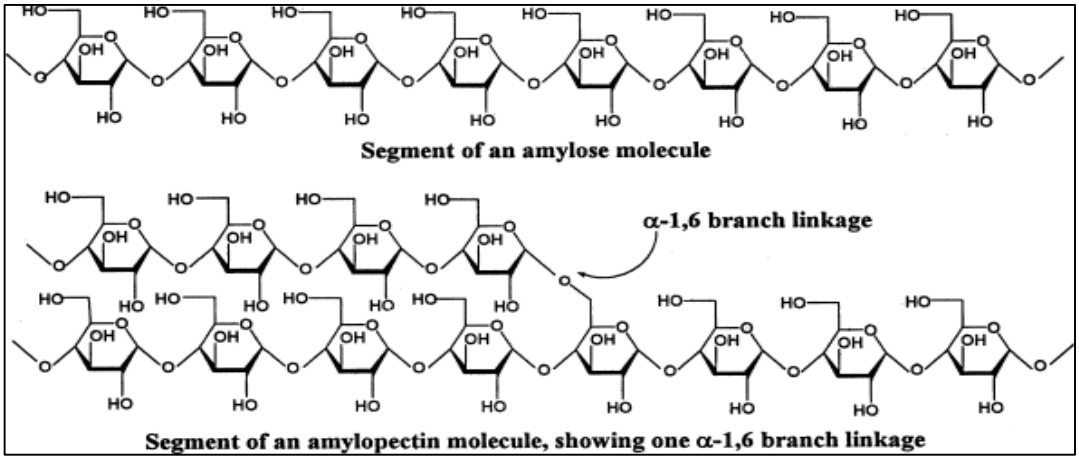

Figure 1.5: Structure of amylose and amylopectin (Robyt, 2008).

Maltooligosaccharides which is obtained by starch hydrolysis is branched or linear oligosaccharides are used in soft drinks, confectionary, meats, packed products, ice-cream, sauces, baby food, canned fruit, preserves because of their high solubility, less sweetness, low calories, less water activity, good moisture level, acid and heat stability properties. Also, these oligosaccharides such as maltotriose, maltotetraose and maltopentaose are used as anti-stalling agent in bakery products because they prevent migration of moisture from the starch, realigning of amylose and amylopectin chains that reduce retrogradation and starch-gluten interaction. Also, monosaccharides and disaccharides which are obtained by starch hydrolysis can are used for glucose, maltose and fructose syrup production. Maltooligosaccharides are taken from the body gradually and provide continuous energy for a long time; also, because of their low calories they can regulate blood glucose levels and decrease post-prandial blood glucose levels. Thus, they are used in healthcare for the treatment of cardiovascular, diabetes and obese patients. Also, they are used as prebiotics because of their soluble non-digestible properties and they help the treatment of patients that have hypoglycemia and brain disorder associated with overconsumption of energy with providing increased body endurance and immunity. Glucose is another starch hydrolysis product that can be converted to fuel alcohols and bio-products (Jòzef, 2007; Satyanarayana & Nisha, 2018).

Enzymes get involved starch hydrolysis process within liquefaction and saccharification steps. In the first step of starch hydrolysis which is called gelatinisation, dissolution of starch is realized to obtain highly viscous slurry of starch granules (~35 %) at high temperatures. Then, dispersion and partial degradation of the gelatinized starch granules make realize in liquefaction step with the help of thermostable α-amylases at 90-110 °C. α-amylases (E.C. 3.2.1.1) decrease the molecular weight of starch rapidly by cleavage of inner α-1,4-glycosidic bonds in amylose and amylopectin. As a result of the reaction, wide variety of oligosaccharides that are consist of 10 to 20 glucose units are obtained. After this point, the enzyme cannot both bind these starch fragments properly and hydrolyze α-1,6-glycosidic bonds and exo α-1,4-glycosidic bonds. To obtain mono- and disaccharides such as glucose, maltose other enzymes are included in the saccharification step. At this process, β-amylases (EC 3.2.1.2) and glucoamylases (EC 3.2.1.3) hydrolyze the oligosaccharides from their non-reducing ends and generate maltose and glucose

respectively. However, β-amylases cannot cleave α-1,6-glycosidic bonds and glucoamylases can cleavage this type of bond very slowly. To exceed this problem debranching enzymes such as pullulanases (EC 3.2.1.41) are incorporated in the saccharification. As can be seen in Figure 1.6, before α-amylase addition to the process, pH is adjusted to the 6.5 because none of the α-amylase cannot stay stable at acidic pH. However, β-amylases and glucoamylases cannot work at pH 6.5; so pH again adjusted to 4.5. For all these pH adjustment, some chemicals are added to the process and also during these pH adjustments salt precipitations are observed. To remove the salts, ion exchange step was added to the process. Keeping their stability at high temperatures and acidic pH, hydrolyzing ability both α-1,4 and α-1,6-glycosidic bonds make amylopullulanases promising candidate of starch hydrolysis process. Usage only one enzyme –amylopullulanase- instead of three or more than three enzymes both reduce production costs and steps of shorten the production steps with the elimination ion exchange and pH adjustment steps. Also, (Jòzef, 2007; Sindhu et al., 2021).

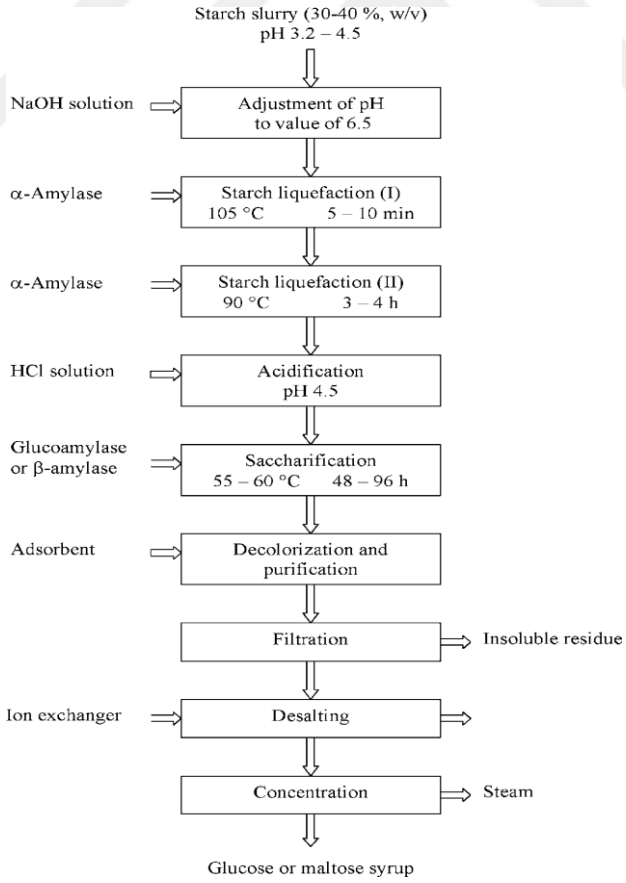

Figure 1.6: Starch hydrolysis process (Jòzef, 2007).

Amylopullulanases are used not only for starch hydrolysis but also in detergent and bakery. In detergent industry, amylopullulanases can be used to remove starch related stains and they can be used in making bread to remove branched maltodextrins to eliminated α-amylase-treated bread and other bakery products. Today, alkolitolerant *G. thermoleovorans* NP33 amylopullulanase is started to use both in detergent and whole wheat doughs for these purposes (Sing et al., 2013).

Thermostable amylopullulanases not only important for industrial applications but also important for protein engineering studies both their thermostable, bifunctional and multidomain properties. With the understanding role of the domains on the enzymes, more protein engineering can be proceeding on the enzymes to create novel property such as thermostability, bifunctionality, enlarge substrate specificity etc.

When looking at the general characteristics of amylopullulanases, amylopullulanases (E.C. 3.2.1.1/41) that are belongs to the glycoside hydrolases (GHs) family can cleave both α-1,4 and α-1,6 glycosidic bonds in starch, pullulan, amylopectin, glycogen, and related oligosaccharides to give glucose, maltose, and maltotriose. They are produced from mesophilic and thermophilic bacteria and archaea. It can be seen in Table 1.8, amylopullulanases can work wide range of high temperatures that is between 50 and 110 °C and some of them can work at acidic pH (Nisha & Satyanarayana, 2013).

Table 1.8: Source and some biochemical characteristics of amylopullulanases (adapted from Nisha et al., 2013).

Source Organism	Source Organism Class	Molecular Weight (Mw)	pH	Temperature (°C)
Bacillus sp. KSM-1378	Mesophilic bacteria	210	10	50
Bacillus circulans F-2	Mesophilic bacteria	220	7-8.5	50
Bacillus subtilis	Mesophilic bacteria	450	7	30
Bacillus sp. US149	Thermophilic bacteria	200	5	60
Bacillus sp. 3183	Thermophilic bacteria	-	5	62
Bacillus sp. TS-23	Thermophilic bacteria	140	9	55
Bacillus sp. DSM 405	Thermophilic bacteria	126	6-6.5	70
Bacillus sp XAL 601	Thermophilic bacteria	224	9	70
Clostridium sp. strain EM1	Thermophilic bacteria	-	5.9	60
Cohnella sp. A10	Thermophilic bacteria	70	6	60

Table 1.8 (continued) : Source and some biochemical characteristics of amylopullulanases (adapted from Nisha et al., 2013).

Source Organism	Source Organism Class	Molecular Weight (Mw)	pH	Temperature (°C)
Desulfucoccus mucosus	Thermophilic bacteria	74	5.8	85
Geobacillus thermoleovorans NP33	Thermophilic bacteria	-	7	80
Geobacillus stearothermophilus L14	Thermophilic bacteria	100	5.5	65
Thermoanaerobacter brockii brockii	Thermophilic bacteria	184	-	-
Thermoanaerobium Tok6-B1	Thermophilic bacteria	120	5.6	70
Thermoanaerobacterium thermosaccharolyticum	Thermophilic bacteria	150	5-5.5	65
Thermoanaerobacter strain B6A	Thermophilic bacteria	450	5	75
Thermoanaerobacter ethanolicus 39E	Thermophilic bacteria	133	5.5	-
Thermobacteroides acetoethylicus	Thermophilic bacteria	-	5-6	75
Thermoanaerobacter finni	Thermophilic bacteria	-	5-6	75
Thermotoga maritima	Thermophilic bacteria	96	-	-
Pyrococcus furiosus	Hyperthermophilic archaea	89	5.5	110
Desulfurococcus amylolyticus JCM9188	Hyperthermophilic archaea	71	5	95
Thermococcus litoralis	Hyperthermophilic archaea	119	6	110
Thermococcus hydrothermalis	Hyperthermophilic archaea	128	5.5	95
Thermococcus profundus	Hyperthermophilic archaea	43	5.5	80-90
Thermococcus celer	Hyperthermophilic archaea	-	5.5-6	90
Thermofilum pendens	Hyperthermophilic archaea	65	3.5	95-100
Pyrococcus woesei	Hyperthermophilic archaea	90	6	100

These enzymes belong to the GH13 or GH57 families according sequence based classification. Enzymes which is in GH13 family have six conserved sequence region at their active site: I (β_3), II(β_4) , III(β_5), IV(β_7),VI(β_2) and VII(β_8) and as a result of directed mutagenesis analysis on *T. ethanolicus* 39E amylopullulanase, three catalytically important amino acid residues were determined: two aspartates (β4, β7)

and glutamate (β5). While aspartate on β4 and aspartate on β7 act as nucleophile and proton donor respectively, glutamate (β5) involved in the transition state stabilizer. Also, secondary structure of the enzyme active site is fold into a $(\alpha/\beta)_8$ which is also known as TIM barrel. However, enzymes that is in GH57 family have catalytically important a glutamic acid and an aspartate residues and secondary structure of the enzyme active site is fold into a $(\alpha/\beta)_7$. While amylopullulanases are produced from mesophilic and thermophilic bacteria belonging to the GH13 family, the GH57 family includes amylopullulanases that are produced from thermophilic archaea. According to active site number, amylopullulanases are divided into two classes. While amylopullulanases which are produced from thermophilic organism have one active site, amylopullulanases which are produced from aerobic microorganisms have one or two active sites.

Amylopullulanases are multidomain proteins. GH13 amylopullulanases possess cyclodextrin (CD) and pullulan-degrading N-terminus domain, α-amylase catalytic domain, amy C domain, fibronectin type 3 domain, CBM 20 domain and some amylopullulanases also have X25 domain. The domain architecture of *Thermoanaerobacter brockii brockii* amylopullulanase (TbbApu) can be seen in Figure 1.7 (Nisha et al., 2013; Mathupala et al., 1993; Nisha & Satyanarayana, 2015).

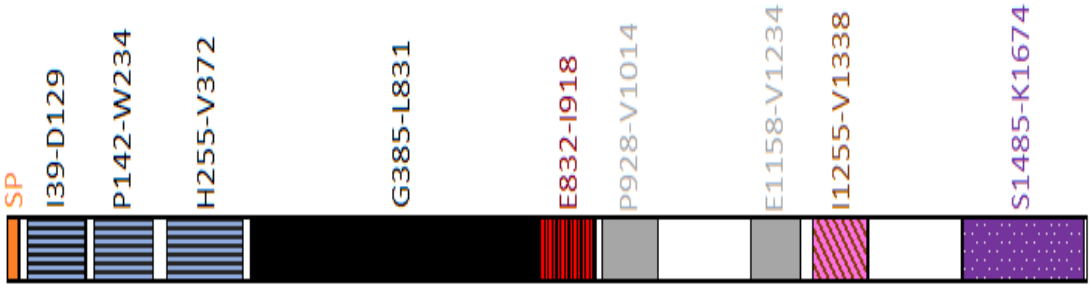

Figure 1.7: Domain architecture of TbbApu:X25 domain (I39-D129 and P142-W234); cyclodextrin (CD) and pullulan-degrading N-terminus domain (H255-V372); α-amylase catalytic domain (G385-L831); amy C domain (E831-I918); fibronectin type 3 domain (P928-Y1014 and E1158-V1234); CBM 20 domain (I1255-V1338) and SH3 domain (S1485-K1674).

Cyclodextrin (CD) and pullulan-degrading N-terminus domain is found at cyclomaltodextrinases (CDases 3.2.1.54), maltogenic amylases (MAases 3.2.1.133), and neopullulanases (NPases 3.2.1.135) which are separated from other α-amylases because of their cyclodextrin hydrolyzing ability together with pullulan and starch. The N domain has a distorted 'β-barrel' like structure consisting of 7 antiparallel β-

strands. According to the studies on *Thermoactinomyces vulgaris* R-47 neopullulanase (TVAII), domain N play important role in position and orientation stabilization of the other domains and is responsible for dimerization. Extra sugar binding site is created as a result of dimerization and this site is important for substrate binding especially cyclodextrin. Also, as a result of protein engineering studies carried out on alpha amylases of *Thermoactinomyces vulgaris* R-47, removal of the N domain caused the enzymes to lose their stability under alkaline conditions and a 10 °C decrease in thermal stability temperature and the activities of the enzymes against starch, pullulan, α-CD, β-CD and γ-CD were decreased (Lee et al, 2002; Park et al., 2000; Kim et al., 1999; Kamitori et al., 1999; Yokota et al., 2001).

α-amylase C-terminal all-β domain (AamyC) is the non-catalytic domain in glycoside hydrolases and may responsible for carbohydrate binding. AamyC may role in breaking the starch granules with the other two binding sites in the catalytic domain. It may provide the opening of the α-glucan chains in the starch and ensures the proper orientation of the active site of the enzyme on the substrate chains. In addition, it is thought that the AamyC domain protects the hydrophobic amino acids of the $(\alpha/\beta)_8$ catalytic domain from solvent and provides stabilization of the domain (Cuyvers et al., 2012; Plou, de Segura & Ballesteros, 2007; Robert et al., 2013; Bozonnet et al., 2007).

Fibronectin type III domain (FnIII) is an evolutionarily conserved domain consisting of 100 amino acids and having a β-sandwich structure. In the SMART database, 33 % of the proteins containing the FnIII domain are of bacterial origin and 19 % of the FnIII domains are carbohydrate related enzymes such as cellulases, amylases, pullulanases, polygalacturonidases and chitinases, or proteins containing CBM. To elucidate role of FnIII domains of the enyzmes, the studies carried on *Bacillus circulans* WL-12 chitinase. As a result of removal one and two of the FnIII domains from the enzyme, chitin binding and enzyme activity did not affect when soluble substrates were used. However, when insoluble colloidal chitin is used as a substrate, removal of an FnIII domain results in 20 % of the enzyme activity and removal of two FnIII domains caused a 50 % decrease in enzyme activity. It is suggested that this domain may have a structural function by creating an optimum distance and orientation between the catalytic domain and the substrate binding domain. Another noteworthy point is that the FnIII domain is found in enzymes that degrade insoluble polymers. This indicates

that the FnIII domain plays a common role in the degradation of insoluble substrates (Valk et al., 2017; Watanabe et al., 1994).

Although various amylases are known to bind to starch granules without containing starch binding domains (SBD), SBDs give amylolytic enzymes the ability to bind to raw, i.e., thermally untreated, granular starch. SBDs belong to the family of carbohydrate binding modules (CBMs). Currently, 15 out of 85 CBM families are considered SBDs due to their ability to bind starch and α-glucans such as glycogen, cyclodextrin, and various maltooligosaccharides and one of these modules is CBM20 module. The common feature of CBM20s is their association with catalytic modules related to starch or glycogen metabolism because enzymatic degradation of starch and other insoluble polysaccharide chains is problematic due to difficulty of reaching these insoluble polysaccharides to the active site. However, the binding of enzymes to insoluble polysaccharide surfaces such as starch granules or crystal cellulose is increased by CBMs and it is also suggested that CBMs disrupt the conformation and packaging of the polymer, thereby facilitating degradation. (Kuchtová et al., 2018).

1.7 Aim of The Study

Today, more than 3000 enzymes are used in many industrial areas such as textile, leather, detergent, pulp and paper industry, health care, and especially in the food and beverage industry. The usage of enzymes in industrial areas is expanding with the developing recombinant DNA technology and it is becoming an important component of the industrial production stages. Together, some disadvantages limit the usage of enzymes in these areas. Most enzymes are unable to maintain their stability under heavy industrial processing conditions such as high pressure and temperature, extreme pH, oxidative conditions and anhydrous solutions. These problems can be overcome in two different ways: using enzymes from extreme organisms and performing protein engineering studies on existing enzymes.

Enzymes obtained from extremophilic organisms can withstand severe industrial conditions and maintain their functionality thanks to their properties and this makes these enzymes potential to be used in industrial stages. As a result of metagenomic studies that are developing day by day, new extremophilic organisms and extremozymes are revealed and they are starting to take place more and more in the industrial field. Also, extremozymes can be used as a guide in protein engineering

studies with their properties and can be a guide in acquiring the desired properties of existing enzymes. In addition to the whole enzyme, it may be possible to design a new enzyme by working on extremozymes domains that provide features such as substrate specificities, stability, and bifunctionality.

One of the promising enzymes in industrial usage and protein engineering studies is amylopullulanase. Because of their ability to cleavage ability both α-1,4 and α-1,6 glycosidic bonds in carbohydrates have an important potential in the food and beverage industry, detergent, pharmaceutical and baking industries. In this study, recombinantly produced amylopullulanase enzyme that originated from a thermophilic organism *Thermoanaerobacter brockii brockii* is being studied.

In the previous studies, thermophilic *Thermoanaerobacter brockii brockii* was obtained with inoculation of sediment where is obtained Washburn thermal spring in Yellowstone National Park by enrichment culture technique. After that, amylopullulanase gene was cloned from genomic DNA of *Thermoanaerobacter brockii brockii* and inserted into pCPC903 chimeric vector (Coleman et al., 1987). Then, the expression of the *apu* gene was done recombinantly at *E. coli* and *Bacillus subtilis*. As a result of the studies, while no significant enzyme activity could be observed in *E. coli*, expression was successfully observed in *B. subtilis*, but there was a thermostability problem. However, the cloned *apu* gene was 3783 bp in length and encodes 1261 amino acids without having a stop codon. Then, the deficient gene fragments were completed by primer walking method using the genome of *T. brockii brockii* (Mumcu, 2018) and expression studies were progressing. Also, two C-term truncated variants of TbbApu which are TbbApuΔSH3 without SH3 and TbbApuΔCBM20 without CBM20 domain were constructed to investigate SH3 and CBM20 domain effects.

In the scope of this thesis, apart from TbbApuΔSH3 and TbbApuΔCBM20, four additional truncated variants which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were obtained to disclose the effects of X25 domains. The biochemical characterisation, raw starch binding ability and kinetic studies of TbbApu and its six truncated variants have been accomplished. Also, the X25 domain function on substrate binding, activity and stability of TbbApu has been revealed for the first time with this study. The domain architecture of TbbApu and its truncated variants can be seen in Figure 1.8.

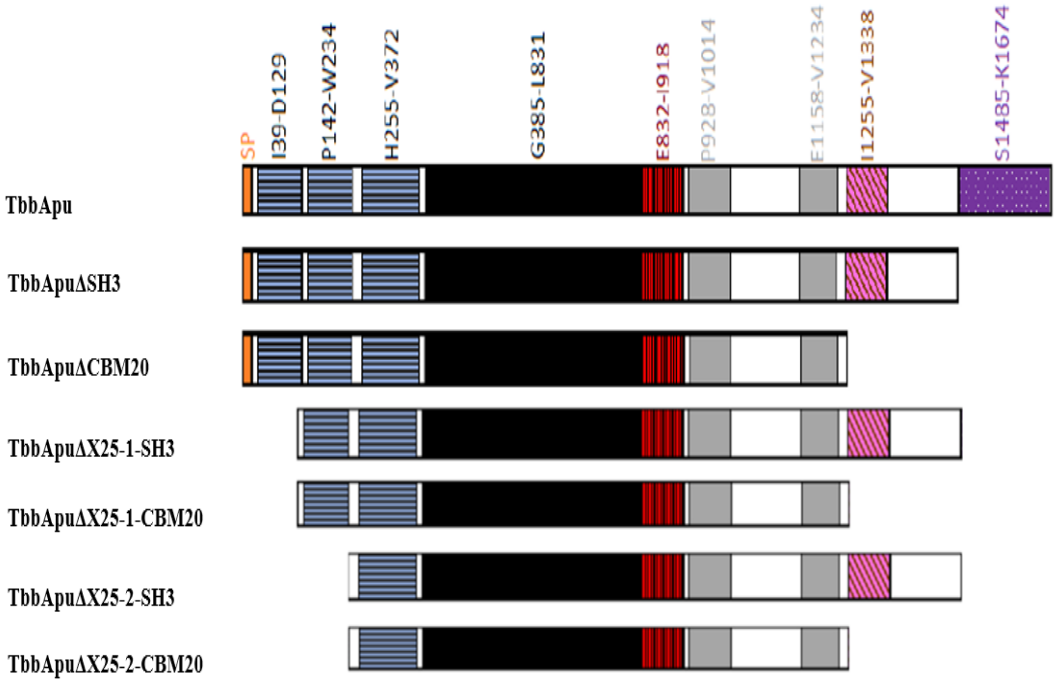

Figure 1.8: Domain architecture of TbbApu and its truncated variants:X25 domain (I39-D129 and P142-W234); cyclodextrin (CD) and pullulan-degrading N-terminus domain (H255-V372); α-amylase catalytic domain (G385-L831); amy C domain (E831-I918); fibronectin type 3 domain (P928-Y1014 and E1158-V1234); CBM 20 domain (I1255-V1338) and SH3 domain (S1485-K1674

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory equipments

The laboratory equipments are given in Appendix A.

2.1.2 Culture media

The ingredients and preparations of the culture media that are used during the study are given in Appendix B.

2.1.3 Buffer and solutions

The composition and preparation of buffers and solutions that are used during the study are given in Appendix C.

2.1.4 Bacterial strains and plasmid

pET-28 a (+) expression vector (Novagen, #69864-3) was chosen for sub-cloning and expression of the Tbbapu and its variants in *E. coli* BL21 (DE3) host cell. pET-28 a (+) expression vector is a bacterial vector and it consists of 5369 base pairs. It allows expression N- and C- terminally 6xHis-tagged of target proteins which provide the purification of proteins with affinity chromatography. KanR gene that allows resistance to kanamycin in the plasmid is the selectable marker. Transcription of the Tbbapu and its variants is under the negative control and the control is provided by a strong, controllable promoters which is T7lac promoter. The promoter includes T7 promoter which is the binding site of T7 RNA polymerase and lac operator is located at the downstraeam of the T7 promoter. The vector carries the natural promoter and coding sequence for the lac repressor and the control of the promoter is provided by lacI repressor. In the absence of allolactose or isopropyl-ß-D thiogalactopyranoside (IPTG) which are the natural and gratuitous inducer of lac operon, lacI inhibits the transcription of the target gene. Also, the vector contains multiple cloning site that includes restriction site for *XhoI, PspXI, PaeR71, NotI, EagI, HindIII, SalI, Eco53Kı, SacI, EcoRI, BamHI* for insertion of the target gene to the vector (Novagen, 2003).

pET-28 a (+) expression vector and the constructed plasmid structure can be seen in Figure 2.1.

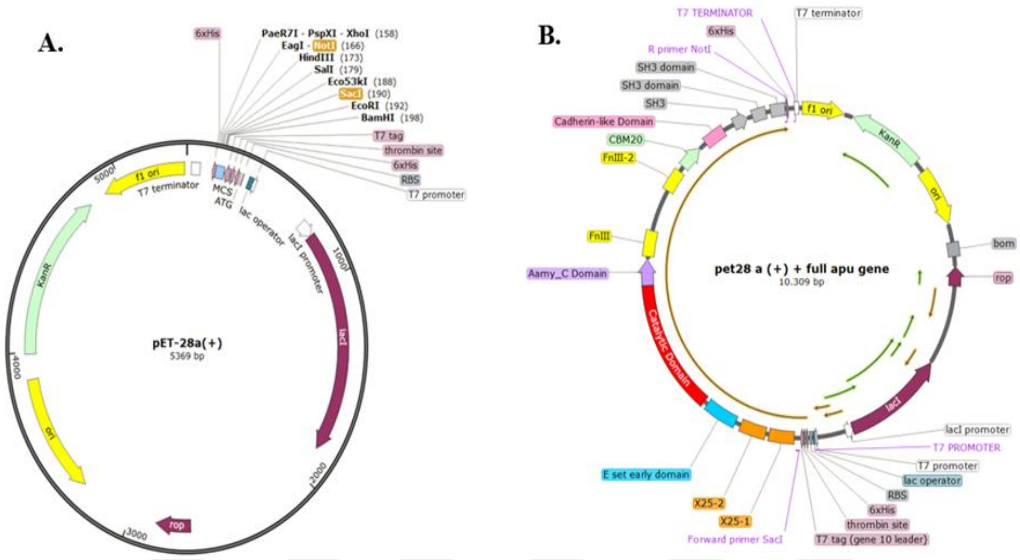

Figure 2.1: Structure of pET-28 a (+) expression vector and the constructed plasmid. **A.** pET-28 a (+) expression vector. **B.** Constructed plasmid that contains full lenght *apu* gene.

In the study, *E. coli* BL21 (DE3) strain was chosen as a suitable host for the pET expression systems. The strain is modified with insertion of achromosomal copy of the T7 RNA polymerase gene. The gene is under control of the lacUV5 promoter which is a mutant lac promoter. Using allolactose or IPTG for the transcription of the target protein encoding gene allows transcription of the T7 RNA polymerase gene at the same time. Also, BL21 strains have some advanced properties for expression of heterologous proteins. These strains have mutation their *lon* and *ompT* gene. *lon* and *ompT* gene encode cytoplasmic and outer membrane protease and mutations of these genes both provide accumulation of heterologous protein in cytoplasm and decrease rate of degredation during purification processes (Novagen, 2003).

2.2 Methods

The method part of the study is divided into three parts. The cloning of truncated *apu* genes into pET-28 a (+) expression vector is explained in the first part. Recombinant expression and purification of TbbApu and its truncated variants are explained in the second part and biochemical characterisation of the enzymes are explained in the third part.

2.2.1 Cloning of truncated *apu* genes

2.2.1.1 Amplification of truncated *apu* genes

To obtain truncated *apu* genes which are *tbbApuΔX25-1-SH3*, *tbbApuΔX25-2-SH3*, *tbbApuΔX25-1-CBM20* and *tbbApuΔX25-2-CBM20* PCR method was chosen.

For the amplification of the genes, full-lenght *apu* gene that is cloned into pET-28 a (+) expression vector was used as template; also, specifically designed forward and reverse primers that have *Sac*I and *Not*I restriction sites at the 5' ends respectively were designed for amplification of truncated *apu* genes (Table 2.1).

Table 2.1: Forward and reverse primers of truncated *apu* genes.

Gene name	Primers	Sequence
tbbApuΔX25-1-SH3	Forward primer	5' GCGAGCTCTCTACAAAATATACACCAATTCCG 3'
	Reverse primer	5'TTGCGGCCGCGGCAGTCACAATGCCATAATT 3'
tbbApuΔX25-2-SH3	Forward primer	5' GGGAGCTCACAGATTACAATCCACCTCTCAC 3'
	Reverse primer	5'TTGCGGCCGCGGCAGTCACAATGCCATAATT 3'
tbbApuΔX25-1-CBM20	Forward primer	5' GCGAGCTCTCTACAAAATATACACCAATTCCG 3'
	Reverse primer	5' TAGCGGCCGCAGGTATTATATCAGGTGTAGC 3'
tbbApuΔX25-2-CBM20	Forward primer	5' GGGAGCTCACAGATTACAATCCACCTCTCAC 3'
	Reverse primer	5' TAGCGGCCGCAGGTATTATATCAGGTGTAGC 3'

For each genes, PCR reaction was set up as 50 µl and contents of the reaction mixtures are as follows: 1.5 µl of template DNA (40 ng/µl), 1 µl of 10 mM Phusion dNTP mixture (Thermo Scientific™, #F530N), 2.5 µl of 10 µM forward and reverse primer, 10 µl of 5x Phusion HF Buffer (Thermo Scientific™, #F518L), (2 U/µL) 0.5 µl of Phusion High-Fidelity DNA Polymerase (Thermo Scientific™, #F-530XL) and 32 µl

of distilled sterile water. Cycling reactions for each genes were adjusted as shown in Table 2.2.

Table 2.2: PCR cycling instruction.

Cycle step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	5 s	35
Annealing	55 °C	30 s	35
Extension	72 °C	101 s	35
Final Extension	72 °C	600 s	1

2.2.1.2 Agarose gel electrophoresis

After the PCR, the products were loaded into 0.7 % agarose gel to control of the amplification. To prepare 0.7 % agarose gel, 0.35 g of agarose was weighed and added into 50 ml of 1X TAE buffer whose ingredients is given in Appendix C . Then, it was boiled microwave oven at 540 W until agarose dissolved in the buffer. The liquid gel was poured onto the electrophoresis plate and the comb was placed. After solidification of the gel, it was placed into a tank and 1X TAE buffer was poured into the tank until it reached to the top of the gel and the comb was removed. After that, 5 µl of sample was mixed with 1 µl of 6X EZ-Vision DNA dye (VWR® Life Science, #97064-190) and loaded into the wells of the gel. Also, to check length of the amplified PCR products, 1 µl of BioLab 1 kb DNA ladder (Thermo Scientific™, #SM0311), 1 µl of 6X EZ-Vision DNA dye, and 1 µl distilled water were mixed and loaded into the gel. The gel was run a constant voltage of 8-10 V/cm for 30 minutes and DNA samples were visualized under the UV-light.

2.2.1.3 DNA extraction from agarose gel

To eliminate non-specific DNA fragments, whole PCR products were loaded into 0.7 % agarose gel and were run in a 1X TAE running buffer. After the running, the gel put in UV-light and DNA fragments relevant with the truncated *apu* genes were cut from the gel using sterilized scalpel. Each excised agarose gel slice was put into a sterile 1.5 ml microcentrifuge tube. Then, the DNA fragments purified from the gel by PCR

clean-up Gel extraction kit (Macherey Negel, # 740609.50). Weight of each fragment were measured and 200 µl of Buffer NTI was added to the tubes in response to 100 mg gel pieces. Then, the samples were incubated for 10 minutes at 50 °C and vortex was applied briefly to the samples every 3 minutes during the incubation. Then, each sample was loaded into a NucleoSpin® Gel and PCR Clean-up Column and centrifugation was applied for 30 s at 11.000 x g. After the flow-through was discarded, 700 µl of Buffer NT3 was loaded to the column and the samples were centrifuged for 30 s at 11.000 x g at washing step. Following the flow-through discarding, to eliminate residual ethanol from Buffer NT3 the columns were centrifuged for 2 minutes at 11.000 x g. Then, each column was transferred to a new 1.5 ml of microcentrifuge tube and 30 µl of Buffer NE was added to the column to elute the DNA fragments from the columns. After that, the columns were incubated for 2 minutes at room temperature and they were centrifuged for 2 minutes at 11.000 x g. Purity of eluted DNA fragments were check on the 0.7 % agarose gel.

2.2.1.4 Double digestion of PCR products and pET-28 a (+) expression vector

For insertion of purified PCR amplicons to pET-28 a (+) expression vector both PCR amplicons and pET-28 a (+) expression vector digested with *SacI* (Thermo Scientific, #ER1135) and *NotI* ((Thermo Scientific, #ER0595) enzyme to create sticky ends. Before the digestion, the samples were run on the 0.7 % agarose gel to determine their amounts. After the amount determination, 30 µl of restriction reactions were prepared both each PCR amplicons and pET-28 a (+) expression vector. The reaction components and their amount can be shown in Table 2.3.

Table 2.3: Double digestion reaction components and their volume.

Component	Volume	Component	Volume
PCR product	20 µl	pET-28 a (+) vector	20 µl
Tango Buffer (10X)	3 µl	Tango Buffer (10X)	3 µl
SacI	1 µl	SacI	1 µl
dH_2O	6 µl	dH_2O	6 µl
Total Volume	30 µl	Total Volume	30 µl

The reactions were incubated for an hour at 37 °C. After the incubation, 3.75 µl of Tango Buffer (10X) (Thermo Scientific, #BY5) and 2 µl of *NotI* enzyme were added to each reaction and the reactions were incubated for an hour at 37 °C.

2.2.1.5 Clean-up of digested truncated *apu* genes and pET-28 a (+) vector for ligation

To remove restriction enzymes and preparation of the digested products to the ligation reaction clean-up was done by Macherey Negel PCR clean-up Gel extraction kit. First of all, volume of the reactions was adjusted to 100 µl with addition of 20 µl distilled water. Then, 100 µl of Buffer NTI was added to the reactions and each sample was loaded into a NucleoSpin® Gel and PCR Clean-up Column. Later, centrifugation was applied for 30 seconds at 11.000 x g. At the washing step, 700 µl of Buffer NT3 was loaded to the columns and they were centrifuged for 30 seconds at 11.000 x g. After the centrifugation, flow-through was discarded from each column and residual ethanol from Buffer NT3 was removed from the columns with centrifugation for 2 minutes at 11.000 x g. After that, each column was placed a 1.5 mL of microcentrifuge tube and 30 µl of preheated Buffer NE at 70 °C for 5 minutes was added to the columns. They were incubated at room temperature for 2 minutes. After the incubation, they were centrifuged for 2 minutes at 11.000 x g to elute DNA from the silica membrane. The amount of the digested truncated *apu* genes and pET-28 a (+) expression vector were check on the 0.7 % agarose gel.

2.2.1.6 Ligation of pET-28 a (+) vector and truncated *apu* genes

To join cohesive ends of truncated *apu* genes which are *tbbApuΔX25-1-SH3, tbbApuΔX25-2-SH3, tbbApuΔX25-1-CBM20* and *tbbApuΔX25-2-CBM20* and pET-28 a (+) expression vector ligation reactions were set up. Insert:vector ratio of the reactions were adjusted as 3:1 and 5:1 and the required insert DNA mass for each reaction was calculated using Ligation calculator (NEB) online web tool. After that, the ligation reaction between truncated *apu* genes and pET-28 a (+) were prepared according to Table 2.4, Table 2.5, Table 2.6 and Table 2.7.

Table 2.4 : *tbbApuΔX25-2-SH3*-pET-28 a (+) ligation reaction ingredients and their volumes.

	3:1 (insert:vector)		5:1 (insert:vector)	
Ligation Reaction	Component	Volume	Component	Volume
tbbApuΔX25-1-SH3-pET-28 a (+)	Vector DNA	7 µl	Vector DNA	5 µl
	Insert DNA	9.38 µl	Insert DNA	11.2 µl
	T4 DNA Ligase Buffer (10X)	2 µl	T4 DNA Ligase Buffer (10X)	2 µl
	T4 DNA Ligase	1 µl	T4 DNA Ligase Buffer	1 µl
	dH_2O	0.62	dH_2O	0.8 µl
	Total Volume	20 µl	Total Volume	20 µl

Table 2.5 : *tbbApuΔX25-1-SH3*-pET-28 a (+) ligation reaction ingredients and their volumes.

	3:1 (insert:vector)		5:1 (insert:vector)	
Ligation Reaction	Component	Volume	Component	Volume
tbbApuΔX25-1-SH3-pET-28 a (+)	Vector DNA	6 µl	Vector DNA	4 µl
	Insert DNA	10 µl	Insert DNA	12.2 µl
	T4 DNA Ligase Buffer (10X)	2 µl	T4 DNA Ligase Buffer (10X)	2 µl
	T4 DNA Ligase	1 µl	T4 DNA Ligase Buffer	1 µl
	dH_2O	-	dH_2O	0.8 µl
	Total Volume	20 µl	Total Volume	20 µl

Table 2.6 : *tbbApuΔX25-1-CBM20*-pET-28 a (+) ligation reaction ingredients and their volumes.

	3:1 (insert:vector)		5:1 (insert:vector)	
Ligation Reaction	Component	Volume	Component	Volume
tbbApuΔX25-1-SH3-pET-28 a (+)	Vector DNA	7 µl	Vector DNA	5 µl
	Insert DNA	8.47 µl	Insert DNA	10.05 µl
	T4 DNA Ligase Buffer (10X)	2 µl	T4 DNA Ligase Buffer (10X)	2 µl
	T4 DNA Ligase	1 µl	T4 DNA Ligase Buffer	1 µl
	dH_2O	1.53 µl	dH_2O	1.95 µl
	Total Volume	20 µl	Total Volume	20 µl

Table 2.7 : *tbbApuΔX25-2-CBM20*-pET-28 a (+) ligation reaction ingredients and their volumes.

	3:1 (insert:vector)		5:1 (insert:vector)	
Ligation Reaction	Component	Volume	Component	Volume
tbbApuΔX25-1-SH3-pET-28 a (+)	Vector DNA	6 µl	Vector DNA	4 µl
	Insert DNA	10 µl	Insert DNA	11.24 µl
	T4 DNA Ligase Buffer (10X)	2 µl	T4 DNA Ligase Buffer (10X)	2 µl
	T4 DNA Ligase	1 µl	T4 DNA Ligase Buffer	1 µl
	dH_2O	1 µl	dH_2O	1.76 µl
	Total Volume	20 µl	Total Volume	20 µl

The prepared reactions were incubated for 16 hours at 15 °C. After the incubation, the ligation reactions were inactivated at 65 °C for 10 minutes to inactivate T4 DNA ligase (BioLabs, # M0202S).

2.2.1.7 Competent cell preperation

1.5 µl of stock *E. coli* BL21 (DE3) strain was streaked on LB agar plates and incubated at 37 °C for 16 hours. After that, a colony was picked and inoculated into a 5 ml LB Broth at 200 rpm and 37 °C for 16 hours. After the incubation, pre-culture was added into a 100 ml LB Broth and it was incubated at 200 rpm and 37 °C until $O.D._{600}$ value was reach between 0.4 and 0.5. Then, the cell culture was transferred into pre-chilled falcon tubes and it was incubated into ice for 10 minutes. Later, it was centrifuged at 5.000 rpm for 10 minutes at 4 °C and supernatant part was removed. After that, pellet was dissolved with 30 ml of pre-chilled solution which consists of 80 mM $MgCl_2$ and 20 mM $CaCl_2$ and it was incubated into ice for 5 minutes. Later, it was centrifuged at 5.000 rpm for 10 minutes at 4 °C and supernatant was removed. Pellet was dissolved again 30 ml of pre-chilled solution that contains of 80 mM $MgCl_2$ and 20 mM $CaCl_2$ and it was incubated into ice for 20 minutes. Then, it was centrifuged at 5.000 rpm for 10 minutes at 4 °C. Supernatant was removed and pellet was dissolved with 1 ml solution which consists of 15 % glycerol and 100 mM $CaCl_2$ and aliquot as 100 µl for the usage.

2.2.1.8 Transformation of the constructed plasmid to *E. coli* BL21 (DE3) cells

After the ligation process, 10 µl of each ligation reaction was transformed into 100 µl competent *E. coli* BL21 (DE3) cells. The cells were incubated on ice for 30 minutes. Then, they were put at 42 °C for 2 minutes than they were transferred immediately on ice for 2 minutes. After that, 1 mL of LB media was added to each cell and they were incubated at 200 rpm for an hour at 37 °C and centrifugation was applied at 13.000 x g for 5 minutes. After the supernatant removal, pellets were dissolved in 100 µl of LB media. The suspended cells were spread on LB Broth with agar containing 40 µg/mL kanamycin and they were incubated at 37 °C for 16 hours.

2.2.1.9 Selection of positive colonies after the transformation

Positive colony selection after the transformation was done with Pullulan Red Reactive (PRR) agar plate. To prepare PRR agar plate, 5 mg PRR was dissolved in 2 ml distilled

water. Then, it was added into 18 ml of melted LB agar containing 40 µg/mL kanamycin and 1 µM IPTG. Then, the prepared PRR agar medium solidified in a petri plate. After that, the half of the colonies were taken and spread on the PRR plates and they were incubated at 37 °C for 16 hours. After the incubation, the plates were put at 60 °C for 4 hours to burst the cells and allow to give reaction of the expressed truncated amylopullulanase enzymes. After the incubation, colonies which gave clear zone were chosen as positive colonies. After the determination of positive colonies with PRR agar, constructed plasmid length of the positive colonies was checked with plasmid isolation and fast digestion of the isolated plasmids. For this purpose, the another half of the colonies were inoculated into 5 ml of LB Broth with agar containing 40 µg/mL kanamycin and they were incubated at 200 rpm for 16 hours at 37 °C. After the incubation, 3 ml were taken from each cell culture and plasmid isolation that was done using High Pure Plasmid Isolation Kit (Roche, 11754777001) was performed. For this purpose, the cell cultures were centrifuged at 13.000 rpm for 10 minutes. After that, the supernatant was removed and the pellets were dissolved with 250 µl of Suspension Buffer + RNase. Later, 250 µl of Lysis Buffer was added to each tube and mixing was applied with inversion of the tubes 6 to 8 inversion. Then, the samples were incubated for 5 minutes at room temperature. After the incubation, 350 µl of pre-chilled Binding Buffer was added and mixing was provided with inversion of the tubes 3 to 6 times. Then, the samples were incubated on ice for 5 minutes. Then, they were centrifuged at 13.000 x g for 10 minutes. After the centrifugation, supernatant parts were taken and they were loaded to a High Pure Filter Tube. Centrifugation was applied at 13.000 x g for a minute. After discard the flow through, 500 µl of Wash Buffer I was added to the tube to eliminate high nuclease content and they were centrifuged at 13.000 x g for a minute. Following the Wash Buffer I, 700 µl of Wash Buffer II was added to the samples and they were centrifuged again at 13.000 x g for a minute. Before the elution of the DNA from the filter tube, to remove residual wash buffers dry spinning was applied to the samples at 13.000 x g for 2 minutes. Then, each filter tube was inserted to a 1.5 ml microcentrifuge tube and 50 µl of Elution Buffer was added. After 2 minutes incubation, to obtain DNA centrifugation was applied at 13.000 x g for 2 minutes. Following the plasmid isolation, the linearization of the isolated plasmids was done to determine the length of the plasmids. For this purpose, fast digestion was chosen and 20 µl of the fast digest reaction that consist of 3 µl of the isolated plasmid, 2 µl of 10X FastDigest Buffer (Thermo Scientific, #B64), 1 µl of FastDigest *SacI*

(Thermo Scientific, #FD1133) and 14 µl of distilled water was set up for each isolated plasmids. After that, the reactions were incubated for 30 minutes at 37 °C. After the incubation, both each isolated plasmids and their fast digestion reaction were loaded to 0.7 % agarose gel and run at 90 V for 30 minutes. After the control of length of the constructed plasmids, glycerol stocks from the remaining 2 ml of the cell cultures that contain right length plasmids were done. For this purpose, the cell cultures were centrifuged at 13.000 rpm for 10 minutes and pellet were dissolved with 164.7 µl of LB medium and 35.3 µl of 85 % glycerol. Also to ensure pullulanase and α-amylase activity of the selected colonies were checked with PRR and starch agar plates. For this purpose, 1.5 µL from the selected colonies were inoculated into a LB Broth with agar that contains 40 µg/mL kanamycin, 1 % PRR and a LB Broth with agar that contains 40 µg/mL kanamycin, 1 % soluble starch and the plates were incubated at 37 °C for 16 hours. Then, the plates were incubated at 60 °C for 4 hours for distruption of the cells and showing activity of the enzymes. After the incubation, clear zone formation can be observed directly on LB Broth with agar that contains 40 µg/mL kanamycin, 1 % PRR; however, the plates which contains LB Broth with agar that contains 40 µg/mL kanamycin, 1 % soluble starch were treated with 2 mL of lugol to observation clear zone formation.

2.2.2 Recombinant protein expression and purification

2.2.2.1 Recombinant protein expression

To check expression of the constructed amylopullulanases which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 1.5 µl of stock culture of the enzymes was streaked on LB Broth with agar that contains 40 µg/mL kanamycin and they were incubated at 37 °C for 16 hours. After the incubation, one colony from each plate was taken and inoculated into on 20 mL Magic Media containing 40 µg/mL kanamycin at 180 rpm for 16 hours at 37 °C. After the incubation, the cultures were centrifuged at 9.000 rpm for 30 minutes at 4 °C and cell pellets were dissolved in 1 mL of 100 mM potassium phosphate buffer (pH 6.5). Then, the suspended cell pellets were sonicated (Bandelin SonoPuls) 10 times with 10 s on / 20 s off cycle and were centrifuged at 9.000 rpm for 30 minutes at 4 °C. Later, the supernatants was checked by SDS polyacrylamide gel electrophoresis to control the expression.

After the expression control, to do recombinant protein expression 1.5 µl of stock culture of TbbApu and its variants was streaked on LB Broth with agar containing 40 µg/mL kanamycin and they were incubated at 37 °C for 16 hours. Then, one colony from each plate was selected and inoculated into 5 ml 40 µg/ml kanamycin containing LB broth at 180 rpm for 16 hours at 37 °C. Later, each 5 mL starter culture was transferred to 100 mL of Magic Media which contains 40 µg/mL kanamycin and they were incubated at 180 rpm for 16 hours at 37 °C. After the incubation, cell cultures were centrifuged at 9.000 rpm for 30 minutes at 4 °C and cell pellets were stored at -20 °C for further experiments.

2.2.2.2 Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) is a protein purification method and it is based on the interaction between recombinant protein that has a short peptide affinity tag and a transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) that is chelated to a solid-phase support (Andjelković et al., 2017). In this study, pET-28 a (+) expression vector was used to obtain N- and C- terminally 6xHis-tagged of recombinant TbbApu and its truncated variants and HisTrap™ HP column (Cytiva 17-5247-01) which is prepacked with precharged Ni Sepharose™ was preferred to purify the recombinant histidine-tagged proteins. In the procedure, induced cell pellets were dissolved with 6 mL of pre-chilled binding buffer whose ingredients was given in Appendix C and cell suspensions were sonicated 9 times with 20 s on / 20 s off cycle. After the sonification, sonicated cell suspensions were centrifugated at 9.000 rpm for 30 minutes at 4 °C to remove the cell debris and purification was performed with cell lysate obtained as a result of the centrifugation. Before loading of the cell lysates to the column, the column was washed with 3 column volumes of distilled water and equilibrated with 5 column volumes of binding buffer. Then, cell lysates were applied to the column and the column was washed with 4 column volumes of binding buffer, 2 column volumes of 20 mM imidazole, 40 mM of imidazole, 60 mM of imidazole, 80 mM of imidazole containing washing buffers whose compositions given in Appendix C to eliminate non-specific bindings. The proteins were eluted with respectively with 2 column volumes of 100 mM, 200mM, 250 mM imidazole containing elution buffers which are given in Appendix C and 5 column volumes of 500 mM imidazole containing elution buffer. The purification result was checked by SDS polyacrylamide gel electrophoresis.

2.2.2.3 Ion exchange chromatography

Ion exchange chromatography (IEX) was applied after the IMAC to obtain targeted protein in high purity. In IEX is rely on reversible interaction with charged samples to oppositely charged groups attached to an insoluble matrix (Cummins et al., 2017). In this study, samples which were obtained observed as a result of IMAC purification were taken in 20 mM L-histidine buffer (pH 6.5) by ultrafiltration. In this pH targeted proteins gain positively charged and will bind to HiTrap Q HP (Cytiva 17115301) anion exchanger. In the procedure, before loading of the sample to the column, the column was washed with 10 column volumes of distilled water, 5 column volume of start buffer and 5 column volume of elution buffer whose composition given in Appendix C. Then, the column was equilibrated with 10 column volumes of start buffer. Then, the samples were applied to the column and the column was washed with 5 column volume of start buffer, 0.1 M, 0.2 M, 0.3 M and 0.4 M NaCl containing washing buffers where the compositions given Appendix C to eliminate protein contaminants. The proteins were eluted with 5 column volume of elution buffer. The purification result was checked by SDS polyacrylamide gel electrophoresis.

2.2.2.4 Ultrafiltration

After the IEX, pure protein fractions were collected and the protein samples were taken in 100 mM potassium phosphate buffer (pH 6.5) by ultrafiltration (UF). For this purpose, ultrafiltration tubes (Amicon®, # UFC901008) with 100 kDa molecular weight cut-off value were used. First of all, two times 10 ml of distilled water and 10 ml of 100 mM potassium phosphate buffer (pH 6.5) which is given in Appendix C were passed from the UF tubes by centrifugation at 3.000 g at 4 °C. Then, collected samples were loaded to the UF tubes and total 30 ml of 100 mM potassium phosphate buffer (pH 6.5) were passed from the UF tubes by centrifugation at 3.000 g at 4 °C to exchange buffer of the samples. After that, 2-4 ml samples were collected from the UF tubes and stored at 4 °C for further processes.

2.2.2.5 Heat purification

Heat purification was chosen as a third step to obtain TbbApu and its variant`s high purity. TbbApu which is an extremozyme is stable at high temperatures. Thus, it can resist high temperature but other protein contaminants that came from expression host cannot resist and denatured. To do this elimination, samples which were obtained as a

result of IEX purification which were taken into 100 mM potassium phosphate buffer (pH 6.5) by UF were incubated at 60 °C for an hour. Then, centrifugation was applied at 11.000 rpm for 20 minutes for precipitation of denatured protein. After the centrifugation, supernatant part of the samples was taken into a new 1.5 mL of Eppendorf tubes. The purification result was checked by SDS polyacrylamide gel electrophoresis.

2.2.2.6 SDS polyacrylamide gel electrophoresis and zymogram analysis

Clear lysates, the obtained fragment as a result of IMAC, IEX and heat purification were electrophoresed on polyacrylamide gel. Stacking gel concentration was adjusted as 5 %, while resolving gel concentration was prepared as 10 %. Components of the gels and buffers were given in Appendix C. For the preparation of the samples, 20 µl of samples were mixed with 5 µl of sample loading buffer where the ingredients were given in Appendix C. Then, they were incubated at 95 °C for 15 minutes. After the incubation, the samples were loaded to the wells of the gels. Also, 3 µl of molecular weight marker (PageRuler^{TM}, #26619) was loaded to the gel without heat denaturation. Electrophoresis was applied at 90 V until the proteins entered to the resolving gel. After that, electrophoresis was carried out at 120 V until the front dye reach the bottom of the gel. After the electrophoresis, stacking gel were removed and resolving gel was stained with staining solution whose composition was given in Appendix C for 2 hours. Later, destaining was applied with destaining solution for an hour to visualize protein bands in the gel. Also, zymogram analysis was done to detect activity of the each purified enzyme. Zymography is based on the activity detection with degredation of a substrate which is impregnated to SDS polyacrylamide gel by non-reduced hydrolytic enzyme (Leber and Balkwill, 1997). In this study, 1 % red pullulan (Megazyme, S-RPUL) and 1 % soluble starch (SIGMA-ALDRICH, S9765) containing two different 8% native SDS PAGE whose preparation were given in Appendix C were prepared to confirm pullulanase and α-amylase activity of full length and truncated TbbApu variants. The purified enzymes were mixed with non-reducing protein sample loading buffer and loaded to the gels without heat denaturation. Electrophoresis was applied at 40 V until proteins entered to the resolving gels. Later, electrophoresis was performed at 190 V until the front dye reach the bottom of the gel. End of the electrophoresis, stacking gel part was removed and the gels were incubated in 100 mM pH 6.5 phosphate buffer at 65 °C an hour. The results were observed as clear zone formation

after the incubation of 1 % red pullulan containing 8 % native SDS PAGE and after the lugol treatment of 1 % soluble starch containing 8 % native SDS PAGE.

2.2.2.7 Bradford assay

The concentration of the purified proteins was determined with Bradford method (Bradford, 1969). In this method, BSA was used as standard at 0.125 mg/ml, 0.250 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.5 mg/ml and 2 mg/ml concentration.

195 µL of Bradford reagent (Sigma, #B6916) was mixed with 5 µL of sample and 5 µL of standards. After that, they were incubated at room temperature for 5 minutes. Absorbance measurement were done at 595 nm. The measurements were replicated three times.

2.2.3 Biochemical characterisation of TbbApu and its truncated variants

2.2.3.1 Enzyme activity assay

The α-amylase and pullulanase activity of TbbApu and its variants were determined according to Bernfeld (1955) using modified dinitrosalicyclic acid (DNSA) reagent. Pullulan (SIGMA, #P4516) and soluble starch (SIGMA, S9765) were used as a substrate for the measurement pullulanase and α-amylase activity of the enzymes respectively and two activities were quantified by the measurement of released reducing sugar during the reaction. The reaction mixture of TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 consists of 0.5 µM enzyme and 1 % pullulan and 1 % soluble starch which were separately dissolved in potassium phosphate buffer in proper pH. The reactions were proceeded for 20 minutes. One unit (U) of the enzyme is defined as the amount of the enzyme that liberates 1 µmol of reducing sugars as maltose per minute under the assay conditions.

2.2.3.2 Effect of temperature and pH on the activity

To determine the optimum pH for pullulanase and α-amylase activity of TbbApu and its truncated variants, reactions were set up with 100 mM buffer solutions with pH value between 3 and 11 (100 mM citrate phosphate buffer pH 3, 4, 5; 100 mM potassium phosphate buffer pH 6, 6.5, 7 and 7.5; 100 mM Tris-HCI buffer pH 8,9; 100 mM glycine-NaOH pH 10,11 whose ingredients are given in Appendix C). Also, to determine the optimum temperature of the enzymes, activity assays were proceeded

between 30-100 °C with 5-10 °C of intervals. Relative activities were determined according to the activity assay method.

2.2.3.3 Effect of modulators

To determine the effect of metals salts which are $MgCI_2$, $FeCI_3$, KCI, $CaCI_2$, $ZnSO_4$, $AlCI_2$, $CuSO_4$, $NiCI_2$, $MnCI_2$, $BaCI_2$, $CoCI_2$, $CrCI_3$, detergents that are sodium dodecyl sulfate (SDS), Tween 20, Tween 80, Triton X-100, cetrimonium bromide (CTAB), polyethylene glycol (PEG), inhibitors which are ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA) and dithiothreitol (DTT) and organic solvents which are isopropanol, acetone, hexane, glycerol, ethanol, methanol, butanol, dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) on the pullulanase and α-amylase activity of the TbbApu and its truncated variants, they were added to the reaction mixture with a final concentration of 5 mM for metal salts, inhibitors and detergents except for PEG, 20 % and 50 % for organic solvents and 2.5 % for PEG. Then, relative activities were determined according to the activity assay method.

2.2.3.4 pH, organic solvent, and thermal seffect on stability

To define the pH, temperature and organic solvent effects on the stability of TbbApu and its truncated variants, the enzymes were incubated with buffer solutions having different pH values (100 mM citrate phosphate buffer pH 3, 4, 5; 100 mM potassium phosphate buffer pH 6, 6.5, 7 and 7,5; 100 mM Tris-HCI buffer pH 8,9; 100 mM glycine-NaOH pH 10,11), varying temperatures (30- 100 °C with 10 °C increments) and 20 % and 50 % organic solvents (isopropanol, acetone, ethanol, methanol, butanol, hexane and glycerol) at room temperature for 24 hours. After that, the samples that were treated with varying pH and organic solvent were diluted at a 1:3 ratios. Then, standard activity assay was performed for each recombinant enzyme.

2.2.3.5 Kinetic assay

Kinetic parameters related to pullulanase and α-amylase activity of TbbApu and six truncated variants were determined by mixing 0.5 µM enzyme with pullulan and starch concentration ranging from 4 to 50 mg/mL and 1 to 30 mg/mL respectively in 100 mM potassium phosphate buffer at optimum reaction temperature value for each enzyme for 22 minutes. One unit (U) of the enzyme is defined as the amount of the enzyme

that liberates 1 μmol of reducing sugars as maltose per minute under the assay conditions.

2.2.3.6 Raw starch adsorption

To detect raw starch binding capacity of TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20, 1 mg/ml enzymes were incubated with different concentrations of raw starch (Sigma Aldrich, #85650) that is between 10-20-40-60-80-100 and 120 mg/ml at 4 °C for an hour on rotary shaker. After that, the samples were centrifuged at 12.000 x g for 5 minutes at 4 °C for prepicipation of raw starch bounded enzymes. After centrifugation, 2 % pullulan was added to the supernanant parts and standard activity assay was performed for each sample.

2.2.3.7 Analysis of end products by thin layer chromatography

Thin layer chromatography was applied for full length and truncated TbbApu to elucidate the released end product as a result of hydrolysis of soluble starch (10 mg/ml) and pullulan (10 mg/ml). Each reaction mixture that includes 1 μM enzyme, soluble starch (5 mg/ml) and pullulan (5 mg/ml) separately was incubated at optimum pH and temperature value for each enzyme for 24 hours. After the incubation, 1.5 μl of digestion products and 5 μg of the standards which are glucose, maltose, maltotriose (20 mM) were loaded on a TLC plate that is activated at 110 °C for 1h. The plates were run in n-butanol/ethanol/water (5:3:2) solvent system and dried for an hour at room temperature. After that, TLC plates were sprayed with ethanol containing H_2SO_4 (10 % by volume) and incubated at 90 °C for 15 minutes to visualize released mono and disaccharides end product.

3.RESULTS AND DISCUSSION

3.1 Cloning Truncated *apu* Gene of *Thermoanaerobacter brockii brockii*

In the previous studies, cloning of the full-length *apu* gene that is 5022 bp and encodes 1674 amino acids and expression optimization studies were done using four hosts which are *E. coli* BL21(DE3), *E. coli* C43 (DE3), *Rosetta 2* (DE3) pLysS and *Rosetta-gami 2* (DE3) pLysS and two expression vectors that are pET-28 a (+) and pET-26 b (+) by our group. Also, two C-term truncated variants of amylopullulanase enzyme which are TbbApuΔSH3 and TbbApuΔCBM20 were constructed to understand the effect of CBM20 and SH3 domains on the expression. As a result of the studies, *E. coli* BL21(DE3) and pET-28 a (+) were chosen as suitable host and expression vectors. In this study, together with TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, four N- and C-term truncated variants which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 of TbbApu were constructed to understand X25, CBM20 and SH3 domains effect on substrate binding, activity and stability of the enzyme. Amino acids sequence and isoelectric point of the constructed enzymes were defined from the *apu* gene with the ExPASy translate tool (http://web.expasy.org/translate/). The general features of TbbApu and its truncated variants can be seen in Table 3.1.

Table 3.1: TbbApu and its truncated variants properties.

Enzyme Name	Gene Size	Sequence lenght	Molecular weight	pI value
TbbApu	5022	1651	184.63	4.83
TbbApuΔSH3	4452	1461	163.8	4.60
TbbApuΔCBM20	3762	1231	138.3	4.59
TbbApuΔX25-1-SH3	4065	1355	152.36	4.63
TbbApuΔX25-2-SH3	3750	1250	140.4	4.61
TbbApuΔX25-1-CBM20	3375	1125	126.76	4.62
TbbApuΔX25-2-CBM20	3060	1020	114.82	4.59

The truncated amylopullulanase genes which are**:** ***tbbApuΔX25-1-SH3*** lacks 387 bp from 5' end and 570 bp from the 3′ end of *tbbApu*; ***tbbApuΔX25-2-SH3*** lacks 702 bp from 5' end and 570 bp from the 3′ end of *tbbApu*; ***tbbApuΔX25-1-CBM20*** lacks 387 bp from 5' end and 1260 bp from the 3′ end of *tbbApu*, ***tbbApuΔX25-2-CBM20*** lacks 702 bp and 1260 bp from the 3′ end of *tbbApu* were amplified by PCR method using designed specific primers (Table 2.1) which possess *SacI* and *NotI* restriction sites at the 5' ends of the forward and reverse and full-length *apu* gene as template DNA.

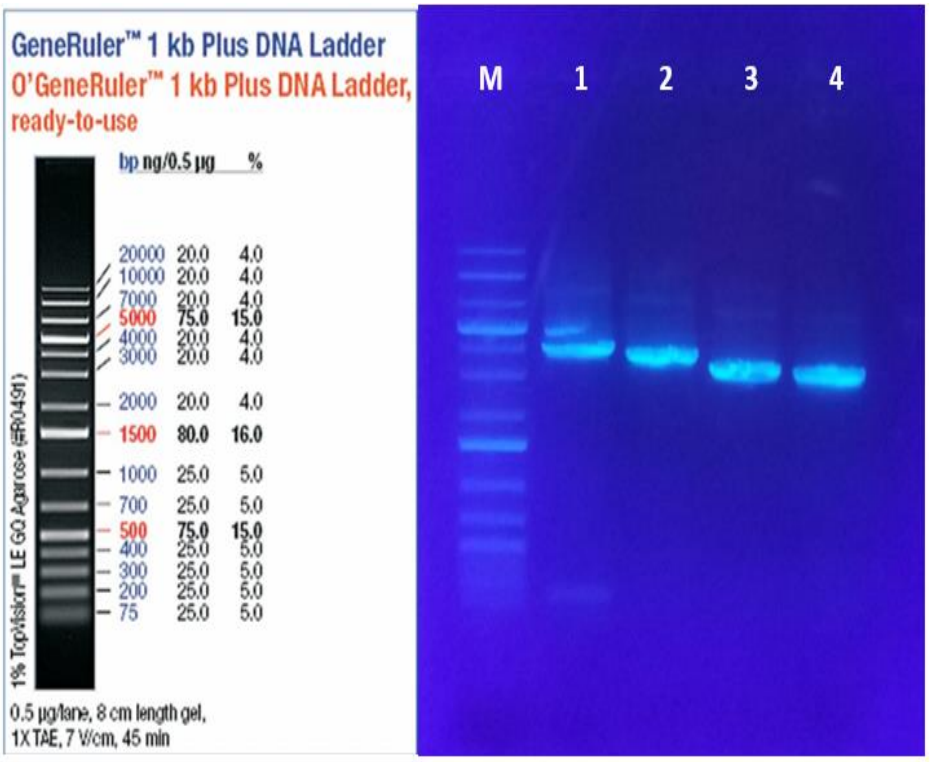

Figure 3.1: PCR amplification results. M: Gene Ruler^{TM} 1 kb Plus DNA ladder; Lane 1: *tbbApuΔX25-1-SH3*; Lane 2: *tbbApuΔX25-2-SH3*; Lane 3: *tbbApuΔX25-1-CBM20*; Lane 4: *tbbApuΔX25-2-CBM20*.

As can be seen in Figure 3.1, *tbbApuΔX25-1-SH3*, *tbbApuΔX25-2-SH3*, *tbbApuΔX25-1-CBM20*, and *tbbApuΔX25-2-CBM20* that are 4065, 3750, 3375 and 3060 bp respectively were successfully amplified by PCR method.

After that, to eliminate non-specific DNA fragments whole PCR reactions were loaded to 0.7 % agarose gel and DNA extraction from the gel was done. Later, both amplified truncated *apu* genes and pET-28 a (+) expression vector were cut with *SacI* and *NotI* restriction enzymes to create sticky ends. After the double digestion, clean-up was applied to the digested products to remove the enzymes and prepare the samples to the ligation reaction. Later, the products were loaded to 0.7 % agarose gel to determine the amount of the digested genes and pET-28 a (+) expression vector (Figure 3.2).

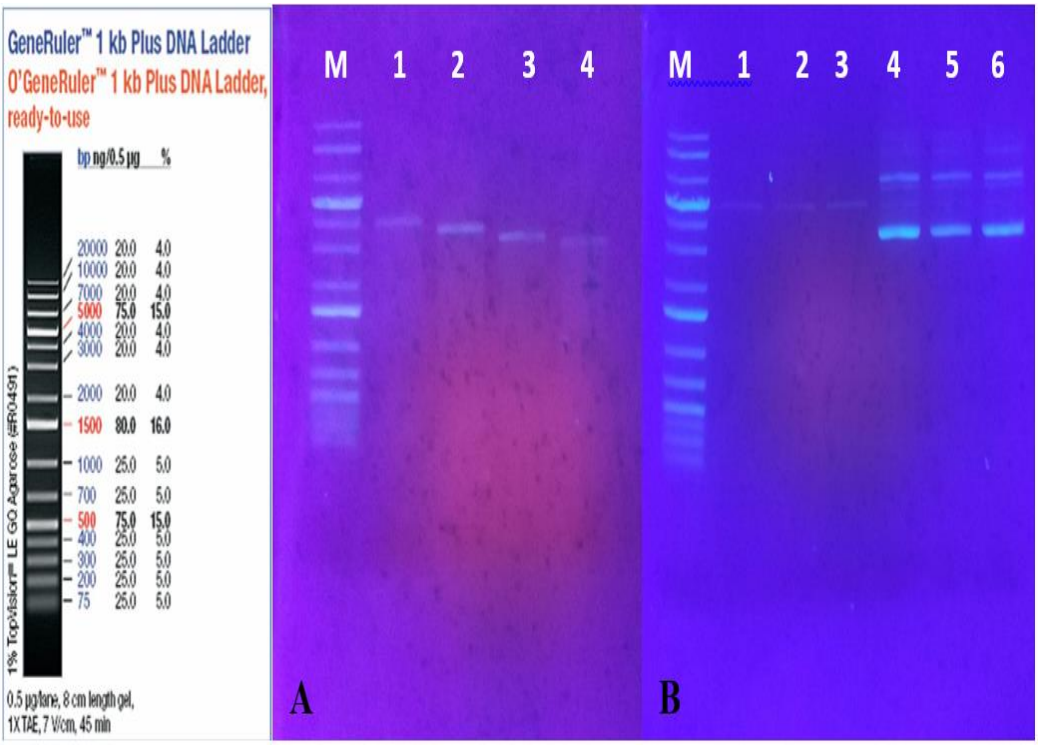

Figure 3.2: Double digestion results of truncated apu genes and pET-28 a (+) vector. **A.** M: Gene RulerTM 1 kb Plus DNA ladder; Lane 1: *tbbApuΔX25-1-SH3*; Lane 2: *tbbApuΔX25-2-SH3*; Lane 3: *tbbApuΔX25-1-CBM20*; Lane 4: *tbbApuΔX25-2-CBM20*. **B.** M: Gene RulerTM 1 kb Plus DNA ladder; Lane 1, Lane 2 and Lane 3: double digested pET-28 a (+) expression vectors; Lane 4, Lane 5, Lane 6: pET-28 a (+) expression vectors as control.

As a result of the agarose gel electrophoresis, the amount of double *digested tbbApuΔX25-1-SH3, tbbApuΔX25-2-SH3, tbbApuΔX25-1-CBM20, tbbApuΔX25-2-CBM20* and pET-28 a (+) expression vector was determined as 6.6 ng/µL, 8.3 ng/µL, 8.3 ng/µL, 6.6 ng/µL and 5.3 ng/µL. Then, according to these results, two sets of ligation reactions were set up with 3:1 and 5:1 insert: vector ratios.

3.2 Transformation and Selection of Positive Colonies with PRR Agar

After the ligation reactions were completed, the reactions were transferred into chemically competent *E. coli* BL21 (DE3) cells. The observed colonies after the transformation can be seen in Figure 3.3.

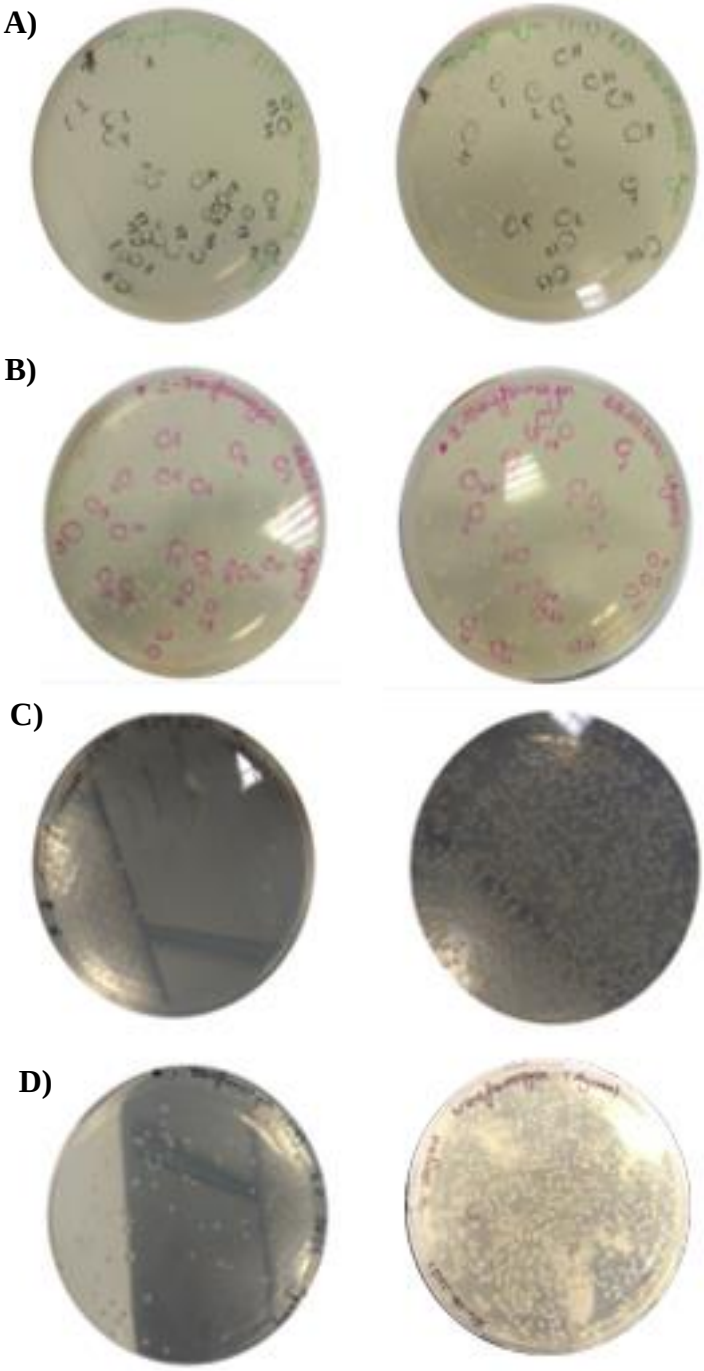

Figure 3.3: Observed colonies after the transformation. **A.** *E. coli* BL21 (DE3) colonies transformed with the plasmid pET-28 a (+) having *tbbApuΔX25-1-SH3* gene. **B.** *E. coli* BL21 (DE3) colonies transformed with the plasmid pET-28 a (+) having *tbbApuΔX25-2-SH3* gene. **C.** *E. coli* BL21 (DE3) colonies transformed with the plasmid pET-28 a (+) having *tbbApuΔX25-1-CBM20* gene. **D.** *E. coli* BL21 (DE3) colonies transformed with the plasmid pET-28 a (+) having *tbbApuΔX25-2-CBM20* gene.

To determine the positive colonies after the transformation, 79, 40, 36 and 64 colonies from the plates that have plasmid pET-28 a (+) having *tbbApuΔX25-1-SH3, tbbApuΔX25-2-SH3, tbbApuΔX25-1-CBM20* and *tbbApuΔX25-2-CBM20* respectively were selected and the half of the selected colonies were inoculated on PRR agar plates

containing 40 µg/mL kanamycin and 1 µM IPTG. IPTG was used to induction of T7lac promoter of pET-28 a (+) expression vector and kanamycin was used for the elimination of negative colonies (Novagen, 2003). Thus, only positive colonies that carry constructed plasmid can grow and express the truncated amylopullulanase enzymes. After the incubation of the PRR plates, the plates were put at 60 °C for 4 hours to burst the cells and the hydrolysis of Pullulan Red Reactive (PRR) from the truncated amylopullulanase enzymes. As can be seen in Figure 3.4, positive colonies gave the clear zone formation as a result of hydrolysis of PRR.

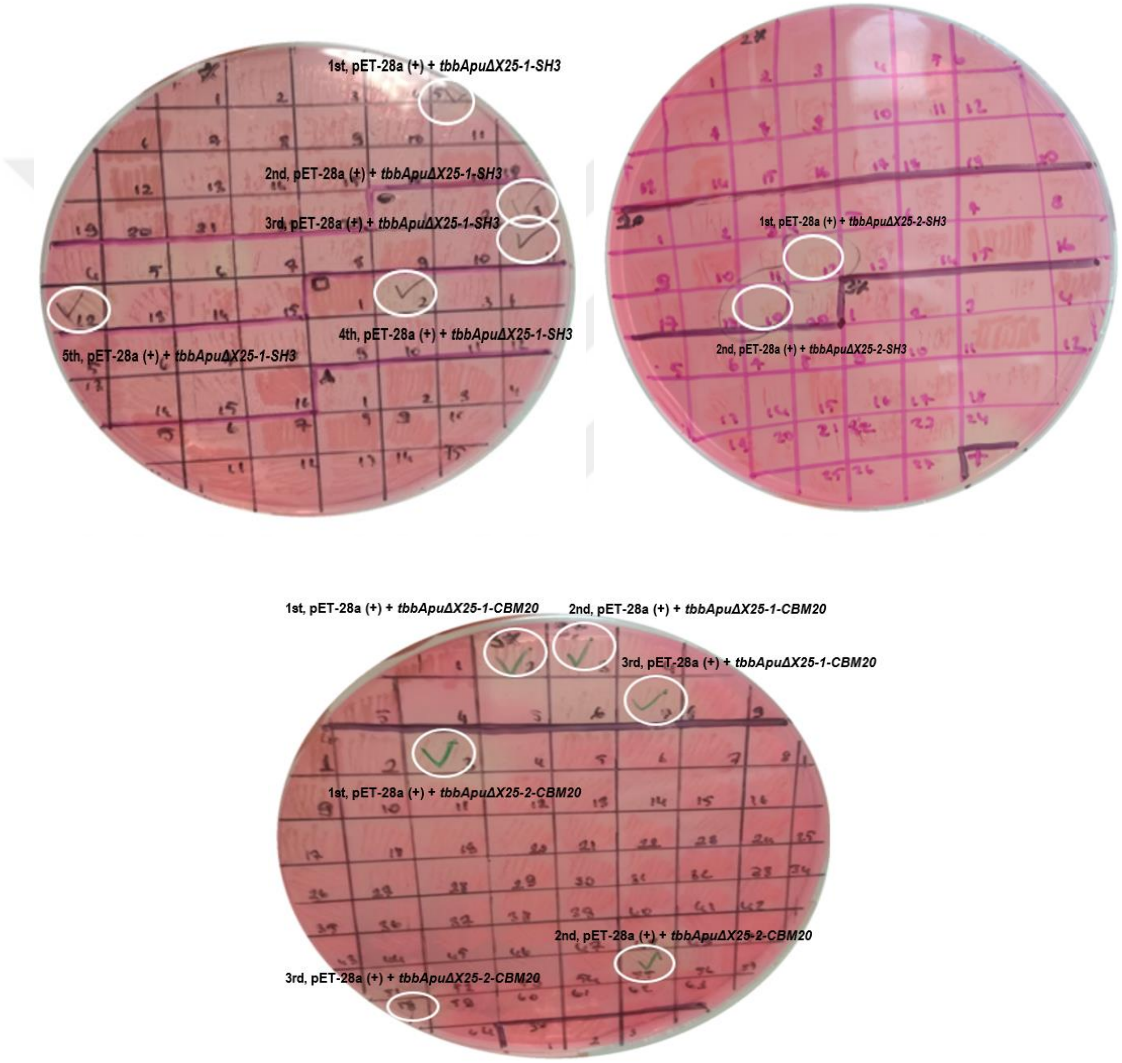

Figure 3.4: Control of the expression of truncated amylopullulanase expressions at the transformed cells in PRR Agar.

As can be seen in Figure 3.4, 5 positive colonies from the 79 colonies, 2 positive colonies from the 40 colonies, 3 colonies from the 36 colonies and 3 colonies from the 64 colonies were observed. The other half of the colonies that showed the clear zone formation at the PRR agar plate were inoculated into LB Broth for the plasmid

isolation to both check the positive colonies whether contain the constructed plasmid and make stock. Plasmid isolation was carried out and fast digestion was applied to the isolated plasmids with FastDigest *SacI* to check the length of the plasmids. Then, the results were checked on the 0.7% agarose gel (Figure 3.5).

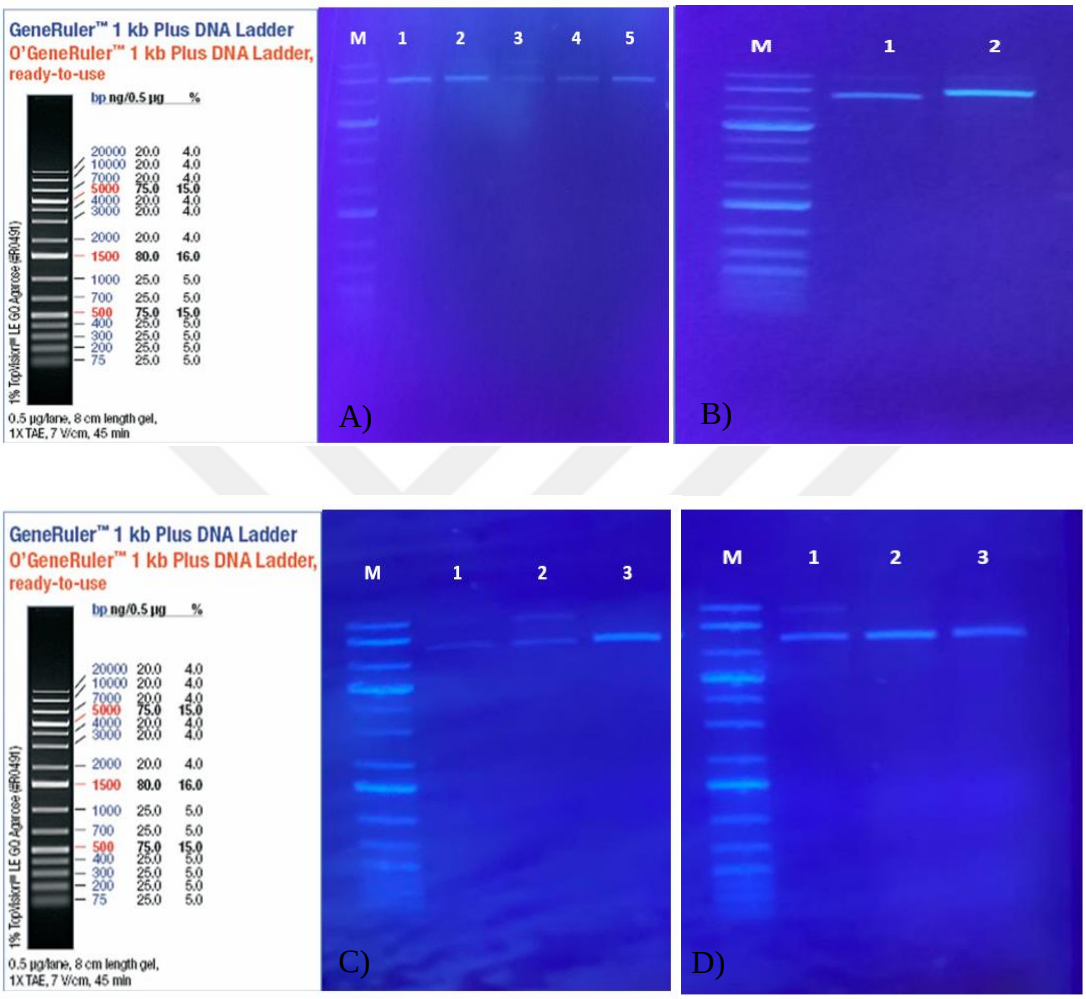

Figure 3.5: Fast digestion results of the isolated plasmids. **A.** M: Gene Ruler™ 1 kb Plus DNA ladder; Lane 1, Lane 2, Lane 3, Lane 4 and Lane 5: double digested plasmids with the plasmid pET-28 a (+) having *tbbApuΔX25-1-SH3* gene. **B.** Lane 1 and Lane 2, double digested plasmids with the plasmid pET-28 a (+) having *tbbApuΔX25-1-SH3* gene. **C.** M: Gene Ruler™ 1 kb Plus DNA ladder; Lane 1 and Lane 2: double digested plasmids isolated from selected 1st, 2nd and 3rd colony transformed with the plasmid pET-28 a (+) having *tbbApuΔX25-1-CBM20* gene respectively. **D.** Lane 1 and Lane 2: double digested plasmids isolated from selected 1st, 2nd and 3rd colony transformed with the plasmid pET-28 a (+) having *tbbApuΔX25-2-CBM20* gene respectively.

When looking at Figure 3.5, the length of the linearized plasmids is between 7.000 bp and 10.000 bp. The total length of pET-28 a (+) expression vector with *tbbApuΔX25-1-SH3* gene, *tbbApuΔX25-2-SH3*, *tbbApuΔX25-1-CBM20* and *tbbApuΔX25-2-CBM20* is 9434 bp, 9119 bp, 8744 bp and 8429 bp respectively. The result affirms that all of the selected colonies carry the pET-28 a (+) expression vector with the interested

truncated *apu* gene. After this verification, α-amylase and pullulanase activities of the cells that are selected as stock were checked on PRR and starch agar plates (Figure 3.6).

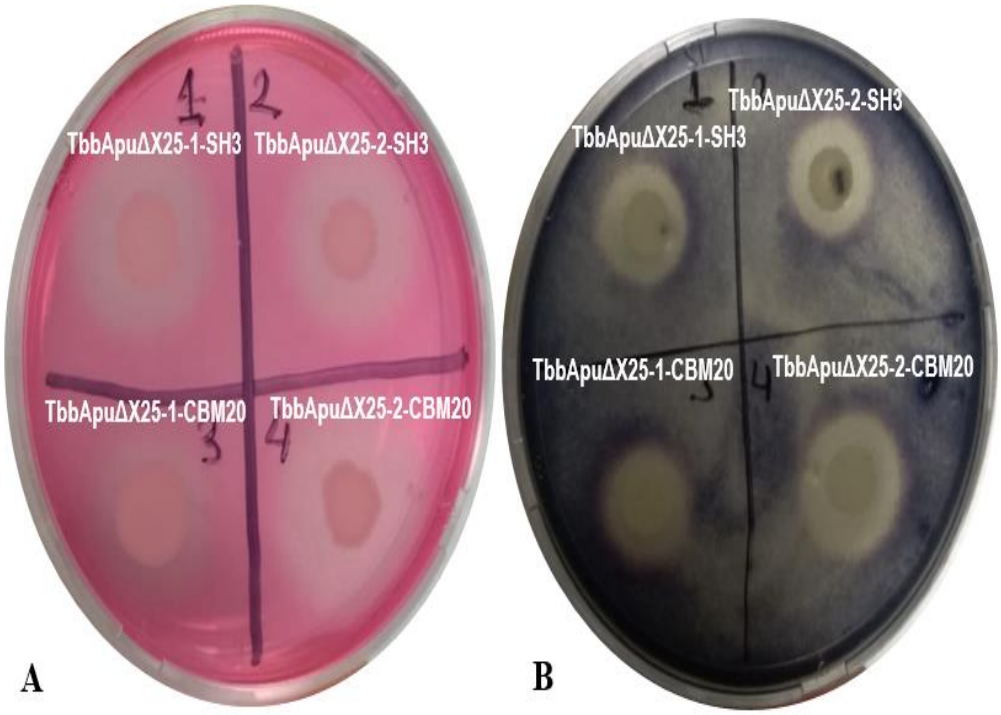

Figure 3.6: Activity assays of truncated amylopullulanases. **A.** Zone formation on PRR agar plate **B.** Zone formation on starch agar plate.

As can be seen in Figure 3.6, all of the truncated amylopullulanase variants which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 are expressed successfully in *E. coli* BL21 (DE3) and they show both pullulanase and α-amylase activity.

3.3 Expression, Purification and Zymogram Analysis of TbbApu and Its Truncated Variants

After the construction of the plasmids was done successfully, the expression of TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were checked in *E. coli* BL21 (DE3) host organism. As can be seen in Figure 3.7, the enzymes were expressed in E. coli BL21 (DE3) successfully. The bands related to TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3 were observed

between 180 and 130 kDa and the bands related to TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were observed between 130 and 100 kDa.

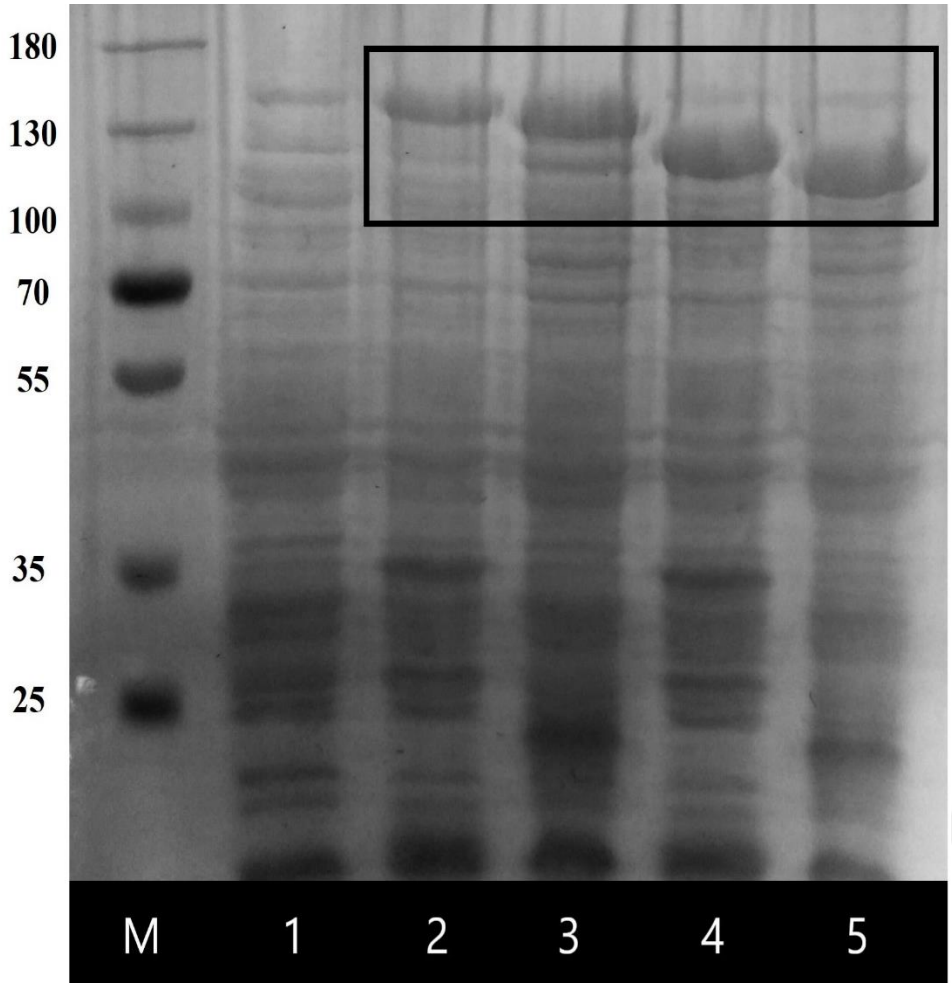

Figure 3.7: Expression results four N- and C-term truncated variants of TbbApu. M: Molecular weight marker; Lane 1: clear lysate of *E. coli* BL21 (DE3) having pET-28 a (+) expression vector; Lane 2: clear lysate of *E. coli* BL21 (DE3) having *tbbApuΔX25-1-SH3* gene ; Lane 3: clear lysate of *E. coli* BL21 (DE3) having *tbbApuΔX25-2-SH3* gene; Lane 4: clear lysate of *E. coli* BL21 (DE3) having *tbbApuΔX25-1-CBM20* gene ; Lane 5: clear lysate of *E. coli* BL21 (DE3) having *tbbApuΔX25-2-CBM20* gene.

Then, TbbApu, TbbApuΔSH3, TbbApuΔCBM20 and four N- and C-term truncated variants which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were produced in *E. coli* BL21 (DE3) host organism. After that, three-step purification was applied to obtain the enzymes with high purity. At the first step, an immobilized metal affinity chromatograph was applied using the HisTrap™ HP column and the purification was carried through the interaction between Ni^{2+} metals that are packaged to the column and 6xHis-tags where is N- and C- terminus of the recombinantly expressed TbbApu and its variants

(Andjelković et al., 2017). However, the enzymes could not be purified with high purity at the end of the process.

It can be seen in Figure 3.8, the bands of overexpressed TbbApu (184.63 kDa), TbbApuΔSH3 (163.8 kDa), TbbApuΔCBM20 (138.28 kDa), TbbApuΔX25-1-SH3 (152.49 kDa), TbbApuΔX25-2-SH3 (140.55 kDa) are between 180 kDa and 130 kDa and the bands of overexpressed TbbApuΔX25-1-CBM20 (126.89 kDa) and TbbApuΔX25-2-CBM20 (114.82 kDa) are between 130 kDa and 100 kDa. Together with these bands, there are some nonspecific bands throughout the gel. The reason for these observed non-specific bands can be the activation of the stress response pathways with the overexpression of the enzymes.

The original organism of amylopullulanase is anaerobic, thermophilic and gram-positive *Thermoanaerobacter brockii brockii;* however, the host organism for the heterologous amylopullulanase expression is facultatively anaerobic, mesophilic and gram-negative *E. coli* BL21 (DE3) (Zeikus et al., 1979; Jeong et al., 2009). *T. brockii brockii* and *E. coli* are evolutionary distant species and distant phylogenetic species generally have different codon usage patterns (Wang et al., 2018). These differences in the codon usage patterns cause the codon bias problem. AGG-AGA-CGA-CGG (Arg), CUA (Leu), AUA (Ile), CCC-CCU-CCA (Pro), UGU-UGC (Cys), ACA (Thr), UCA-AGU-UCG-UCC (Ser), GGA-GGG (Gly) are rare codons at *E. coli* (Kane, 1995). However, the amylopullulanase enzyme contains 36 arginine- 20 of them encoded by AGA codon, 7 of them encoded by AGG, 2 of them encoded by CGA- and 36 isoleucine encoded by AUA. With the overexpression of the amylopullulanase, depletion of rarely produced tRNAs that couple to rare codons occurs in the cytoplasm and these depletions can cause miss-translation of the overexpressed protein as a result of premature translational termination, frameshift formation, mistranslation amino acid substitutions. With the miss-translation, the expressed protein cannot fold correctly and the incorrect folding causes both losing functionalities of the expressed proteins and aggregation and inclusion body formation (Sørensen & Mortensen, 2005). Also, overexpression can cause depletion of host organism sources such as energy, amino acids, rare codons etc. and the organisms cannot carry out normal metabolic functions and the metabolic burden is observed. With both accumulation of misfolded proteins and metabolic burden event, *E. coli* activates some stress response pathways such as heat shock-like response, stringent response, SOS response and overlapping

stress response (Hoffmann & Rinas, 2004). Together with the activation of the pathways, the expression of the proteins that are in these pathways is realized such as chaperons ClpB, DnaK/J and GroEL/S, proteases Lon, ClpP, ClpC, HsIV (ClpY), HsIU, ClpQ and FtsH. According to research, some native *E. coli* proteins show high affinity to divalent cations such as nickel, cobalt, etc. and many of these proteins are stress-responsive proteins. Thus, they are copurified with the recombinantly expressed proteins during IMAC. These native binding proteins eliminate from the IMAC columns with high imidazole concentrations. To give an example, Fur, Crp, SlyD, ArgE, Cu/Zn-SODM and YodA proteins require higher than 80 mM imidazole concentration for the elution from the IMAC columns (Bolanos et al., 2006).

Another factor that activates these pathways may be due to amylopullulanase itself. Previous studies that are carried out on the recombinant expression of TbbApu showed the inclusion formation (Mumcu, 2018) of the enzyme and the reason for the inclusion body formation can be a domain of the enzyme. Amylopullulanase consists of seven domains and one of the domains which called the SH3 domain is located at the C terminus of amylopullulanase. SH3 domain is responsible for protein-protein interaction and the domain binds two motif classes which are Class I and Class II. While Class I consists of (R/K)xΦPxΦP where x and Φ show -any amino acid and hydrophobic amino acid respectively, Class II consists of ΦPxΦPx(R/K), Pro-X-X-Pro motif (Carducci, 2012). Besides these classes, SH3 domain can make interactions some unconventional motifs: ΦxxPxxP, PxxDY, RxxPxxxP, PxxxPR, PxRPx(R), RxxK consensus, RKxxYxxY. As a result of the examination of the amino acid sequence of amylopullulanase, the enzyme has Pro-X-X-Pro and some conventional motifs. While P-N-I-P (187-190), P-D-N-P (403-406), P-N-A-P (627-630) and P-G-M-P (752-755) are Pro-X-X-Pro motifs in the amylopullulanase, M-G-Y-P-G-M-P (749-755), P-A-G-D-Y, R-D-Y-K (145-148), R-G-I-K (489-492), R-Y-G-K (511-514) and R-E-D-K (781-784) are the unconventional motifs in the enzyme. Thus, the SH3 domain of the enzyme makes interactions with these motifs and in consequence of these interactions, aggregation and inclusion body formation can be observed from overexpressed amylopullulanase enzyme (Carbonell & Villaverde, 2002). As a result of inclusion body formation, some stress response pathways are activated, so the expression of the proteins that show high affinity to divalent metal ions natively in these pathways is realized.

Also, when looking at Figure 3.8, the purity of the enzymes was increased both by the truncation of the SH3 domain from the enzymes and reaching a smaller size. TbbApuΔX25-2-CBM20 has the lowest molecular weight obtained highly pure as a result of IMAC and heat treatment was sufficient to obtain the enzyme in high purity after the IMAC.

Anion exchange chromatography that was carried out using the HiTrap Q HP column was chosen as the second purification step to eliminate the nonspecific proteins. The purification mechanism is based on the interaction between the negatively charged proteins to the positively charged $CH_2N+(CH_3)_3$ group that is attached to an insoluble matrix in the column (Cummins et al., 2017). The isoelectric point of TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 is 4.84, 4.6, 4.59, 4.63, 4.61, 4.62, 4.59 pH value respectively. To gain a net negative charge to the enzymes, L-histidine (pH 6.5) was chosen as the IEX buffer. In the process, the ionic strength of the buffer was increased stepwise to eliminate the contaminants and the final elution was carried out with 1M NaCl. As can be seen in Figure 3.8, some non-specific bands elimination was realized; however, the eluted samples contained contaminants again. For these reasons, heat treatment was applied as the third purification step. Samples obtained as a result of anion exchange chromatography are incubated at 60 °C for denaturation of *E. coli* native proteins. Amylopullulanase enzyme originated from the thermophilic organism T. *brockii brockii* (Coleman et al., 1987); so amylopullulanase enzyme is a thermostable protein. While the amylopullulanase enzyme keeps stable and active, *E. coli* proteins denatured as a result of incubation at 60 °C. Thus, purification of both amylopullulanase and truncated variants was provided.

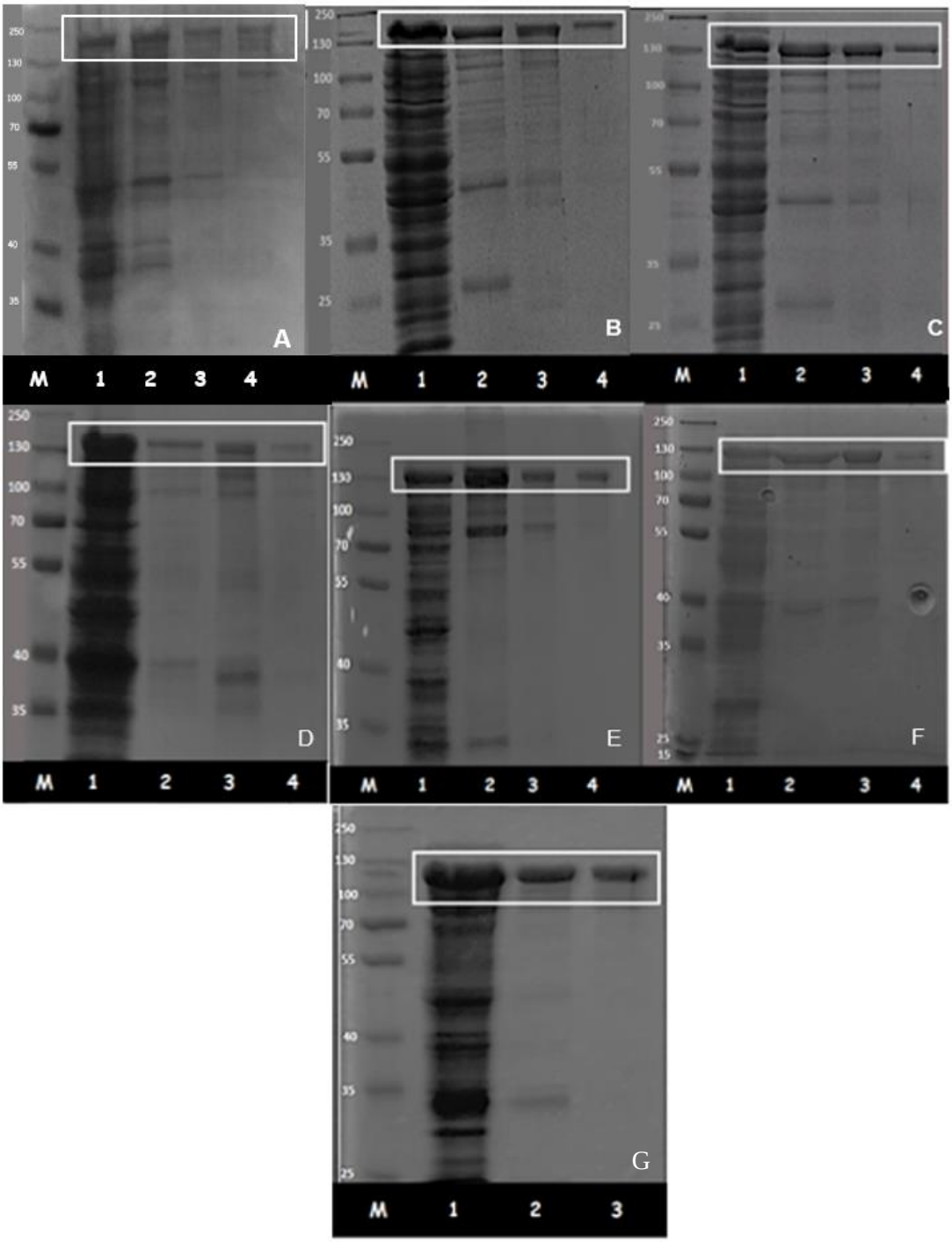

Figure 3.8: Purification results of TbbApu and its truncated variants. **A.** Purification results of TbbApu M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: IEX chromatography; Lane 4: Heat purification. **B.** Purification results of TbbApuΔSH3 M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: IEX chromatography; Lane 4: Heat purification. **C.** Purification results of TbbApuΔCBM20 M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: IEX chromatography; Lane 4: Heat purification. **D.** Purification results of TbbApuΔX25-1-SH3 M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: IEX chromatography; Lane 4: Heat purification. **E.** Purification results of TbbApuΔX25-2-SH3 M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: IEX chromatography; Lane 4: Heat purification. **F.** Purification results of TbbApuΔX25-1-CBM20 M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: IEX chromatography; Lane 4: Heat purification. **G.** Purification results of TbbApuΔX25-2-CBM20 M: M: Molecular weight marker; Lane 1: clear lysate; Lane 2: IMAC; Lane 3: Heat purification.

After the purification, to detect the hydrolysis ability TbbApu and its truncated variants pullulan and soluble starch zymography were applied. Reactive red bounded pullulan and soluble starch were used as substrates. The bond that is between reactive red and pullulan is broken with pullulanase activity. As a result of this, colourless zone formation is observed (Yang and Coleman, 1987). In the case of α- amylase activity of the enzymes, starch molecules in the gel were degraded and starch degraded regions keep unstained with the treatment of the zymography gel with lugol (Mishra & Behera, 2008). As can be seen in Figure 3.9, all of the N- and C-terminal truncated amylopullulanases which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 hydrolyzed α-1,6 glycosidic bonds in pullulan and α-1,4 and α-1,6 glycosidic bonds in soluble starch. Thus, it can be said that TbbApu and its variants have bifunctionality with the ability to break two types of bonds which are α-1,4 and α-1,6 glycosidic bonds.

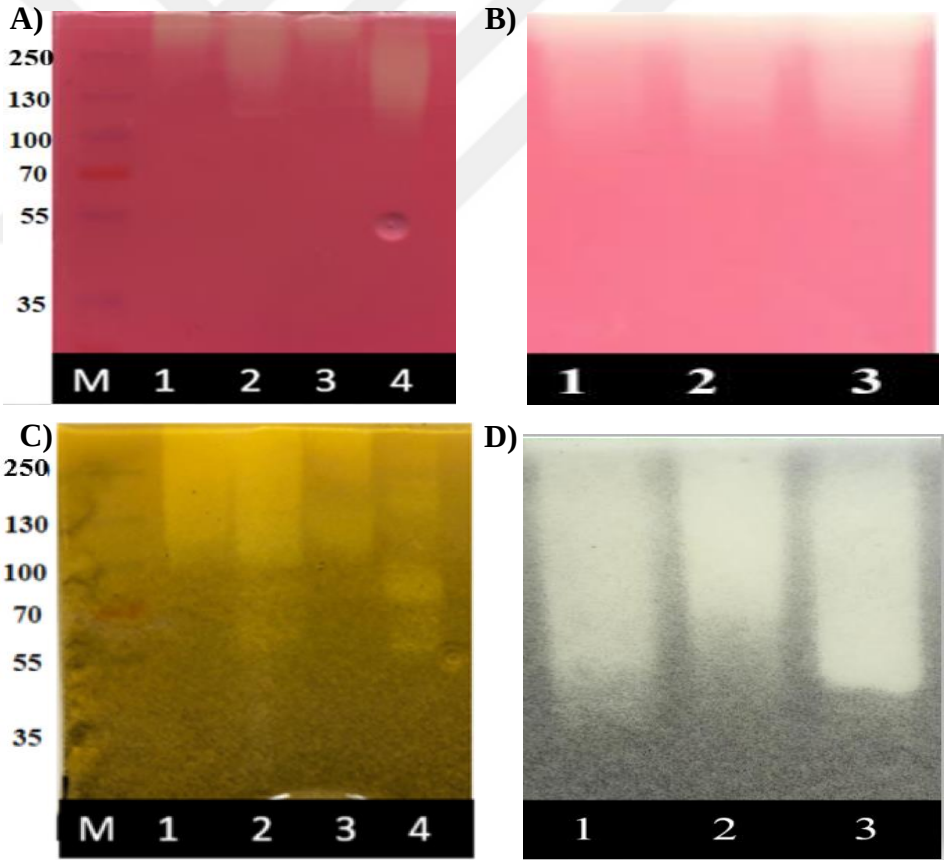

Figure 3.9: Zymography results of TbbApu and its truncated variants. **A-C.** M: Molecular weight marker; Lane 1: TbbApuΔX25-1-SH3; Lane 2: TbbApuΔX25-2-SH3; Lane 3: TbbApuΔX25-1-CBM20; Lane 4: TbbApuΔX25-2-CBM20 in the presence of pullulan and starch respectively **B-D.** Lane 1: TbbApu; Lane 2: TbbApuΔSH3; Lane 3: TbbApuΔCBM20 in the presence of pullulan and starch respectively.

3.4 Biochemical Characterisation of TbbApu and Its Truncated Variants

3.4.1 Effect of temperature and pH

Temperature and pH effect on α-amylase and pullulanase activity of purified TbbApu, TbbApuΔSH3 and TbbApuΔCBM20 and four N- and C-terminus truncated variants which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were measured by standard activity assay. The optimum reaction temperature for pullulanase activity was found at 70 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 75 °C for TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and 80 °C for TbbApuΔX25-2-CBM20 and the optimum reaction temperature for α-amylase activity was found 75 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 80 °C for TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 (Figure 3.10).

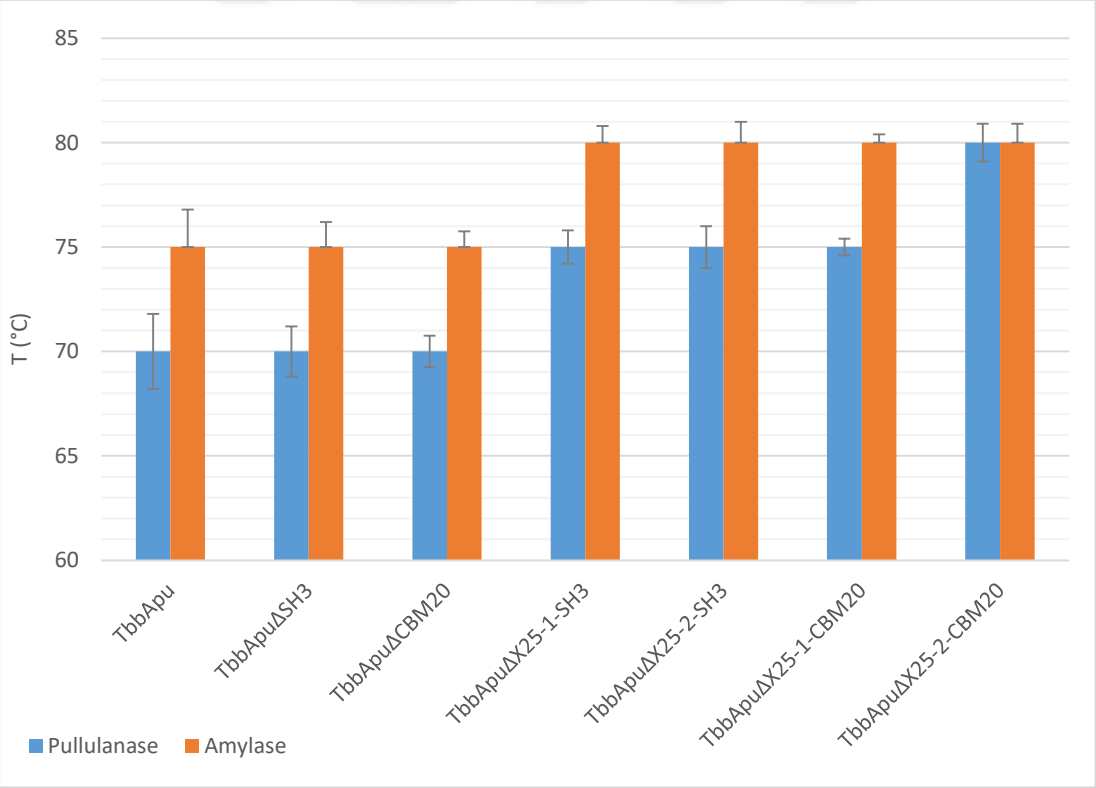

Figure 3.10: The optimum temperature of TbbApu and its variants for pullulanase and α-amylase activity.

According to the measurements, the enzymes reached ≥ 80% relative pullulanase activity after 60 °C and protected their 80 % activity up to 85 °C. Most remarkably TbbApuΔX25-2-CBM20 showed 66 % pullulanase activity at 100 °C while the other enzymes can show barely pullulanase activity at 100 °C. TbbApu, TbbApuΔSH3 and

TbbApuΔCBM20 reached their > 80% relative α-amylase activity after 60 °C; however, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 reach their > 80% relative α-amylase activity after 75 °C. All of the enzymes protect their 80 % relative activity up to 85 °C. Together with these results, TbbApu, TbbApuΔSH3 and TbbApuΔCBM20 show different relative α-amylase activity profiles than TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20. While TbbApu, TbbApuΔSH3 and TbbApuΔCBM20 reach their 50 % relative α-amylase activity at 40 °C, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 reach to this activity percentage at 65 °C. The main differences between TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 from TbbApu, TbbApuΔSH3 and TbbApuΔCBM20 is the absence of X25 domain(s). Thus, it can be said that the X25 domain is important for the α-amylase activity of the amylopullulanase enzyme at temperatures lower than the optimum temperature. When looking at the temperatures that are higher than optimum α-amylase temperature, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 whose X25, SH3 and CBM20 domains were truncated lose their nearly 80 % relative α-amylase activity at 90 °C, while other enzymes which lack only SH3 domain, SH3 and CBM20 domains, SH3 and X25 domains protect their ≥ 65 % relative α-amylase activity. The difference between TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 from the other enzymes is the lack of X25 domain(s) together with SH3 and CBM20. Thus, we can say that the absence of both X25 and CBM20 domains affects the α-amylase activity of the enzyme at high temperatures and the presence of X25 or CBM20 domain is required for the α-amylase activity of the enzymes at high temperatures (Figure 3.11). When we compare optimum reaction temperature with *Thermoanaerobacter sp.* amylopullulanases (Table 3.5), TbbApu shows a similar optimum temperature with *Thermoanaerobacter Strain* B6A, *Thermoanaerobacterium saccharolyticum* B6A-RI and *Thermoanaerobacter thermohydrosulfuricus* E101. However, *Thermoanerobacter ethanolicus* 39E shows a higher optimum reaction temperature (90 °C) thanTbbApu and TbbApu has a higher optimum reaction temperature than *Thermoanaerobacterium saccharolyticum* DSM 571 (65 °C) and *Clostridium thermosulfurogenes* EM1 (60-65 °C).

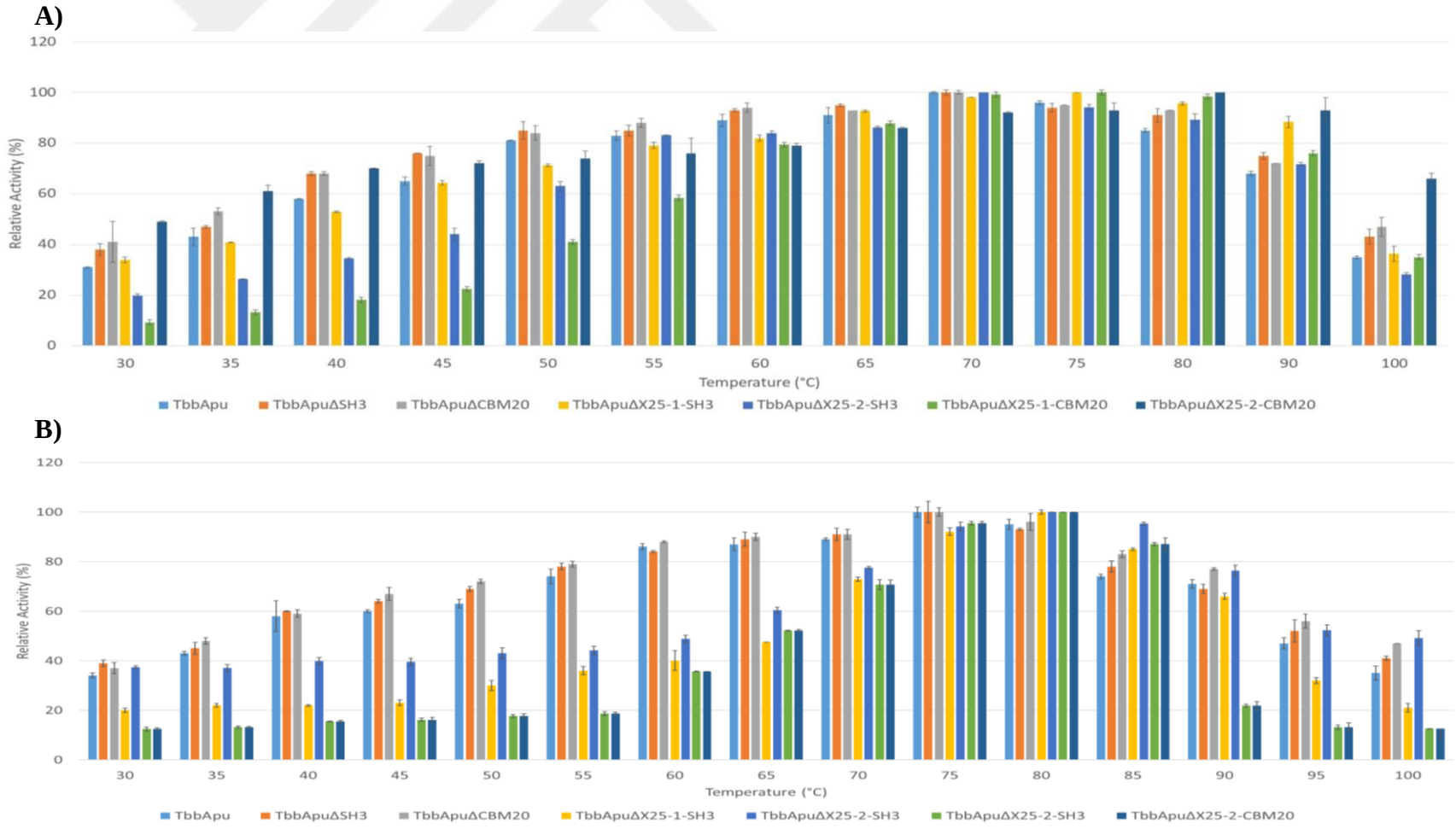

Figure 3.11: Effect of temperature on activity of TbbApu and its truncated variants **A.** pullulanase **B.** α-amylase activity.

The optimum pH value for both pullulanase and α-amylase activity was defined as 6.5 for TbbApu, TbbApuΔX25-1-SH3 and TbbApuΔX25-1-CBM20, 6 for TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-2-SH3, 7 for TbbApuΔX25-2-CBM20 (Figure 3.12).

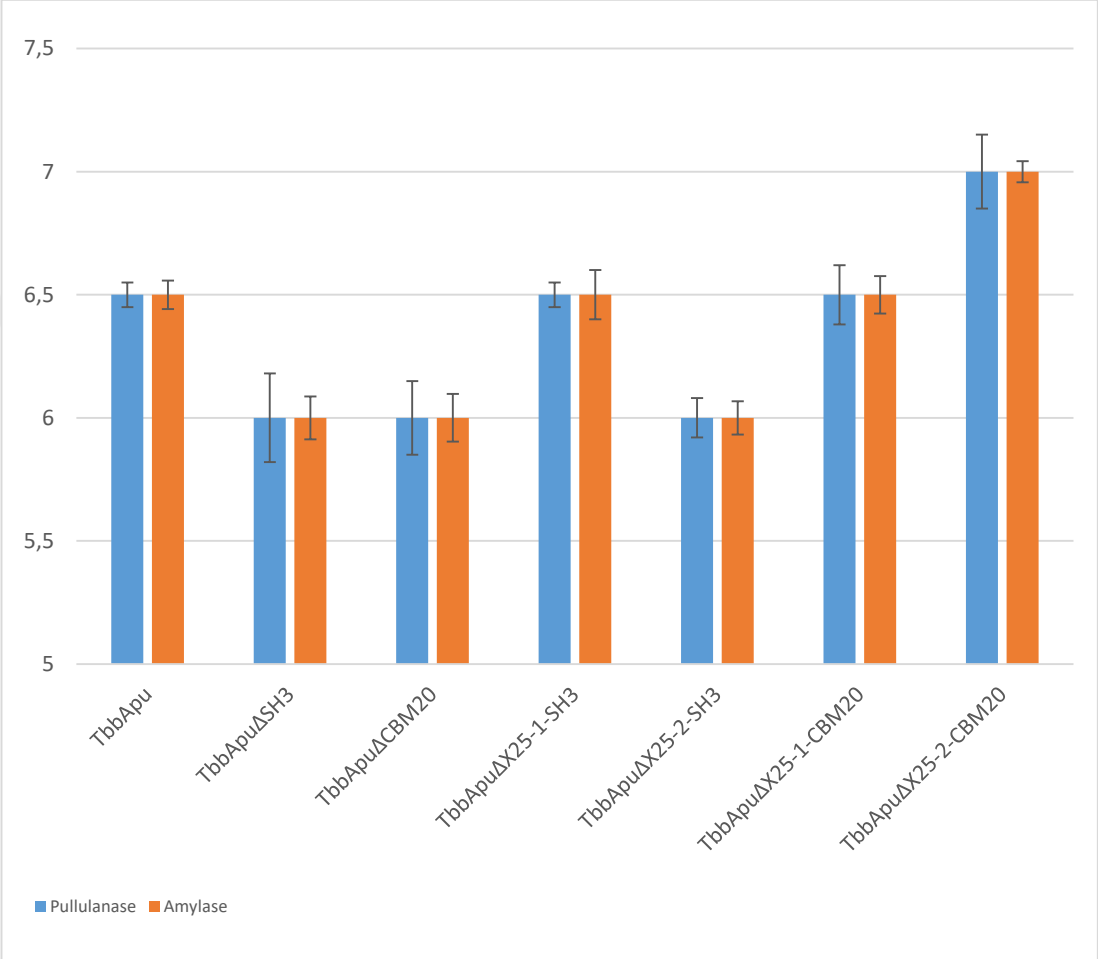

Figure 3.12: The optimum reaction pH for pullulanase and α-amylase activity of TbbApu and its truncated variants.

According to Figure 3.13, both TbbApu and its truncated variants reach nearly 80 % relative pullulanase and α-amylase activity at pH 5 and lose a significant percentage of their pullulanase and α-amylase activity very sharply at pH 8. It can be seen that while the enzymes work very efficiently at acidic pH, they lose their activities at basic pH. When we compare optimum reaction pH with *Thermoanaerobacter sp.* amylopullulanases (Table 3.5), TbbApu shows higher optimum pH value than *Thermoanaerobacter sp.* amylopullulanases.

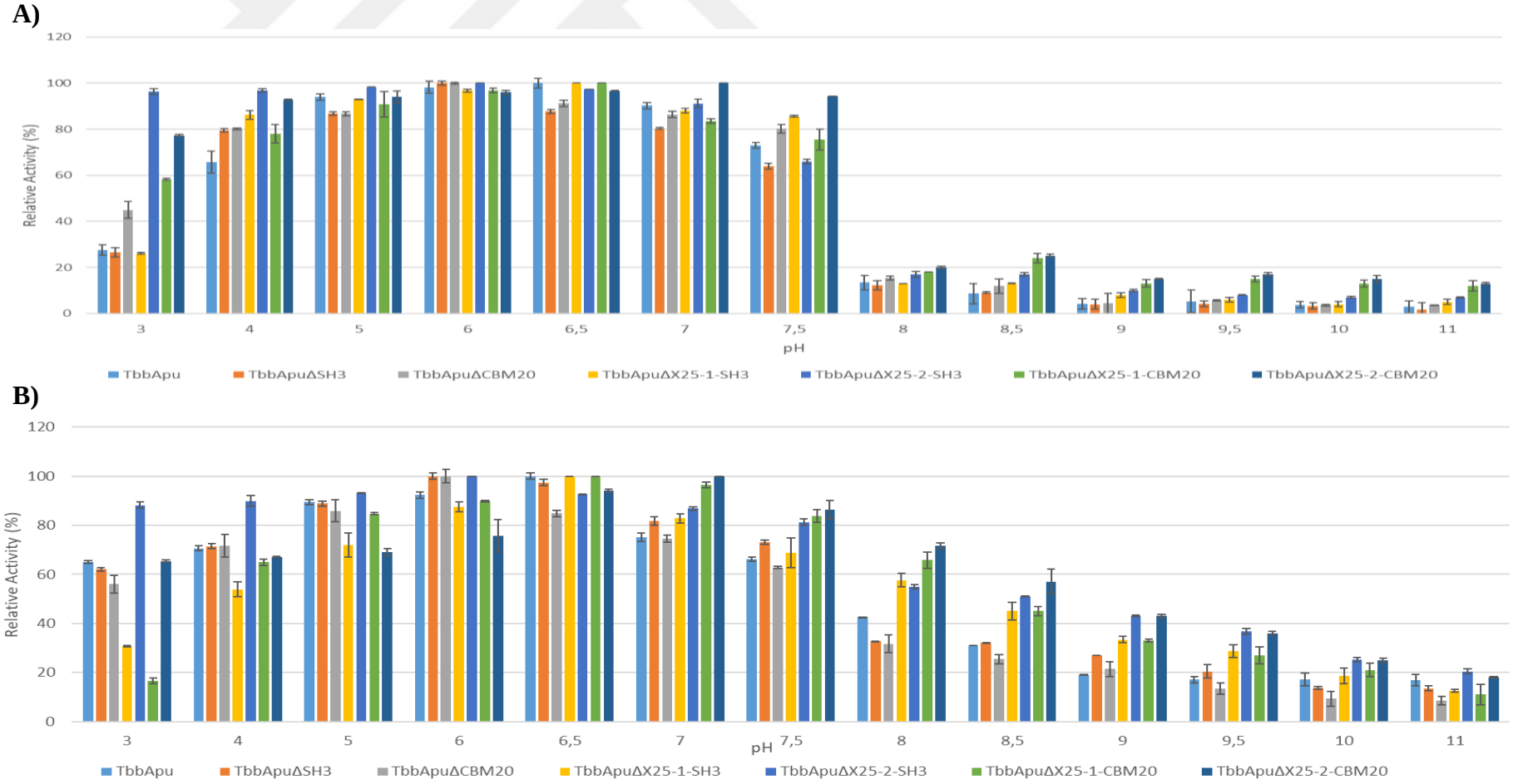

Figure 3.13: Effect of pH on TbbApu and its truncated variants **A.** pullulanase **B.** α-amylase activity.

3.4.2 Effect of modulators

3.4.2.1 Effect of metal ions

To investigate metal ions` effect on pullulanase and α-amylase activity of the enzymes, metal salts were added to the reaction mixture with a final 5 mM concentration. When looking at Table 3.2, Mn^{2+} and Co^{2+} increased both pullulanase and α-amylase activity of TbbApu and its all variants. On the other hand, Mg^{2+}, Zn^{2+} and Al^{3+} ions decreased the pullulanase activity of the enzymes by 5 % to 36 % except for TbbApuΔCBM20 and α-amylase activity of the enzymes by 2 % to 37 %. Thus, it can be said that Mn^{2+} and Co^{2+} are the activators and Mg^{2+}, Zn^{2+} and Al^{3+} are the inhibitors of TbbApu and its truncated variants. Cr^{3+}, Ba^{2+}, Ni^{2+} and K^{+} do not have much effect on the pullulanase and α-amylase activity of the enzymes. In the case of Fe^{2+} ion, pullulanase activity of the enzymes increased by 9 % to 21 % except for TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 and it caused to decrease in α-amylase activity by 3 % to 34 % except for TbbApuΔX25-2-CBM20. In the presence of Cu^{2+} ion, pullulanase activity of the enzymes increased by 3 % to 124 % except for TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 and α-amylase activity of the enzymes increased by 3 % to 20 % except for TbbApuΔX25-1-CBM20, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20. The comparison of TbbApu and amylopullulanase of *T.ethanolicus* 39E that share 98.7 % similarity with TbbApu, while Ca^{2+} is required for the stability and the activity of the amylopullulanase of *T.ethanolicus* 39E (Lin & Leu, 2002), both activities of the TbbApu and its variants moderately decreased except TbbApuΔCBM20 and TbbApuΔX25-2-SH3. Another difference between these two amylopullulanases is the effect of Ba^{2+}. While pullulanase and α-amylase activity of amylopullulanase of *T.ethanolicus* 39E increased in the presence of Ba^{2+} (Lin et al., 2002), both activities of TbbApu and its variants do not affect by Ba^{2+} ion. The independence of TbbApu and its truncated variants to the metal ions for activity and stability is an important feature because all α-amylases that are used in the food industry require Ca^{2+} ions for activity and stability during the process (Jòzef, 2007). The addition of Ca^{2+} ions to the process both increases the cost of the process and requires an additional step to remove Ca^{2+} ions from the final products.

Table 3.2 : Metal ions' effect on pullulanase and α-amylase activity of TbbApu and its truncated variants.

	TbbApu		TbbApuΔSH3		TbbApuΔCBM20		TbbApuΔX25-1-SH3		TbbApuΔX25-2-SH3		TbbApuΔX25-1-CBM20		TbbApuΔX25-2-CBM20	
	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase
Mg^{2+}	73±0.69	89±0.54	77±0.25	87±0.17	95±0.59	63±0.50	79±1.29	80±2.36	85±0.27	87±0.34	69±0.50	80±1.86	65±1.64	88±0.44
Fe^{2+}	118±1.13	93±0.91	121±4.14	93±1.25	121±1.19	66±0.20	105±0.21	66±2.33	109±0.49	97±0.88	81±1.09	87±1.62	73±2.06	100±0.74
K^{+}	98±2.38	99±0.52	97±3.49	95±0.19	108±5.39	59±0.41	91± 0.94	85±1.85	103±1.73	105±1.07	88±1.01	88±0.15	70±2.76	99±0.86
Ca^{2+}	80±0.91	81±1.04	89±4.01	83±0.10	106±2.99	62±0.23	95±0.22	98±1.17	106±0.23	108±2.29	101±1.07	110±2.25	76±1.63	94±3.31
Zn^{2+}	89±1.58	87±0.15	89±2.17	90±0.58	106±2.59	67±0.30	83±0.15	85±1.85	94±1.66	98±0.75	71±2.51	79±0.10	71±1.17	88±1.16
Al^{3+}	83±0.98	88±0.91	85±1.39	81±0.25	100±1.24	64±0.20	78±1.11	73±2.71	78±1.26	93±0.07	68±0.59	66±0.09	67±1.68	89±1.29
Cu^{2+}	116±0.45	116±0.40	224±1.94	120±0.42	247±2.81	103±0.39	107±0.65	90±2.90	125±1.78	115±1.12	83±5.36	87±0.42	70±1.12	97±3.38
Ni^{2+}	98±2.98	98±2.22	105±2.81	101±0.50	117±3.95	71±0.41	92±1.11	91±1.74	101±2.13	98±0.37	79±2.70	76±2.59	73±0.89	98±3.01
Mn^{+2}	180±5.77	200±1.13	224±1.55	205±0.44	318±2.40	129±0.23	161±0.69	163±4.28	175±2.06	153±2.32	151±2.92	149±3.18	132±2.85	158±2.64
Ba^{2+}	104±2.68	121±0.40	105±2.53	116±0.42	105±1.24	85±0.23	100±0.54	97±1.74	92±0.23	101±4.19	84±2.11	99±0.90	80±1.96	101±3.81
Co^{2+}	152±2.14	186±0.93	206±5.00	183±1.01	205±3.38	147±0.50	153±0.70	132±2.32	155±3.16	143±0.83	124±3.19	127±2.81	171±1.17	148±3.63
Cr^{3+}	91±3.96	109±2.34	90±1.80	116±0.67	106±0.59	82±0.50	83±0.35	81±1.90	93±0.51	94±0.65	78±2.18	85±3.14	89±2.84	91±1.72

3.4.2.2 Effect of organic solvents

To examine organic solvents` effect on pullulanase and α-amylase activity of the enzymes, organic solvents were added to the reaction mixture with a final 20 % and 50 % concentrations. Pullulanase and α-amylase activities of both TbbApu and its truncated variants increased in the presence of hexane and acetone (20 %, 50 %) with some exceptions. Isopropanol (20 %, 50 %) presence in the reaction mixtures enhanced both pullulanase and α-amylase activity of TbbApu, TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-1-SH3 (except 50 %); however, decrease both activities of TbbApuΔX25-1-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20. In the case of ethanol and methanol, while 20 % and 50 % presence of the solvents enhanced pullulanase activity of TbbApuΔSH3, TbbApuΔCBM20 and α-amylase activity of TbbApuΔCBM20 and TbbApuΔX25-1-SH3 (except 50 % ethanol presence), they caused to decrease both activities of the other enzymes. On the other hand, the presence of butanol, DMF and DMSO (20 % and 50 %) in the reactions caused to loss of both pullulanase and α-amylase activity strongly. Especially, the presence of DMF cause to loss of at least 70 % of both activities of all enzymes. Also, the presence of 20 % and 50 % glycerol in the reaction mixtures causes to loss of both pullulanase and α-amylase activity of all the enzymes with two exceptions. Pullulanase activity of TbbApuΔSH3 and TbbApuΔCBM20 increased by 68 % and 58 % with glycerol (50 %) (Table 3.3). In the literature, it can be seen that some amylopullulanases can preserve their activity and stability in the presence of organic solvents. Amylopullulanase originated from *Alkalilimnicola sp.* preserved its pullulanase and α-amylase activity in the presence of 20 % methanol and DMSO. Also, amylopullulanase that originated from *Geobacillus thermoleovorans* NP33 is resistant to 10 % acetone, butanol, ethanol and methanol.

Table 3.3 : : Organic solvents' effect on pullulanase and α-amylase activity of TbbApu and its truncated variants.

		TbbApu		TbbApuΔSH3		TbbApuΔCBM20		TbbApuΔX25-1-SH3		TbbApuΔX25-2-SH3		TbbApuΔX25-1-CBM20		TbbApuΔX25-2-CBM20	
		Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase
Isopropanol	20%	113±1.34	100±0.18	149±2.92	98±0.70	124±5.50	190±0.42	86±1.27	80±0.30	101±2.45	114±1.76	50±3.57	45±1.88	64±4.84	128±3.87
	50%	120±1.01	105±0.18	272±6.53	102±0.23	262±0.86	170±0.14	89±2.46	87±2.17	96±0.69	77±0.76	27±2.30	28±5.49	35±1.28	12±2.13
Acetone	20%	122±1.63	94±0.73	152±0.32	97±1.17	136±3.47	195±0.42	97±2.41	94±1.79	103±2.48	130±1.80	76±4.00	96±2.30	70±5.65	114±1.01
	50%	202±2.42	116±0.73	338±2.61	123±0.47	331±0.58	179±0.56	132±1.56	115±4.61	149±2.89	153±1.81	115±0.19	107±2.60	84±1.61	105±3.93
Ethanol	20%	80±1.21	83±0.18	128±5.84	84±0.59	135±0.87	132±1.25	66±1.62	75±1.67	95±3.15	117±0.87	45±0.67	34±3.34	38±0.70	86±4.18
	50%	100±4.24	61±0.55	226±1.63	64±0.23	217±1.44	89±0.42	50±0.22	58±3.46	71±2.76	62±4.07	24±0.05	26±1.65	18±4.57	11±1.38
Methanol	20%	80±0.96	73±1.28	126±2.92	71±0.47	112±0.00	125±0.28	68±1.21	63±0.25	87±0.60	122±1.82	52±3.60	58±0.98	43±1.33	92±5.63
	50%	105±2.02	89±0.55	232±1.31	86±0.23	222±1.44	119±1.67	68±0.39	68±1.85	88±1.84	121±3.25	29±1.59	26±1.91	27±0.35	23±2.40
Butanol	20%	47±0.56	41±0.92	93±2.59	44±0.35	87±1.45	39±0.56	45±0.94	34±0.27	71±2.78	85±2.37	26±1.53	29±1.92	22±0.05	18±0.03
	50%	55±0.81	49±0.37	177±3.92	51±0.59	149±5.18	77±0.14	28±0.92	36±3.78	39±1.74	34±0.21	26±2.53	25±1.36	31±3.67	19±1.01
DMF	20%	19±0.15	28±0.18	30±0.65	21±0.59	26±2.03	14±0.28	19±0.07	18±0.12	23±1.73	24±1.51	25±1.22	29±1.06	12±0.09	11±0.31
	50%	15±0.40	29±0.37	21±0.65	22±0.12	18±0.00	25±0.28	15±0.46	17±2.72	21±0.24	37±3.07	21±1.48	26±0.27	10±0.19	11±0.69
DMSO	20%	76±2.02	39±0.73	111±2.59	49±0.12	115±0.58	13±0.98	72±1.97	39±0.26	89±2.88	84±0.17	56±0.70	44±1.02	60±0.42	52±3.01
	50%	16±1.01	53±0.55	29±1.63	56±0.12	25±0.86	22±0.28	16±0.17	44±0.53	22±0.40	27±0.45	23±0.52	25±1.37	19±0.61	10±0.80
Hexane	20%	142±4.82	93±1.47	176±2.92	97±0.12	152±0.87	133±0.14	110±2.40	85±1.23	117±3.52	113±1.53	94±2.85	103±0.29	82±0.03	151±4.32
	50%	213±0.40	116±0.73	340±6.20	131±0.35	337±8.06	204±0.56	150±1.24	130±2.35	98±0.96	124±2.00	98±0.23	103±5.75	131±0.95	116±2.56
Glycerol	20%	71±4.82	73±0.37	101±1.30	75±0.59	97±0.29	78±0.56	76±0.37	68±0.74	94±0.72	65±2.83	78±2.14	57±3.76	35±2.26	35±4.11
	50%	95±4.84	82±0.37	168±4.24	84±0.35	148±3.17	86±0.42	58±0.31	83±0.26	72±2.04	79±0.10	29±1.68	35±4.07	36±0.67	33±1.41

3.4.2.3 Effect of detergents and inhibitors

To elucidate inhibitors and detergents` effect on α-amylase and pullulanase activity of the enzymes, 5 mM modulators were added to the reaction mixtures separately (Table 3.4). EDTA, EGTA, DTT and β-mercaptoethanol were used as inhibitors and the inhibitors caused decreased pullulanase activity of the enzymes moderately by 3% to 41% with some exceptions. These exceptions are that the activity of TbbApuΔCBM20 increased against all inhibitors and the presence of DDT and β-mercaptoethanol improved pullulanase activity of TbbApuΔSH3 and TbbApuΔX25-1-CBM20. When looking at the effects of the inhibitors on the α-amylase activity of the enzymes, the activity of the enzymes decreased moderately by 8 % to 50 % with two exceptions. The α-amylase activity of the TbbApuΔX25-1-CBM20 increased by 6 % and 10 % in the presence of DTT and β-mercaptoethanol respectively. When the compare TbbApu and its truncated variants, truncation of the domains provided to improve both pullulanase activity except TbbApuΔX25-2-SH3 and α-amylase activity of TbbApu except TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20. The resistance of TbbApu against the inhibitors can be explained structural properties of thermostable enzymes. Thermostable enzymes have a more rigid and compact structure than enzymes which originated from mesophilic organisms because this rigidity and compactness allow the protection of the catalytically active structure of the enzymes at high temperatures. These structural properties also provide to gain resistance of thermophilic enzymes against inhibitors (Li et al., 2005). This explains the resistance of TbbApu to inhibitors.

When looking at the effects of detergents on activities of the enzymes, it can be seen that non-ionic (Tween 20, Tween 80, TritonX-100, and PEG) and anionic detergents (SDS) caused to decrease in pullulanase and α-amylase activity moderately by 3 % to 43 % and 1 % to 63 % respectively. However, the α-amylase activity of TbbApu and TbbApuΔX25-1-SH3 increased in the presence of PEG and Tween 80 respectively. However, CTAB which is a cationic detergent inhibited both pullulanase and α-amylase activity of the enzymes strongly. This inhibition can result from the biochemical characteristics of the enzymes. The isoelectric point of the enzymes is between 4.59 and 4.83 and at the reaction pH, the total charge of the enzymes is negative. Thus, they can make salt bridges with the cationic detergent CTAB. As a

result of these interactions, the enzymes can lose their native structure. Also, these results explained the ghost bands in the SDS-PAGE gels. It can be seen that the enzymes can keep their α-amylase and pullulanase activity in the presence of SDS, β-mercaptoethanol and DTT that are used in the SDS-PAGE sample loading buffer, so they can preserve their active folded structure. Proteins can be run faster on SDS-PAGE if it was not denatured enough so multiple protein conformations can be seen (Guan et al., 2013). The comparison of the truncated variants with full-length TbbApu revealed that truncation of the domains from TbbApu (except TbbApuX25-2-SH3 variant) improved the pullulanase activity against non-ionic (except PEG) and anionic detergents and SH3 and X25 domain(s) truncation enhanced α-amylase of the enzymes in the presence non-ionic (except PEG) and anionic detergents. However, truncation CBM20 domain and X25 domain(s) with CBM20 caused to decrease in the α-amylase activity of the enzymes against all of the detergents. When we look at the literature, some amylopullulanases are resistant to detergents and inhibitors. Amylopullulanase originated from *Alkalilimnicola sp*. (Mesbah & Wiegel, 2018), *Pyrococcus furiosus* (Dong et al, 1997), *Exiguobacterium sp.* (Rajaei et al., 2015) and *Geobacillus thermoleovorans* NP33 (Nisha et al., 2013) are resistant to SDS and TritonX-100. Also, the activity of the amylopullulanase from *Pyrococcus furiosus* (Dong et al., 1997), *Exiguobacterium sp*. (Rajaei et al., 2015) increased in the presence of DTT and β-mercaptoethanol. Also, compare of TbbApu with amylopullulanase of *Thermoanaerobacterium ethanolicus* 39E amylopullulanase shares 98.7 % similarity with TbbApu, while amylopullulanase of *Thermoanaerobacterium ethanolicus* 39E lose its pullulanase activity completely and α-amylase activity significantly, TbbApu preserves its pullulanase and α-amylase activity (Lin et al., 2002).

Table 3.4: Inhibitors and detergents' effect on pullulanase and α-amylase activity of TbbApu and its truncated variants.

	TbbApu		TbbApuΔSH3		TbbApuΔCBM20		TbbApuΔX25-1-SH3		TbbApuΔX25-2-SH3		TbbApuΔX25-1-CBM20		TbbApuΔX25-2-CBM20	
	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Amylase
Inhibitors														
EDTA	72±2.13	73±0.91	75±1.49	76±0.17	123±2.09	67±0.39	78±1.31	76±2.06	66±2.29	68±0.38	79±0.29	21±0.28	97±2.91	83±0.16
EGTA	68±2.61	59±0.52	73±0.86	63±0.42	114±3.56	50±0.39	82±2.95	88±4.12	59±0.04	68±2.46	73±5.03	30±2.04	88±4.84	89±1.82
DTT	92±1.15	69±0.65	110±2.17	73±0.42	136±1.37	66±0.30	93±2.33	119±2.61	66±1.28	79±1.68	106±1.84	64±1.31	97±3.07	91±0.71
βME	90±0.72	81±0.26	129±0.25	85±0.17	147±1.24	74±0.20	100±3.50	121±1.81	64±1.12	85±2.33	110±2.06	64±1.66	101±2.98	92±0.90
Detergents														
SDS	66±1.79	62±0.15	89±6.16	69±0.91	101±2.25	49±0.60	101±2.92	20±3.16	66±1.49	66±2.20	85±6.7	18±4.06	93±3.46	37±1.27
Tween 20	74±0.79	65±0.40	86±0.86	71±0.42	113±1.49	53±0.20	100±1.66	94±5.16	60±0.79	79±0.25	95±0.02	57±1.33	97±2.01	89±2.23
Tween 80	74±0.57	64±0.54	102±4.19	68±1.00	127±7.66	59±0.20	96±2.38	107±1.06	60±1.00	71±0.25	93±1.26	69±0.65	87±3.5	80±0.85
TritonX-100	69±0.68	64±0.40	75±0.66	70±0.35	124±1.24	59±0.39	103±3.32	90±8.85	67±1.47	69±0.12	101±5.4	85±1.68	95±4.01	99±0.82
CTAB	12±0.47	49±0.26	17±0.25	44±0.67	31±1.03	30±0.82	18±2.35	18±4.25	17±0.02	16±0.78	13±3.8	16±2.01	20±0.26	14±0.33
PEG	107±2.83	102±0.40	173±6.47	91±0.51	238±2.93	94±0.23	69±1.32	79±3.35	79±2.54	87±2.45	44±2.1	75±3.04	106±1.23	87±2.56

3.4.3 Effect of temperature, pH and organic solvents on stability of TbbApu and its truncated variants

To assess the temperature effect on the stability of TbbApu and its truncated variants, the enzymes were incubated at between 30 °C and 100 °C for 24 hours. While TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 preserved ≥ 70 % of their pullulanase activity up to 70 °C, TbbApu, TbbApuΔSH3, and TbbApuΔCBM20 kept 90 % of their pullulanase activity up to 70 °C. The most stable temperature for pullulanase activity is 60 °C for TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3 and TbbApuΔX25-2-SH3, 50 °C for TbbApuΔX25-1-CBM20 and 40 °C for TbbApuΔX25-2-CBM20. In the case of α-amylase activity, all of the enzymes preserved their ≥ 87 % of α-amylase activity up to 70 °C and the most stable temperature for α-amylase activity is 70 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 60 °C for TbbApuΔX25-1-SH3, 50 °C for TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-SH3, 40 °C for TbbApuΔX25-2-CBM20 (Figure 3.14). All of the enzymes lost their pullulanase and α-amylase activity significantly after 70 °C. When looking at the domain truncation effect on the temperature stability of TbbApu according to pullulanase and α-amylase activity, truncation of only SH3 and CBM20 domain from the enzyme does not have any effect on temperature stability according to both activities. However, truncation of X25 domain(s) together with CBM20 domain affected negatively of temperature stability of TbbApu for its pullulanase activity and truncation of X25 domain(s) together with SH3 and CBM20 domains reduced the temperature stability of TbbApu for its α-amylase activity

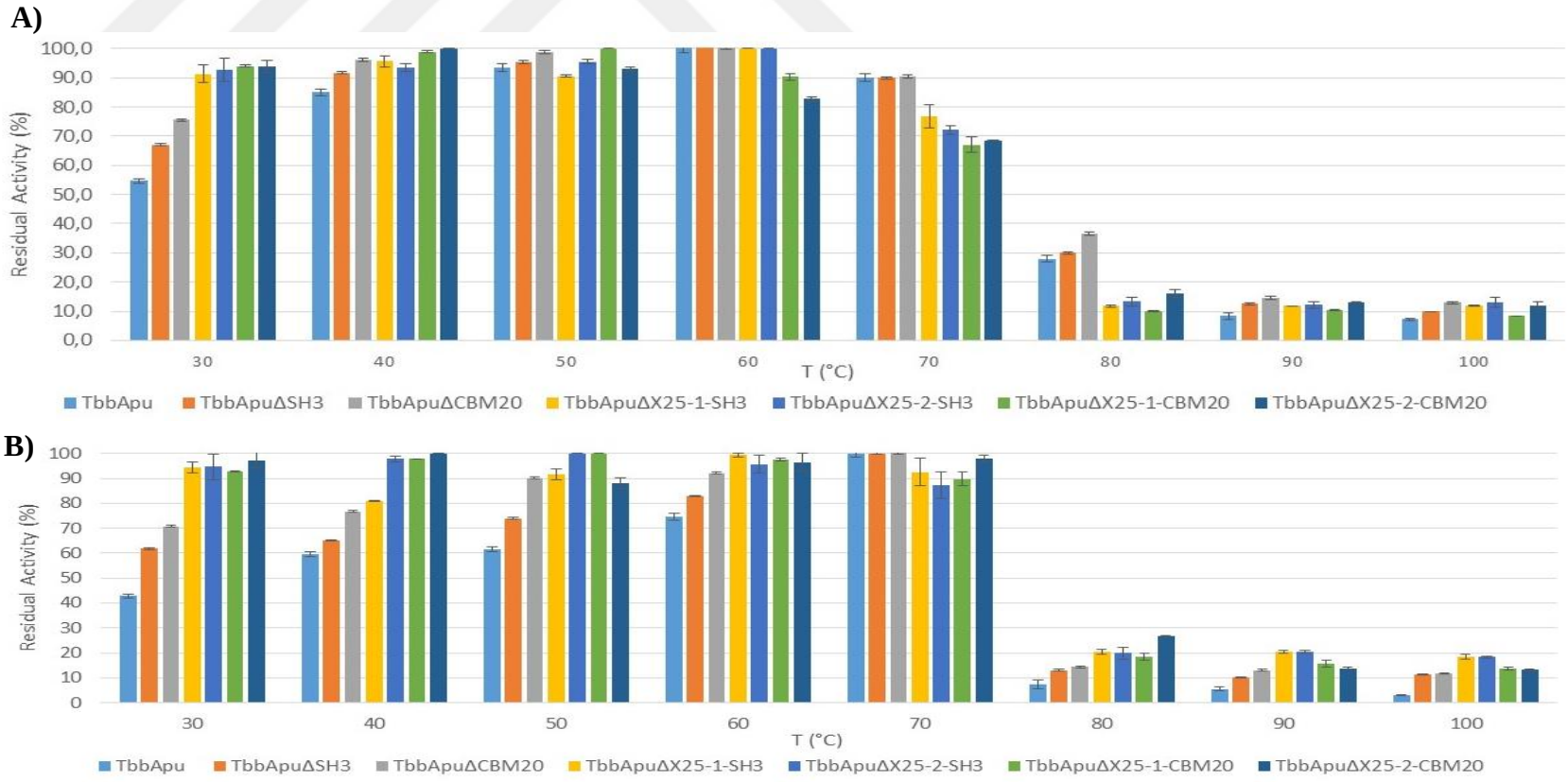

Figure 3.14: Temperature effect on stability of TbbApu and its truncated variants according to **A.** pullulanase **B.** α-amylase activity.

After that, to elucidate the pH effect on the stability of TbbApu and its truncated variants, the enzymes were incubated at pH between 3-10 for 24 hours. While TbbApu, TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-1-SH3 preserved ≥ 80 % of their pullulanase activity up to pH 8, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were kept ≥ 90 % of their pullulanase activity up to pH 7.5. The most stable pH for pullulanase activity is pH 4 for TbbApu, TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-2-CBM20, pH 6 for TbbApuΔX25-2-SH3 and pH 6.5 for TbbApuΔX25-1-SH3 and TbbApuΔX25-1-CBM20. In the case of α-amylase activity, while TbbApu, TbbApuΔSH3 and TbbApuΔCBM20 were preserved ~80 % of their α-amylase activity up to pH 5, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were kept ≥ 80 % of their α-amylase activity up to pH 7. The most stable pH for α-amylase activity is pH 3 for TbbApu, TbbApuΔSH3, TbbApuΔCBM20, pH 6 for TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20, pH 7 for TbbApuΔX25-1-SH3. While TbbApu, TbbApuΔSH3 TbbApuΔCBM20, and TbbApuΔX25-1-SH3 lost their pullulanase activity significantly at pH 10, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 lost its pullulanase activity at pH 9. However, all of the enzymes lost their ≥ 40 % α-amylase activity at pH 9 (Figure 3.15). When looking at the domain truncation effect on pH stability according to pullulanase and α-amylase activity of TbbApu, truncation of SH3, CBM20 and X25 domains from the enzyme did not cause any effect on the stability of the enzymes at acidic and alkali pH and both TbbApu and its truncated variants can keep their stability wide range of pH that is between pH 3 and 8. However, truncation of X25 domain(s) together with SH3 and CBM20 caused to shift optimum stable pH value of the enzymes at near to neutral pH.

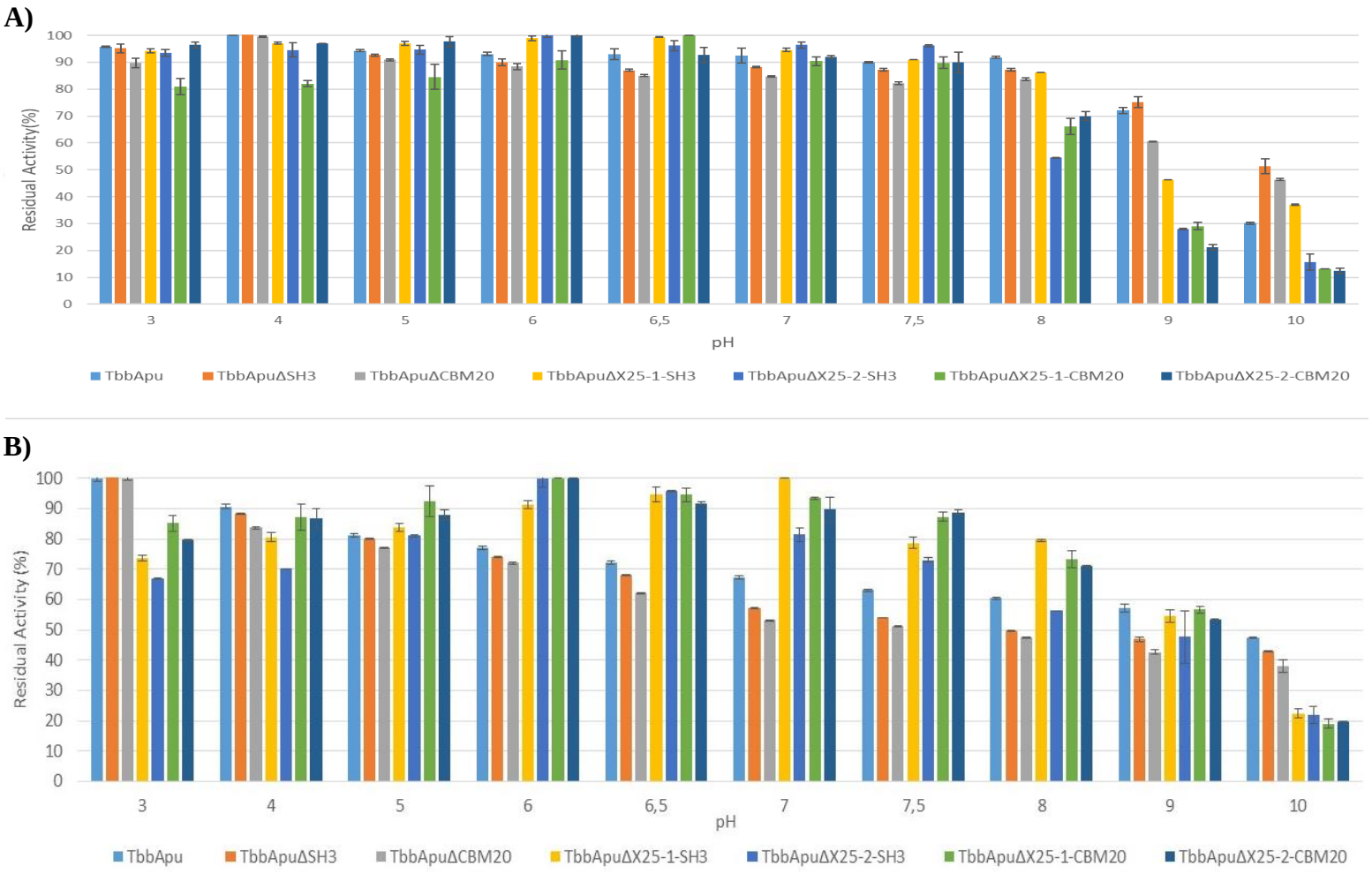

Figure 3.15: pH effect on stability of TbbApu and its truncated variants according to **A.** pullulanase **B.** α-amylase activity.

When we compare the temperature and pH stability of TbbApu with *Thermoanaerobacter sp.* amylopullulanases (Table 3.5), TbbApu shows similar temperature stability to other *Thermoanaerobacter sp.* amylopullulanases except for *Thermoanerobacter ethanolicus* 39E and *Thermoanaerobacter thermohydrosulfuricus* E101. However, TbbApu shows higher pH stability which is between pH 3 and 8 than the other *Thermoanaerobacter sp.* amylopullulanases. Also, when we compare the temperature stability of recombinantly produced (*E. coli* BL21(DE 3) and *B. subtilis*) and native TbbApu, it can be seen that native TbbApu and recombinant TbbApu which is produced in *B. subtilis* were stable at 70 °C for 241 min. and for 109 minutes respectively. However, TbbApu which is produced recombinantly in *E. coli* BL21(DE 3) host in this study was still stable at 75 °C for 24 hours.

Table 3.5: *Thermoanaerobacter sp.* amylopullulanases properties.

Originated Organism	Optimum Temperature (°C)	Optimum pH	Stable Temperature (°C)	Stable pH	Reference
Thermoanaerobacter Strain B6A	75	5	75	4-6	(Saha et al., 1990)
Thermoanaerobacterium saccharolyticum B6A-RI	70-75	5,5-6	65		(Ramesh et al., 1994)
Thermoanaerobacterium saccharolyticum DSM 571	65	5-5,5	60		(Ganghofner et al., 1998)
Thermoanaerobacterium saccharolyticum NTOU1 (truncatated)	70	5-6			(Lin et al., 2011)
Thermoanerobacter ethanolicus 39E	90	3-5	90	6.2	(Lin et al., 2002)
Thermoanerobacter ethanolicus 39E (truncated)	80-90	6	80	3-6	(Lin et al., 2008)
Clostridium thermosulfurogenes EM1	60-65	5,5-6	70-75	5-6.5	(Spreinat et al., 1990)
Thermoanaerobacter thermohydrosulfuricus E101	80-85		85 in the presence of Ca^{2+} ion		Melasniemi et al., 1989)

In conclusion, we can say that amylopullulanase is a promising candidate for starch hydrolysis process other than amylopullulanases of *Thermoanaerobacter sp. and* archaea because of its characteristics. TbbApu can keep its stability at high temperatures like other amylopullulanases of *Thermoanaerobacter sp. and* archaea.

Also, it is stable with both high acidic pH and a wide range of pH values (3-8); however, stability and activity of *Thermoanaerobacter sp. and* archaea-originated amylopullulanases decrease greatly when pH value was less than 5.0. On the other hand, some amylopullulanase of *Thermoanaerobacter sp.* and archaea need Ca^{2+} ion for their activity and stability but TbbApu is Ca^{2+} independent (Li et al., 2018). Thus, no need for pH adjustment and Ca^{2+} addition to the process eliminates some steps of the process. Also, the bifunctionality of the enzymes can provide a one-step liquefaction saccharification process. Thus, both the production steps can be shortened and the production cost can be reduced.

To elucidate the organic solvent effect on the stability of TbbApu and its truncated variants, the enzymes were incubated at 20 % and 50 % isopropanol, acetone, ethanol, methanol, butanol, hexane and glycerol for 24 hours. Then, their stabilities were compared (Figure 3.16). According to pullulanase activity measurements, both TbbApu and its truncated variants have higher stability in the presence of acetone and hexane (20 %, 50 %) than their controls with one exception. TbbApuΔSH3 showed higher stability in the presence of 20 % of all of the organic solvents and TbbApuΔX25-2-SH3 was more stable at 20 % isopropanol, ethanol and methanol together with acetone and hexane. According to α-amylase activity measurements, all of the enzymes were more stable at hexane (20 %, 50 %). Also, TbbApuΔSH3 and TbbApuΔCBM20 showed higher stability in the presence of isopropanol (20 %), acetone (20 %, 50 %), ethanol (20 %, 50 %). Together with these, TbbApuΔX25-1-CBM20 and TbbApu showed higher stability at 20 % acetone and methanol and 50 % acetone respectively. Also, based on pullulanase and α-amylase activity, both TbbApu and its truncated variants preserved $\geq$ 50 % of their stability toward to isopropanol (20 %), acetone (20 %, 50 %), ethanol (20 %), methanol (20 %) and hexane (20 %, 50 %). TbbApuΔSH3 was the most stable variant against organic solvents. When looking at the domain truncation effect on organic solvent stability of TbbApu, truncation of the SH3 domain and two X25 domains together with the SH3 domain provide to improve organic solvent stability of TbbApu according to pullulanase activity and truncation of the SH3 domain and CBM20 domain enhanced organic solvent stability of TbbApu according to α-amylase activity. In conclusion, it can be said that TbbApu and its truncated variants have higher activity and stability in hexane solution than in aqueous solution.

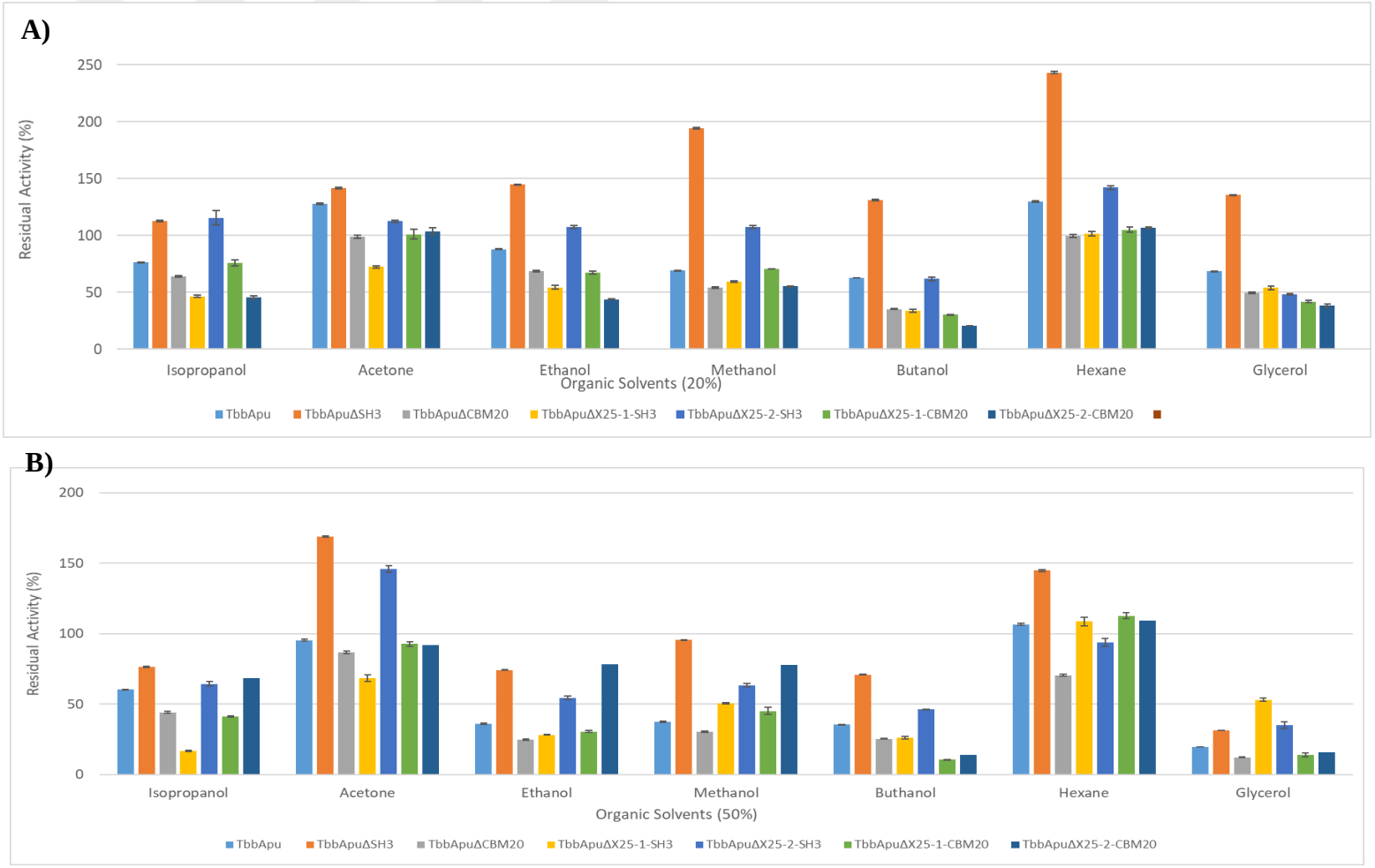

Figure 3.16: Organic solvents effect on stability of TbbApu and its truncated variants. **A-B.** pullulanase activity of the enzymes in the presence 20% and 50% organic solvents respectively. **C-D.** α-amylase activity of the enzymes in the presence 20% and 50% organic solvents respectively.

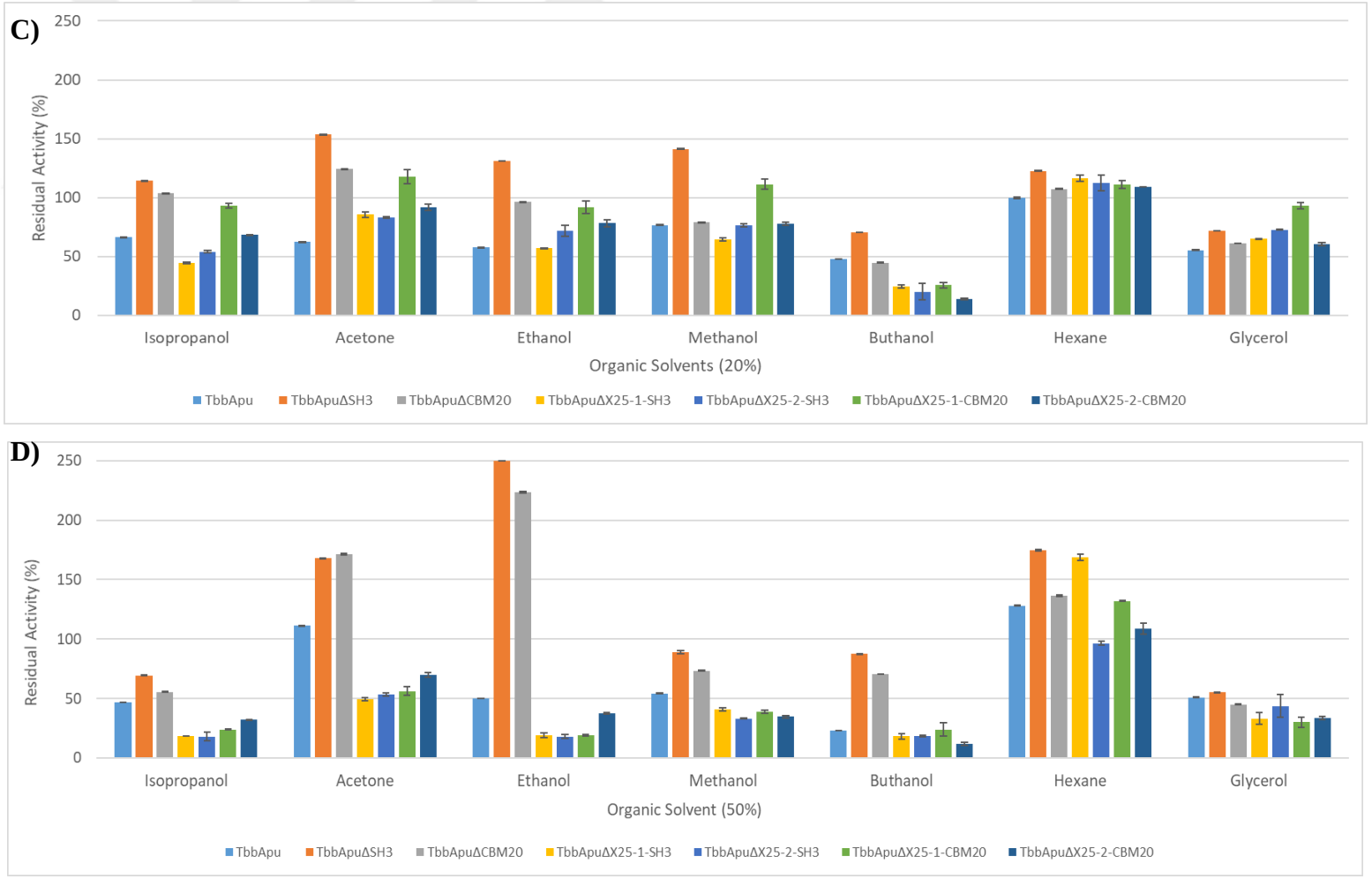

Figure 3.16 (continued): organic solvents effect on stability of TbbApu and its truncated variants. **A-B.** pullulanase activity of the enzymes in the presence 20% and 50% organic solvents respectively. **C-D.** α-amylase activity of the enzymes in the presence 20% and 50% organic solvents respectively.

Organic solvents affect the activity and stability of enzymes by causing some changes in the microenvironment and structure of the enzyme. The presence of a certain level of water around enzymes is important for their natural conformation and functionality and there is an optimum water content for each enzyme. Below this percentage, enzymes reach a very rigid structure; at a rate higher than this percentage, enzymes reach a more flexible structure and begin to unfold. The reason why TbbApu and its variants showed less activity and stability in hydrophilic solvents such as ethanol, methanol and butanol than in aqueous solution may be that such hydrophilic solvents remove water molecules that provide the enzyme-dependent activity from the enzyme and cause to lose natural conformation from the enzyme. Thus, they may cause to loss of activity and stability of TbbApu. As a result of the studies, it has been seen that isopropanol (1-propanol), whose functional group is attached to the terminal carbon, has a higher inhibitory effect than the C atom attached to the internal C atom. The reason for this situation may be because the –OH group attached to the internal carbon atom is exposed to more steric effects and interacts less with the enzyme. This study explains the reason why n-butanol has a higher inhibitory effect than 2-propanol may be because the functional –OH group is attached to the terminal carbon instead of the internal C atom (Wang et al., 2016).

In addition to the hydrophilicity of the solvents, organic solvent hydrophobicity also significantly affects the activity and stability of enzymes. As a result of molecular dynamics simulation studies, as the hydrophobicity of the solvents increases, the flexibility of the enzymes decreases and they acquire a more rigid structure. In this study, the increase in activity and stability of TbbApu and its variants in the presence of a hydrophobic solvent such as hexane may have been obtained as a result of the enzyme's attaining a less flexible structure. We also know that thermozymes have a more rigid structure than mesozymes and they can maintain their catalytically active structures at high temperatures with high rigidity. Therefore, the interaction of a hydrophobic solvent such as hexane with TbbApu and its variants may have provided the enzyme to have a more rigid structure by removing water molecules from its surroundings. Another reason why hexane increases the activity of TbbApu may be that the enzyme's substrate specificity and affinity may have changed as a result of hydrophobic interactions with side groups of amino acids near the active site and in the hydrophobic region of the enzyme. The inference obtained from the studies

performed was that the stabilization of the enzymes increased with the interactions of the hydrophobic groups in the enzyme. The highest activity was achieved in the presence of 10 % water. This explains why the activity and stability of TbbApu increase with increasing hexane concentration. With the increasing hexane concentration, the optimum water content may have been reached and the stabilization of the enzyme's structure may have increased (Wang et al., 2016; Li et al., 2005).

TbbApu and its variants are stable up to 80 °C and they are stable at highly acidic pH. Also, they show higher stability and activity in the presence of hexane rather than the aqueous solution. These results can be explained by original organism habitat conditions. *Thermoanaerobacter brockii brockii* was isolated from Washburn thermal spring in Yellowstone National Park. The spring is classified as 'acid sulfate water' and the pH of the spring is less than 5 and the temperature of the spring is between 75-90 °C. Also, the spring contains alkanes such as methane, ethane, propane, etc. (Bergfeld et al., 2014).

3.4.4 Kinetic paramaters of TbbApu and Its Truncated Variants

To determine the kinetic parameters of α-amylase and pullulanase activities of TbbApu and its truncated variants, enzymatic reactions were set up with 0.5 μM enzyme and different concentrations of pullulan and soluble starch that are ranging from 4 to 50 mg/mL and 1 to 30 mg/mL respectively. According to Lineweaver Burk graphs, the kinetic parameters for pullulanase and α-amylase activity of each enzyme were calculated (Table 3.6). Lineweaver Burk graphs for pullulanase and α-amylase activity of each enzyme are given in Figure 3.17.

Table 3.6: Kinetic parameters of TbbApu and its truncated variants.

	Pullulanase			α-amylase		
Enzyme	Km (mg/ml)	kcat (1/s)	kcat/Km (ml/(mg*s))	Km (mg/ml)	kcat (1/s)	kcat/Km (ml/(mg*s))
TbbApu	4,35	117,33	26,97	1,72	120,00	69,77
TbbApuΔSH3	2,27	328,33	144,64	1,35	205,00	151,85
TbbApuΔCBM20	5	390,33	78,07	6,49	276,00	42,53
TbbApuΔX25-1-SH3	4,27	833,33	195,16	8,26	900,00	108,96
TbbApuΔX25-2-SH3	3,8	393,00	103,42	7,09	600,67	84,72
TbbApuΔX25-1-CBM20	5,88	548,33	93,25	16,1	1058,00	65,71
TbbApuΔX25-2-CBM20	6,71	772,67	115,15	18,88	821,00	43,49

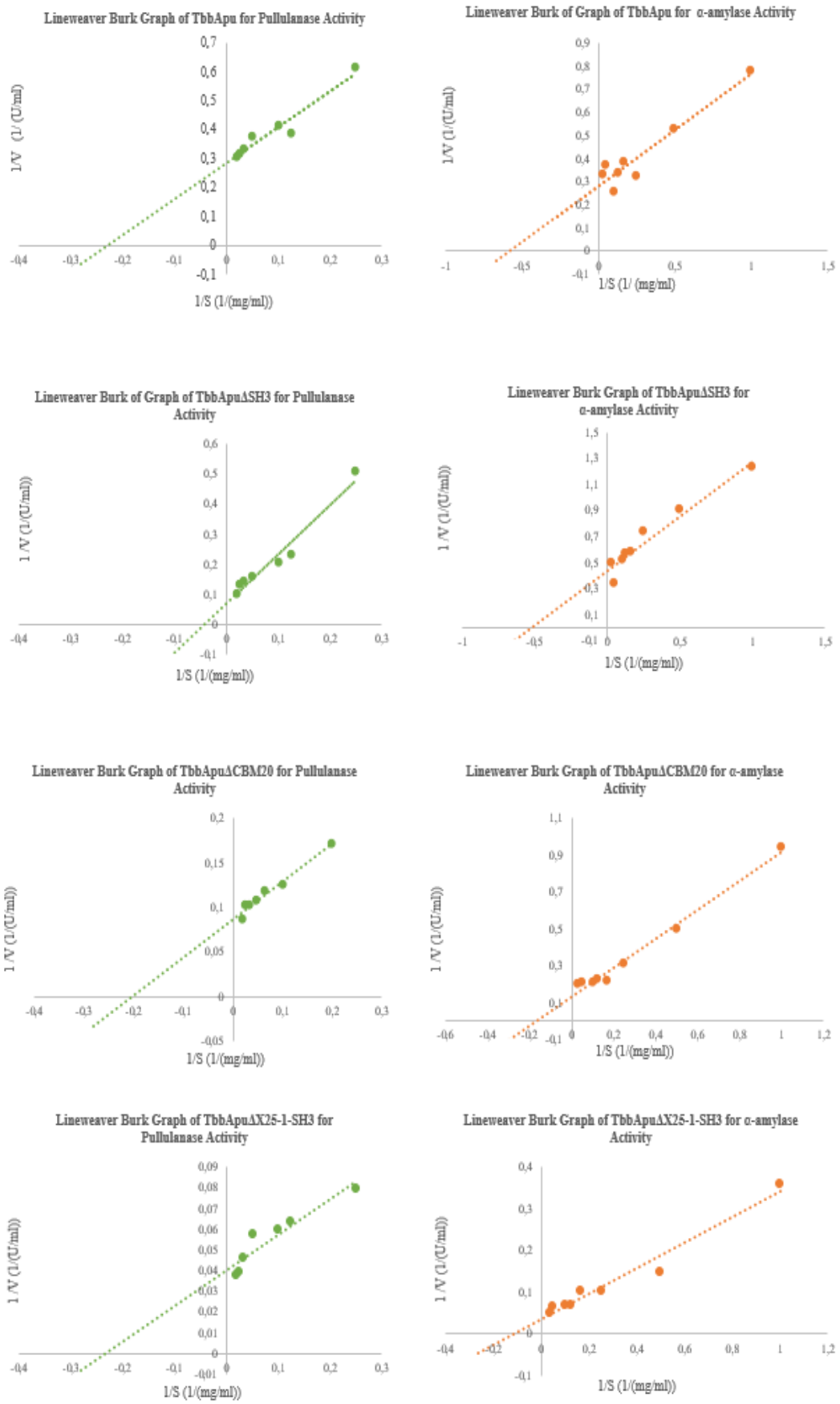

Figure 3.17: Lineweaver Burk graphs of TbbApu and its truncated variants.

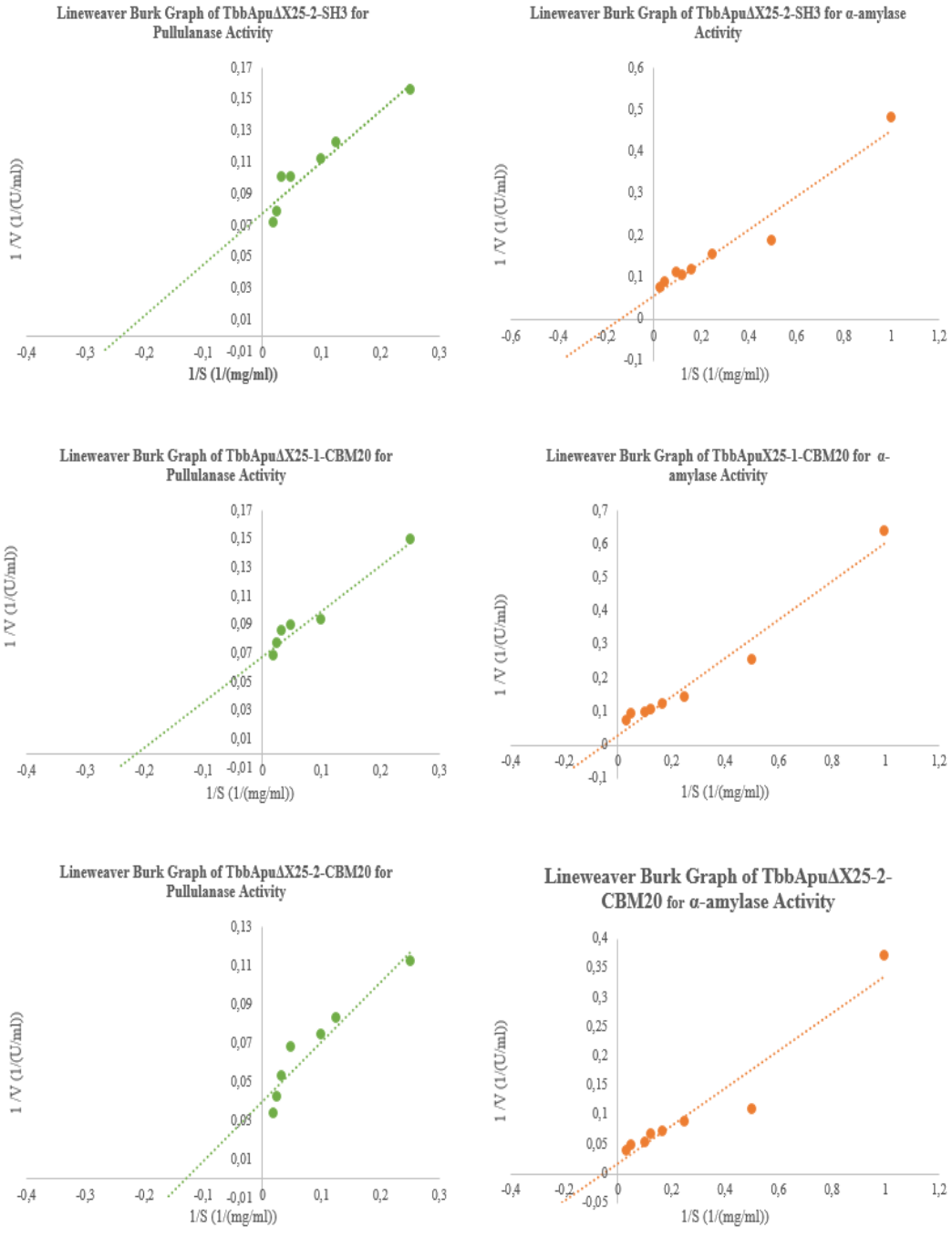

Figure 3.17 (continued): Lineweaver Burk graphs of TbbApu and its truncated variants.

According to k_{cat} / K_m values, the catalytic efficiency of TbbApuΔSH3 with pullulan and soluble starch was 4.25 and 2.18 fold, respectively, higher than wild type TbbApu. K_m values of pullulan hydrolysis for TbbApu and TbbApuΔSH3 were 4.35 mg/ml and 2.27 mg/ml respectively and K_m values of soluble starch hydrolysis for TbbApu and TbbApuΔSH3 were 1.72 mg/ml and 1.35 mg/ml respectively. It can be seen that the affinity of TbbApuΔSH3 increased approximately 1.92 and 1.27 fold for pullulan and

soluble starch respectively. Thus, truncation of the SH3 domain from TbbApu increased the affinity and specificity of the enzyme to both soluble starch and pullulan and it can be said that SH3 domain truncation improved both α-amylase and pullulanase activity of the enzyme. Truncation CBM20 from the enzyme increased the catalytic efficiency to pullulan 2.89 fold and decreased the catalytic efficiency to soluble starch 1.64 fold according to k_{cat} / K_m values. Also, CBM20 domain truncation caused the decreased affinity of the enzyme to soluble starch 3.77 fold. A significant increase in the K_m value of TbbApuΔCBM20 for soluble starch with a concomitant decrease in k_{cat} / K_m value beside TbbApu indicates that the CBM20 domain is important for soluble starch binding and degradation of TbbApu. The results are supported by the literature. CBM20 domain is a carbohydrate-binding module and is responsible for binding starch and other insoluble polysaccharides to the enzyme. Thus, it assists degradation of these substrates from enzymes (Christiansen et al., 2009). Not only the CBM20 domain but also the X25 domain is important for the α-amylase activity of TbbApu. As can be seen in Table 3.6, truncation sequential removal of X25 domains from the enyzme together with the SH3 domain improved the affinity of the enzyme to pullulan 1.02 and 1.14 fold and caused to decrease in the affinity of the enzyme to soluble starch 4.80 and 4.12 fold compared to wild type enzyme. At the same time, X25 domain(s) truncation improved the substrate specificity of the enzyme for pullulan over soluble starch. Thus, it can be seen that the X25 domain whose function is not known in literature assists in soluble starch degradation of the enzyme. The results are also supported by Nisha et al. (2015). Truncation N1 domain that was identified as the X25 domain of *B. acidopullulyticus* pullulanase from *Geobacillus stearothermophilus* NP33 amylopullulanase (gt-apu) caused to increase affinity and specificity of the truncated enzymes to pullulan. Also, sequential removal of X25 domains together with CBM20 domain from TbbApu caused to 9.36 and 10.97 fold increase in K_m value and a 1.06 and 1.60 decrease in k_{cat} / K_m for soluble starch compared to TbbApu. At the same time, CBM20 and X25 domain(s) truncation caused to decrease in specificity to soluble starch 1.06 fold for TbbApuΔX25-1-CBM20 and 1.60 fold for TbbApuΔX25-2-CBM20. To sum up, truncation CBM20 domain together with X25 domain(s) caused to improvement in the substrate specificity for pullulan over starch. Thus, it can be seen that CBM20 and X25 domains have crucial role α-amylase activity of TbbApu.

3.4.5 Raw starch adsorption of TbbApu and its truncated variants

TbbApu and its truncated variants were treated with different concentrations of raw starch (1-120 mg/ml) to understand the role of SH3, CBM20 and X25 domains in the raw starch binding of the enzyme. As can be seen in Figure 3.18, while TbbApuΔSH3 and TbbApuΔCBM20 showed similar raw starch binding ability with full-length TbbApu, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 lost their raw starch binding ability significantly according to TbbApu. Especially, TbbApuΔX25-2-CBM20 lost nearly all of the raw starch adsorption ability.

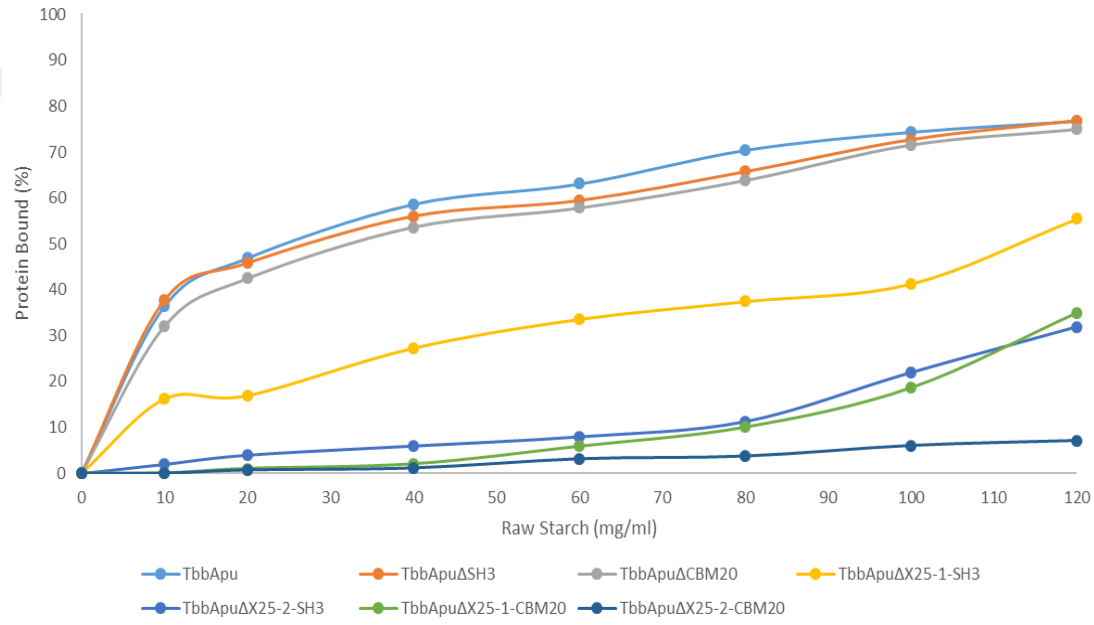

Figure 3.18: Raw starch binding percent of TbbApu and its truncated variants.

However, when the domains are examined, it is found that CBM20 is a carbohydrate-binding module and binds and disrupts the structure of starch and other insoluble polysaccharides to facilitate both degradation and reaching the polysaccharides to the active site. The CBM20 family consists of 90 to 130 amino acids, and analysis of several CBM20s shows that some of these amino acids are more highly conserved than other amino acids. These amino acids that are directly involved in interactions with glucan. The 3D structures of this module suggest that CBM20 contains two distinct glucan binding sites: 'binding site 1' and 'binding site 2'. Binding site 1 and binding site 2 contain critical aromatic amino acid residues that interact with the substrates directly (Christiansen et al., 2009; Ngo et al., 2019; Janeček et al., 2019). When the

CBM20 domains of TbbApu of other hydrolyze enzymes are compared, it can be seen that, the CBM20 domain of TbbApu contains highly conserved amino acid residues Phe1259, Trp1283, Tyr1299, Lys1313 and Trp1319 which are responsible for raw starch binding (Figure 3.19).

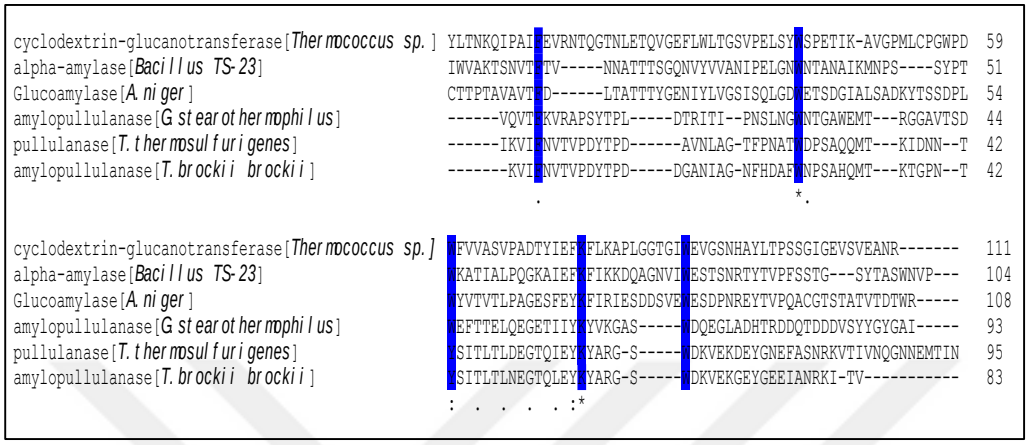

Figure 3.19: CBM20 domain sequence alignment of hydrolyze enzymes.

Although the CBM20 domain is responsible for adsorption of the raw starch, truncation of the domain did not any effect on the raw starch binding ability of TbbApu. However, truncation of the CBM20 domain from *Aspergillus niger* glucoamylase that consists of catalytic and CBM20 domain caused to loss of the binding ability of the enzyme to the raw starch (Dalmia & Nikolov, 1991). Thus, it can be concluded that other domains can compensate for the truncation of the CBM20 domain. One of the possible domains that compensate this function is the X25 domain. X25 domain is found in some amylopullulanases and pullulanases at their N-terminal region such as amylopullulanase of *Geobacillus stearothermophilus* TS-23 and pullulanase of *B. Acidopullulyticus.* Studies that carried out on *B. Acidopullulyticus* pullulanase and *Geobacillus stearothermophilus* NP33 amylopullulanase (gt-apu) suggest that X25 domains plays role in starch adsorption. Although its exact role is not known, studies on gt-apu, with the truncation of X25 domains from the enzyme caused decreased specificity against starch while it increased against pullulan and the raw starch adsorption rate decreased 1.5 fold (Turkenburg et al., 2009; Nisha et al., 2015). This result suggests that this domain may play a role in starch adsorption. In this study, it can be seen in Figure 3.18 that truncation of X25 domains together with CBM20 caused to loss of the binding ability of the enzyme completely. As a result of sequence alignment of X25 domains of TbbApu with gt-apuX25 domains, gt-apu CBM20 domain and TbbApu CBM20 domain, it can be seen that conserved amino acid

sequences which interact with raw starch in CBM20 are found in the same position at X25 domains of TbbApu which are Trp55, Tyr72, Lys88 and Trp95 in the TbbApu X25-1 domain, the Trp158, Tyr176, Lys190 and Trp197 X25-2 domain (Figure 3.20).

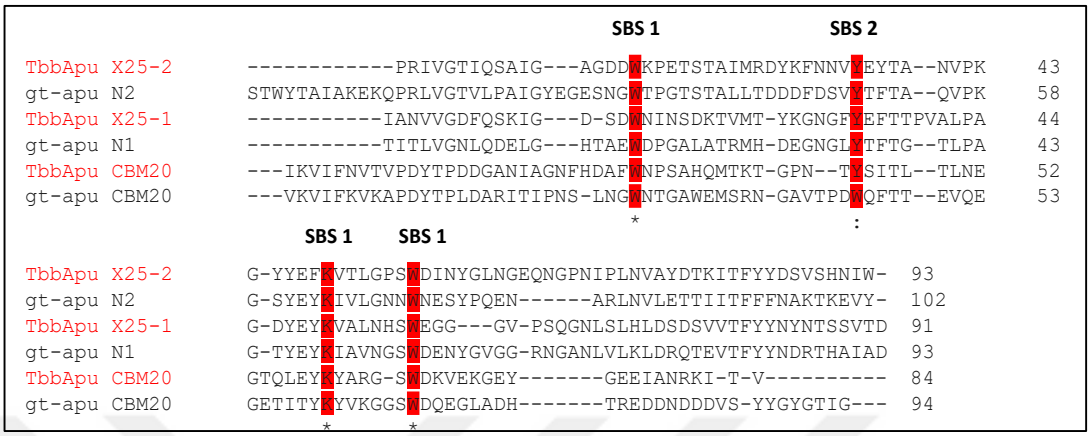

Figure 3.20: Sequence alignment of X25 domains with gt-apuX25 domains, gt-apu CBM20 domain and TbbApu CBM20 domain.

Truncation of the CBM20 domain with SH3 did not cause any effect on the raw starch binding ability of the enzyme; however, truncation of one and two X25 domains with SH3 caused to decrease of 27.3 % and 58.4 % of the raw starch binding ability of the enzyme respectively. Also, truncation CBM20 in the presence of X25 domains did not cause to change in the binding ability, while truncation of CBM20 with two X25 domains caused to loss of 90% binding ability of the enzyme. Thus, it can be said that the X25 domain plays the main role in raw starch adsorption than CBM20 at TbbApu and CBM20 domain cannot compensate for the absence of X25 domains.

3.4.6 End product analysis

To determine the hydrolysis product of TbbApu and its truncated variants, the thin layer chromatography method was used. Thin layer chromatography allows the identification of breakdown products of mono-, di-, tri- and oligosaccharides of enzymatic hydrolysis of carbohydrates (Hansen et al., 1975). When looking at Figure 3.21, both TbbApu and its truncated variants gave maltotriose as a breakdown product of pullulan. Pullulan consists of maltotriose units which are linked to each other via α-1,6 glycosidic bonds (Trinetta & Cutter, 2016). Thus, we can say that the enzymes cleavage α-1,6 glycosidic bonds in pullulan and hydrolyse pullulan to its maltotriose units. Also, as a result of hydrolysis of soluble starch, both TbbApu and its truncated variants gave maltose and maltotriose. It can be said that the enzymes can break down both α-1,4 glycosidic bonds and α-1,6 glycosidic bonds in starch. Maltose may be

given as products as a result of the breakdown of α-1,4 glycosidic bonds in linear amylose, maltose and maltotriose may be observed as a result of the breakdown of α-1,4 glycosidic bonds and α-1,6 glycosidic bonds. Also, glucose was not observed as a breakdown product of both pullulan and soluble starch and it can be explained that TbbApu and its truncated variants can only hydrolyse inner α-1,4 glycosidic bonds, not at the end of the α-1,4 glycosidic bonds.

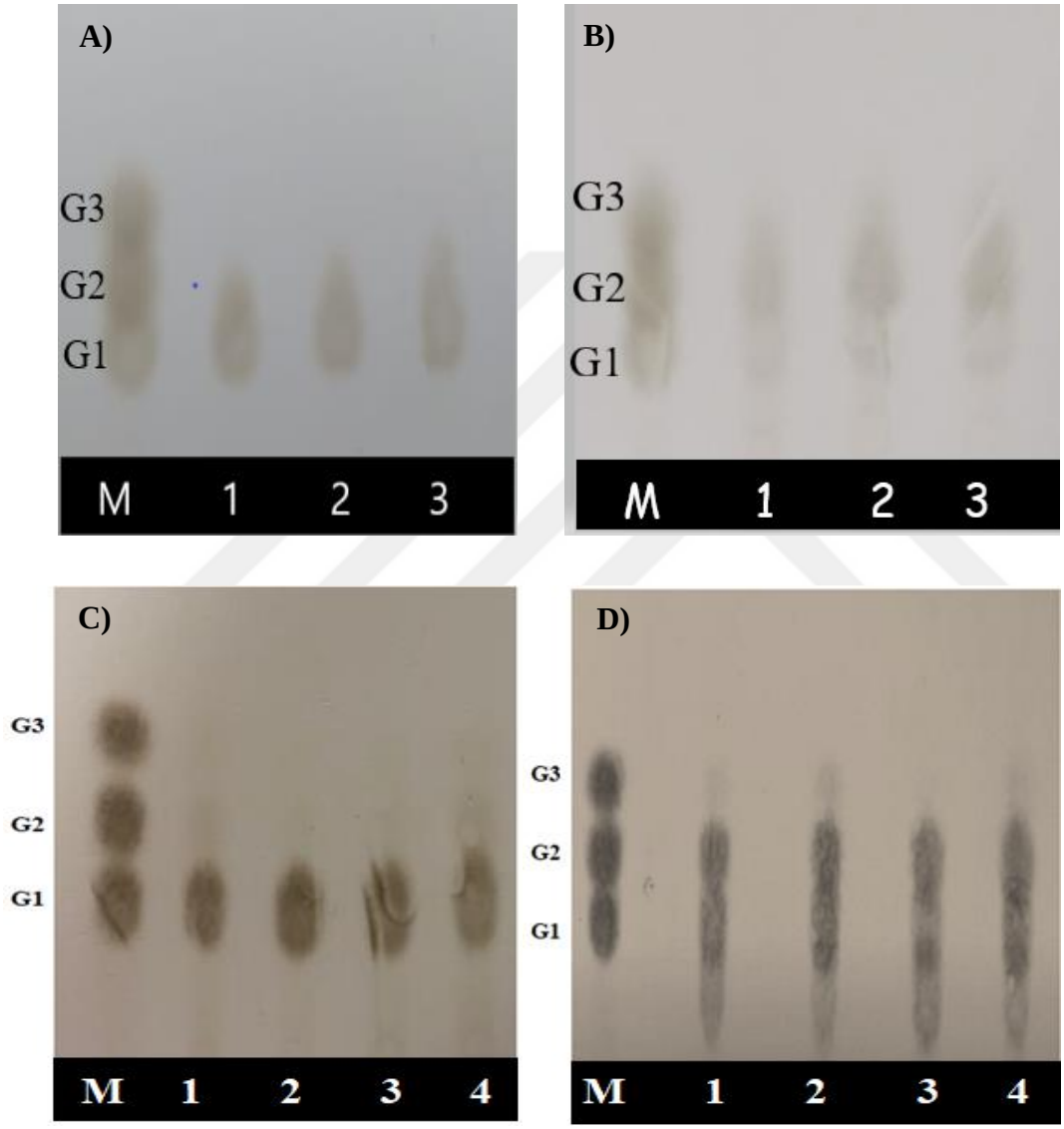

Figure 3.21: The end product analysis of starch and pullulan hydrolysis reaction catalyzed by TbbApu and its truncated variants. **A.** M: G1-G3 maltooligosaccharide markers (maltotriose, maltose and glucose); Lane 1: pullulan/ TbbApu; Lane 2: pullulan/ TbbApuΔSH3; Lane 3: pullulan/ TbbApuΔCBM20. **B.** M: G1-G3 maltooligosaccharide markers (maltotriose, maltose and glucose); Lane 1 soluble starch/TbbApu; lane 2: soluble starch/TbbApuΔSH3; lane 3: soluble starch/TbbApuΔCBM20. **C.** M: G1-G3 maltooligosaccharide markers (maltotriose, maltose and glucose); Lane 1: pullulan/ TbbApuΔX25-1-SH3; Lane 2: pullulan/ TbbApuΔX25-2-SH3; Lane 3: pullulan/ TbbApuΔX25-1-CBM20; Lane 4: pullulan/ TbbApuΔX25-2-CBM20. **D.** M: G1-G3 maltooligosaccharide markers (maltotriose, maltose and glucose); Lane 1: soluble starch/ TbbApuΔX25-1-SH3; Lane 2: soluble starch/ TbbApuΔX25-2-SH3; Lane 3: soluble starch/ TbbApuΔX25-1-CBM20; Lane 4: soluble starch/ TbbApuΔX25-2-CBM2

4. CONCLUSION

People had benefited from the reactions that carried out by enzymes to obtain various products in many fields since they did not know the existence of these catalysts. Today, more than 3.000 enzymes are used in industrial fields. However, usage of enzymes in the industry is limited because of the loss the stability of enzymes under harsh industrial process conditions. Together discovery of extremophilic organisms and their extremozymes and protein engineering studies help to exceed this problem.

In this study, amylopullulanase (TbbApu) a promising candidate for the starch hydrolysis process was studied. In the previous studies, cloning of the full-length gene of amylopullulanase (TbbApu) which originated from thermophilic *Thermoanaerobacter brockii brockii* and expression optimization were performed using four different hosts and two different expression vectors. Also, the variants of TbbApu which are TbbApuΔSH3 without SH3 domain and TbbApuΔCBM20 without CBM20 domain were constructed to understand the effect of SH3 and CBM20 domain on the enzyme. As a result of the studies, *E. coli* BL21 (DE3) and pET-28 a (+) expression vector were chosen as suitable host and as suitable vector, respectively. Also, truncation of SH3 and CBM20 domain from the TbbApu improves the expression of the enzyme. Although CBM20 is responsible for starch and other insoluble polysaccharides binding to the enzyme, truncation CBM20 domain from TbbApu did not cause any effect on the raw starch binding ability of the enzyme. At this point, it is hypothesized that there may be another domain in the enzyme that performs this function. It was seen that amino acid residues which are responsible for interaction with raw starch in CBM20 were found in the same position at X25 domains of TbbApu as a result of amino acid sequence alignment of X25 domain of TbbApu with CBM20 domains of TbbApu and *Geobacillus thermoleovorans* NP33 amylopullulanase. In this study, four C-term and N-term truncated variants which are TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 of TbbApu were constructed and the effects of X25, CBM20 and SH3 domains on the substrate binding, activity and stability of the enzyme were elucidated.

In the first part of the study, four truncated variants genomes were amplified with PCR using specific forward and reverse primers. As a result of the PCR, 4065 bp, 3750 bp, 3375 bp and 3060 bp truncated amylopullulanase genes which are *tbbApuΔX25-1-SH3, tbbApuΔX25-2-SH3, tbbApuΔX25-1-CBM20* and *tbbApuΔX25-2-CBM20* were amplified respectively. After that, both the amplified genes and the pET-28 a (+) expression vector were cut with *SacI* and *NotI* restriction enzymes to create a sticky end. After the double digestion, to insert the genes into pET-28 a (+) expression vector ligation reactions were set up. Then, the constructed plasmids were transferred to *E. coli* BL21 (DE3) cells with chemical transformation and the positive colony selection was done PRR activity assay.

In the second part of the study, both TbbApu and six truncated variants which are TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20 were over-expressed using magic media. Then, purification of the expressed protein was supplied with three purification steps: metal affinity chromatography, ion-exchange chromatography and heat purification. At the end of these three steps, both TbbApu and its truncated variants were purified.

In the third part of the study, biochemical characterisation of purified TbbApu and its truncated variants was performed. As a result of the studies, the optimum reaction temperature for pullulanase activity was determined as 70 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 75 °C for TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and 80 °C for TbbApuΔX25-2-CBM20 and the optimum reaction temperature for α-amylase activity was determined as 75 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 80 °C for TbbApuΔX25-1-SH3, TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20. The enzymes reached their > 80 % relative pullulanase and α-amylase activity after 60 °C and 75 °C respectively and preserved % 80 activity up to 85 °C. Another important result is that TbbApuΔX25-2-CBM20 preserved its 66 % pullulanase activity at 100 °C. The optimum pH values for both pullulanase and α-amylase activity were found at 6.5 for TbbApu, TbbApuΔX25-1-SH3 and TbbApuΔX25-1-CBM20, 6 for TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-2-SH3, 7 for TbbApuΔX25-2-CBM20. Both TbbApu and its variants kept their 80 % relative pullulanase and α-amylase between pH 5 and 8. After pH 8, they lost both activities significantly. In the

case of metal ion effects on the activity of the enzymes, it was seen that while Mn^{2+} and Co^{2+} ions increased both activities of the enzymes, Mg^{2+}, Zn^{2+} and Al^{3+} ions decreased both activities of the enzymes moderately. In the case of organic solvent effects on both activities, while the presence of hexane and acetone (20 %, 50 %) in the reaction mixture caused to increase in both activities of the enzymes with some exceptions, the presence of butanol, DMF and DMSO (20 % and 50 %) in the reactions caused to loss both activities of the enzyme significantly. In the case of detergents and inhibitors, while the used inhibitors and non-ionic and anionic detergents caused decreased both activities of the enzymes moderately with some exceptions, CTAB which is a cationic detergent caused to lose of both activities of each enzyme significantly.

After that, temperature, pH and organic solvent effect on the stability of TbbApu and its truncated variants were elucidated. While the most stable temperature was determined as 60 °C for TbbApu, TbbApuΔSH3, TbbApuΔCBM20, TbbApuΔX25-1-SH3 and TbbApuΔX25-2-SH3, 50 °C for TbbApuΔX25-1-CBM20 and 40 °C for TbbApuΔX25-2-CBM20 according to pullulanase activity of the enzymes, the most stable temperature was found 70 °C for TbbApu, TbbApuΔSH3 and TbbApuΔCBM20, 60 °C for TbbApuΔX25-1-SH3, 50 °C for TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-SH3, 40 °C for TbbApuΔX25-2-CBM20 according to the α-amylase activity of the enzymes. All of the enzymes preserved their ≥ 70 % pullulanase and α-amylase activities up to 70 °C. Also, it was concluded that truncation of X25 domain(s) together with SH3 and CBM20 domains affected the temperature stability of TbbApu according to both activities. In the case of the pH effect on the stability of the enzymes, the most stable pH was determined as pH 4 for TbbApu, TbbApuΔSH3, TbbApuΔCBM20 and TbbApuΔX25-2-CBM20, pH 6 for TbbApuΔX25-2-SH3 and pH 6.5 for TbbApuΔX25-1-SH3 and TbbApuΔX25-1-CBM20 according to their pullulanase activities, the most stable pH was found as pH 3 for TbbApu, TbbApuΔSH3, TbbApuΔCBM20, pH 6 for TbbApuΔX25-2-SH3, TbbApuΔX25-1-CBM20 and TbbApuΔX25-2-CBM20, pH 7 for TbbApuΔX25-1-SH3 according to their α-amylase activities. Both TbbApu and its truncated variants have kept their stability between pH 3 and 8 according to both activities. In the case of organic solvents, all of the enzymes showed higher stability than their control in the presence of acetone and hexane (20 %, 50 %) according to pullulanase activity and

had higher stability than their control in the presence of hexane (20 %, 50 %) according to α-amylase activities. Also, TbbApuΔSH3 was founded as the most stable variant against organic solvents. Resistance of TbbApu against inhibitors, detergents and organic solvents can be explained structural feature of the enzyme. TbbApu is isolated from a thermophilic organism; hence, TbbApu is a thermozyme. Thermozymes have some structural adaptations to resist high temperatures. These enzymes have a more rigid structure than mesophilic enzymes. Becoming a rigid, less flexible structure not only provides resistance to high temperatures but also provides resistance to inhibitors and detergents. At the same time, the increase in the stability of TbbApu in the presence of hexane also can be explained that hexane is a hydrophobic solvent and hydrophobic solvents remove water molecules from the microenvironment of the enzyme. Thus, enzymes gained rigidity.

After these studies, kinetic parameters of both TbbApu and its truncated variants were determined. According to the results, truncation of the SH3 domain from TbbApu increases both affinity and specificity to both pullulan and soluble starch. However, truncation CBM20 domain from the enzyme caused to decrease in affinity and specificity of the enzyme to soluble starch. While truncation of X25 domain(s) together with CBM20 domain caused decreased affinity and specificity to soluble starch and increased specificity to pullulan. It can be seen that CBM20 and X25 domains have crucial role α-amylase activity of the enzyme. Also, while truncation CBM20 domain did not cause any effect on the raw starch binding ability of the enzyme, truncation X25 domain(s) together with CBM20 domains caused to loss of 90 % raw starch binding ability of the enzyme. Thus, it can be said that X25 domain(s) have the main role in the raw starch binding ability of the enzyme.

Finally, end product analysis was done with the TLC method. According to results, both TbbApu and its truncated variants gave maltotriose as a result of hydrolysis pullulan. Thus, it can be said that enzymes can hydrolyze α-1,6 glycosidic bonds in pullulan. Also, the enzymes gave maltose and maltotriose as a consequence of soluble starch hydrolysis, so it can be seen that enzymes can cleavage both α-1,4 and α-1,6 glycosidic bonds in starch.

In conclusion, both biochemical characterisation of TbbApu and SH3, CBM20 and X25 domains function were elucidated in this study. Besides, it was realised that

TbbApu is a strong candidate for the starch hydrolysis process due to its stability at highly acidic pH values and high temperatures and also Ca^{2+} independence.

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APPENDICES

APPENDIX A: Laboratory equipments
APPENDIX B: Culture media composition
APPENDIX C: Buffers and solutions

APPENDIX A: Laboratory equipments

Analytical Balances: Precisa BT 610C

Autoclave: TOMY SX-700E HighPressure Steam Sterilizer

Centrifuges: Centurion Scintific K280R; Microfuge 18, Beckman Coulter; NF 800R, Nuve

Deep freezers and refrigerators: Ultra low freezer MDF-U4086S, Sanyo; Ultra low freezer MDF-U333, Sanyo; 1061 M refrigerator, Arcelik.

Electrophoresis equipment: Horizon 11.14, Whatman, Biometra Gel Casting System; Horizon 20-25, Whatman, Biometra Gel Casting System; Biorad Mini-PROTEAN® Gel Casting System.

Gel Documentation System: UVIpro GAS7000; Biometra UVStar Transilluminator

Ice maker: AF 10, Scotsman

Magnetic stirrer: Heidolph Standard

Microplate reader: Biorad Benchmark Microplate Reader

Orbital shaker: Forma orbital shaker, Thermo Electron Corporation

pH meter: SevenCompact pH meter S220, Mettler Toledo

Pipettes: Eppendorf

Rotator: Rotator SB series Model SB 2, Stuart

Sonicator: Sonoplus, Bandelin

Thermal Cyclers: Biometra Thermal Cycler; MiniAmp Plus

Thermomixer: Thermoshaker Ts1, Biometra

UV-Visible light spectrophotometer: UV Star Biometra

Vortex: SI-D256 Daigger

APPENDIX B: Culture media compositions

Luria Bertani (LB) Agar Medium (100 ml): 1 g tryptone, 0.5 g yeast extract and 0.5 g NaCl were dissolved in 100 mL of dH_2O and 1.5 g agar was added. The mixture was autoclaved at 121^oC for 15 minutes.

Luria Bertani (LB) Medium (100 ml): 1 g tryptone, 0.5 g yeast extract and 0.5 g NaCl were dissolved in 100 mL of dH_2O and autoclaved at 121^oC for 15 minutes.

PRR Agar Medium (100 ml): 1 g tryptone, 0.5 g yeast extract and 0.5 g NaCl, 50 mg red pullulan were dissolved in 100 mL of dH_2O and 1.5 g agar was added. The mixture was autoclaved at 121^oC for 15 minutes.

Magic Media (1000 ml): 39 g MagicMedia™ Component A was dissolved in 950 mL distilled water and autoclaved at 121^oC for 20 minutes. Before the usage, 50 ml MagicMedia™ Component B was added to the medium.

APPENDIX C: Buffers and solutions

Citrate/Phosphate Buffer (100 mM, pH 3): 37.1 ml of 0.2 M citric acid and 12.9 ml of 0.4 M Na2HPO4 were mixed and completed to 100 ml with dH2O.

Citrate/Phosphate Buffer (100 mM, pH 4): 30.7 ml of 0.2 M citric acid and 19.3 ml of 0.4 M Na2HPO4 were mixed and completed to 100 ml with dH2O.

Citrate/Phosphate Buffer (100 mM, pH 5): 24.3 ml of 0.2 M citric acid and 25.7 ml of 0.4 M Na2HPO4 were mixed and completed to 100 ml with dH2O.

Glycine-NaOH Buffer (100 mM, pH 9.5): 25 mL of 0.4 M glycine and 9.8 mL of 0.4 M NaOH were mixed and completed to 100 Ml with dH2O.

Glycine-NaOH Buffer (100 mM, pH 10): 25 mL of 0.4 M glycine and 16 mL of 0.4 M NaOH were mixed and completed to 100 Ml with dH2O.

Glycine-NaOH Buffer (100 mM, pH 11): 25 mL of 0.4 M glycine and 22.75 mL of 0.4 M NaOH were mixed and completed to 100 Ml with dH2O.

HisTrap Binding Buffer: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, pH 7.4

HisTrap Elution Buffer 1: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 100 mM imidazole, pH 7.4

HisTrap Elution Buffer 2: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 200 mM imidazole, pH 7.4

HisTrap Elution Buffer 3: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 250 mM imidazole, pH 7.4

HisTrap Elution Buffer 4: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 500 mM imidazole, pH 7.4

HisTrap Wash Buffer 1: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, pH 7.4

HisTrap Wash Buffer 2: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 40 mM imidazole, pH 7.4

HisTrap Wash Buffer 3: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 60 mM imidazole, pH 7.4

HisTrap Wash Buffer 4: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 80 mM imidazole, pH 7.4

IEX Elution Buffer: 20 mM L-histidine, 1 M NaCl, pH 6.5

IEX Start Buffer: 20 mM L-histidine, pH 6.5

IEX Wash Buffer 1: 20 mM L-histidine, 0.1 M NaCl, pH 6.5

IEX Wash Buffer 2: 20 mM L-histidine, 0.2 M NaCl, pH 6.5

IEX Wash Buffer 3: 20 mM L-histidine, 0.3 M NaCl, pH 6.5

IEX Wash Buffer 4: 20 mM L-histidine, 0.4 M NaCl, pH 6.5

IPTG (1M): 238 mg IPTG was dissolved in 1 mL of dH2O, filter sterilized and stored at -20ºC.

Kanamycin stock solution: 40 mg of kanamycin was dissolved in 1 mL of dH2O, filter sterilized and stored at -20ºC.

KH_2PO_4 (1M): 13.6 g KH2PO4 was dissolved in 100 ml dH2O.

K_2HPO_4 (1 M): 17.4 g K2HPO4 was dissolved in 100 ml dH2O.

NaH_2PO_4 (1M): 15.6 g NaH2PO4 was dissolved in 100 ml dH2O.

Na_2HPO_4 (1M): 17.8 g Na2HPO4 was dissolved in 100 ml dH2O**.**

Potassium Phosphate Buffer (100 mM, pH 6): 1. 32 ml of 1 M K2HPO4 and 8.68 ml of 1 M KH2PO4 were mixed and completed to 100 ml with dH2O.

Potassium Phosphate Buffer (100 mM, pH 6.5): 2.78 ml of 1 M K2HPO4 and 7.22 ml of 1 M KH2PO4 were mixed and completed to 100 ml with dH2O.

Potassium Phosphate Buffer (100 mM, pH 7): 6.15 ml of 1 M K2HPO4 and 3.85 ml of 1 M KH2PO4 were mixed and completed to 100 ml with dH2O.

SDS-PAGE Destaining solution: 40 mL of methanol, 10 mL of acetic acid, 50 mL of dH2O.

SDS-PAGE Resolving gel (10 %): 2320 mL of dH2O, 1.275 mL of 40% Acrylamid/Bisacrylamid (37.5:1), 1.3 mL of Tris-HCl pH 8.8, 50 µL of 10%SDS, 50 µL of 10%APS, 5 µL of TEMED.

SDS-PAGE Running buffer (10X-1L): 30 g of Tris, 144 g of glycine and 10 g of SDS were

dissolved in 1 L distilled water.

SDS-PAGE Sample loading buffer (5X): 90 mM TRIS-HCl, pH 6.8, 20 % Glycerol, 2 % SDS, 0.02 % Bromophenol blue, 0.1 M DTT

SDS-PAGE Stacking gel (5%): 2262 mL of dH2O, 300 µL of 40% Acrylamid/Bisacrylamid (37.5:1), 375 µL of Tris-HCl pH 6.8, 30 µL of 10% SDS, 30 µL of 10%APS, 3 µL of TEMED.

SDS-PAGE Staining solution (100 mL): 0.1 g of Coomassie Brilliant Blue R-250, 40 mL of methanol, 10 mL of acetic acid, 50 mL of dH2O

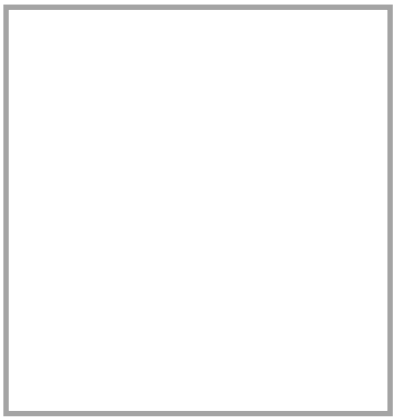
Sodium Phosphate Buffer (20 mM, pH 7.4): 8.1 mL of 1 M Na2HPO4 and 1.9 mL of 1 M NaH2PO4 were mixed and completed to 1000 mL with dH2O.

TAE Buffer (50X-1L): 242 g of Tris, 57.1 mL of glacial actic acid, 100 mL of 0.5 M EDTA (pH 8) was mixed and completed to 1L with dH2O.

Tris-HCl Buffer (100 mM, pH 8, 8.5, 9): 1.2114 g Tris was dissolved in 90 ml of dH2O and pH was adjusted 8, 8.5 and 9 with HCl. Then, the volume of the buffers completed to 100 ml with dH2O.

Zymography (8%): 2620 mL of dH2O, 975 mL of 40% Acrylamid/Bisacrylamid (37.5:1), 1.3 mL of Tris-HCl pH 8.8, 50 µL of 10%SDS, 50 µL of 10 % APS, 5 µL of TEMED, 5 mg red pullulan or 5 mg starch.

CURRICULUM VITAE



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