KARADENİZ TECHNICAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF MOLECULAR BIOTECHNOLOGY

CLONING, EXPRESSION, AND CHARACTERIZATION OF THE β-GLUCOSIDASE FROM *JIANGELLA UREILYTICA* KC603

MASTER THESIS

Arife KAÇIRAN

AUGUST 2020
TRABZON

KARADENİZ TECHNICAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MOLECULAR BIOTECHNOLOGY

CLONING, EXPERSSION, AND CHARACTERIZATION OF THE β-GLUCOSIDASE FROM *JIANGELLA UREILYTICA* KC603

Arife KAÇIRAN
ORCID : 0000 - 0002 - 6881 - 8568

This thesis is accepted to give the degree of
"MASTER OF SCIENCE"
By
The Graduate School of Natural and Applied Sciences at
Karadeniz Technical University

The Date of Submission : 31 / 08 /2020
The Date of Examination : 28 / 08 /2020

Supervisor : Prof. Dr. ALİ OSMAN BELDÜZ
ORCID : 0000 - 0003 - 2240 - 7568

Trabzon 2020

ACKNOWLEDGEMENT

The thesis study was carried out at the Molecular Biology Research Lab, Department of Biology at Karadeniz Technical University.

This study was supported by TUBITAK-PSF Bilateral cooperation call (118Z494) titled as “Specific designing of β-glucosidase for modification of natural products with the aim to enhance their cancer preventing potential”.

I express my deepest gratitude and sense of obligation to my research supervisor, Professor Dr. Ali Osman BELDÜZ, for his supervision and support. His constructive comments and valuable suggestions throughout the experiments and literature search have contributed to the success of this research. I have been in fact blessed to have the supervisor who cares about my work and who answers back to my queries and demands so promptly. I would also like to extend my thanks to Dr. Fulya AY ŞAL, Ass. Prof. Kadriye İNAN, Dr. Öğr. Üyesi Dilşat Nigar ÇOLAK, for providing me all the privileges during my research work. I would like to express my appreciation to all my lab fellows for their help. I appreciate all my friends and research fellows especially Miray ŞAHİNKAYA, for moral support during my work. I would also like to extend my thanks to Noman SALEH ZADA and Mariun SABA who supported me alot in writing thesis.

In particular, I would be honored to offer my gratitude to my dear family, who always empowers me with their spiritual support. Finally, I would also like to extend my thanks to my ex but never forgotten friends, who always know what I love and want, and who I pray to their existence.

Arife KAÇIRAN

Trabzon 2020

STATEMENT OF ETHICS

I declare that all the parts of master thesis entitled "Cloning, Expression, and Characterization of the B-Glucosidase from *Jiangella Ureilytica* Kc603" have been accomplished under the supervision of my advisor **Prof. Dr. Ali Osman BELDÜZ**. I also declare that I have collected the data myself, completed the experiments / analysis in the related laboratories, and observed the highest ethical and scientific standards during the development of thesis. I avoided plagiarism and fully acknowledged the work of others to which I have referred in this thesis. I admit all the legal consequences if the falsehood of this declaration is proven. 28/08/2020.

Arife KAÇIRAN

TABLE OF CONTENTS

ACKNOWLEDGEMENT III

STATEMENT OF ETHICS IV

TABLE OF CONTENTS V

SUMMARY VIII

ÖZET IX

FIGURE LIST X

TABLE LIST XI

SYMBOL LIST XII

1. INTRODUCTION 1

1.1. Actinobacteria 3

1.1.2. General features of the Genus *Jiangella* 4

1.1.2.1. *Jiangella ureilytica* KC603 6

1.2. Hydrolases (EC 3) 6

1.2.1. Glycoside Hydrolases (EC.3.2) 7

1.2.2. β-Glucosidase (EC3.2.1.21) 7

1.2.2.1. General Features of β-Glucosidases 8

1.2.2.2. Catalysis Mechanisms of β-Glucosidases 9

1.2.2.3. Use of β-Glucosidases in Industrial Processes 13

1.2.2.4. Substrate Specificity of β-glucosidases 15

1.3. Antioxidant Activity 18

1.3.1. Polyphenolic and Phenolic Compounds 20

1.3.2. The effect of β-glucosidases on Antioxidants 22

1.4. Glucose Tolerance of β-glucosidase 22

1.5. Purpose of the Study 24
2. MATERIAL AND METHOD 25
2.1. Material 25
2.1.1. Chemicals, Enzymes and Kits 25
2.1.2. Vectors and Bacteria 26
2.1.3. Bioinformatic Analysis 26
2.2. Method 27
2.2.1. Esculin Assay 27
2.2.2. Cloning and Expression of β-glucosidase 27
2.2.2.1. Primer Design and Amplification of β-Glucosidase Gene 27
2.2.2.2. Cloning of the β-glucosidase Gene into pGEM®-T Easy Vector System I 28
2.2.2.3. Cloning of the β-glucosidase Gene into pET28a (+) Expression Vector 29
2.2.2.4. Expression of β-glucosidase 29
2.2.2.5. Purification of β -glucosidase 30
2.2.2.6. SDS Polyacrylamide Gel Electrophoresis 31
2.2.2.7. Enzyme Assay 31
2.2.3. Characterization of the β-glucosidase 32
2.2.3.1. Optimum Temperature 32
2.2.3.2. Optimum pH 32
2.2.3.3. Enzyme Kinetics 32
2.2.3.4. Thermal Stability 33
2.2.3.5. pH Stability 33
2.2.3.6. Metal Ion Effect 33
2.2.3.7. Effects of Inhibitors and Organic Solvents 34
2.2.3.8. Glucose Tolerance 34
2.2.4 Analysis of Polydatin by Thin Layer Chromatography (TLC) 34
3. RESULTS 35

3.1. Esculin Assay 35
3.2. β-Glucosidase Amplification of *Jiangella ureilytica* KC603 35
3.3. Results of Bioinformatic Analysis 36
3.4. Cloning of the β-glucosidase Gene into pGEM®-T Easy Vector System I and pET28a (+) Expression Vector 38
3.5. Gene Expression and Protein Purification 39
3.6. Characterization of β-Glucosidase 40
3.6.1. Optimum Temperature 40
3.6.2. Optimum pH 41
3.6.3. Thermal Stability 42
3.6.4. pH Stability 42
3.6.5. Metal Ion Effect 43
3.6.6. Effects of Inhibitors and Organic Solvents 44
3.6.7. Glucose Tolerance 46
3.6.8. Enzyme Kinetics 47
3.6.9. Analysis of Polydatin by Thin Layer Chromatography 49
4. DISCUSSION 51
5. CONCLUSIONS 58
6. RECOMMENDATIONS 61
7. REFERENCES 62
CURRİCULUM VİTAE 68

Master Thesis / PhD. Thesis

SUMMARY

CLONING, EXPRESSION, AND CHARACTERIZATION OF THE B-GLUCOSIDASE FROM *JIANGELLA UREILYTICA* KC603

Arife KAÇIRAN

Karadeniz Technical University
The Graduate School of Natural and Applied Sciences
Molecular Biotechnology Graduate Program
Supervisor: Prof. Dr. Ali Osman BELDÜZ
2020, 80 Pages,

Carbohydrates within organic molecules are the most common molecules in the biosphere. The variety of these molecules is provided by the synthesis and demolition processes of many enzymes. Among these enzymes, β-glucosidases (EC.3.2.1.21) is mostly in the glycoside hydrolase family 1 and 3. β-glucosidases hydrolyzes oligosaccharides or the glycosidic bond between two groups, one carbohydrate and the other non-carbohydrate (aryl or alkyl). β-glucosidases are potenial candidate for use in deglycosylation of bulky phenolic compounds. In this study, deglycosylation activity of JurBglKC603 strain on phenolic compounds was investigated. First, the DNA sequences encoding for β-glucosidase of *Jiangella ureilytica KC603* (*JurBglKC603*) was cloned into the p-GEM®-T easy cloning vector and then it was subcloned into the pET28a (+) expression vector. JurBglKC603 was characterized and effects on phenolic compounds were investigated. The results obtained after the studies are as follows. The optimum temperature of JurBglKC603 was 35 $^{\circ}$C and the optimum pH was 6,0. Para-nitrophenyl β-D-glucopyranoside (PNPG) related kinetics parameters were examined for the substrate of the enzyme and for this substrate, kinetic parameters were determined as K_m = 0.448757 mM, V_{max} = 3790.176 U / mg, k_{cat} = 3217.405119 s^{-1} and k_{cat} / K_m = 7169.596 $s^{-1}mM^{-1}$. The effects of Mg^{2+}, Ni^{2+}, Ca^{2+}, K^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Na^{2+}, Hg^{2+} and Co^{2+} on this enzyme were examined. In the next step, the effect of JurBglKC603 on Cucubitacin (Cucurbitacin E-2-O-glucoside), Myricetin (myricetin-3-O-glucoside) and Resveratrol (piceid) substrates with phenolic properties was determined by TLC analysis. It was observed that JurBglKC603 deglycolysated only resveratrol out of these substrates. Thus, resveratrol, which is used in cancer treatment, has become more antioxidant by liberating its functional group as a result of deglycosylation.

Key Words: Jiangella ureilytica KC603, β-glucosidase, Cucubitacin, Resveratrol, Myricetin

Yüksek Lisans/Doktora Tezi

ÖZET

CLONING, EXPRESSION, AND CHARACTERIZATION OF THE B-GLUCOSIDASE FROM *JIANGELLA UREILYTICA* KC603

Arife KAÇIRAN

Karadeniz Teknik Üniversitesi
Fen Bilimleri Enstitüsü
Moleküler Biyoteknoloji Anabilim Dalı
Danışman: Prof. Dr. Ali Osman BELDÜZ
2020, 80 Sayfa,

Organik moleküller içinde yer alan karbohidratlar biyosferde en yaygın olan moleküllerdir. Bu moleküllerin çeşitliliği birçok enzimin sentez ve yıkım işlemleri ile sağlanır. Bu enzimlerden glikozid hidrolaz ailesinden 1. ve 3. aile içinde büyük bir grup olan β-glukozidazlar (β-D-glukozid glukohidrolazlar), oligosakkaritleri ya da biri karbohidrat diğeri karbohidrat olmayan (aril veya alkil) iki grup arasındaki glikozidik bağı hidrolizler. β-glukozidazların bilinen bir diğer özelliği de fenolik bileşikler üstüdeki etkisidir. Bu çalışmada *Jiangella ureilytica KC603* suşundan elde edilen β-glukozidaz (JurBglKC603) enziminin belirlenen fenolik bileşikler üzerindeki etkisi incelendi. İlk olarak *Jiangella ureilytica KC603* suşundan elde edilen β-glukozidaz genini kodlayan DNA dizisinin p-GEM®-T easy klonlama vektörüne ve ardından pET28a(+) ekspresyon vektörüne klonlama işlemi gerçekleştirildi. Saf olarak elde edilen β-glukozidaz enziminin karakterizasyonu gerçekleştirildi. JurBglKC603'nin optimum sıcaklığı 35 ºC ve optimum pH değeri 6,0 olduğu tespit edildi. Enzimin substratı olarak paranitrofenil butirat ile ilgili kinetik parametreleri incelendi ve bu substrat için kinetic parametreler, K_m = 0,448757 mM, V_{max} = 3790,176 U / mg, k_{cat} = 3217, 405119 s^{-1} ve k_{cat} / K_m = 7169,596 $s^{-1}mM^{-1}$ olarak belirlendi. Mg^{+2}, Ni, Ca^{+2}, K, Zn^{+2}, Fe^{+2}, Cu^{+2}, Mn^{+2}, Na^{+2}, Hg^{+2} ve C^{+2} iyonlarının bu enzim üzerindeki etkisi incelendi. İnhibitörler ve aktivatörlerin JurBglKC603 üzerindeki etkileri belirlendi. Sonraki aşamada fenolik özellik gösteren Cucubitasin (Cucurbitasin E-2-O-glucoside), Mrisetin (myricetin-3-O-glucoside) ve Resveratrol (Piceid) substratları üzerinde JurBglKC603'nın etkisi, TLC analizi ile belirlendi. JurBglKC603'ün, bu substratlardan sadece resveratrolü deglikolize ettiği gözlendi. Böylece kanser tedavisinde kullanılan resveratol, deglikolizasyon sonucu fonksiyonel grubunun açığa çıkarılması ile daha antioksidan hale getirilmiş oldu.

Anahtar Kelimeler: *Jiangella ureilytica* KC603, β-glukozidaz, Cucubitasin, Resveratrol, Mirisetin

FIGURE LIST

Page Number

Figure 1. Three-dimensional structure of β-glucosidase obtained from Streptomyces sp..9

Figure 2. General catalysis reaction scheme of β-glucosidases.. 10

Figure 3. Deconstruction of cellulose by cellulolytic enzymatic system. 11

Figure 4. General flow scheme of hydrolysis of cyanogenic β-glucosidases..... 12

Figure 5. Chemical structure of substrates specific to β-glucosidases.. 17

Figure 6. Phenolic compounds used in this study. 21

Figure 7. It is shows a schematic view of two enzyme active sites and the mechanism proposed for glucose tolerance in β-glucosidases;..... 23

Figure 8. β-glucosidase activity of JurBglKC603 by esculin assay. 35

Figure 9. *Jiangella ureilytica* KC603 β-glucosidase gene. 36

Figure 10. Conserved active region of JurBglKC603. 37

Figure 11. *Jiangella ureilytica* KC603 β-glucosidase gene fragment digestion with *EcoRI*, *NcoI* and *XhoI* restriction enzymes.. 39

Figure 12. Expression and and activity assay results of JurBglKC603 with substrate, p-nitrophenyl-β-D-glucopyranoside.. 40

Figure 13. Optimum temperature of JurBglKC603. 41

Figure 14. Optimum pH of JurBglKC603. 41

Figure 15. Thermal stability of JurBglKC603. 42

Figure 16. pH stability of JurBglKC603. 43

Figure 17. Effect of metal ions on JurBglKC603. 44

Figure 18. Effects of Inhibitors and Organic Solvents. 45

Figure 19. The glucose tolerance of JurBglKC603 47

Figure 20. Michaelis-Menten curve of JurBglKC603 (by OriginPro 2020b). 48

Figure 21. Lineweaver-Burk plot of JurBglKC603. 48

Figure 22. Analysis of deglycosylation of polydatin by JurBglKC603. 49

Figure 23. Analysis of deglycosylation of myricetin-3-O-glucoside and Cucurbitacin E-2-O-glucoside by JurBglKC603. 50

TABLE LIST

Page Number

Table 1. Enzyme groups and reactions that enzymes catalyze. 2

Table 2. General information about strains of *Jiangella* genus and genomes of their species 4

Table 3. The use of β-glucosidases obtained from microorganisms in some industrial process. 13

Table 4. Natural substrates obtained in different organisms 15

Table 5. Some antioxidant structures and the effect of mechanisms. 19

Table 6. Vectors and bacteria strains to used in the study. 26

Table 7. The designed primers for β-glucosidase of *Jiangella ureilytica* KC603 27

Table 8. Expression conditions tested for appropriate expression conditions 30

SYMBOL LIST

E. coli	: *Escherichia coli*
BglKC603	: β-glucosidase of *Jiangella ureilytica* KC603
μM	: Micromolar
kD	: Kilodaltons
mA	: Milliamp
mM	: Millimolar
mg	: Milligram
nm	: Nanometer
OD	: Optimum Density
PNPB	: p-nitrophenylbutyrate
p-NPG	: 4-Nitrophenyl β-D-glucopyranoside
SDS- PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SDS	: Sodium Dodecyl Sulfate
EDTA	: Ethylene Diamine Tetraacetic Acid
EGTA	: Ethylene Glycol Tetraacetic Acid
PMSF	: Phenylmethylsulfonyl Fluoride
DTT	: Dithiothreitol
DEPC	: Diethyl pyrocarbonate
ROS	: Reactive Oxygen Species
Tris	: Tris (hydroxymethyl) Aminomethane
V_{max}	: Maximum velocity
ε	: Molar absorption coefficie

1. INTRODUCTION

All living organisms on earth have been classified in to three groups: which includes Bacteria, Archea and eukaryotes. Bacteria are most widely distributed everywhere in the environment. On the basis of temperature conditions bacteria are categorized as: 1) Psychrophiles; can live between -10 ºC and 15 ºC, 2) mesophiles that can tolerate temperature range between 15 ºC and 50 ºC, however, 3) thermophiles can survive above 50 ºC (Trent, 2000).

Bacteria exist in various habitats such as soil, seawater, deep in the ocean, crust of earth, skin, intestines, and hides of animals, acidic hot water springs and radioactive waste. Bacteria play significant role in various industrial sectors, such as wastewater treatment; cheese, soy sauce, vinegar, wine and yoghurt production, as well as the production of pharmaceuticals agents, antibiotics and other chemicals essential for biotechnological fields. Bacteria are also of great importance in the elimination of industrial toxic wastes from chemical industry and environment, also for the production of pharmaceuticals and agrochemicals (Dworkin vd., 2006). Similarly, bacteria that can hydrolyze hydrocarbons in petroleum are often used to remove petroleum scattering. Moreover, enzyme productions by bacteria are also being exploited and their properties can be enhanced by modifying structure of DNA and plasmids through mutation by several means. Bacteria have been used as an experimental entity for comprehending biochemistry of cell by using enormous amounts of enzyme kinetics and gene expression in mathematical models of living organisms (Angelin-Bonnet vd., 2018).

Research on enyzmes have occupied most of the history of biochemistry. First important trials on biological catalysis by enzymes were carried out between 1760-1825. S.S.Berzelius, a Swedish chemist, conducted the first study on a particular enzyme in 1835, showing that diastasis hydrolyzes starch *'in vivo'* at a higher yield than sulfuric acid. Similarly, in 1860, L. Pasteur proved by experimentation that enzymes carried out the fermentation event. Therefore, the term 'ferment' has been used instead of the term “enzyme”. However, Pasteur supposed that enzymes can only function within the cell. The most important development in the field of enzyme technology was made in 1962 when J.

B. Sumner revealed that it was a protein compound after crystallizing the urease enzyme from the 'Jack Bean' plant. These findings, which were approached with suspicion in the past, was confirmed between 1930 and 1936 by J. Northrop crystallizing the pepsin, trypsin and chymotrypsin enzymes and demonstrating that they were in protein structure (Küfrevioğlu and Keha, 2012).

Several studies had carried out on enzymes by scientists and still vast amount of knowledge is being added into this field. Enzymes obtained as a result of these studies are classified in six different categories according to their structure and function, and specific enzyme commission (EC) number is alloted to them. According to International Biochemistry and Molecular Biology (IUBMB), the designation and classification of enzymes is based on the function of the enzyme in certain catalysis reaction mechanism (McDonald vd.,2008). At present, six main enzyme groups exist and are briefly named as Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases and Ligases (Table 1) (Orhan, 2003).

Table 1. Enzyme groups and reactions that enzymes catalyze (Işbakan, 2006).

Enzymes / EC numbers	**Reactions that enzymes catalyze**
Oxidoreductases (EC.1)	Enzymes that catalyze redox reactions between two substrates
Transferases (E.C.2)	Enzymes that catalyze the transfer of groups without hydrogen between two substrates.
Hydrolases (E.C.3)	They are enzymes that catalyze the hydrolytic reaction by releasing water molecule
Lyases (E.C.4)	Specific molecules are joined by forming double bonds is catalysed by these enzymes
Isomerases (E.C.5)	Enzymes that catalyze the conversion of geometric, optical or structural isomers to each other. It provide reorganization of bonds in a single molecule.
Ligases (E.C.6)	Enzymes that catalyzes the binding reactions of two molecules with the help of energy that results from the breaking of the phosphate bond from high energy phosphate compounds such as ATP and GTP.

1.1. Actinobacteria

Actinobacteria, present in both aquatic and terrestrial environments, are gram-positive bacteria with high GC content, which constitutes one of the largest bacterial phyla. When the sequencing of the actinobacterial genomes are performed, it is assumed that they belong to the relevant organisms in the field of medical, veterinary, biotechnology and ecology. Similarly, these phyla reflect the biological diversity of the observed genomic heterogeneity. Moreover, the fact that actinobacteria has a wide secondary metabolism, makes this group widely used in the pharmaceutical industry and agriculture. Actinomycetes are used in the production of most naturally derived antibiotics in the current clinical conditions, as well as many antifungal, anticancer and insecticidal compounds (Barka vd., 2016).

It is known that many of the actinobacteria have a free-living and undergo complicated morphological differentiation. Actinomycetes, which can grow through the combination of hyphae elongation and branching, have received their names due to these properties. Actinomycetes derived from the Greek words ray (actisor beam) and fungus (mucosa) and considered as properties between fungi and bacteria. Because of the formation of mycelium of most actinobacteria and the many of these mycelium actinomycetes reproduce by sporulation, it is similar to filamentous fungi (Bhatt vd., 2017). On the other hand, it is superficial as compared to mushrooms. The cells of actinomycetes are thin like all bacteria, as well as they have a prokaryotic nucleotide and a chromosome arranged on a peptidoglycan cell walls. At the same time, their cells are sensitive to antibacterial agents. When viewed physiologically and ecologically, most Actinobacteria are aerobic (Barka vd., 2016).

In the last decade, due to development of genomic sequencing and metabolic engineering, obtaining natural products and using their derivatives in the treatment of many diseases, research on actinobacteria have increased. The actinobacteria that is considered microbial cell factory used to produce most of the natural antibiotics. With the introduction of new bioinformatic tools and methods of metabolic engineering, synthetic reaction pathways of chemicals such as fatty acids and biofuels have been arranged in model organisms. With the development of these methods, regulations have been made on the production of natural antibiotics by actinobacteria (Tan and Liu, 2016).

1.1.2. General features of the Genus *Jiangella*

The members of the *Jiangella* located in the *Jiangellaceae* are in the Actinobacteria. *Jiangella* can be distinguished by morphological and chemotaxonomic features such as LL-diaminopimelic acid in the cell wall incorporated in the peptidoglycan and MK9 (H4) as the main menaquinone. *Jiangella* genus has limited tolerance to high sodium chloride level compared to other family types. There are eight species belong to the genus *Jiangella* in the literature (Table 2). These are *Jiangella gansuensis* isolated from desert soils, *Jiangella alba, Jiangella alkaliphila, Jiangella endophytica* and *Jiangella rizosphaerae* isolated from plant tissues, *Jiangella anatolica* obtained from the crater lake and mangrove beaches, and *Jiangella muralis* isolated from a mangroves soil environment (Saygın vd., 2019).

Table 2. General information about strains of *Jiangella* genus and genomes of their species (Saygın vd., 2019).

	Genbank accession number	**contig**	**Size (bp)**	**Protein coding sequences**	**RNA**	**DNA G + C content (mole%)**
***Jiangella asiatica* 5K138**	SMKZ00000000	134	7034063	6786	51	71.0
***Jiangella aurantiaca* 8K307**	SMLB00000000	102	6620775	6289	50	71.8

Table 2. continues

***Jiangella ureilytica* KC603**	**SMKL00000000**	**168**	**6345964**	**6104**	**50**	**72.5**
***Jiangella alba* DSM 45237**	FNUC00000000	4	7681885	7160	54	72.8
***Jiangella alkaliphila* DSM 45079T**	LT629791	1	7716600	7399	53	72.1
***Jiangella anatolica* GTF31**	POTW01000000	256	7005973	6785	51	72.5
***Jiangella endophytica* KE2-3**	QVAI00000000	89	7850704	5344	51	72.3
***Jiangella gansuensis* DSM 44835**	AZXT01000000	9	5584980	6945	50	70.9
***Jiangella asiatica* 5K138**	SMKZ00000000	134	7034063	6786	51	71.0
***Jiangella aurantiaca* 8K307**	SMLB00000000	102	6620775	6289	50	71.8

Although Jiangella can be isolated from different habitats, arid and desert habitats draw attention for their potential and metabolic abilities to host new Actinobacteria. Therefore, *Jiangella ureilytica* KC603 isolated from soil from the Karakum Desert was used in the present study **(**Saygın vd., 2020).

1.1.2.1. *Jiangella ureilytica* KC603

Jiangella ureilytica KC603 strain is aerobic and grouped into gram-positive actinomycetes that form a substrate mycelium fragment to short or extended rods. According to the studies in the literature, it is known that strains ISP3, ISP4 and Czapek show good growth in agar environment, while ISP2, ISP5 showed growth in tryptic soy and nutrient agar medium growth, and do not produce dispersible pigment on any tested medium. Optimum temperature required to grow is 28-37 ºC, while the optimum pH range is 6.0-9.0. It has a 3% tolerance against NaCl **(**Saygın vd., 2020).

1.2. Hydrolases (EC 3)

Enzymes are bio-catalysts, produced by all domain of lives. They accelerate chemical reactions in living organisms and proudce essential products at the end. Enzymes have been used outside of the living cells in adequate enviroment which have replaced many conventially used chemical reagents. For this reason, production, purification and revealing effects of enzyme in biochemical reactions and investigating their kinetic properties are of great importance over the past few decades (Hamza, 2017).

Based on the literature, hydrolases are used more often in polymer studies than other enzymes. Hydrolases that act on ester, glycosyl, ether, peptide, acid, anhydride C-C, C-Halogen and P-N bonds catalyze the hydrolysis reactions of these compounds. Hydrolases are known as macromolecular organic catalysts that can hydrolyze substrates in the presence of water (Pandey vd.,2017). Hydrolases are widely used to perform polymerization and polymer modification reactions in addition to their natural function of hydrolyzing substrates. Many hydrolases are not specific and can be divided into many subgroups. Lipases, phosphatases, glucosidases, peptidases, nucleosidases and esterases are some commonly used hydrolase enzymes (Gübitz and Paulo, 2003).

1.2.1. Glycoside Hydrolases (EC.3.2)

Many enzymes such as polysaccharide ligases, carbohydrate esterases and glycoside hydrolases are involved in the complete decomposition of polysaccharides. Glycoside hydrolases are group of enzymes hydrolyze the glycoside bond between two or more carbohydrates or a carbohydrate and a non-carbohydrate moiety. This group of enzymes breaks down the glycoside bonds in polysaccharides and oligosaccharides and produces shorter metabolizable products. According to International Biochemistry and Molecular Biology (IUBMB), the designation of glycoside hydrolases is done by looking at the substrate specificity and sometimes its molecular mechanism rather than the structural features of the enzyme. However, some glycoside hydrolases, such as GH16, exhibit mixed substrate specificity. Identification of sequenced genomes and specific glycoside hydrolase sites in metagenomes provide estimation of the structural analysis potential of molecules such as starch, cellulose, chitin and dextran (Pandey vd., 2017).

1.2.2. β-Glucosidase (EC3.2.1.21)

Carbohydrates within organic molecules are the most common molecules in the biosphere. The variety of these molecules is provided by the synthesis and demolition processes of many enzymes. Among these enzymes, β-glucosidases (β-D-glucoside glycohydrolase), a large group in the 1st and 3rd family of the glycoside hydrolase family, hydrolyzes oligosaccharides or the glycosidic bond between two groups which is one carbohydrate and the other non-carbohydrate (aryl or alkyl). These enzymes are found in all living things, from bacteria to humans, and they have great importance in basic biological and biotechnological processes. Most of the microbial beta-glucosidases belong to family 1 and 3 of glycosyl hydolyses family. These enzymes, β-O-glucosidases (EC 3.2.1.21) that hydrolyze oxygen-bound β-glycosides, and β-S- glucosidases (3.2.3.1) which are largely in the *Brassicaceae* family in plants that hydrolyze sulfur-bound β-glycosides (glucosinolates, β-thioglucosides), can be grouped in two (Gemici, 2006).

There have been many studies intended to understand the structure and functions of β-glucosidases. Although plant β-glucosidases have high sequence homology between

them, researchers suggest that there are many differences in their substrate specificities. Therefore, plant β-glucosidases are used as a model system especially for the solution of problems related to substrate specificity (Esen,1993).

1.2.2.1. General Features of β-Glucosidases

Besides the aryl and alkyl β-D-glucosides, β- glucosidases are known as β- D - glucoside glycohydrolases. They catalyze glycoside bond that have a carbohydrate moiety such as cellobiose and oligosaccharides. These enzymes are known as cellulolytic β-glucosidases found in bacteria and yeasts, lysosomal glucocerebrosidases found in animals and β-glucosidases found in plants. The cellulolytic β-glucosidases found in bacteria and yeast are involved in the reduction of cellulose to glucose, while the β-glucosidases in the plant are involved in defense in plant membranes, lignification, phytohormone activation and cell wall component catabolism. According to Ergöçen; bacteria, fungi, human and such as *Sinapis alba* (white mustard), some binary plant that had the β-glucosidases were glycosylated, but some monochromatic plant such as *Zea mays* (maize) and *Sorghum bicolor* (millet) were not glycosylated (Ergöçen, 2013).

In the basic structure of each of the β-glucosidase family 1 monomers, high conservation peptide motifs are situated. These motifs are known as SAYQI, YRFSI, TFNEP, LGLNYY, YITENG and DNFEW. Of these motifs, the TFNEP and YITENG region are part of the active site of the enzymes and contain two catalytic glutamates (Ekmekci, 2016).

In general, it is accepted that the structural and functional properties of β-glucosidase enzyme are common. The active forms of these enzymes, which are composed of subunits with molecular weights of 50-70 kD, vary from monomeric structure to homomultimeric structure. When the three-dimensional structures of β-glucosidases obtained in different origins were examined, although there was 17-44% sequence similarity between them, it was observed that the structures formed bearingly with the (α/β)8 turn were the same (Figure 1) (Ekmekci, 2016).

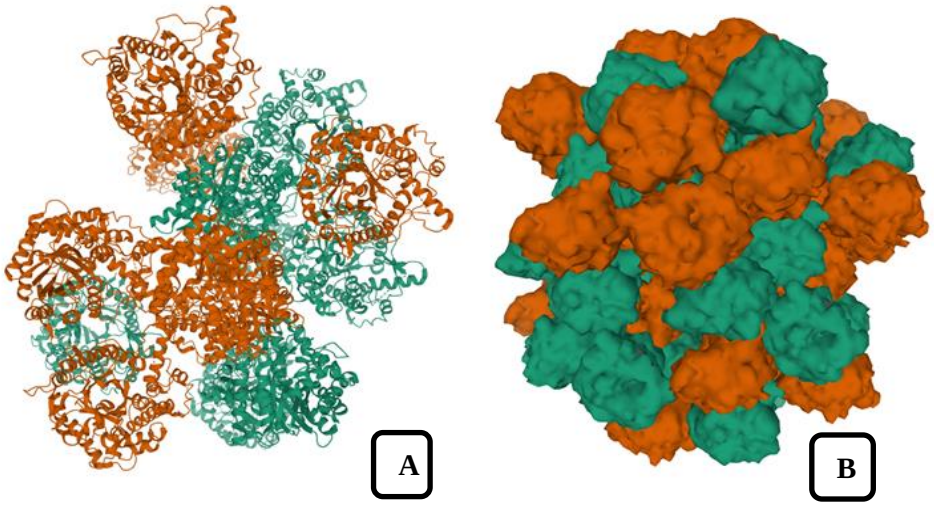


Figure 1. Three-dimensional structure of β-glucosidase obtained from *Streptomyces* sp. **A)** Schematic representation of the protein backbone shape, colored by secondary structure types: **B)** Surface representation accessible by the solvent, colored by residue types (Guasch vd., 2002).

Substrates of β-glucosidases contain glycon and aglycone (aryl or alkyl) groups and aglycone parts of the substrates are bonded by β-glycoside bond or oligosaccharide that has β-bond. Beta glucosidases need substrates with glycon and aglycone (aryl or alkyl) groups in order to complete their catalytic functions. The maximum pH range that the enzyme β-glucosidase can make catalyis is generally 4-7. β-glucosidases from different organisms have been shown to have high substrate specificity with β-glycosides aglycon groups (Sönmez, 2013).

1.2.2.2. Catalysis Mechanisms of β-Glucosidases

It is known that β-glucosidases perform hydrolysis of β-glucoside bonds in oligosaccharides or glucosides. These enzymes biochemically perform hydrolysis of the glycosidic bonds of aryl or alkyl β-glucosides and release a β-glucose and an aglycon (Figure 2) (Kara, 2010).

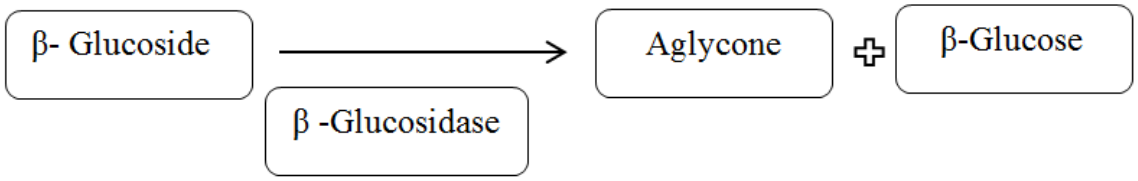


Figure 2. General catalysis reaction scheme of β-glucosidases. Hydrolysis of the glycosidic bonds of aryl or alkyl β-glucosides by β-glucosidases release a β-glucose and an aglycon.

β-glucosidases are ubiquitous to all domain of lives and are involved in many biochemical reactions in living cells. β-glucosidases along with other hydrolyases of cellulolytic enzymatic system deconstruct cellulose. It has been found that β-glucosidases of bacteria, fungi, humans and dicotyledon plants such as *Sinapis alba* (white mustard) are glycosylated, whereas those of monocot plants such as *Zea mays* (maize) and *Sorghum bicolor* (millet) are not glycosylated (Gemici, 2006). These enzymes are mainly exo-gluconases/exo-celluloses and endo-gluconases. Exo-gluconases acts on terminal ends of the cellulose chains, producing short chains which are in turn acted upon by endogluconases to produce cellobioses. β-glucosidases in turn acts on cellobioses to produce glucose (Figure 3) (Artzi vd., 2016).

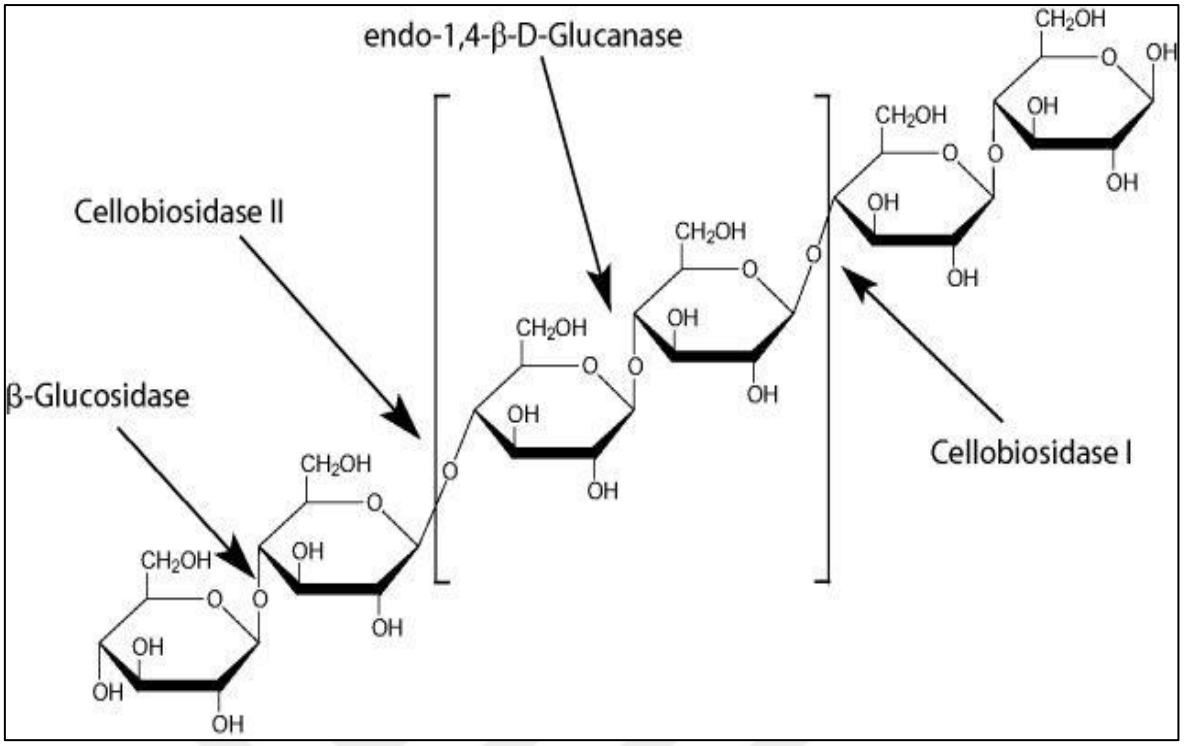


Figure 3. Deconstruction of cellulose by cellulolytic enzymatic system. β-glucosidase degrade the cellulose that is intermediate product to glucose (Uzun, 2015).

The second task of β-glucosidases is involved in the hydrolysis of the β-glucosidic bond in endogenous cyanogenic glycosides. In this reaction, β-glucosidases catalyze the formation of aglycan and cyanohydrins by hydrolyzing the β-glucosidic bonds found in endogenous cyanogenic glycosides. Cyanohydrins in the aglycan part of cyanogenic glycosides are known as the source of HCN in nature and also take part in nitrogen transport metabolism. HCN, which is used as a nitrogen source in the biosynthesis of L-asparagine, protects the plant in tissue injuries caused by herbivore and fungal attacks. The demolition of cyanogenic glucosides, which are largely in plants, is known as cyanogenesis, and in this event, glucosides occur in a two-stage decomposition reaction (Kara, 2010). The reaction steps are shown in Figure 4.

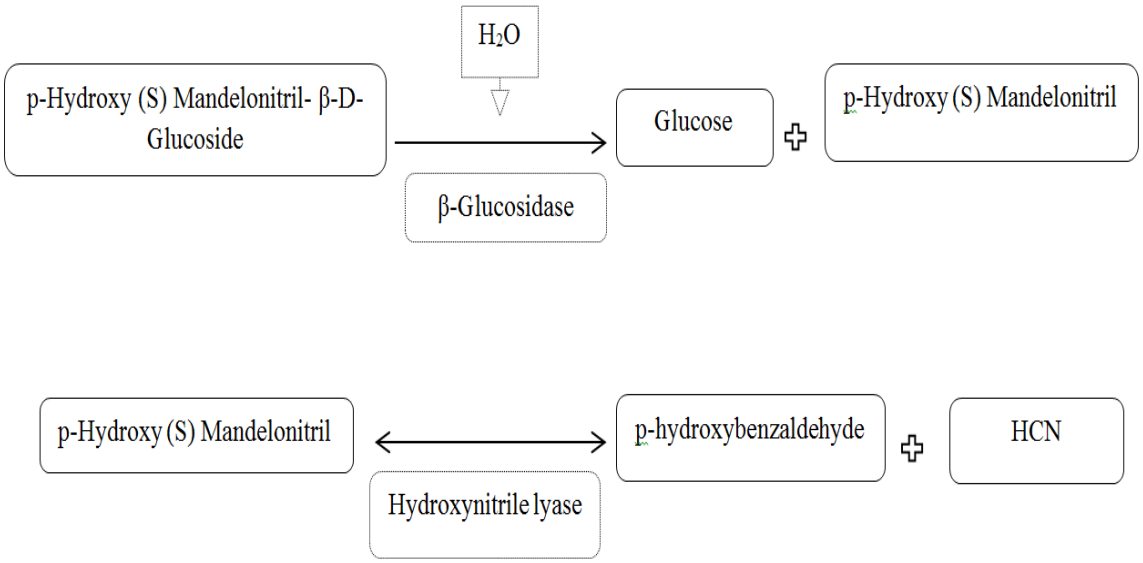


Figure 4. General flow scheme of hydrolysis of cyanogenic β-glucosidases. In the first step, it has been shown that cyanogenic glycosides are hydrolyzed to glucose and hydroxynitrile by β-glucosidase. Second step, the hydroxynitrile is separated into ketone and HCN by a hydroxynitrile lyase.

In the first step, cyanogenic glucosides are hydrolyzed into glucose and hydroxynitrine by β-glucosidase. In the second step, hydroxynitrile is divided into ketone and HCN compounds by a hydroxynitrile lyase. The catalysis mechanism performing the hydrolysis of the glycosidic bonds in the β-glucosides, used as substrate by the β-glucosidases, is called “retention”. In this mechanism, firstly the β-glucosidic bonds are hydrolyzed, the enzyme is glycosylated and the aglycon is released; Secondly, glucose is separated from the enzyme and glucon is released (Ekmekci, 2016).

Another important task of β-glucosidases is that it plays a role in the regulation of biological activities of plant hormones. β-glucosidases regulate the biological activity of hormones such as cytokinin, gibberellin and auxine, by releasing active hormone forms from hormonal glucoside compounds found inactivated in plants (Morant vd., 2008).

1.2.2.3. Use of β-Glucosidases in Industrial Processes

β-glucosidases, which are of great importance for biological systems, are used in many fields such as biomass, food detoxification, beverage flavoring, pharmaceutical and detergent industry if *in vitro* ambient conditions are provided. It is known that β-glucosidases are responsible for the hydrolysis of cellobiose, the formation of intermediates necessary for cell wall lignification in plants and plant defense against biotic stress (Singh vd., 2016). β-glucosidases in mammals are thought to play a role in the metabolism and signal functions of glycolipids and dietary glycosides. β-glucosidases are of great importance both in terms of their functions and industry. These enzymes are used in various biotechnological applications in the food, cosmetics, pharmaceutical and detergent industries, such as the synthesis of different glycosides, surfactants, biofuels and the agricultural industry (Singh vd., 2016). Besides, many β-glucosidase enzymes have important biological activities such as showing antioxidant properties, lowering blood glucose level, protecting against harmful freezing and thawing damage, and inhibiting tyrosinase. The use of β-glucosidases in some industrial areas is given in detail in the Table 3 (Singhania vd., 2013).

Table 3. The use of β-glucosidases obtained from microorganisms in some industrial process (Singhania vd., 2013).

Microorganism used	Method used	Application areas
Debaryomyces Pseudopolymorphus	Shake-Flask (Shaking) Fermentation	Increase of Wine Flavor
Trichoderma atroviride	Shake-Flask (Shaking) Fermentation	Biomass Hydrolysis
Penicillium pinophilum	Smf, (Fermentor with Working Volume Less Than 200ml)	Cellobiose Hydrolysis
Penicillium citrinum	Solid-State (solid state) Fermentation	Cellulosic Biomass Conversion
Periconia Sp.	Smf	Biomass Transformation

Table 3. continues

Penicillium decumbens	Smf	Biomass Transformation(from corncob)
Penicillium echinulatum	Smf	Biomass Hydrolysis
Stachybotrys sp	Fed-Batch Smf	Cellobiose Hydrolysis
Humicola insolens	Shake Flask	Cellulosic Biomass Hydrolysis
Fomitopsis palustris	Shake Flask	Cellobiose Hydrolysis
Fomitopsis sp	Solid-State Fermentation	Rice and Wheat Straw Hydrolysis
Aspergillus niger	Smf, Shake Flask	Biomass Hydrolysis
Debaryomyces Pseudopolymorphus	Smf, Shake Flask	Biomass Hydrolysis

β-glucosidases are used in many fields in industrial process. The most striking part of these areas of use is biofuel production. Biofuel production is a technique developed to solve existing environmental problems that are originate from fossil fuels. β-glucosidases in biofuel production from biomass produce monomeric sugars from cellulose based oligosaccharides, providing hydrolysis of biomass. At the same time, the production and availability of this enzyme plays an important role in increasing the total production cost in biofuel production technology (Srivastava vd., 2018). Therefore, β-glucosidases are highly needed enzymes in biogas production. β-glucosidases also play a major role in the conversion of biomass into liquid biofuels. β-glucosidases are the important enzyme component found among cellulases and complete the final stage by converting cellobiose to glucose during cellulose hydrolysis. Considering this function of the β-glucosidase enzyme, it plays a key role in the production of bioethanol from biomass by enzymatic route. In a sample study, a β-glucosidase gene obtained from *Saccharomycopsis fibuligera* was transferred to the selected host cell and its expression was achieved. During the studies, the amount of enzymes produced using different host cells and their effect on bioethanol production were examined and the results presented new information applicable to bioethanol production (Gurgu vd., 2011).

1.2.2.4. Substrate Specificity of β-glucosidases

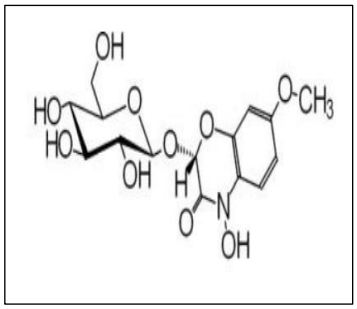
Representing the most important group of glycoside hydrolases, β-glucosidases play an important role in various basic biological and biotechnological processes. β-glucosidases, which are abundant in nature, show specificity in terms of substrate recognition and binding ability. Products such as steroid β-glucosides and β-glucoside ceramides produced by mammals, cyanogenic β-glucosides and glucosinolates of plant secondary metabolism and oligosaccharides released from the demolition of cellulose in the plant cell wall are known as natural substrates of β-glucosidases. β-glucosidases are highly specific to these naturally occurring substrates. (Some natural products are included in Table 4.) Substrate specificity in β-glycosidases generally means aglycon specificity, as the glucose group in β-glycosidic substrates is unchanged. Since the glycon part (glucose) of the substrate does not change, it cannot be considered as the basis of substrate specificity (Kim and Ma, 2018).

Table 4. Natural substrates obtained in different organisms (Gemici, 2006).

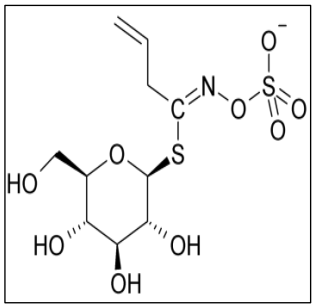
Substrate	Mono-/ Di glucoside	Source Organism
Amygdalin	di-	*Prunus serotina*
Prunasin	mono-	*Manihat esculenta Crantz*
Linustatin	di-	*Linum usitatissimum*
Neolinustatin	di-	*Linum usitatissimum*
Dihurin	mono-	*Sorghum bicolor Moench*
Linamarin	mono-	*Linum usitatissimum*
Gentiobiose3	mono-	*Prunus serotina*
Coniferin	di-	*Hevea brasiliensis* *Prunus serotina*
Cellobiose *reesei*	di-	*Trichoderma reesei*
Cello-oligosaccharides	di-	*Trichoderma reesei*
DIMBOAGlc	mono-	*Zea mays Hordeum vulgare*
DIBOAGlc	mono-	*Secale cereale*

Hydrolysis of the β-glycosidic bond of β-glucosidases in the glucose hydrolase 1 family is of great importance for substrates. This catalysis mechanism takes place in two steps (glycosylation and deglycosylation) with the hydrolysis of the β-glycosidic bond, the maintenance of the anomeric configuration (Withers, 1996). The reaction is usually carried out by protein donor region and nucleophilic active region that are conserved. Nucleophile active region which have conserved amino acids, take part in the substrate hydrolysis, whereas protein donor region take part in the formation of hydrogen bond and catalysis (Graebin vd., 2016). In the first stage, the nucleophile active region plays the role of a nucleophile, attacking the anomeric centre to displace the aglycone and form a glycosyl enzyme intermediate. At the same time protein donor region functions as an acid catalyst and protonates the glycosidic oxygen. In the second step (known as the deglycosylation step), the glycosyl enzyme is hydrolyzed with water, and the active regions of the enzyme return to their normal form (Xu vd., 2004).

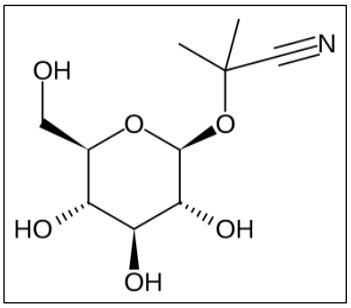
Although natural substrates are pure and easily obtainable, they are not used during enzyme purification and characterization. Synthetic substrates are commonly used to determine the activity of enzymes in *in vitro* conditions and to identify and characterize natural substrates (Czjzek vd., 2000). It is frequently used in biochemical analysis since the speed of enzymatic reactions with artificial substrates and the relevance of the enzyme to the substrate can be easily tested. On the other hand, β-glucosidases have limited ability to hydrolyze their artificial substrates qualitatively and quantitatively. These artificial substrates are chromogenic in nature, such as benzyl and nitrophenylglycosides, or fluorogenic in nature, such as methylumbelliferyl-glycoses (Kim and Ma, 2018). These synthetic substrates have the chromogenic structure, such as benzyl and nitrophenylglycosides, or fluogenic structure, such as methylumbelliferyl-glycoses. In Figure 5, natural and artificial substrates are given as examples (URL-1).



DIMBOA-β-glucoside

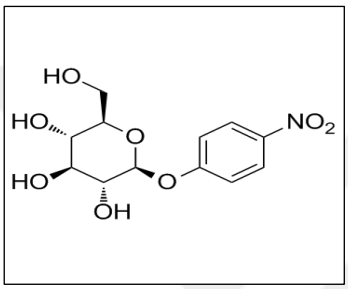


Sinigrin



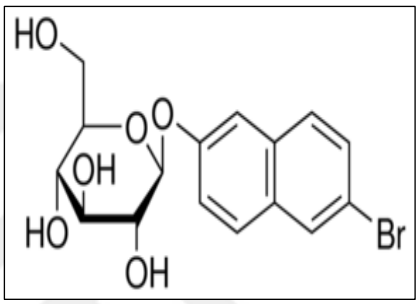
Linamarin

(**A**)



4-Nitrophenyl α-D-glucopyranoside

(PNPG)



6-Bromo-2-naphthyl β-D-glucopyranoside

(**B**)

Figure 5. Chemical structure of substrates specific to β-glucosidases. **A)** Chemical structure of some natural substrates**. B)** Chemical structure of chromogenic synthetic substrates.

Substrate specificity is of great importance in the investigation of the biotechnological and clinical effect of enzymes. Substrate specificity in β-glucosidases is qualified as the aglycone specificity, since the substrates are unchanged in the glucon portion. There is insufficient information about the catalysis mechanisms of β-glucosidase, therefore, researchers have done various studies on β-glucosidase enzyme and its substrates. In parallel with these studies, β-glucosidases with different substrate specificity were taken into account. Mutant enzyme was obtained by directed mutagenesis studies between enzymes showing different substrate specificity (Czjzek vd., 2000). These mutant enzymes were searched for answering the questions about substrate specificity and the glycon-aglycone regions that provide this specificity, with using X-ray crystallography. In a study related to this; in order to answer questions about the mechanism and location of

substrate specificity, an inactive form of maize β-glucosidase isozyme Glu1 was produced by site-directed mutagenesis, and enzyme substrate, enzyme aglycon and enzyme crystals were obtained with X-ray crystallography. As a result of this study, β-glucosidase presented new information about the understanding the functional analysis of the enzyme.

1.3. Antioxidant Activity

The oxidation process, which is an essential part of aerobic life and its metabolism, occurs by transferring electrons between two atoms. Although this electron flow reaches sufficient quantity, free radicals and reactive oxygen species are formed in the environment (ROS). The molecule or molecular fragment that has one or more unpaired electrons in the medium is considered free radicals. In this case, the presence of unpaired electrons provides significant reactivity on free radicals. Reactive oxygen types formed in this case show much higher chemical reactivity than normal and damage many biological materials such as protein, lipid, DNA and nucleotide coenzymes. These damages are created by excessive reactive molecules (reactive oxygen species (ROS) and reactive nitrogen species (RNS)), at the cellular level and biomolecular functions, trigger aging in the body and also many diseases such as cardiovascular diseases, various types of cancer, ingestion, slimming in the intestinal system and digestive system diseases (Topal vd.,2015).

Many studies have been done to balance the overproduction of ROS and RNS. Antioxidants are the major part of these studies. The process of a bioactive compound that is able to sustain cell structure and function by effectively cleaning free radicals, inhibition of lipid peroxidation reactions and preventing other oxidative damage is called antioxidation activity (Zeu, 2016).

Antioxidants have the ability to capture and stabilize free radical groups in the environment. According to their mechanism of action antioxidants are divided into two groups. Primary antioxidants react with existing radicals and prevents these molecules from turning into a more harmful form and the formation of new reactive molecules (Wang vd., 2015). Primary antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase enzymes. Secondary antioxidants break radical chain reactions that capture oxygen radicals. Examples of these antioxidants are vitamin C, vitamin E and polyphenols.

When the mechanism of action of antioxidants is examined, it is seen as the basis of many biological functions such as anticancer, anti inflammation and anti aging. Therefore, many studies have been done on antioxidants *in vitro* and *in vivo* conditions. Antioxidants naturally occurring in nature are at the top of these studies. Many fruits and vegetables are products rich in natural antioxidant molecules. In particular, citrus fruits A, C and E are the source of many phytochemicals such as vitamins, mineral elements, flavonoids, coumarins, limonoids, carotenoids and pectins. The phytochemicals found in fresh fruits have many biological functions, including antioxidants, anti-inflammation and anticarcinogenic (Liang, vd.,2018). The chemical formulas and mechanism of some structures in fruits are given briefly in the Table 5 (Zou vd., 2016).

Table 5. Some antioxidant structures and the effect of mechanisms (Zou vd., 2016).

Compound Name		Antioxidant activity
vitamins	A	Reacts with free radicals, especially O_2 Reacts with peroxyl radicals
	C	Scavenge variety species of ROS Clear O_2 Reduce sulfur radicals
	E	Synergistic effect with selenium to protect mitochondria against free radical damage and their membranes against peroxidation damage Prevent the oxidation of carotenoid, enhancing their antioxidant capacity
minerals	Selenium (Se)	Se: Destroy free radicals in the cytoplasm as an essential component of antioxidant enzyme glutathione peroxidase (GSH-Px) Se: Synergistic effect with vitamin E to protect mitochondria against free radical damage and their membranes against peroxidation damage
	Zinc (Zn)	
	Copper (Cu)	
	Iron (Fe)	
	Manganese (Mg)	

Table 5. continues

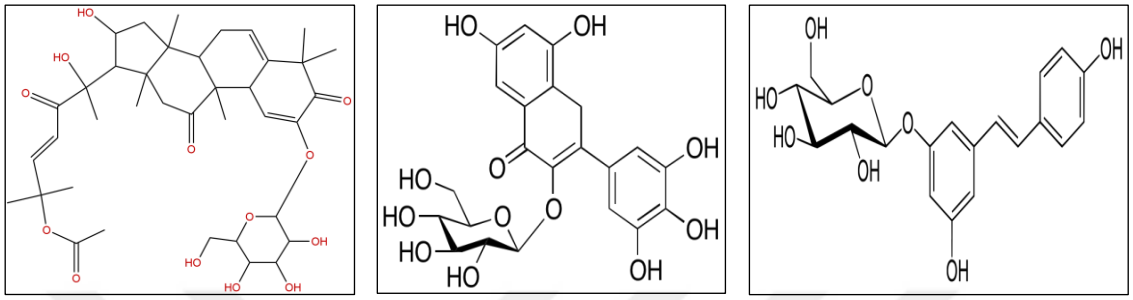
Phenolic compounds	Flavonoids	Naringin Naringenin Hesperidin Quercetin Rutin Myricetin	Inhibit the body's oxidant enzymes Improve the body's antioxidant enzyme activity Scavenge ROS directly Anti-lipid oxidation in vitro Decrease quality of peroxide formation in vivo
	Phenolic acid		Have different levels of free radical scavenging effect The dehydrogenation capacity of hydroxyl group The effect of ortho substitution on benzene ring
	Coumarins		Coumarins have been shown to possess strongly antioxidant activities because of their phenolic hydroxyl groups in molecule structure

1.3.1. Polyphenolic and Phenolic Compounds

Plants that survive in nature synthesize some compounds within their bodies. These compounds can be examined in two different groups. Of them connected with photosynthesis, respiration, growth and development, and the group that includes phytosterols, acyl lipids, nucleotides, amino acids and organic acids, are called primary metabolites. Phytochemical compounds that are outside this group and many of them accumulate in high concentration in the plant are called secondary metabolites. Secondary metabolites are structurally diverse and can be diagnosed by chemotaxonomic studies, many of which are distributed among plants in a limited number of species. Although antibiotics are seen as potential sources of natural medicines and herbicides today, they are also widely used in paints, fibers, glues, oils, flavors, medicines and perfumes (Tapas vd., 2008).

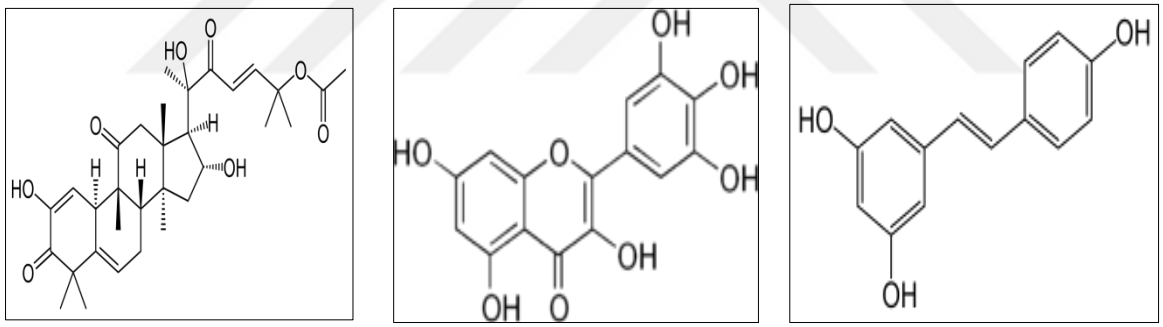
Phenolic compounds contained in secondary metabolites are of great importance in the sensory and nutritional quality of fruits, vegetables and other plants. Phenolic compounds have an aromatic ring that carries one or more hydroxyl groups, and its structure can vary from the complex state of a phenolic molecule to polymer molecules with high molecular weight (Wang vd., 2015).

Three different phenolic compounds were selected for use in this study. These are; Cucurbitacin E-2-O-glucoside, Myricetin-3-o-glucoside and Polydatin (Resveratrol-3-O-β-glucoside). Chemical formulas are shown in Fig. 6. β-glucosidases hydrolyze a glucose from these substrates to release the functional group, thus, increase the antioxidant effects of these substrates (Fig 6).



Cucurbitacin E-2-O-glucoside | Myricetin 3-O-glucoside | Polydatin

(A)



Cucurbitacin | Myricetin | Resveratrol

(B)

Figure 6. Phenolic compounds used in this study. **A)** Cucurbitacin E-2-O-glucoside, Myricetin-3-O-glucoside and Polydatin phenolic compounds can be deglycolysated by β-glucosidase. Cucurbitacin E-2-O-glucoside, Myricetin-3-O-glucoside and Polydatin are glycosylated phenolic compounds, while Cucurbitacin, Myricetin and Resveratrol do not have a glucose moiety (URL-2). B) Cucurbitacin E, Myricetin and Resveratrol phenolic compounds have high antioxidant activity (URL-3).

1.3.2. The effect of β-glucosidases on Antioxidants

There are many studies on the effect of β-glucosidases on antioxidants. These studies are directed towards increasing the effect of flavanoid compounds in the environment or increasing the flavonol accumulation rather than the antioxidant effect. Many studies have been done on this. Plants were generally used in these studies. In examining the effect of the β-glucosidase enzyme on antioxidants, soybean appears a lot (Lee vd., 2018).

The process of glycosylation and deglycosylation in plants are important mechanisms involved in regulating the biological activity of a number of secondary metabolites. Despite increasing glycosylation solubility and creating a favorable environment for transporting and separating metabolites, deglycosylation activates them to perform biological functions. Site-directed mutagenesis either of the two conserved catalytic glutamic acid residues (Glu200 and Glu414) of the active site completely eliminates β-glucosidase activity (Baba vs., 2017). Excessive amounts of β-glucosidases in the environment allow the accumulation of antioxidant flavonols and abiotic stress. Many studies have been done on this subject. Studies and antioxidant analyzes have shown that the accumulation decreases of reactive oxygen species during abiotic stress conditions in the accumulation of flavanol. β-glucosidases are known to cause dehydration in abiotic stresses, especially through abscisic acid, but their role in the accumulation of flavanols is not fully known. A study associated with this subject explains the role of a β-glucosidase (CsBGlu12) obtained from *Crocus sativus* effect on ROS in abiotic stress. β-glucoside provides the accumulation of cleansing flavonols for reactive oxygen species by deglycosization of flovanols (Baba vd., 2017).

1.4. Glucose Tolerance of β-glucosidase

B-glucosidases are known to hydrolyze the glycosidic bonds of alkyl-, amino- or aryl-β-D-glucosides, cyanogenic glucosides, disaccharides and short oligosaccharides. Moreover, these enzymes carry out the hydrolysis of glycosyl bonds between different molecules by transglycosylation. The ubiquitous phylogenetic distribution, substrate

diversity and their ability to both hydrolysis and synthesis glycosidic bonds increase the importance of these enzymes. Although beta glucosidases are used in much industrial process, their tolerance to glucose limits the use of this enzyme. Because many β-glucosidases are inhibited by the reaction product glucose. The reduction of the catalytic activity of beta glucosidases by glucose causes to the use of these enzymes in the industrial field to impose restriction. Therefore, beta glucosidases that are high glucose tolerance are ordinarily used in the industrial field. There are many studies on glucose tolerance of beta glucosidases. In a study related to this, enzymes that are tolerant to glucose and enzyme that are not tolerant to glucose were compared structurally (Giuseppe vd., 2014) (Fig 7).

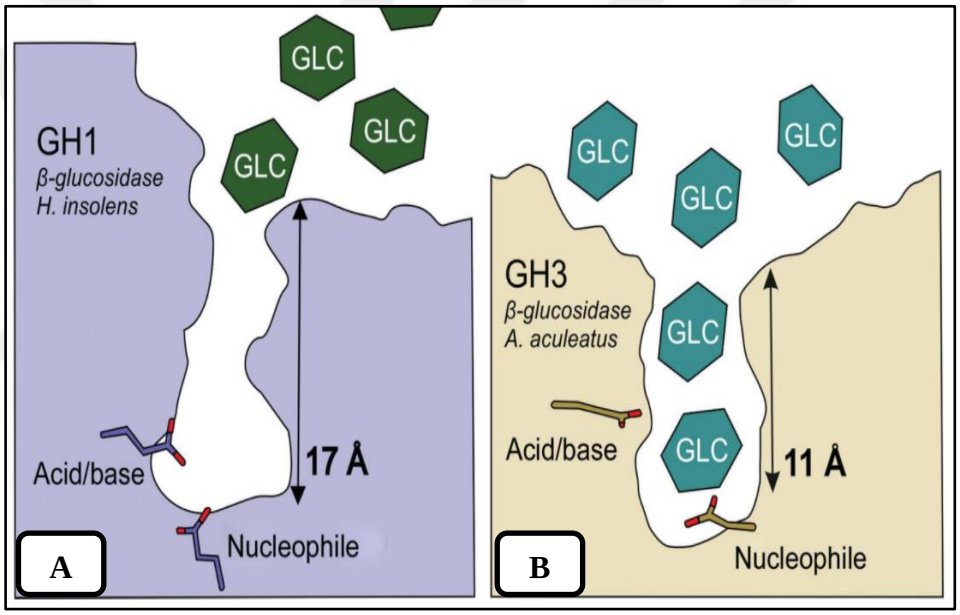


Figure 7. A schematic view of two enzyme active sites and the mechanism proposed for glucose tolerance in β-glucosidases; A) Structure of the active site of the β -glucosidases that is tolerant to glucose; B) The structure of the active site of the β -glucosidases that is intolerant to glucose (Giuseppe vd., 2014).

1.5. Purpose of the Study

The factors such as environmental pollution, alcohol and tobacco using, forest fires, X-Ray rays and UV radiation cause to increase the free radicals in the human body. This increase in free radical sources causes damage to carbohydrates, fats, proteins and DNA in humans. This situation is accepted as the cause of many diseases, especially cancer (Zou vd., 2016). Therefore, consumption of exogenous antioxidants is required. As the need for antioxidants increases, new approaches are being developed by scientists. One of them is increasing the activity of antioxidants with biocatalysts such as β-glucosidase. The use of β-glucosidase as biocatalysis is an alternative and environmentally friendly way to strengthen the antioxidant potential of phytochemicals with great potential for cancer prevention and use in neurodegenerative diseases.

Enzymes of Actinomycetes are preferred in the industrial field due to their small size, low K_m and high activity and stable structure. In this study, we aim to characterize β-glucosidase of *Jiangella ureilytica* KC603 (kindly provided from Ondokuzmayıs University) from actinomycetes group. In this direction, the gene fragment was amplified by specific primers, cloned into the expression vector, and then protein was expressed, purified and characterized.

We aim to obtain more antioxidant phytochemical substrates (Cucurbitacin E, Myricetin and Resveratrol) by liberating functional groups with deglycosylation of phytochemical such as Cucurbitacin E-2-O-glucoside, Myricetin-3-o-glucoside and Polydatin.

2. MATERIAL AND METHOD

2.1. Material

2.1.1. Chemicals, Enzymes and Kits

Yeast extract (Merck), NaCl (Merck), Tripton (Merck), Taq DNA Polymerase (Promega), dNTP (Promega), pGEM®-T Easy Vector System I (Promega), EDTA (Merck), X-Gal (Applichem), IPTG (Applichem), $CaCl_2$ (Aktar Kimya), $CuSO_4$ (Merck), Kanamycin (Applichem), Ethyl Alchol, Ampicillin (Applichem), *EcoRI* (Promega), *NcoI* (Promega), T4 DNA Ligase (NEB), *XhoI* (Promga), Gel Extracting Kit (Thermoschintific), Na_2HPO_4 (Merck), Methylene Blue (Merck), β-mercaptoethanol (Merck), Methanol(Isolab), Acetic acid (Isolab), Glyserol (Merck), Amonium Sulfate (Merck), TEMED (Janssen Chimica), Protein Ladder (Biolab), KCl (Merck), $MgCl_2$ (Sigma), LiCl (Sigma), $CuCl_2$ (Sigma), Commassie Brillant Blue G-250, Commassie Brillant Blue R-250 (Merck), Phosphoric Acid (Merck), BSA (NEB), Sodium Acetate (Merck), *Trisma* Base (Sigma), Acrylamide (Sigma), Bis-Acrylamide (Promega), Bromophenol Blue (Gerbu), NaOH (Merck), KOH (Sigma-Aldrich), Citric Acid (Merck), Cucurbitacin (89613) (Sigma-Aldrich), Myricitin (M6760) (Sigma-Aldrich), Resveratrol(A1020B) (Sigma-Aldrich), pNPG (4-Nitrophenyl-β-D- glucopyranoside) (Sigma-Aldrich).

2.1.2. Vectors and Bacteria

Information be related to bacteria and vectors used in the study is given in Table 6.

Table 6. Vectors and bacteria strains to used in the study.

Bacteria/ vectors	Referance	Speciality
***Jiangella ureilytica* KC603 (NZ_SMKL000000001)**	19 Mayıs University, Samsun, TURKEY	
E.*coli* K12 JM101	NEB-Laboratory stock	F', *tra*D36, *pro*A^+B^+, Laclq, Δ(lacZ), M15/Δ(lacproAB),*gln*V, *thi*$^+$
E. *coli* BL21(D3)	Novagen	F', *omp*T, *hsd*S_B, ($r_B^-m_B^-$), *gal*, *dcm*(D3), pLysS, Cam^R
pET28a(+)	Novagen	High expression level, T7 promoter, His*Tag®, *lac*I gene, Kan^R and MCS (multiple cloning region) in C terminal region
pGEM®-T Easy I	Promega	High copy number, T7 and SP6 RNApolimerase promoters, an MCS (multiple cloning site) located within the α-peptide cloning region of the β-galactosidase enzyme, 3'-T end and Amp^R

2.1.3. Bioinformatic Analysis

In this study, the presence of β-glucosidase gene in genome of *Jiangella ureilytica* KC603 was searched in databases by using bioinformatics methods such as NCBI, Esembl, PDB. *Jiangella ureilytica* KC603 was kindly provided by Ass. Prof. Dr. Hilal Ay at Ondokuzmayıs University, Molecular Biology and Genetics Department. Presence of β-glucosidase is demonstrated by esculin test and after approvel of β-glucosidase activity, cloning, expression and characterization processes were performed. Following the databank scanning, the three-dimensional structure and active regions of the β-glucosidase belonging to *Jiangella ureilytica* KC603 (JurBlgKC603) were examined in the PyMOL program.

2.2. Method

2.2.1. Esculin Assay

Jiangella ureilytica KC603 is grown in ISP2 medium at 30 ºC. The β-glucosidase activity was determined by plate assay using Luria-Bertani (LB) agar supplemented with %1 Esculin and 0.5 g/L ferric ammonuim chloride. β-glucosidase act on esculin to produce esculetin and glucose with in turn is reduced in the presence of iron and produce black color as zone of hydrolysis indicating β-glucosidase activity (Saqib and Whitney, 2005).

2.2.2. Cloning and Expression of β-glucosidase

2.2.2.1. Primer Design and Amplification of β-Glucosidase Gene

In order to amplify β-glucosidase gene, forward and reverse primers are designed according to genome of *Jiangella ureilytica* KC603 by Snapgene software. The designed primers are given in Table 7.

Table 7. The designed primers for *JurBglKC603*. The restriction enzyme digestion sites are underlined.

Primer name	Primer sequence
JurBglu_KC603_F	5'- C<u>CCATg g</u>CC TTC CgC TTC CCg -3' *NcoI*
JurBglu_KC603_R_Histag	5'<u>- CTC GAG</u> CGC GTC CTC ACT CTC C -3' *XhoI*

For the genomic DNA isolation from *Jiangella ureilytica KC603*, Promega DNA isolation kit was used. For lysis step, *Jiangella ureilytica* KC603 was inoculated into ISP2 liquid medium and incubated at 30 °C for 2-4 day. Then, the grown bacteria was pelletted by centrifugation and pellet was saved. The pellet was dissolved in TE buffer (10 mM) and

kept at -80 ºC for 20 minutes. Then, heat shock was applied at 100 ºC for 5 minutes. Then, the product was taken to 37 ºC and kept for 15 minutes. Afterwards, 120 µl lysozyme (50 mM) and 50 µl proteinase K (20 mM) were added and kept for one day at 37 ºC. The lysis step was completed and the genomic DNA was isolated by Promega DNA isolation kit. The isolation procedure determined by the company was taken into account while performing the isolation. The product that was run at 1% agarose gel electrophoresis was monitored with Biorad Gel Imaging System.

The genomic DNA obtained from *Jiangella ureilytica* KC603 was used as template in PCR application. The PCR conditions are as following table 8.

Table 8. Cycle of PCR

Step	**Temperature**	**Time (second)**	**Cycle**
Heating	95 °C	120	1
Denaturation	95 °C	50	
Annealing	49 °C	50	35
Extension	72 °C	90	
Final Extension	72 °C	300	1

The DNA fragment formed as a result of PCR run on agarose gel was viewed by Biorad Gel Imaging System. The resulting DNA was extracted from the gel using the gel extraction kit.

2.2.2.2. Cloning of the β-glucosidase Gene into pGEM®-T Easy Vector System I

pGEM®-T Easy Vector System I was used to clone the gene fragment amplified by PCR. Firstly, the ligation was performed under the conditions prescribed by the company (1 µl pGEM®-T Easy vector, 1 µl T4 DNA ligase, 5 µl PCR product, 1 µl 10X DNA ligase

and 2 µl dH_2O), and incubated overnight at 16 °C. The ligation product was transformed into *E. coli* JM101 competently prepared by $CaCl_2$ method. The transformation process was carried out as follows; 10 µl of ligation product was added to the tube containing 200 µl of competent cells and kept on ice for 30 minutes. After incubation, the samples were shocked in 42 ºC heater block for 2 minutes to allow the plasmid to enter the cell. After incubation for two hours at 37 °C, samples were spread on LB plates containing 50 µg/µl ampicillin by adding 40 µl of X-Gal and 40 µl of 100 mM and incubated overnight at 37 °C. White colonies were selected among blue-white colonies that are formed after transformation. The plasmid DNA samples were isolated according to alkali lysis method. The isolates which were digested with the *EcoRI* restriction enzyme were checked and the recombinant clones were determined. The plasmid clones that were isolated by plasmid isolation kit (Promega) was sequenced by Macrogen (Netherlands), and identified by the Advanced Blast Program of GenBank (NCBI, NIH, Washington DC).

2.2.2.3. Cloning of the β-glucosidase Gene into pET28a (+) Expression Vector

The ends of β-glucosidase gene amplified by PCR contains *NcoI* and *XhoI* restriction endonuclease regions. Therefore, the recombinant pGEM-T Easy vector and pET28a (+) expression vector were digested with *XhoI* and *NcoI* restriction endonucleases under the conditions recommended by the company. The gene fragment and linearized pET28a(+) vector were extracted by gel extraction kit separately and mixed and ligated. The product of ligation transformed to *E. coli* JM101 competent cell by $CaCl_2$ method. The recombinant plasmids were isolated by alkali lysis method, digested with *NcoI* and *EcoRI* restriction endonucleases and run on agarose gel. The recombinant plasmid was determined and transformed into *E. coli* BL21 (DE3) pLys.

2.2.2.4. Expression of β-glucosidase

Different conditions were tested since the expression conditions were not fully known. Firstly, in order to precisely adjust its duration, expression was performed under

different time conditions in LB containing 1 mM of IPTG and 0.5 mM of kanamycin. Secondly, different IPTG concentrations were tested. Finally, different temperature conditions were tried (conditions are given in detail in Table 9). As a result of these trials, the best expression conditions were determined. According to the determined expression conditions; a 16-hour expression in the range of 20-30 ºC was performed at 1% IPTG, 1% kanamycin and. After expression, 5 min centrifuge was performed at 10,000 rpm and pellet was saved. Pellet was treated with pH 7,0 sodium citrate buffer, sonicated with 80% amplitude for 5 min, 0.6 cycle by Sartorius Labsonic M. And then, lysate was centrifuged at 14,000 rpm for 15 minutes and the supernatant was saved.

Table 9. Expression parameters tested for most suitable expression conditions

	Trial 1	Trial 2	Trial 3	Trial 4
Kanamycin (mM)	0.5	0.5	0.5	0.5
IPTG (mM)	0.1 0.5 0.7 1	0.1 0.5 0.7 1	0.1 0.5 0.7 1	0.1 0.5 0.7 1
Temperature	18 ºC	24 ºC	28 ºC	30 ºC
Time (hour)	Overnight	4 6 9 Overnight	4 6 9 Overnight	4 6 9 Overnight
O.D.	0.1 0.5 0.7 1	0.1 0.5 0.7 1 2	0.1 0.5 0.7 1 2	0.1 0.5 0.7 1 2

2.2.2.5. Purification of β -glucosidase

The purification of the β-glucosidase carrying HisTag in its C terminal was performed by the Ni-affinity chromatography. The buffer A and buffer B solutions were adapted to achieve optimal conditions for a target protein from procedure recommended by

the company (Buffer A: 20 mM, pH 7.5 Tris HCl, 200 mM NaCl, Buffer B: 20 mM, pH 7.5 Tris HCl, 200 mM NaCl, 500 mM imidazole). The column was equilibrated with 10 ml of buffer A. Protein was diluted 1:2 with buffer A and passed through the column. The column was washed with 10 ml of buffer A and eluted with 1 ml buffer B. The pure fraction was stored at +4 ºC for characterization.

2.2.2.6. SDS Polyacrylamide Gel Electrophoresis

Protein gel electrophoresis was performed under a current of 15 mA using 12% gel. The purity and molecular weight of the β-glucosidase protein was determined after running the fractions in SDS polyacrylamide gel electrophoresis. The sample buffer was prepared by mixing 0.15 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol and 6% β-mercaptoethanol. 15 µl protein fraction and 5 µl loading dye were mixed and incubated for 5 minutes at 95 °C. The denatured proteins were loaded into 12% SDS-PAGE. After running, the gel was stained with Commansie Brillant Blue R-250 (0.125% Commassie Brillant Blue R-250, 50% methanol, 10% acetic acid) for one hour. Proteins were fixed into gel with the solution I (50% methanol, 40% ddH_2O, 10% acetic acid) for an hour and, finally, gel was washed with solution II (7% methanol, 10% acetic acid, 83% ddH_2O).

2.2.2.7. Enzyme Assay

The activity of β-glucosidase against pNPG substrates was determined by monitoring absorbance of colorimetric product (yellow). 250 ml reaction solution consisting of 50 mM sodium citrat buffer (pH 6.0), 0,8 µg enzyme and 1 mM substrate were incubated at 35 ºC for 30 min. Reaction was stopped by adding 250 ml $NaHCO_3$ (50 mM), subsequently, measured at 405 nm (Garcia, vd., 2020).

2.2.3. Characterization of the β-glucosidase

2.2.3.1. Optimum Temperature

The optimal temperature value of JurBglKC603 was determined by a series of reactions performed in the heater block set at 20, 25, 30, 35, 40, 45, 50 and 55 ºC. In next characterization steps, determined optimum temperature value was used.

2.2.3.2. Optimum pH

In order to determine the effect of pH on the activity of JurBglKC603, 100 mM sodium citrate buffer (pH 3-6), 100 mM sodium phosphate buffer (pH 7-8), 100 mM Gly-NaOH (pH 9-10), 100 mM CAPS buffer (pH 11) was used. The reactions were carried out at the determined optimum temperature. The optimum pH activity value observed was used as the reaction pH in studies such as determining the kinetic parameters to be performed later.

2.2.3.3. Enzyme Kinetics

The kinetic data of JurBglKC603 were determined by serial reactions performed with substrate (p-NPG) concentrations between 0.1 and 7 mM, determined as a result of preliminary studies. Reactions were carried out with 50 mM sodium citrate buffer (pH 6) at 35 ºC. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined as the inverse of the values corresponding to the points where the x and y axes intersect in the Lineweaver – Burk curve (Lineweaver and Burk, 1934).

2.2.3.4. Thermal Stability

JurBglKC603 was incubated to examine the effect of heat on the stability of the enzyme at 20, 25, 30, 35, 40, 45, 50, and 55 ºC with sodium citrate (pH-6.0) buffer at different time intervals. Remaining activity was determined by using p-NPG substrate at a 30-minute reaction and checked for stability.

2.2.3.5. pH Stability

In order to determine the pH stability of JurBglKC603, enzyme was incubated in 50 mM sodium citrate buffer (pH 3-6), 50 mM sodium phosphate buffer (pH 7-8), 50 mM Gly-NaOH (pH9-10), 50 mM CAPS buffer (pH 11) in the temperature at which the enzyme is stable at 25 °C. The activity was measured along continuing incubation time, until it decreases below 50%. Remaining activity was measured as a result of a 30-minute reaction with the p-NPG substrate and checked for stability. After the activity measurement, the pH at which the enzyme is stable was determined.

2.2.3.6. Metal Ion Effect

Effects of metal ions on the activity of JurBglKC603 were carried out with chloride salts of following metal ions; Mg^{2+}, Li, Ca^{2+}, K, Zn^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+}, Na^{2+}, Co^{2+} and Fe^{2+}. After the addition of 1 mM, and 5 mM metal ions, the enzyme was incubated for 30 minutes in the temperature at which the enzyme is stable at 25 °C. The enzyme activities were measured after 30 minutes reaction with p-NPG substrate. The activity of the enzyme on optimal conditions was considered as 100 % and the relative activity of the enzyme incubated with metal was calculated.

2.2.3.7. Effects of Inhibitors and Organic Solvents

The effects of detergents and organic solvents on the activity of JurBglKC603 were investigated. 1%, 5% of detergents (SDS, Triton X-100, Tween 20), 1 mM, 5 mM of inhibitors (EDTA, DTT, PMSF, EGTA, DEPC) and 5%, 10% of organic solvents (DMSO, Dioxane, Methanol, Butanol, Ethanol, Isopropanol) were treated with enzyme and incubated for 30 min at room temperature. Then, remaining activity was measured at the optimum pH and temperature with a reaction of 30 min with p-NPG substrate. The activity of the enzyme on optimal conditions was considered as 100 %, and the remaining activity of the enzyme incubated with chemicals was calculated.

2.2.3.8. Glucose Tolerance

The effect of glucose on the activity of JurBglKC603 was examined. 200 mM, 400 mM, 600 mM, 800 mM, 1 M, 2 M, 3 M and 4 M glucose were treated with enzyme and incubated for 30 minutes in the temperature at which the enzyme is stable at 25 °C. The activity of enzymes with p-NPG substrate was measured and the effect of glucose was determined.

2.2.4 Analysis of Polydatin by Thin Layer Chromatography (TLC)

TLC analysis was performed to determine the effect of β-glucosidase on substrates (Cucurbitacin, Myricetin and Piceid). The reaction solution consisting of 50 mM sodium citrate buffer (pH 6.0), 0.8 µg enzyme and 100 µM were inoculated at 35 °C for 1 hour. TLC analysis was carried out on pre-coated silica gel plates. Polydatin, Myricetin and Cucurbitacin were run with ethyl acetate/hexane/glacial acetic acid (8:1:0.5, v/v/v) on silica gel plates, and visualized by spraying with 10% phosphomolybdic acid, 90% ethanol. They was oxidized by phosphomolybdic acid and appeared in green as colour after heating up for 3 min at 110 °C.

3. RESULTS

3.1. Esculin Assay

On esculin assay plate, black color formation was observed as a sign of β-glucosidase activity (Fig 8).

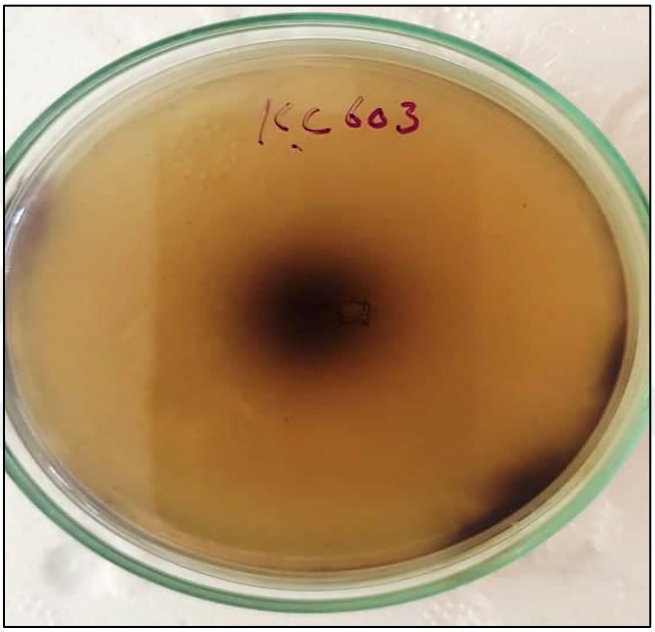


Figure 8. β-glucosidase activity of JurBglKC603 by esculin assay.

3.2. β-Glucosidase Amplification of *Jiangella ureilytica* KC603

Genomic DNA of *Jiangella ureilytica KC603* was used as a PCR template. As a result of the PCR reaction, ~1400 fragment was obtained as a product, indicating the presence of β-glucosidase. (Fig 9).

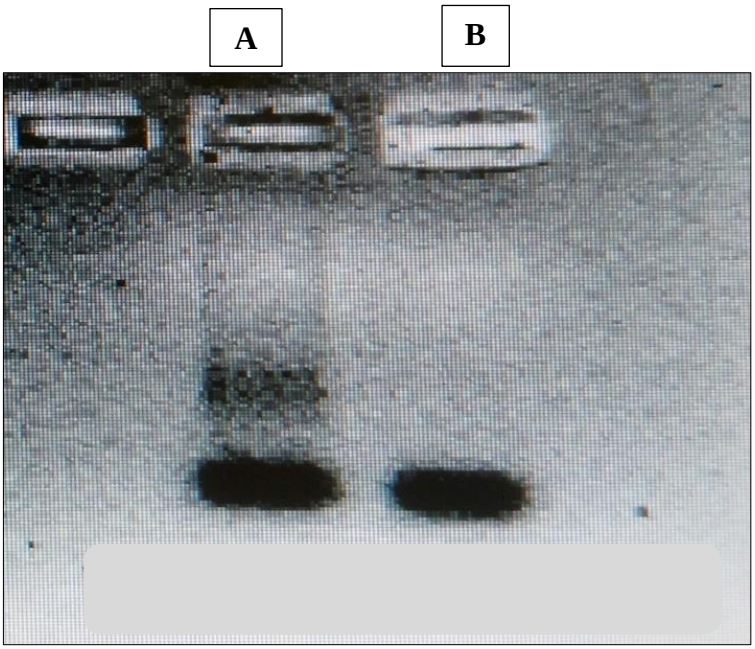


Figure 9. *Jiangella ureilytica* KC603 β-glucosidase gene. A) PCR known to be 1374. B) PCR product

3.3. Results of Bioinformatic Analysis

The amino acid sequence of JurBglKC603 was determined by translating DNA sequence with the ExPASy program. The determined amino acid sequence was aligned with a few β-glucosidase of *Actinobacteria* from NCBI with Clustal Omega software and two conserved active sites were identified (Fig. 10).

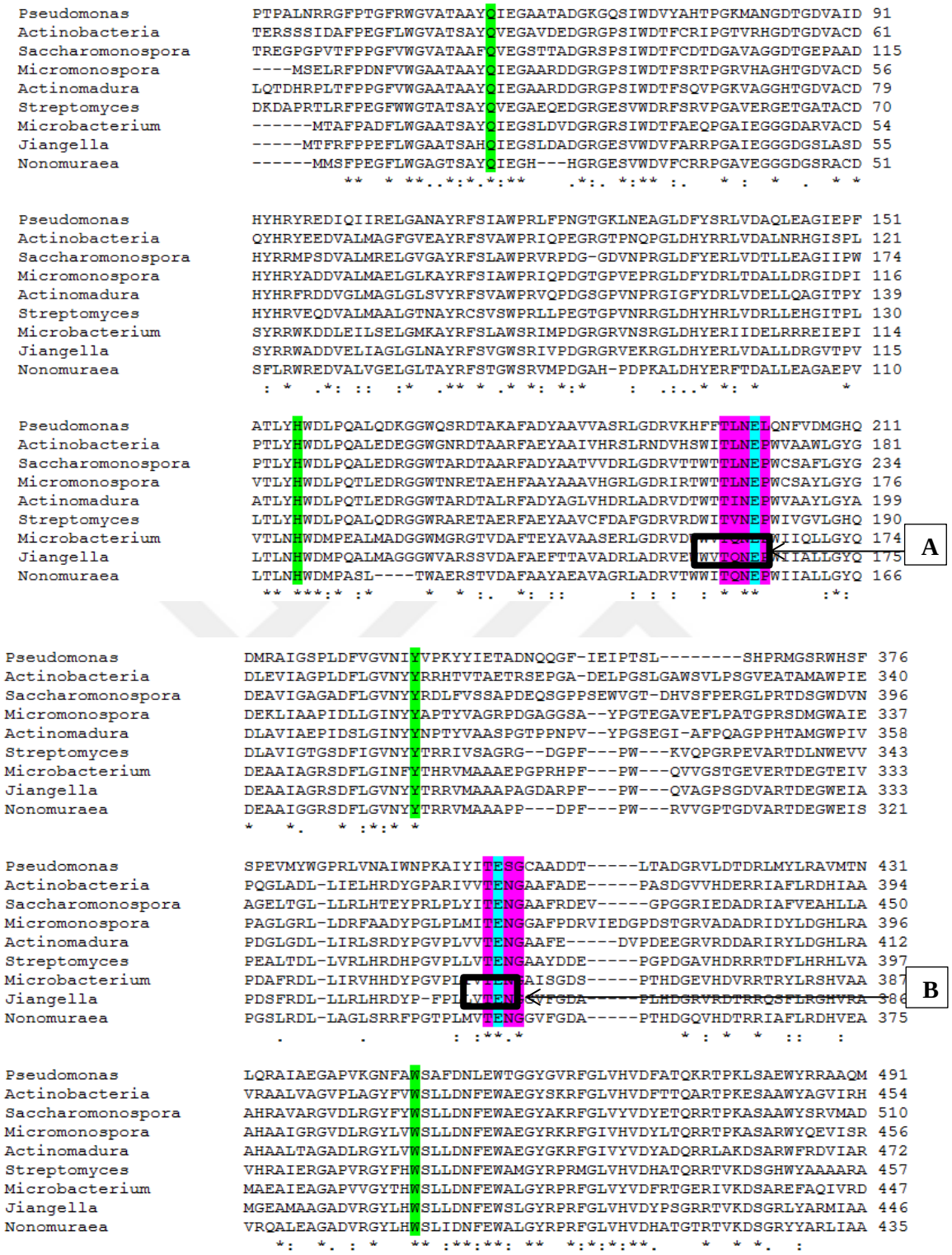


Figure 10. Conserved active region of JurBglKC603; A) Protein donor site B) Nucleophile site.

The *Jiangella ureilytica KC603* amino acid sequence was blasted in the Uniprot program. TQNEP protein donor site, TENG nucleophile active site and also five substrate binding sites were determined from the conserved regions. According to these data, it was confirmed that *Jiangella ureilytica KC603* has β-glucosidase belonging to the glycosyl hydrolase I family.

3.4. Cloning of the β-glucosidase Gene into pGEM®-T Easy Vector System I and pET28a (+) Expression Vector

For the confirmation of the cloning of β-glucosidase gene into pGEM-T easy cloning vector, isolated plasmids were digested by the *EcoRI* restriction enzyme. As a result, the DNA fragment, which is the same size as the PCR product, was observed (Fig. 11A).

The partial digestion with *NcoI* and *XhoI* restriction enzymes was performed to check whether the β-glucosidase gene was cloned into the pET28a (+) expression vector. After digestion, ~1200 bp and ~1400 bp fragment were observed since β-glucosidase gene contains internal *NcoI* restriction enzyme recognition site in ~1200th bp region (Fig. 11B).

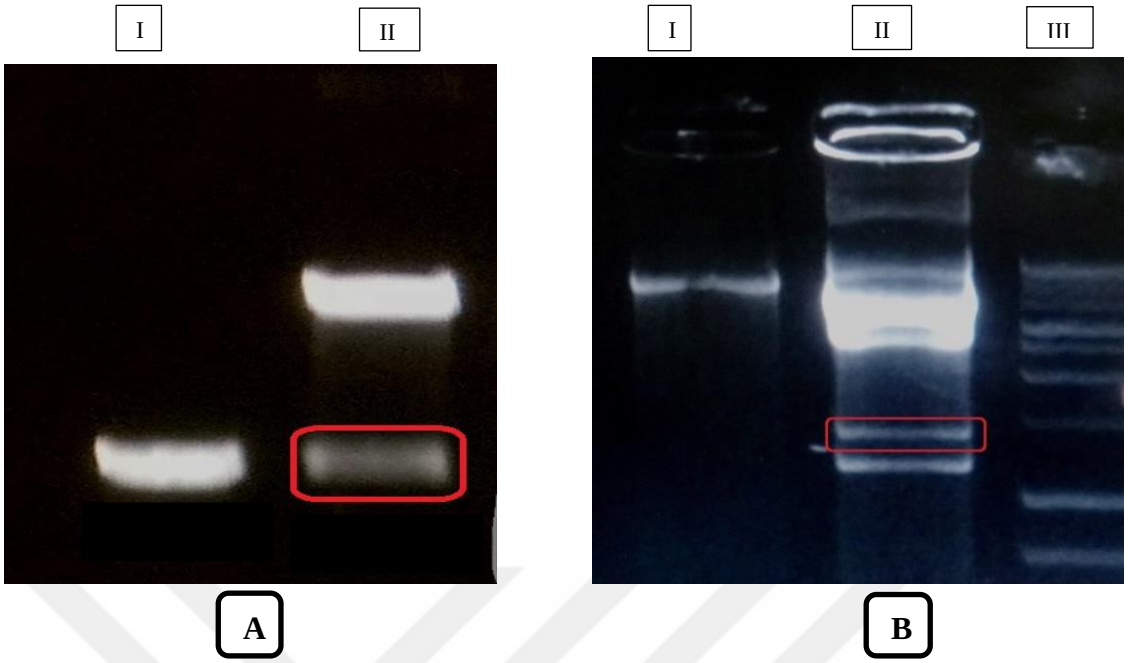


Figure 11. *Jiangella ureilytica* KC603 β-glucosidase gene fragment digestion with *EcoRI*, *NcoI* and *XhoI* restriction enzymes. A) I: PCR product, II: Digestion of pGEM-T easy cloning vector containing β-glucosidase gene. B) I. Digestion of pET28a (+) expression vector, II: Digestion of pET28a (+) expression vector containing β-glucosidase gene.

3.5. Gene Expression and Protein Purification

The crude extract and purified protein by Ni-affinity chromatography were run on SDS-PAGE gel electrophoresis and about 50.1 kDa protein fragment was observed (Fig. 11B).

After purification, JurBglKC603 activity was determined by a reaction in which β-glucosidase hydrolyzes p-nitrophenyl-β-D-glucopyranoside resulting in the formation of a colorimetric (yellow) product, proportional to the β-glucosidase activity present (Fig 12A).

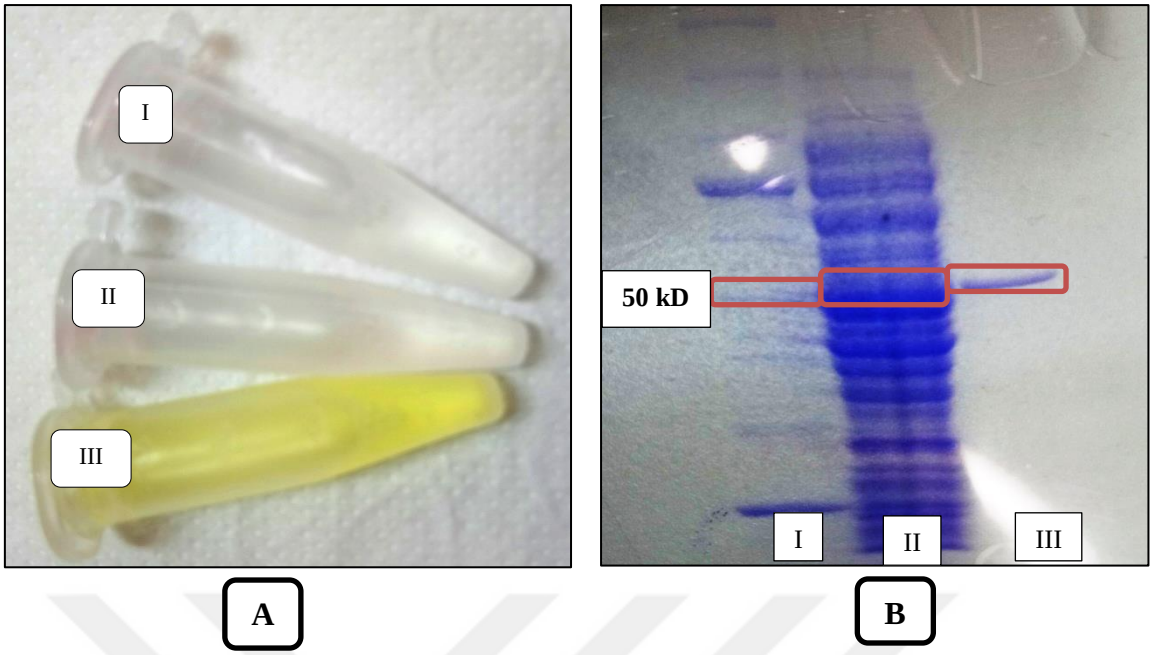


Figure 12. Expression and and activity assay results of JurBglKC603 with substrate, p-nitrophenyl-β-D-glucopyranoside. A) I: Enzyme control without substrate; II: Substrate control without enzyme; III: Active β-glucosidase forms yellow color because of hydrolysis of the substrate p-nitrophenyl-β-D-glucopyranoside B) SDS–PAGE visualization of crude cell extract and purified β-glucosidase; I: Marker; II: Crude cell extract; III: Pure enzyme.

3.6. Characterization of β-Glucosidase

3.6.1. Optimum Temperature

According to experimental results, optimum temperature of the enzyme was determined as 35 ºC and a temperature-activity graph was created (Fig 13).

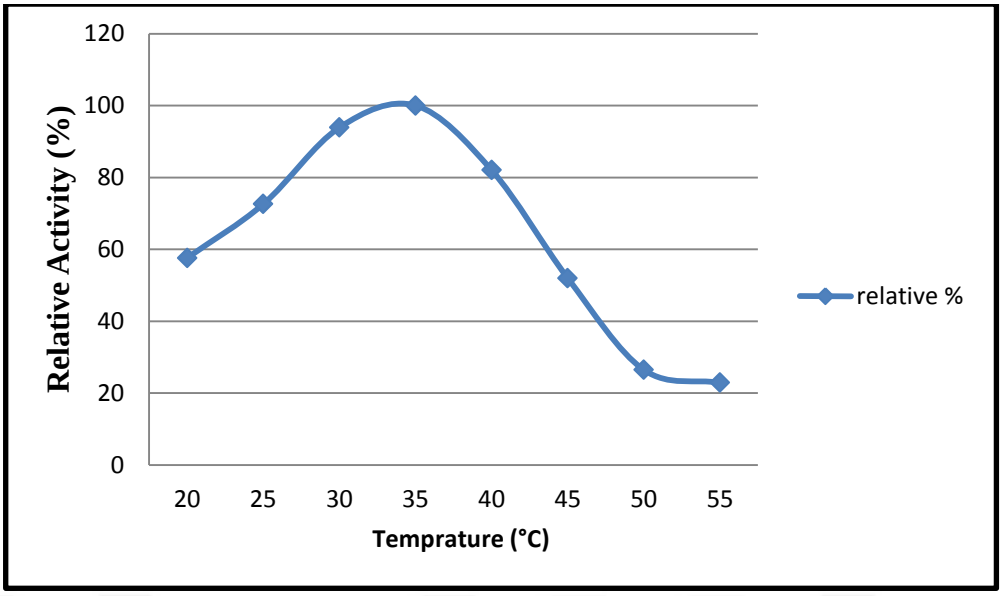


Figure 13. Optimum temperature of JurBglKC603.

3.6.2. Optimum pH

By creating a pH-activity graph, optimum pH of the enzyme was determined as 6.0 (Fig. 14).

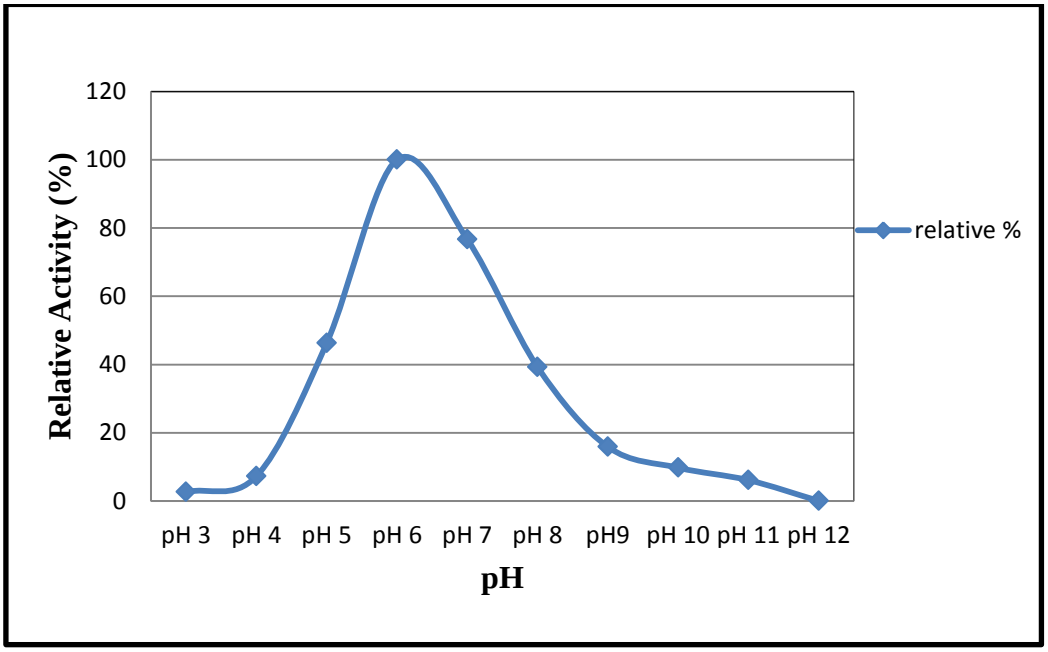


Figure 14. Optimum pH of JurBglKC603.

3.6.3. Thermal Stability

Thermal stability of JurBglKC603 was determined by creating activity-time graph. According to graph, activity of enzyme decreased to half within 216 h at 25 °C, completely, lost within 6 h at 35 °C and within 30 min at 55 °C (Fig 15).

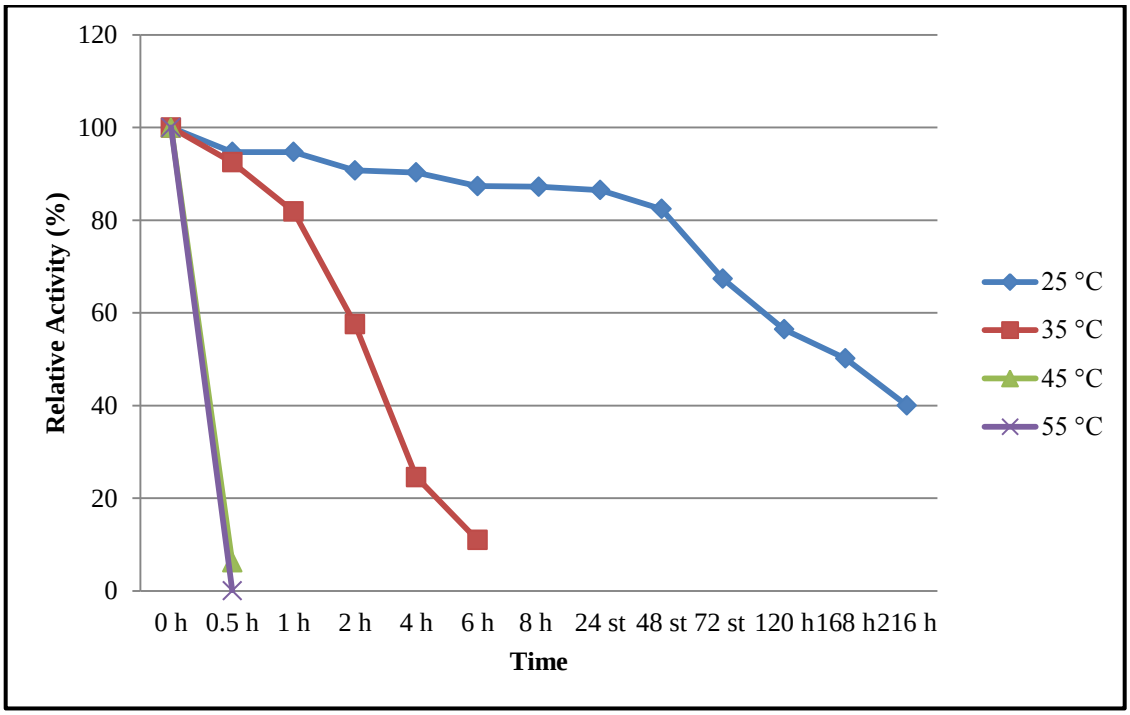


Figure 15. Thermal stability of JurBglKC603.

3.6.4. pH Stability

When pH stability of JurBglKC603 was analyzed at room temperature at different pH conditions; in the first 30 minutes, the activity of the enzyme at pH 3 decreased approximately 50%. The enzyme lost 50% of its activity at pH 4 after 8 hours. The activity of the enzyme fell to 50% at pH 10-11 after 72 hours, and the enzyme lost 50% of its activity at pH 8-9 on the 5th day (Fig. 16).

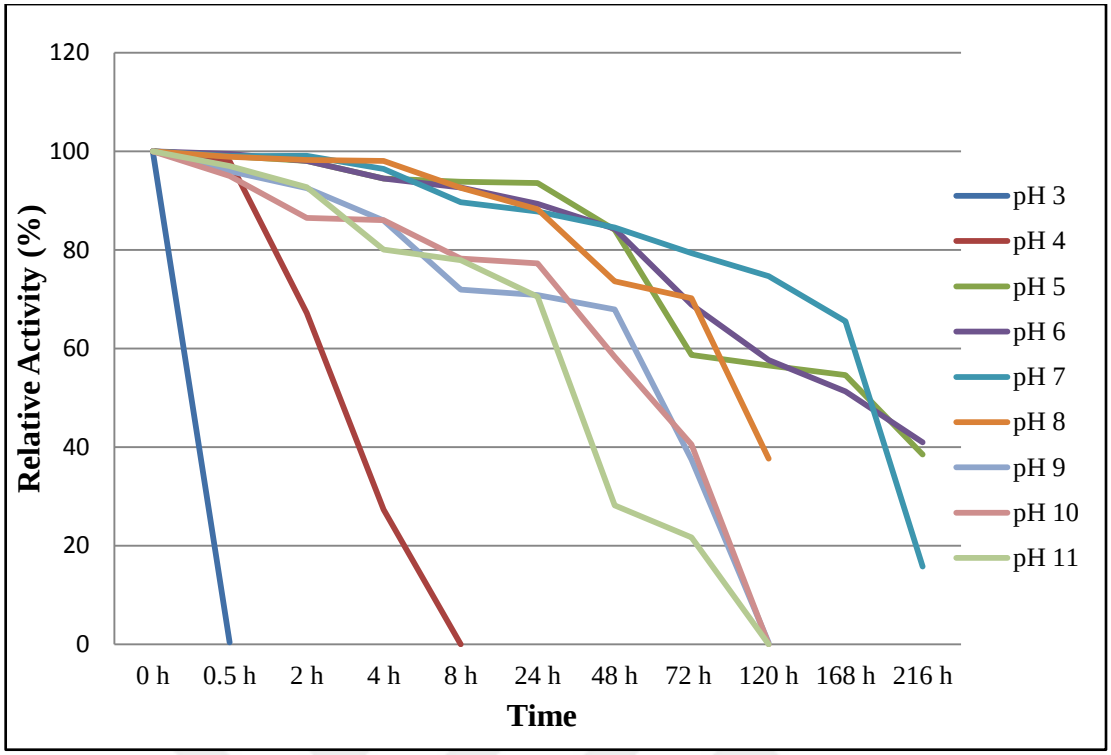


Figure 16. pH stability of JurBglKC603.

3.6.5. Metal Ion Effect

The 3-axis bar graph was created to observe JurBglKC603 activity in 1 mM and 2 mM metal concentrations. In general, any effect of 0.5 mM metal ions on JurBglKC603 activity was not observed, while activity in 2 mM metal ions fell. While Ca^{2+}, Cu^{2+}, Fe^{2+}, Na^{2+} increased the activity of JurBglKC603 in rate of 3-17%, Ni^{2+} and Mg^{2+} did not change and Hg^{2+} almost reduced the activity of JurBglKC603 by 76-84 % (Fig. 17).

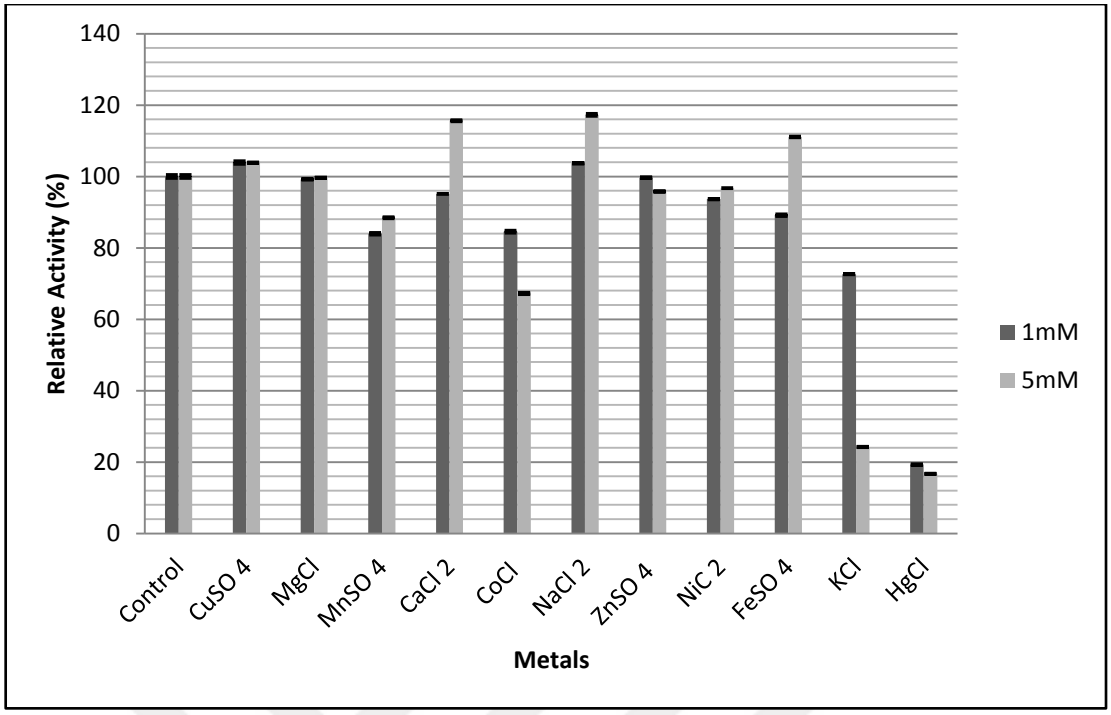


Figure 17. Effect of metal ions on JurBglKC603.

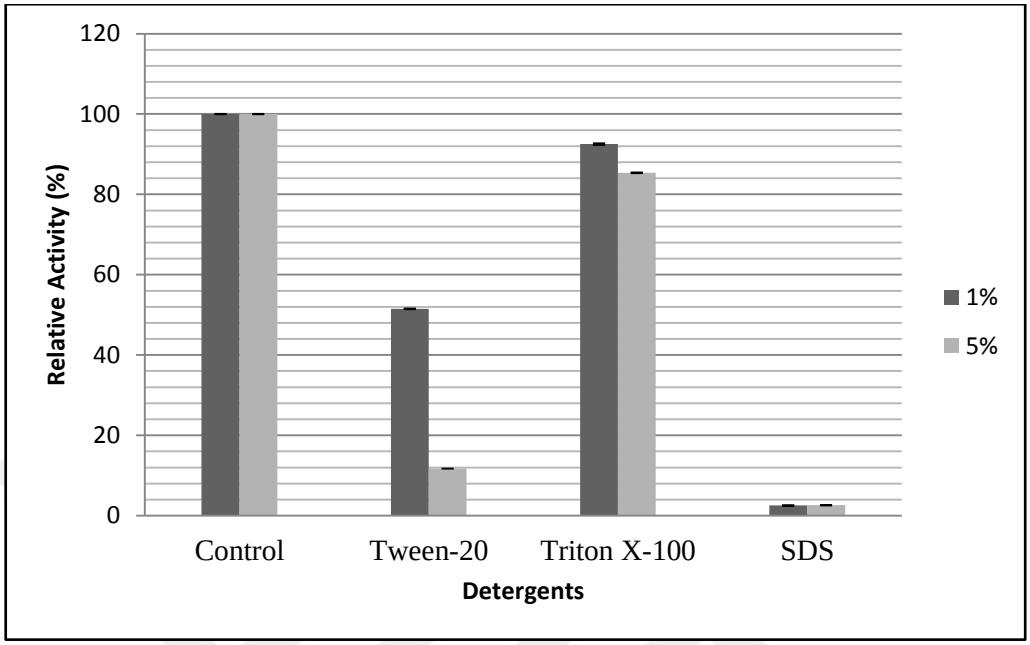
3.6.6. Effects of Inhibitors and Organic Solvents

The 3-axis bar graph was created to observe effect of detergents in 1% and 5%, concentrations on JurBglKC603 activity. While Triton X-100 reduced the activity by ~8-15%, Tween-20 reduced by ~50-90% and SDS 98% (Fig 18A)

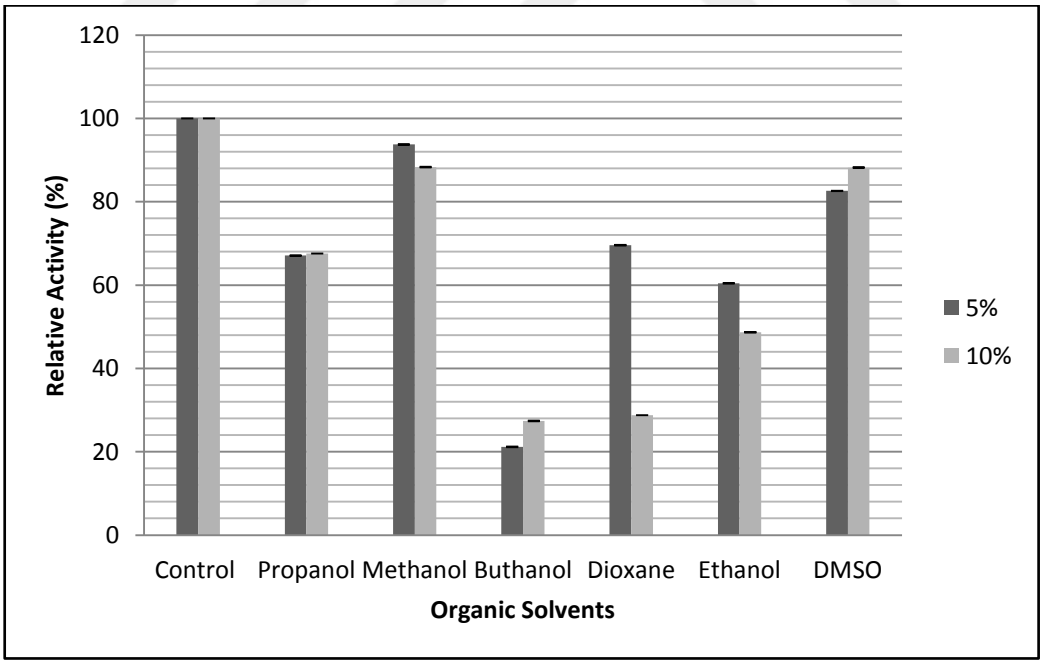
The 3-axis bar graph was created to observe effect of organic solvents in 5% and 10% concentrations on JurBglKC603 activity. JurBglKC603 activity was overall generally decreased by 5% and 10% organic solvent except methanol and DMSO. 10% of methanol and DMSO reduced the activity by 9-11% JurBglKC603 activity. According to results of experiment, 5 % and 10% of ethanol reduced the activity by 41-52%. While propranol reduced the activity by ~33%, butanol decreased the activity by ~80%. While 5% of Dioxane reduced the activity by ~30%, 10 % of Dioxane reduced the activity by ~73%. (Fig 18B).

The 3-axis bar graph was created to observe effect of inhibitors in 1 mM and 5 mM concentrations on JurBglKC603 activity. According to graph, EGTA is seen a strong

inhibitor for JurBglKC603. PMSF, EDTA, DEPC and DTT did not show a very high effect on JurBglKC603 (Fig 18C).



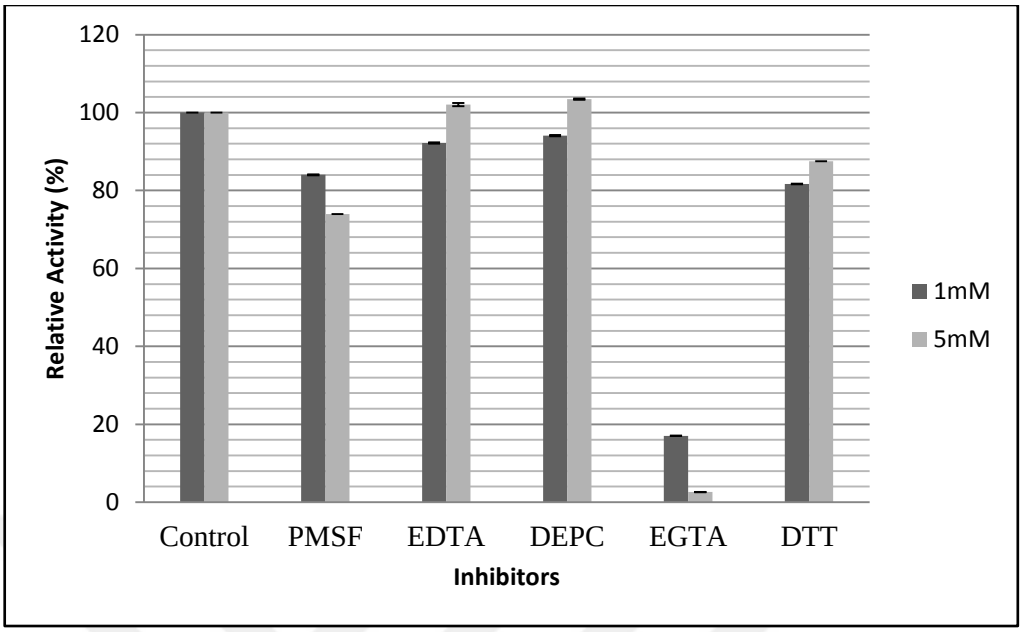
A



B

Figure 18. Effects of Inhibitors and Organic Solvents. A) The effect of detergents on the β-glucosidase. B) The effect of organic solvents on the β-glucosidase. C) The effect of inhibitors on the β-glucosidase.

Figure 18. continues



C

3.6.7. Glucose Tolerance

Glucose tolerance graph was created by measuring the β-glucosidase activity in 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1 M, 2 M, 3 M and 4M glucose. According to tolerance analysis, it was observed that JurBglKC603 has 80% glucose tolerance in 0.2-2 M glucose and ~70% glucose tolerance in 3-4 M glucose (Fig. 18).

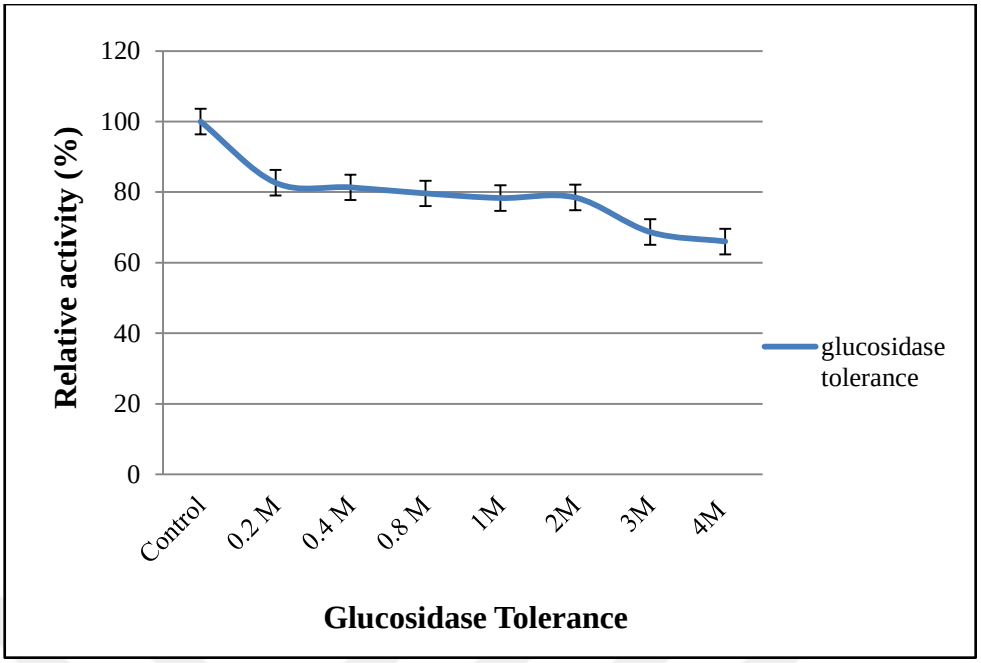


Figure 19. The glucose tolerance of JurBglKC603

3.6.8. Enzyme Kinetics

The Lineweaver – Burk curve was created by using the inverse of the values corresponding to the points where the x and y axes from Michaelis-Menten plot (Fig 20). Kinetic parameters were calculated as K_m = 0.448757 mM, V_{max} = 3790.176 U/mg using Lineweaver – Burk equation. Finally, the turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were calculated as 3217.405119 s^{-1}, 7169.596 for 0.8 µg enzyme used in 1 ml reaction volume (Fig. 21).

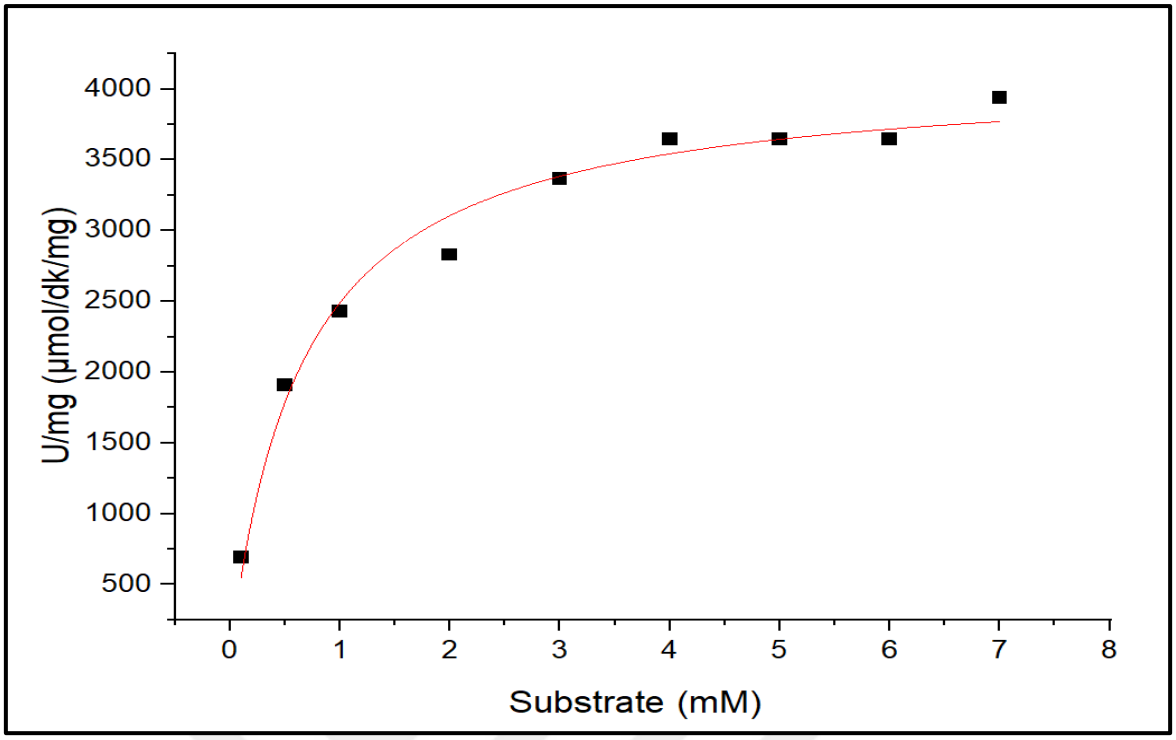


Figure 20. Michaelis-Menten curve of JurBglKC603 (by OriginPro 2020b).

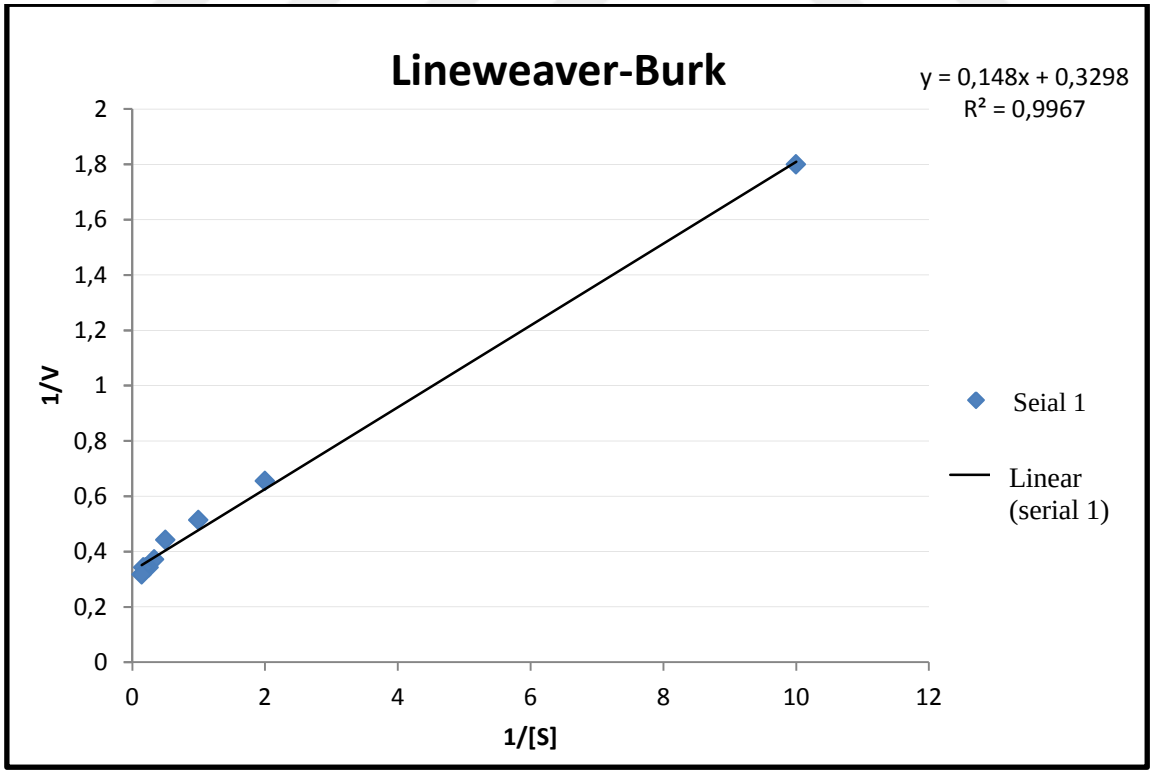


Figure 21. Lineweaver-Burk plot of JurBglKC603.

3.6.9. Analysis of Polydatin by Thin Layer Chromatography

When polydatin substrate was hydrolyzed by JurBglKC603, Resveratrol (Piceid) is produced. Following TLC plate indicates that polydatin was deglycosylated (hydrolyzed) by JurBglKC603. The reaction solution consisting of 50 mM sodium citrate buffer (pH 6.0), 0,8 μg enzyme and 100 μM substrates (Cucurbitacin, Myricetin and Piceid) were inoculated at 35 C for 1 hour.

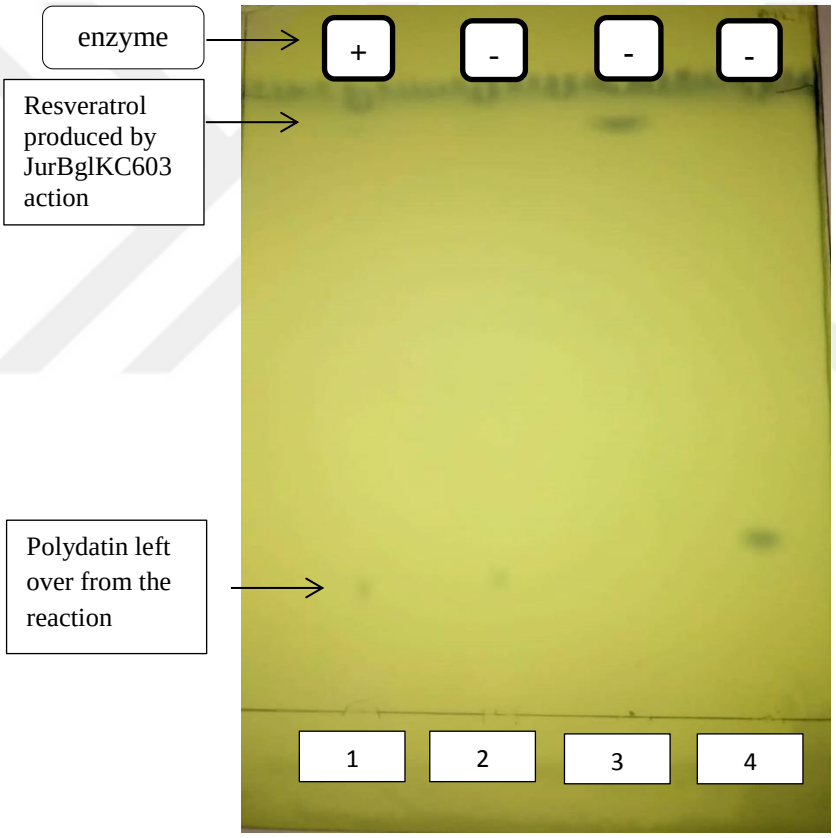


Figure 22. Analysis of deglycosylation of polydatin by JurBglKC603. Lane 1: Reaction mixture; Lane 2: Reaction mixture with no enzyme; Lane 3: Resveratrol as a control 4: Polydatin substrate as a control.

When myricetin-3-o-glucoside substrate was hydrolyzed by JurBglKC603, myricetin must be produced. Following TLC plate (Fig. 22) indicates that myricetin-3-O-glucoside was non-hydrolyzed by JurBglKC603. Cucurbitacin must also be produced, when Cucurbitacin E-2-O-glucoside substrate was hydrolyzed by BglKC603 (Fig. 22). However, no cucurbitacin deglycosylation was observed by TLC.

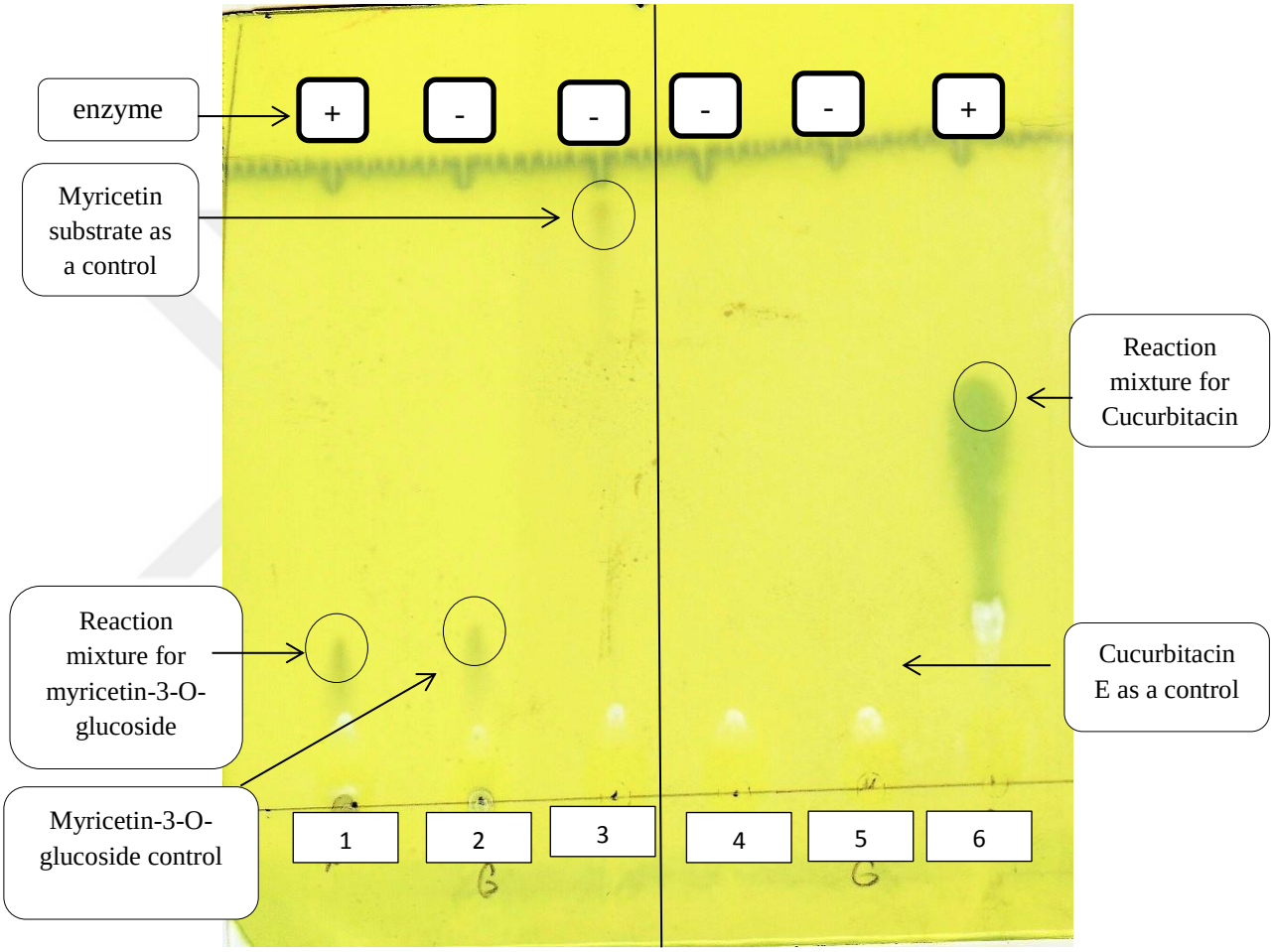


Figure 23. Analysis of deglycosylation of myricetin-3-O-glucoside and Cucurbitacin E-2-O-glucoside by JurBglKC603. Lane 1: Reaction mixture for myricetin-3-O-glucoside; Lane 2: myricetin-3-O-glucoside control; Lane 3: myricetin substrate as a control; Line 4: Cucurbitacin E as a control; Line 5: Cucurbitacin E-2-O-glucoside control; Line 6: Reaction mixture for Cucurbitacin E-2-O-glucoside.

4. DISCUSSION

In current study, β-glucosidase gene was detected in the genome of mesophilic bacterium *Jiangella ureilytica KC603*. Primers were designed to amplify full length β-glucosoidase from genome of *Jiangella ureilytica* KC603 (*JurBglKC603*). Amplified gene sequence was first cloned into pGEM-T easy cloning vector followed by the subcloning into pET28a (+) expression vector. *E. coli* BL21 (DE3) cells were used for expression for recombinant enzyme. Recombinant protein was purified by NTN-NI affinity column and then the recombinant enzyme was biochemically characterised. The effect of deglycosylation activity of recombinat enzyme was analysed using cucurbitacin, myricitin and resveratrol as substrates.

Macromolecules are biological entities that are essential for biochemical processes of life. With the advances in recombinant technology and protein engineering, enzymes have become important molecules widely used for different industrial purposes. Microbial enzymes, which attracted great attention with the development of enzyme technology, are used in more fields due to their features such as economic feasibility, high efficiency, consistency, ease of product modification and optimization. β-glucosidase is one of the industrially important enzymes because of the usability in increasing the quality of food and paper, lignin biosynthesis, obtaining renewable fuel production, secondary plant metabolism and anticarcinogenic effects. The optimum temperature of JurBglKC603 was determined as 35 ºC. Since *Jiangella ureilytica* KC603 is mesophilic bacterium, JurBglKC603 has low optimum tempreture. In the literature review, while the optimum temperature of β-glucosidase of *Micrococcus antarcticus* strain was determined as 25 ºC, *Exiguobacterium antarcticum* B7 strain was determined as 30 ºC (Sun, vd., 2020). The other β-glucosidases reported in the literature are usually thermophilic and exhibit higher temperature optima. For example, temperature optima for β-glucosidase of *Oenococcus oeni* was determined as 50 ºC. Generally, enzymes resistant to high temperatures are used in the industrial field. In this case, the use of enzymes at low temperatures is important in terms of providing production at low costs. Such enzymes can be adapted to the industry and used more efficiently with recombinant DNA and fermentation technologies and

protein modification processes. β-glucosidases are used in many industrial areas and have therefore drawn attention. In literature, there are β-glucosidase studies conducted above 400 different organisms on β-glucosidase and approximately the gene of 100 of these microorganisms have been cloned.

After the optimum pH studies, the optimum pH of the JurBglKC603 was determined as 6.0. When the studies in the literature are examined, the optimum pH value of the β-glucosidase of *Neotermes koshunensis* and *Myceliophthora thermophila* M.7.7 are 5.0, the optimum pH value of the β-glucosidase of *Bacillus subtilis* is 6.0, and the optimum pH value of the β-glucosidase of *Metschnikowia pulcherrima* is 4.5. The optimum pH range of the β-glucosidases in general is 4-7. JurBglKC603 is more alkaline than the β-glucosidases of *Neotermes koshunensis* and *Myceliophthora thermophila* M.7.7. Considering the studies in the literature (Şenol, vd., 2017), JurBglKC603 can be used in studies related to biomass fermentation since biomass fermentation processes are usually performed at the pH between 6 and 8.

By drawing substrate-activity graphs for JurBglKC603, it was determined that the enzyme fits the simple Michaelis-Menten kinetics. As a result of the calculations, K_m = 0.448757 mM, V_{max} = 3790.176 U / mg, k_{cat} = 3217.405119^{s-1} and k_{cat} / K_m = 7169.596 $s^{-1}mM^{-1}$ values were obtained. The kinetic parameters of β-glucosidase form *Bacillus tequelensis* in the literature was determined as K_m = 7.43, V_{max} = 1,462.25 U /mg, k_{cat} = 454.05 s^{-1} and k_{cat} / K_m= 61.12 $s^{-1}mM^{-1}$. *Thermotoga naphthophila* β-glucosidase is K_m = 0.45 mM, V_{max} = 153 × 10^3U / mg k_{cat} = 20,238.08 and k_{cat} / K_m = 2,698,413 $s^{-1}mM^{-1}$. *Bacillus sp. SJ-10* β-glucosidase has K_m = 4.6 mM, V_{max} = 1.526 U/mg, k_{cat} = 13.6 × 10^5 s^{-1} and k_{cat} / K_m = 3.0 × 10^5 $s^{-1}mM^{-1}$. Considering the researches on enzyme kinetics, the results of JurBglKC603 are better compared to the reported Beta-glucosidase enzyme (Raza, 2019).

Considering the pH stability of JurBglKC603 at room temperature, according to experimental results, activity of enzyme fell to half within 9th day at pH 5-7 and completely lost within 30 min at pH 3, within 8 hr at pH 4, within 5th day at pH 9-11. The enzyme lost 50% of its activity at pH 5, 6 and 7 on the 9th day. JurBglKC603 showed stability and activity between pH 5 and 7. A previous study showed that stability of two β-glucosidases AaBGL1 and AaBGL2 of *Actinomadura amylolytica* YIM 77502 T, were in the pH range of 4 to 9 (Yin vd., 2018). β-glucosidase AaBGL1 retained 95% of its activity

after 12 hours of incubation at room temperture while AaBGL2 couldn't, AaBGL2 activity dropped to 60% after 24 hours. When looking at the pH stability of the β-glucosidase of *Metschnikowia pulcherrima,* the enzyme half-life was about 5 hours (pH 4.5) at 23 ºC. On the other hand, JurBglKC603 retained 70% of its activity between pH range of 5 to 11 after 24 hours of incubation. JurBglKC603 has a wider pH range and mora stability than these two enzymes. Therefore, JurBglKC603 has the potential to be used in a wider pH range in the industrial processes than others.

Metal ions play significant role in increasing and decreasing enzyme activity by altering structure through binding amino acids having negative charge in specific domains. Metal ions tend to bind protein sites with high affinity and site specificity. Generally, heavy metals can alter enzymatic activities by linking functional groups containing carbonyl, carboxyl and sulfhydryl. The effects of metal ions on beta glucosidase activity were examined. In the presence of 5 mM Zn^{2+}, maximum enzyme activity was measured as 95%. The enzymatic activity of *Myceliophthora thermophila* M.7.7 fell to 63% at a concentration of 2.5 mM Zn^{2+}. While *Bacillus subtilis* treated with Zn^{2+} showed 56% enzyme activity, Marine bacterium showed 8.7 % relative activity (1 mM). While Zn^{2+} decreased the activity of β-glucosidase I of *Trichoderma reesei* upto 92%, it increased the activity of β-glucosidase II of the same bacterium upto 106%. Zn^{+2} is a strong inhibitor that acts on enzymes and it can inhibit the activities of enzymes. Zn^{+2} inhibited the activity of JurBglKC603 lesser than other enzymes. JurBglKC603 showed 99% relative activity with Mg^{2+}. Same results were obtained from *Myceliophthora* and *Bacillus subtilis* within a range of 100% and 92% respectively (Bofa, vd., 2018). While *Bacillus subtilis* showed 92% activity in the presence of 1 mM Mg^{2+}, JurBglKC603 showed 99% activity. While *Myceliophthora thermophila* M.7.7 treated with K^{+} showed 76% enzyme activity, JurBglKC603 showed 72% relative activity (1 mM). However, K^{+} increased the activity of Marine bacterium to 106%. Fe^{2+} has reduced enzyme activity of JurBglKC603 (89%) as compared to *Bacillus subtilis*, which showed 142% of activity. However, the activity of JurBglKC603 increased with increasing Fe^{2+} concentration (5 mM) upto to 111%. In case of *Myceliophthora thermophila* M.7.7, all of its activity was lost in the presence of Fe^{2+}. In the presence of Ca^{2+} (1 mM), JurBglKC603 represented 95% activity while in the case of *Bacillus subtilis* only 98% activity was observed. However, while the activity of JurBglKC603 increased with increasing Ca^{2+} concentration (5 mM) upto to 115%, the activity of *Bacillus subtilis* decreased with increasing Ca^{2+} ion concentration (10 mM) and

fell to 88%. Moreover, marine bacterium showed no activity in the presence of Ca^{2+} (1 mM). Cu^{2+} increased the activity of JurBglKC603 to 103% while in case of *Bacillus subtilis* only 17% activity was observed. Moreover, *Myceliophthora thermophi*la M.7.7, showed no activity in the presence of Cu^{2+}. In the presence of Ni^{2+}, JurBglKC603 and *Bacillus subtilis* showed 93% and 67% activity respectively (Raza, 2019). JurBglKC603 showed 83% activity in Mn^{2+} ion, while *Bacillus subtilis* and *Myceliophthora thermophila* M.7.7, showed 116% and 49% activity respectively. Na^{2+}, increased relative activity of JurBglKC603 to 103%, while *Myceliophthora thermophila* M.7.7 showed 70% and *Bacillus subtilis* 40% activity. However, the activity of JurBglKC603 increased with increasing Na^{2+} concentration (5 mM) upto 117%. JurBglKC603 showed 85% activity in the presence of Co^{2+} (1 mM), while *Soy Cotyledon* showed 90% activity. While, in the presence of Hg^{2+} (1 mM), enzymatic activity decreased to 19% for JurBglKC603, β-glucosidase of *Soy Cotyledon* was decreased to 16% (Santos vd., 2011).

Heavy metals such as Cu^{2+}, Zn^{2+}, and Hg^{2+} usually exhibit high affinity for thiol groups (Sulfhydryl group (-RSH)). These heavy metal ions oxidize functional groups of cysteine residues and inhibit the enzymatic activity of some proteins (Ganesan vd., 2020). These heavy metals did not show much effect on JurBglKC603 except for Hg^{2+}. As the destructive property of Hg^{2+} affects the 3D structure of the enzyme, its activity was decreased. The reason why other ions do not show much effect is that there are no thiol groups in the active region of the enzyme we have studied. As a result of bioinformatics screening, there are glutamic acid and histidine groups in the active region of JurBglKC603. Metal ions generally affect these groups in the active area. In addition, while Ca^{2+} increases the activity of the enzyme, Mn^{2+} has not shown to have much effect on the enzyme. It is known that Ca^{2+} and Mn^{2+} ions can stabilize enzymes. Also, the Mg^{2+} ion had no significant effect on enzyme activity. An increase in the activity of the enzyme was observed in the presence of Fe^{2+} and Na^{2+} ions (San vd., 2020). Most of the metal ions has no negative effect on the activity of the enzyme and it will be used for the hydrolysis of natural product which often contain different ions, so such condition makes it an ideal enzyme to use in industrial field.

When the effects of modifiers on the JurBglKC603 was evaluated, significant change was observed. Detergents (%1 v/v) SDS, Tween-20 and Triton-X100 reduced enzyme activity. The activity of JurBglKC603 fell upto 51% in the presence of 5% Tween-

20, wheareas the activity was decreased upto 92% in the presence of %1 Triton X-100. In the presence of 1-5% SDS, approximately complete inhibition occurs because the activity of the enzyme fell upto 1%.

In the literature, it is seen that these chemicals have different effects on the activities of β-glucosidase obtained from various sources. The activity of the β-glucosidase of *Bacillus subtilis*; in the presence of Triton X-100 decreased by 5% , however, for Tween-20 decreased by 19% and for SDS 9%. The activity of the β-glucosidase of *Myceliophthora thermophila* M.7.7 decreased by 55% in the presence of Triton X-100 and 18% in the presence of SDS (Bofa vd., 018). The activity of the β-glucosidase from *Aspergillus fumigatus* decreased by 76% in the presence of SDS. SDS is a powerful surfactant and it can inhibit enzymes by disrupting the 3D structures of enzymes. As a result of this study, JurBglKC603 activity was decreased by SDS to 2%. Tween-20 also significantly affected the activity of the JurBglKC603. Based on the results, JurBglKC603 cannot maintain its stability in the presence of strong detergents.

Effect of organic solvents (5% v/v) on the activity of JurBglKC603 was determined. Butanol decreased the activity of enzyme by 80%, ethanol by 41%, propanol by 33%, DMSO by 18%, Dioxane by 31% and methanol by 7%. Effect of organic solvents (10% v/v) on the activity of JurBglKC603 was determined. Butanol decreased the activity of enzyme by 73%, ethanol by 52%, propanol by 33%, DMSO by 11%, Dioxane by 72% and methanol by 9%. The activity of β-glucosidase of *Bacillus subtilis* was decreased by methanol upto 86%, ethanol upto 82% and propanol upto 93%. The activity of the β-glucosidase of *Gongronella butler* increased by 30% in the presence of ethanol. β-glucosidase activity of *Myceliophthora thermophila* M.7.7; in the presence of isopropanol and ethanol, was decreased upto 70% while the one of DMSO was decreased upto 92% by (Chamoli vd., 2016).

Efffects of several inhibitors (1 mM) on the activity of *Jiangella ureilytica* KC603 β-glucosidase was determined. PMSF decreased the activity of enzyme by 16%, EDTA by 8%, DPEC by 6%, DTT by 19%, EGTA by 83%. Effect of inhibitors (5 mM) on the activity of JurBglKC603 was determined. PMSF decreased the activity of enzyme by 27%, DTT by 13% and EGTA by 98%, but EDTA and DEPC increased the activity of enzyme by 2-3%. According to the previous studies, relative activity of the β-glucosidase of *Myceliophthora thermophila M.7.7* in the presence of EDTA decreased by 32%, while

18% by DTT. *Sporothrix schenckii* β-glucosidase activity was decreased upto 98% in the presence of EDTA and it was decreased upto 97% in the presence of EGTA. β-glucosidase of *Leuconostoc mesenteroides* showed 42% activity reduction for DMSO while EDTA increased the activity by 19%. While EDTA (1 mM) increased the activity of β-glucosidase of Marine bacterium by 16%, β-glucosidase of *Trichoderma reesei* was increased the activity by 2%. While DTT (1 mM) increased the activity of β-glucosidase of Marine bacterium by 17%, β-glucosidase of *Oenococcus oeni* was decreased by 16 %. While PMSF decreased the activity of β-glucosidase of *Soy cotyledon* by %22, EDTA decreased the activity by %21 (Santos vd., 2011).

When the effects of inhibitors on JurBglKC603 were examined, EDTA increased the enzyme activity by 2% while EGTA decreased by 98%. Both inhibitors are chelating agents, which have similar structures and functions. Regarding their structure, EGTA contains two oxygen atoms with unshared electrons conversely EDTA. Other than this, EGTA has higher affinity for Ca^{2+} ion than EDTA. Considering this situation, the structure of *Jiangella ureilytica* KC603 β-glucosidase is more affected by EGTA. Two oxygens with unshared electrons in EGTA can tend to bond more easily with this enzyme and this can inhibit the enzyme's activity.

Glucose effect, on varying range of glucose (200 mM to 4000 mM), on the activity of JurBglKC603 was investigated. At glucose concentration 200 mM, the activity of the enzyme dropped to 82%. There was not much change in the enzyme activity up to 3 M glucose in the reaction mixture. But 3 M glucose dropped the enzyme activity down to 68%. However, it decreased to 66% in 4 M glucose concentration.

In a previous study, activity of β-glucosidase (Cen205) of *Bursaphelenchus xylophilus* changed in various concentrations of glucose. The activity of Cen205 retains approximately 80% activity at 100 mM glucose concentration. As the molarity of glucose increased, enzyme activity decreases linearly and the activity was approximately 55% at 500 mM. β-glucosidase of *Gongronella butleri*, while giving 100% activity at 5 mM glucose concentration, the activity of the enzyme decreased as the glucose concentration increased. At 15 mM glucose concentration, the activity of enzyme reduced to 50% (Zhang vd., 2017). Glucose generally inhibit β-glucosidases. Therefore it is important that these enzymes tolerate glucose. In general, β-glucosidase having tolerance to glucose are preferred in studies such as biomass and biofuel production (Liter vd., 2020). Therefore,

the high glucose tolerance of the JurBglKC603 indicates that it is a preferable enzyme for the industrial process.

Summing up the study, JurBglKC603 was cloned and characterized for the first time. Compared to the enzymes discussed, the K_m of this enzyme is low and the V_{max} is high. This means that high yields can be obtained with less substrate. The high reaction rate of the enzyme means that the rate of product formation in the environment is high. Because of the high reaction rate of the enzyme, high efficiency can be obtained with a small amount of enzyme in the industrial field and in a short time. The high thermal stability and pH stability of the enzyme and its effect on some phenolic compounds indicate that this enzyme can be used industrially.

Myricetin and resveratrol have great antioxidative and cytoprotective potentials, anti-carcinogenic antioxidant, anti-inflammatory actions, antiviral and antimicrobial properties, and anti-platelet activity (Semwal vd., 2016). The cucurbitacins from plants of Cucurbitaceae are important compound for treatment of cancer, fever, amenorrhea, leukemia, jaundice, rheumatism and tumour (Al-Snafi, 2016). According to the results of TLC analysis, there was no deglycosylation as a result of the reaction with myricetin-3-O-glucoside. It was determined that Polydatin was deglycosylated by JurBglKC603 and antioxidant resveratrol was formed. As a result of the reaction performed, JurBglKC603 formed resveratrol with higher antioxidant activities by removing a glucose from Polydatin.

5. CONCLUSIONS

As to best of our knowledge, JurBglKC603 is not reported previously. In this study, β-glucosidase characterization and its effect on phenolic compound modification was investigated.

Optimum temperature of JurBglKC603 was determined as 35 ºC while optimum pH as 6.0. Kinetics parameters of paranitrophenyl β-D-glucopyrinoside as a chromogenic substrate were examined, K_m value was determined as 0.448757 mM, V_{max} value was 3790.176 U /mg, k_{cat} value was 3217.405119^{s-1} and k_{cat} / K_m value was 7169.596 s^{-1} mM^{-1}.

pH stability of JurBglKC603 was evaluated. Enzyme activity at pH 3 at room temperature fell below 50% in the first 30 minutes, however, at pH 4 after 8 hour's activity reduction below 50% was observed. At pH 10-11, relative enzyme activity reduction at 50% was observed after 72 hours. While at pH 8 and 9, enzyme was stable and retain its activity untill 5 days. However, at pH 5-7, the activity of the enzyme decreased below 50% after 9th day. It was concluded that enzyme was stable at high pH range and its maximum activity was observed at more alkaline conditions when comparing with many β-glucosidases

Temperature stability of JurBglKC603 has decreased below 50% after 216 hours at 25 °C while 50% at 35 °C after 4 hours. In addition, it reduces 50% of its activity after first 30 minutes at 45 °C and 55 °C. So, it can be suggested that enzyme cannot withstand at high temperatures.

Effects of metals of Mg^{2+}, Ni^{2+}, Ca^{2+}, K^{+}, Zn^{2+}, Fe^{2+}, Cu^{2+} , Mn^{2+} , Na^{+} , Hg^{2+} and Co^{+2} on enzyme activity was investigated. Metal ions in various concentrations were treated with enzymes and their activities were measured. The change in enzyme activity at 1 mM and 5 mM metal ion concentrations is as follows; enzyme activity decreased 1% in the presence of Mg^{2+}. In the presence of Ni^{2+}, the enzyme retained 93% and 96%, activity, respectively. The enzyme showed 95% and 115% activity under the influence of Ca^{2+}. Enzyme maintained 72% and 24% activity under the influence of K^{+}. The enzyme showed 107% and 95% activity under the influence of Zn^{2+}. The enzyme retained 98% and 111%

activity under the influence of Fe^{2+}. The enzyme possessed 103% and 111% activity under the influence of Cu^{2+}. The enzyme maintained 83% and 88% activity under the influence of Mn^{2+}. The enzyme retained 19% and 16% activity under the effect of Hg^{2+}.

To study the effect of detergents on the enzyme, 1% and 5% concentration were included in reaction medium. According to the results; Tween-20 reduced the activity of the enzyme by 49%, 89% whereas Triton X-100 reduced activity of the enzyme only about 8-15%. On the other hand, SDS lost enzyme's activity by about 98%.

1 mM and 5 mM final inhibitor concentrations were included in reactions and their effects on the activity of the enzyme were examined. EDTA and DEPC showed an effect similar to original enzyme activity. When EDTA and DEPC were at a concentration of 5 mM, the activity of the enzyme increased up to 102-103%. Enzyme activity decreased to 84-81% while PMSF and DTT were at a concentration of 1 mM. Inhibitors used generally reduced the activity of the enzyme, except EDTA and DEPC. The enzyme activity increased with increasing the EDTA and DEPC concentrations.

Effect of organic solvents of methanol, butanol, propanol, ethanol, dioxane and DMSO on enzyme activity was investigated. Organic solvent used in various concentrations were treated with enzymes and their activities were measured. The change in enzyme activity at organic solvents concentrations of 5% and 10% is as follows; enzyme activity is reduced by 33% in the presence of propanol. In the presence of butanol, the enzyme showed 20% and 27% activity, respectively. The enzyme showed 93%, 91% activity in the presence of methanol. Enzyme showed 69% and 28% activity in the presence of dioxane. The enzyme showed 59% and 48% activity in the presence of ethanol. The enzyme showed 82%, and 89% activity in the presence of DMSO.

Activity of JurBglKC603 was measured by treating several concentrations of glucose for determination of glucose tolerance of the enzyme. Eighty-two percent activity of JurBglKC603 has been maintained in 200 mM glucose. Up to 3 M, enzyme activity did not change much, but at 3M glucose, 68% of the enzyme activity has been retained. Activity dropped to 66% in 4 M glucose concentration. These findings suggest that glucose tolerance of JurBglKC603 is high.

Three different substrates previously known to have antioxidant effects have been analyzed. These determined substrates were treated with JurBglKC603 and TLC analysis

was performed. Depending on our results, we suggest that JurBglKC603 has increased the antioxidant activity of Polydatin (Piceid). But Myricetin -3-O-glucoside was not deglycosylated by JurBglKC603. Also, Cucurbitacin is produced if Cucurbitacin E-2-O-glucoside substrate is hydrolyzed by JurBglKC603. However, no Cucurbitacin E-2-O-glucoside deglycosylation was observed on TLC.

6. RECOMMENDATIONS

1) JurBglKC603 is stable at low temperatures but not stable at high temperatures. Thermal stability of the enzyme can be enhanced by site directed mutagenesis following the determination of the residues that are possibly important for temperature stability by bioinformatic analysis and literature review.

2) Proline-tag can also be incorporated to improve thermal stability of enzyme as conducted by Irfan et al 2016 for xylanase from *Geobacillus thermodenitrificans* C5.

3) Myricetin-3-O-glucoside, that is used as antioxidant, is a flavanoid compound and JurBglKC603 can not deglycosylate this compound. Considering bioinformatics analyses and β-glucosidase studies, some amino acid substitution studies on JurBglKC603 can be made by site directed mutagenesis and its effect on the Myricetin -3-O-glucoside might be changed.

4) Whether JurBglKC603 is effective on cucurbitacin substrate has not been determined. HPLC analysis can be performed to understand whether the cucurbitacin is deglycosylated by enzyme.

5) Stability analysis of JurBglKC603 activity at lower temperatures has not been performed. The stability of the enzyme can be examined at lower temperatures such as +4 °C and -20 °C.

6) Kinetic analysis is carried out only with pNPG, it could be also performed on natural substrates such polydatin.

7. REFERENCES

Acar, M., 2013. Mandalina (*Citrus Reticulata*) Meyvesinden Saflaştırılan Beta-Glukosidaz Enziminin Nanopartiküllere İmmobilizasyonu ve Meyve Suyu Aroma Arttırma Etkisi, Yüksek Lisans Tezi, Balıkesir Üniversitesi Fen Bilimleri Enstitüsü, Balıkesir.

Al-Snafi, A. E.,2016. Chemical constituents and pharmacological effects of Citrullus colocynthis-A review, IOSR Journal of Pharmacy, 6,3, 57-67.

Angelin-Bonnet, O., Biggs, P., J., and Vignes, M., 2019. Gene Regulatory Networks: A Primer in Biological Processes and Statistical Modelling, Methods in Molecular Biology, Humana Press, 1883, New York, 347–383.

Artzi, L., Bayer, A., and Moraïs, S., 2016. Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides, Nature Reviews Microbiology, 15,2, 83–95.

Baba, S., A., Vishwakarma, R., A. and Ashraf, N., 2017. Functional Characterization of CsBGlu12, a -Glucosidase from Crocus sativus, Provides Insights into Its Role in Abiotic Stress through Accumulation of Antioxidant Flavonols, The Journal of Biological Chemistry, 292, 11, 4700-4713.

Barka, E.,A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Kelenk, H., P., Clément, C., Ouhdouch, Y. and Wezel, G., P., V., 2016. Taxonomy, Physiology and Natural Products of *Actionabacteria*, Microbiology and Molecular Biology Reviews, 80, 1-44.

Bhatti, A., Haq, S., Bhat, R., 2017. Actinomycetes Benefaction Role in Soil and Plant Health, Microbial Pathogenesis, 111, 458-467.

Bofa, E.C., Moretti,M., Gomes, E., Orlando G. and Rodriguez, B., 2018. Biochemical Characterization Of An İsolated 50kda Beta-Glucosidase From The Thermophilic Fungus *Myceliophthora Thermophila M.7.7*, Biocatalysis And Agricultural Biotechnology, 13, Brazil, 313-318.

Boudabbous, M., Hmad, I., Saibi, Walid., Mssawra, M., Belghith, H. and Gargori, A., 2017. Trans-Glycosylation Capacity Of A Highly Glycosylated Multispecific β-glucosidase From *Fusarium Solani*, Bioprocess Biosyst Eng, 40, Tunisia, 559–571.

Chen, P., Fu, X., Ng, T. and Ye, X., 2011. Expression Of A Secretory β-glucosidase From Trichoderma Reesei İn Pichia Pastoris And its Characterization, Biotechnol Lett, 33, 2475–2479.

Chamoli, S., Kumar, P., Navani, N. and V, A., 2016. Secretory Expression, Characterization And Docking Study Of Glucose-Tolerant-Glucosidase From *B. Subtilis*, International Journal Of Biological Macromolecules, 85, 425-433.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. and Stackebrandt, E., 2006. The Prokaryotes: Vol. 1: Symbiotic Associations, Biotechnology, Applied Microbiology. Springer Science and Business Media, 509-937.

Eduardo, A., Ximenes, Carlos R., Felix, Cirano J. and Ulhoa, 1996. Production of cellulases by *Aspergillus fumigatus* and characterization of one β-glucosidase, Current Microbiology, 32, 3, 119-123.

Ergöçen, G., 2013. B-Glukozidaz Enziminin Kayısı (*Prunus Armeniaca*) Çekirdeklerinden Saflaştırılması ve Karakterizasyonu, Yüksek Lisans Tezi, Çukurova Üniversitesi, Fen Bilimleri Enstitüsü, Adana.

Esen, A., 1993. β-Glucosidases. Biochemistry and Molecular Biology, Acs Symposium Series 533, American Chemical Society, Washington. D.C.

Ganesan, M., Vinayakamoorthy, R. M., Thankappan, S., Muniraj, I., and Uthandi, S., 2020. Thermotolerant glycosyl hydrolases-producing Bacillus aerius CMCPS1 and its saccharification efficiency on HCR-laccase (LccH)-pretreated corncob biomass. Biotechnology for biofuels, 13, 1, 1-14.

Garcia, R., Porrellia, A., Fernandeza, P., Carreteroa, A. and Curiel, J., 2020. Identification, Purification And Characterization Of A Novel Glycosidase (Bglm1) From Leuconostoc Mesenteroides, LWT - Food Science And Technology, 122, 108829.

Gemici-Palabıyık, B., 2004. Bitki Genlerinin *Schizosaccharomyces Pombe*'de Anlatımı, Doktora Tezi, İstanbul Üniversitesi, Fen Bilimleri Enstitüsü, İstanbul.

Giuseppe, P. O., Souza, T. D. A., Souza, F. H. M., Zanphorlin, L. M., Machado, C. B., Ward, R. J. and Murakami, M. T. 2014. Structural basis for glucose tolerance in GH1 β-glucosidases, Acta Crystallographica Section D: Biological Crystallography, 70, 6, 1631-1639.

Graebin, N., Schöffer, J., Andrades, D., Hertz, P., Ayub, M. and Rodrigues, R., 2016. Immobilization of Glycoside Hydrolase Families GH1, GH13, and GH70: State of the Art and Perspectives, Molecules, 21,8,1074.

Grabowski, G., A., Gatt, S. ve Horowitz, M., 1990. Acid β-glucosidase: Enzymology and Molecular Biology of Gaucher Disease, Critical Reviews in Biochemistry and Molecular Biology, 25, 6, 385-414.

Gurgu., L., Lafraya, A., Polaina, J. ve Marin-Navarro, J., 2011. Fermentation od cellobiase to ethanol by industrial *Saccharomyces* strains carrying the β-glucosidase gene (BGL1) from *Saccharomycopsis fibuligera*, Bioresource Technology, 102, 5229-5236.

Guasch, A., Perez-Pons, J.A., Vallmitjana, M., Querol, E., Coll, M., 2002. β-glucosidase from Streptomyces sp., RCSB PDB, 10-22

Gübitz, G. and Paulo, A., 2003. New substrates for reliable enzymes: enzymatic modification of polymers, Elsevier, 14, 577–582.

Hamza, T., 2017. Bacterial Protease Enzyme: Safe and Good Alternative for Industrial and Commercial Use, American Institute of Science, 3, 1-10.

Hernandez-Guzmán, A., Flores-Martínez, A., Ponce-Noyola, P. and Villagómez-Castro, J. C., 2016. Purification and characterization of an extracellular β-glucosidase from *Sporothrix schenckii*, FEBS Open Bio, 6, 11, 1067-1077.

Irfan, M., Güler, H., Ozer, A., Sapmaz, M., Belduz, A.O., Hasan, F. and Shah,A., 2016, C-Terminal Proline-Rich Sequence Broadens the Optimal Temperature and pH Ranges of Recombinant Xylanase from *Geobacillus thermodenitrificans* C5, Enzyme and Microbial Technology, 91,34-41.

Işbakan, N., 2006. Lipaz Enzimi Biyokatalizörlüğünde Enantiyomerik Saflıkta 1-Fenil-1-Propanolün Transesterleşme Tepkimesiyle Kinetik Rezolüsyonu, Yüksek Lisans, Ankara Üniversitesi, Fen Bilimleri Enstitüsü, Ankara.

James, R., Cairns, K. and Esen, A., 2010. β-Glucosidases, Cellular and Molecular Life Sciences, 67, 3389–3405.

Jiang, K., Asami, T., 2018. Chemical Regulators of Plant Hormones and Their Applications in Basic Research and Agriculture , Bioscience, Biotechnology, and Biochemistry, 82, 8, 1265-1300.

Lee, J. H., Hwang, C. E., Son, K. S., and Cho, K. M., 2019. Comparisons of nutritional constituents in soybeans during solid state fermentation times and screening for their glucosidase enzymes and antioxidant properties. Food chemistry, 272, Korea, 362-371.

Li, G., Jiang, Y., Fan, X., Liu, Y., 2012, Molecular Cloning And Characterization Of A Novel β-glucosidase With High Hydrolyzing Ability For Soybean İsoflavone Glycosides And Glucose-Tolerance From Soil Metagenomic Library, Bioresource Technology,123, China, 15-22.

Karabulut, H. and Gülay, M., Ş., 2016. Antioksidanlar, MAE Veterinerlik Fakültesi Dergisi, 1, 1, 65-76.

Kim, Y., S. and Ma, J., Y., 2018. Insight Into the Hdrolytic Selectivity of β- Glucosidase ro Enhance the Contects of Desired Active Phytochemicals in Medicinal Plants, Hindawi BioMed Research International, Korea, 10.

Koca, N. ve Karadeniz, F., Serbest Radikal Oluşum Mekanizmaları ve Vücuttaki Antioksidan Savunma Sistemleri, Gıda Mühendisliği Dergisi, 32-37.

Liter, J., Dominguez, M., Toro, B., Santana, A., Prieto, A., Asensio, J., Cañada, F., Eugenio, L., and Martinez, M., 2020, A Glucotolerant β-Glucosidase From the Fungus Talaromyces Amestolkiae and Its Conversion Into A Glycosynthase for Glycosylation of Phenolic Compound, Microbial Cell Factories, 19, 1, 1-13.

Lobo, V., Patil, A., and Phatak, A., 2010, Chandra, N., Free radicals, antioxidants and functional foods: Impact on human health, Pharmacognosy Reviews, 4,8-118.

Mcdonald, A. G., Boyce, S. And Tipon, K. F., 2009, Explorenz: The Primary Source Of The IUBMB Enzyme List, Nucleic Acids Research, 37, 593-597.

Miao, L., Hou, Y., Fan, H., Qu, J., Qi, C., Liu, Y., Li, D., and Li, Z., 2016. Molecular Structural Basis for the Cold Adaptedness of the Psychrophilic β-Glucosidase BglU in Micrococcus antarcticus, Applied and Environmental Microbiology 82, 2021–2030.

Morant, A. V., Jorgensen, K., Jorgensen, C., Paquette, S. M., Sanchez-Parez, R., Moller, B. L., and Bak, S., 2008. β-Glucosidases as detonators of plant chemical defense. Phytochemistry, 69, 9, 1795–1813.

Ni, J., Tokuda, G., Takehara, M., and Watanabe, H. 2007. Heterologous expression and enzymatic characterization of β-glucosidase from the drywood-eating termite, Neotermes koshunensis, Applied Entomology and Zoology, 42, 3, 457-463.

Raza, A., Pothula, R., Abdelgaffar, H., Bashir, S., Luis, J., and Fuentes, J., 2020. Identification and Functional Characterization of a β-Glucosidase from *Bacillus tequelensis* BD69 Expressed in Bacterial and Yeast Heterologous Systems, PeerJ8:e8792.

Pino-Garcia,R., Porrelli, A., Rus-Fernandez, P., Carreterol, A., and Curiel,A., 2020. Identification, Purification and Characterization Of A Novel Glycosidase (Bglm1) From *Leuconostoc Mesenteroides*, LWT, 122, 108829.

Pombo, P., Perez,G., Carrau, G., Guisan, J., Viera, F., Brena, B., One-step Purification and Characterization of an Intracellular β-Glucosidase from *Metschnikowia pulcherrima*, Biotechnology letters, 30:1469–1475

Saha, B. C., and Bothast, R. J., 1996. Production, purification, and characterization of a highly glucose-tolerant novel β-glucosidase from Candida peltata. Applied and Environmental Microbiology, 62(9), 3165-3170.

Salgado, J., Meleiro, L., Carli, S., and Ward, R., 2018. Glucose tolerant and glucose stimulated β-glucosidases – A review, Bioresource Technology, 267, 704-713.

Santos, S., Olıveıra, C., Varea, G., Orradıdasılva, G., Ida, I., Mandarıno, J., Carrão-Panızzı, M., and Rıbeıro, M., 2011. Purification And Characterization of *Soy Cotyledon* β-glucosidase, Journal of Food Biochemistry, 37, 302–312.

Saqib, A., and Whitney, P., 2006. Esculin Gel Diffusion Assay (EGDA): A Simple Andsensitive Method for Screening-Glucosidases, Enzyme and Microbial Technology, 39, 182–184

Saygin, H., Ay, H., Güven, K., and Şahin, N., 2020. Genome-based classification of three novel actinobacteria from the Karakum Desert: *Jiangella asiatica* sp. nov., *Jiangella aurantiaca* sp. nov. and *Jiangella ureilytica* sp. nov., International Journal of Systematic and Evolutionary Microbiology, 70, 1993-2002.

Semwal, D., Semwal, R., Combrinck, S., and Viljoen, A., 2016. Myricetin: A Dietary Molecule with Diverse Biological Activities, Nutrients, 8, 90.

Sigel, R., and Pyle, A., 2006, Alternative Roles for Metal Ions in Enzyme Catalysis and the Implications for Ribozyme Chemistry, Chemical Reviews, 107, 97−113.

Singhania, R., R., Patel, A., K., Pandey, A. and Ganansounou, E., 2017. Genetic modification: A tool for enhancing beta-glucosidase production for biofuel application, Biosource Technology, 245, 1352-1361.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., and Pandey, A., 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresource technology, 127, 500-507.

Singh, G., Verma, A., K. and Kumar, V., 2016. Catalytic properties, functional attributes and industrial applications of β-glucosidases, 3 Biyotech, 6, 1, 3.

Srivastavaa, N. , Srivastavac, M., Mishrab, P., Guptad, V., Molinaf, G., Rodriguez-Coutog, S., Manikantab, A., and Ramteke,A., 2018. Applications of fungal cellulases in biofuel production: Advances and limitations, Renewable and Sustainable Energy Reviews, 82, 2379-2386.

Srivastava, N., Rathour R., Jha, S., Pandey, K., Srivastava, M., and Thakur, V., K., 2019. Microbial Beta Glucosidase Enzymes: Recent Advances in Biomass Conversation for Biofuels Application, Biomolecules, 9, 220, 1-23.

Sönmez, G., D., 2013. Zeytin β-Glukozidaz ve Metalotionein Genlerinin Moleküler, Fizyolojik ve Biyokimyasal Karakterizasyonu, Doktora Tezi, Balıkesir Üniversitesi Fen Bilimleri Enstitüsü, Balıkesir.

Sun, J., Wang, W., Ying, Y., and Hao, J., 2020. A Novel Glucose-Tolerant GH1 β-Glucosidase and Improvement of Its Glucose Tolerance Using Site-Directed Mutation. Applied Biochemistry and Biotechnology, 1-17.

Tan, G. Y., and Liu, T.,2017. Rational synthetic pathway refactoring of natural products biosynthesis in actinobacteria. Metabolic engineering, *39*, 228-236.

Tapas, A. R., Sakarkar, D. M., and Kakde, R. B., 2008. Flavonoids as nutraceuticals: a review, Tropical journal of Pharmaceutical research, *7*, 3, 1089-1099.

Topal, F., Nar, M., Göçer, H., Kalin, P., Koçyiğit, U., M., Gülçin, İ., and Alwasel, S., H., 2015. Antioxidant activity of taxifolin: an activity–structure relationship, Journal of Enzyme Inhibition and Medicinal Chemistry, 31, 4, 674-683.

Trent, D., J., 2000. Extremophiles in Astrobiology: Per Ardua Ad Astra, Gravitational And Space Biology Bulletin, 13, 2, 5-11.

URL-1,2 . https://www.sigmaaldrich.com/ prodMerch10-3, 2020

URL-3. https://www.wikipedia.org.com.tr, 2020

Yin, Y. R., Sang, P., Xian, W. D., Li, X., Jiao, J. Y., Liu, L., and Li, W. J. 2018. Expression and characteristics of two glucose-tolerant GH1 β-glucosidases from Actinomadura amylolytica YIM 77502T for promoting cellulose degradation, Frontiers in microbiology, *9*, 3149.

Wang, H., Lin, X., Li, J., Xie, C., Liu, D., and Yao, D., 2020. Enzyme and MicrobialTechnology, Science Direct, China,

Wang, J., Hu, S., Nie, S., Yu, Q., and Xie, M., 2016. Reviews on mechanisms of in vitro antioxidant activity of polysaccharides, Oxidative Medicine and Cellular Longevity, 2016.

Withers, S. G., 1996. Identification of Active-Site Residues in Glycosidases. Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry, 365–380.

Zhang, L., Fu, Q., Li, W., Wang,B., Yin, X., Liu,S., Xu, Z. and Niu,Q., 2017. Identification and Characterization Of A Novel B-Glucosidase Via Metagenomic Analysis Of Bursaphelenchus Xylophilus And Its Microbial Fora, Scientific Reports, 7, 14850.

Zhang, J., Zhao, N., Xu, J., Qi, Y.,, Wei, X., and Fan, M., 2020. Homology analysis of 35 β-glucosidases in *Oenococcus oeni* and biochemical characterization of a novel β-glucosidase BGL0224, Food Chemistry, 0308-8146.

Zhiwei Xu,Z., Escamilla-Trevin, L., Zeng, L., Lalgondar, M., Bevan, R., Winkel, B., Mohamed, A., Cheng, C., Shih, M., Poulton, J., and Esen, A., 2004. Functional genomic analysis of Arabidopsis thaliana glycoside hydrolase family 1, 104, Plant Molecular Biology, 343–367.

Zou, Z., Xi, W., Hu, Y., Nie, C., and Zhou, Z., 2016. Antioxidant activity of Citrus fruits, Food chemistry, 196, 885-896.

CURRİCULUM VİTAE

She was born in 1995 in Hekimhan/Malatya. She studied his primary and secondary education at the Yukarı Selimli köyü Şehit On Başı Münir Kılıç Primary School. After completing her high school education at Hekimhan High School, she started her undergraduate education in the Genetics and Bioengineering Department of Gümüşhane University Faculty of Engineering and Natural Sciences in the 2013-2014 academic year. During her undergraduate education, she studied as a student in the TUBITAK student project on the Research of Antioxidant Properties of Flower Parts of Some Endemic Plant Species in Gümüşhane and its surroundings. After completing her undergraduate education in 2017, she started her master's degree in Molecular Biotechnology Graduate Institute of Natural and Applied Sciences at KTU in the same year. During her graduate education, she studied on gene cloning, protein expression and characterization. She studied education about occupational health and safety, corporate entrepreneurship, B1 level english and python robotic coding. She has four certificates that is Occupational Health and Safety certificate, KOSGEB certificate, KTÜ School of Foreign Languages English B1 Certificate and Python Robotic Coding Training Certificate. She knows English at intermediate level.