R.T.
UNIVERSITY OF DICLE
INSTITUTE OF NATURAL AND APPLIED SCIENCES

CLONING and CHARACTERIZATION of α-AMYLASE from *Bacillus circulans* ATCC 61

Aram Jalil TAWFIQ

MASTER'S THESIS

DEPARTMENT OF BIOLOGY

DİYARBAKIR
February 2016

T.C.
DİCLE ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

Bacillus circulans ATCC 61'den α-AMİLAZIN KLONLANMASI ve KARAKTERİZASYONU

Aram Jalil TAWFIQ

YÜKSEK LİSANS TEZİ

BİYOLOJİ ANABİLİM DALI

DİYARBAKIR
Şubat 2016

ACKNOWLEDGMENT

This master thesis was conducted at DICLE University, under respectable supervision **Prof. Dr. Fikret UYAR**. I'm thankful to him for his supervision, which made wonderful influence on me in learning crucial aspects of my work. He gave me a new chance at all possible situations to study and follow things during critical and interesting situations, encouraged me to think like a scientist, who helps me to draw out my inner strengths, thank you again.

I wish to thank my co-supervisor **Assistant Prof. Dr. Cem ÖZİÇ** at KAFKAS University for providing, exceptionally laboratory facilitates and continuous help and support to bring my project materials successfully.

I also express my sincere thanks to my teacher **Dr. Besi SERİN** for always being helpful with technical and practical advice in the laboratory. In addition, she told me what to do when my works were not in compliance with the plan in the lab.

I would also like to thank my **Parents** for their encouragement and support through all these years, their prayers and countless effort made this work possible. Also, Spatial thanks to my **sisters** and my **brothers** for their timely co-operation and encouragement during this work.

My profound thanks to **Mr. Farman OTHMAN**, at CHARMO University, he suggested me to start **Msc** degree, thanks a lot to **Dr. Hero M. Ismael** at SALAHADDIN University, she was a good teacher and I will never forget her help. And thanks to my best friend **Mr. Hüsamettin AYGÜN**.

I wish to thank Mr**. Mahir BİNİCİ** and **Assistant Prof. Dr. Selahattin TEKEŞ** for all the possible help during the practical work. Thanks for **Prof. Dr. Ali SATAR**, he helped me when I had registration problems.

I would like to thank my friends **Mr. Sura A. Mahmood, Mr. Bassam A. Jameel, Mr. Barham J. Fatah, Mr. Peshawa Muhammad, Mr. Soran O. Khalifa, Mr. Bestoon A. Shexani, Mr. Aso Asad, Mr. Sarwar M. Rashid,** and **Mr. Farhad O. Mhamadameen** for their help throughout my working period. Thanks to everybody in the lab for their any day support and conversation.

Aram Jalil TAWFIQ

CONTENTS

		Page no.
ACKNOWLEDGMENT		**I**
CONTENTS		**II**
ABSTRACT		**IV**
ÖZET		**V**
LIST OF TABLES		**VI**
LIST OF FIGURES		**VII**
LIST OF ABBREVIATION		**VIII**
1.	**INTRODUCTION**	**1**
1.1.	Discovery of Amylase and the Development of Enzymology	1
1.2.	Sources of α- Amylase	2
1.3.	Cloning of the α-Amylase Gene	4
1.4.	Industrial Application of α-Amylase	7
1.4.1.	Detergent Industry	7
1.4.2.	Textile Industry	8
1.4.3.	In Bread Making & anti-staling	8
1.4.4.	Medical	8
1.4.5.	Production of Sweeteners from Starch	9
1.4.6.	Paper Industry	9
1.4.7.	Alcohol Industry	9
1.4.8	Feed Industry	9
1.5.	α- Amylase	9
2.	**PREVIOUS STUDY**	13
3.	**MATERIAL AND METHODS**	21
3.1.	MATERIALS	21
3.1.1.	Used of Lab Instruments	21
3.1.2.	Media Preparation	21

3.1.3 LB (Luria Bertani).. 22

3.1.4. LA (Luria Bertani Agar)... 22

3.1.5. LB Ampicillin 22

3.1.6. LB plates with Ampicillin/IPTG/X-Gal 23

3.1.7. SOC (Super Optimal Catabolite Repression) Media 23

3.1.8. Buffers and Solutions.. 23

3.1.9. Primer, Vectors and Bacterial Strains 25

3.1.10. DNA Marker 26

3.2. METHODS 27

3.2.1. Extraction of Genomic DNA from Bacteria 27

3.2.2. Genomic DNA Concentration Measurement... 27

3.2.3. PCR (Polymerase Chain Reaction) 28

3.2.4. Agarose Gel Electrophoresis 30

3.2.5. Gel Purification Steps: We Used Quick Gel Extraction Kit. 31

3.2.6. DNA Sequence Analyses.. 32

3.2.7. Cloning of the PCR product into pGEM®-T Easy Vectors......................... 33

3.2.8. DNA Ligation... 33

3.2.9. Transformation 34

3.2.10. Plasmid Isolation... 36

3.2.11. Screening of Clones by PCR, Restriction Enzyme Digestion and Sequenc Analysis 37

4. **RESULT** 39

5. **DISCUSSION** 41

6. **REFERENCES** 43

ABSTRACT

CLONING and CHARACTERIZATION OF α-AMYLASE

from *Bacillus circulans* ATCC 61

MASTER'S THESIS IN MOLECULAR BIOLOGY

Aram Jalil TAWFIQ

DEPARTMENT OF BIOLOGY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

UNIVERSITY OF DICLE

2016

Amylases are one of the most important enzymes and have a great significance in the present industry of biotechnology. Enzymes obtained from microorganisms are widely used in the industrial field. In the present work, *Bacillus circulans* ATCC 61 for α-amylase production has been used. Because well known as a good producer of α-amylase and its genome structures has been known, it was used for cloning for this purpose, the genomic DNA of *Bacillus circulans* ATCC 61 was isolated and the gene encoding α-amylase enzyme was amplified with specific primer by PCR consist of 1400 bp long. The specific primer was designed while detecting the gene belonging to *Bacillus* α-amylase from gene library. The gel-purified PCR product was inserted into the pGEM®-T Easy vector 3015 bp long, which linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of A-tailing on the PCR products. *Taq* DNA polymerase independently generates single deoxyadenosine at both ends of PCR product. Bioinformatics analysis of the cloned gene showed that there is 100% similarity between this gene and amylase gene of *Bacillus* sp. These results display that the target gene was cloned successfully. Finally for the gene expression, the vector was transferred to JM109 high efficiency *E. coli* competent cells. This study is the first record in term of the cloning α- amylase enzyme from *Bacillus circulans* ATCC 61 in the literature.

Keywords: Cloning, Characterization, *Bacillus circulans* ATCC 61.

ÖZET

Bacillus circulans ATCC 61'den α-AMİLAZIN KLONLANMASI ve KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Aram Jalil TAWFIQ

DİCLE ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

BİYOLOJİ ANABİLİM DALI

2016

En önemli enzim grubunu oluşturan amilazlar, biyoteknolojide de büyük önem kazanmaktadır. Mikroorganizmalardan elde edilen enzimler endüstriyel alanlarda yaygın bir şekilde kullanılır. Mevcut çalışmada *Bacillus circulans* ATCC 61 α-amilaz üretimi için kullanılmıştır. Bakterinin α-amilaz üretmesi ve genom yapısı iyi bilindiğinden klonlama işlemi için kullanıldı. *Bacillus circulans* ATCC 61'in genomik DNA'sı izole edildi ve α-amilaz enzimini kodlayan amyE geni 1400 bç uzunluğundaki spesifik primerler kullanılarak PCR yöntemi ile çoğaltıldı. *Bacillus* α-amilaz'ına ait genler gen kütüphanesinde tarandıktan sonra uygun primer tasarlandı. PCR protokolünden sonra jelden saflaştırılan gen ürünleri 3015 bç uzunluğunda ve her iki 3' ucunda terminal timidin bulunan lineer pGEM®-T Easy vektörüne aktarıldı. İnsersiyon bölgesinde bulunan tek zincirli T kuyruklar, A-kuyruğuna sahip PCR ürünlerinin etkin bir şekilde ligasyonunu sağlar. *Taq* DNA polimeraz, PCR ürünlerinin sonuna bağımsız bir şekilde tek zincirli deoksiadenozinler sentezler. Klonlanan gene ait bioinformatik analizler, bu genin *Bacillus sp.* α-amilaz geni ile %100 oranında bir benzerliğe sahip olduğunu göstermiştir. Son olarak gen eksperesyonu için vektör, yüksek etkiye sahip JM109 *E. coli* competent hücrelerine transfer edildi. Bu çalışma *Bacillus circulans* ATCC 61'den α- amilaz enziminin klonlanması bakımından literatürdeki ilk kayıttır.

Anahtar Kelimeler: Klonlama, Karakterizasyon, *Bacillus circulans* ATCC 61.

LIST OF TABLES

Table No.	Description	Page no.
Table 1.1.	Properties of some amylases from different sources ……………..…….…….	3
Table 1.2.	Cloning α-amylase genes from different sources. ……………………….…...	5
Table 3.1.	LB Media (pH 7.5) ………………………………………………………..	22
Table 3.2.	LA Media (pH 7.5) ……………………………………..…………………	22
Table 3.3.	LB Ampicillin (pH 7.5) ………………………………….......................	22
Table 3.4.	SOC Media ……………………………………..………………………	23
Table 3.5.	Primer list of used ……………………………………………………………	25
Table 3.6.	Vectors and Bacterial Strains ……………………………….....................	25
Table 3.7.	Standard PCR reaction ……………………………………………………	29
Table 3.8.	PCR program ……………………………………….……………………..	29
Table 3.9.	Ligation reactions set up ……………………….…………………….	34

LIST OF FIGURES

Figure No.	Description	Page no.
Figure 1.1.	Diagram shows the steps involved in molecular cloning ………………….	6
Figure 1.2.	Catalytic mechanism of retaining glycosyl hydrolases. (I) Protonation of the glycosidic oxygen and attack on the glucose C1 by D231. Departure of the reducing end of the substrate. (II) Activation of a water molecule, cleavage of C1-D231 covalent bonds. (III) Regeneration of the initial protonation states………………………………………………………………………..	10
Figure 1.3.	Attack sites and breakdown products for various starch degrading enzyme…	11
Figure 3.1.	The promoter and multiple cloning sequence of the pGEM®-T Easy Vector..	25
Figure 3.2	Sketch showing the pGEM®-T Easy Vector Sequences, Multi-Cloning Sites and Circle Maps…………………………………………………….	26
Figure 3.3.	Sketch of the (Lambda DNA/EcoRI+HindIII marker, 3) …………….……..	26
Figure 3.4.	Showed genomic DNA concentration measurement …………………….....	28
Figure 3.5.	Blast analysis results, sequence displays that similarity of the amylase gene as we obtained..………………………………..	32
Figure 4.1.	**a.** Show PCR product size. 1 well Marker (Lambda DNA/EcoRI+HindIII ladder), 3 well show PCR product. **b.** Show total genomic DNA of Bacillus circulans ATCC 61………………...	39
Figure 4.2.	Positive clone after digestion with EcoRI ………………………………...	40

LISTOFABBREVIATION

%	: Percentage
A, G, C, T	: Adenine, Guanine, Cytosine, Thymine
ATP	: Adenosine triphosphate
Bp	: Base pair
Da	: Dalton
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleic triphosphate
DP	: Degree of polymerization
E. coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
IPTG	: Isopropyl-α-D- thiogalactopyranosi
Kb	: Kilobase
kDa	: Kilodalton
L	: Litre
LB	: Luria Bertani
M	: Molar
Mg	: Milligram
Min	: Minutes
Ml	: Millilitre

^oC	: Centigrade Celsius
OD	: Optical Density
PAGE	: Polyacrylamide gel electrophoresis
PCR	: Polymerase Chain Reaction
RNA	: Ribonucleic acid
RPM	: Revolutions per minute
SDS	: Sodium Dodecyl Sulfate
SOC	: Super Optimal Catabolite Repression
SSF	: Solid State Fermentation
TBE	: Tris Borate EDTA
TE	: Tris-EDTA
Tm	: Melting Tempreture
U	: Unite
UV	: Ultraviolet
X-Gal	: 5-Bromo-4-Chloro-3-Indolyl-D-Galactoside
A	: Alpha
B	: Beta
µL	: Microlitre

1. INTRODUCTION

1.1. Discovery of Amylase and the Development of Enzymology

It is difficult to determine the exact discovery of enzymes, in the earliest times of civilization, human's use of enzymes for the basic needs of life (Salis et al., 2007). In 1783 Spallanzani noted the gastric juice of hawks can liquefy meat (Sumner and Somers, 2014). In the following years, numerous similar observations were made. For example, in l814 Russian chemist Kirchhoff observed that a "glutinous" (i.e., proteinaceous) wheat component was capable of converting starch to sugar (Segel, 1975). Thus, Kirchhoff laid the foundation for the discovery of Amylase.

Some of the earliest studies were performed in 1830 Robiquet and Boutron and also Chaland discovered the hydrolysis of amygdalin by bitter almonds (Ehrlich and Newman, 2008). Swedish chemist Jons Jakob Berzelius, who created the term "catalysis" when he noticed that certain chemicals help speed up the rate of a reaction (Singh, 2007). Leuchs, in 1831, described the diastatic action of salivary ptyalin (Segel, 1975). The term 'enzyme' first used by Kühne, is derived from the Greek term meaning 'in yeast'. Generally the first discovery of an enzyme is credited by scientists Anselme Payen and Jean-François Persoz, who, in 1833, treated an aqueous extract of malt with ethanol and precipitated a heat-labile substance which promoted the starch hydrolysis. They called their fraction "diastase," (Whitehurst and Van Oort, 2010). Diastase, is the Greek word which means separation, it can transform the starch into maltose, after that, it converts into glucose. http://worldofenzymes.info/enzymes-introduction/diastase/. Today, scientists recognize that the diastase of Payen and Persoz was an impure preparation of amylase.

In 1894 amylase in the fungal source is the first enzyme produced industrially, which was used as a pharmaceutical drug for the treatment of digestive disorders (Pandey et al., 2000). In 1917 Biodin and Effront were the first to use *B. mesentericus* and *B. subtilis* for the production of α-amylase (SINGH et al., 2011). Hydrolysis product of α-amylase in the alpha configuration, in 1925 α-amylases were named by Kuhn. Also Ohlsson in 1930 discovered another amylase, He named it β-amylase (Kumari et al., 2012). The three dimensional crystal structures of each form were determined in the 1990's (Qian et al. 1995).

In 1950 Myrb‘ack and Neiimuler proposed additional classification for the amylases. Namely. a) Exoamylases and b) Endoamylases. Scarifying amylase or β-amylase was included into the former and starch liquefying or α-amylase. Into the latter amylases, Their criterion for the amylase classification was based on the mode of action (Pergamon, 1988). A large-scale starch processing industry has developed in the last century. In the past decades, we have seen a shift from the acid hydrolysis of starch to the use of starch-converting enzymes in the production of maltodextrin, modified starches, or glucose and fructose syrups (Van Der Maarel et al., 2002).

1.2. Sources of α- Amylase

Amylases can be derived from different sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. The major advantage of using microorganisms for the production of amylases is cost-effectiveness, efficient production, consistency, and microbes are easy to manipulate to obtain enzymes of desired characteristics, among bacterial amylases, thermophilic, thermostable, and acidic enzymes isolated from *Bacillus* strains have attracted interest because of their widespread usage on starch processing, It is estimated that enzymes compromised about 50% of the total global enzyme market (Afzal-Javan, 2013; Ozturk et al., 2013; Lonsane and Ramesh, 1990). As well as, Amylases derived from several sources will be subject to genetic variation in relations of structure and associated variation with respect to activity (Planchot et al., 1995).

Bacteria belonging to the genus *Bacillus* produce large amounts of extracellular enzymes such as amylase and protease and, therefore, they have been one of the microorganisms used extensively in the fermentation industry. Recently, a great number of papers have been published on the cloning and expression of the amylases from the genus Bacillus, and some of their nucleotide sequences have been determined (Ohdan et al., 1999). Various physiological and biochemical characteristics of the different sectors with α-amylase enzyme with the demands of the new features requires the search and development. Enzyme engineering is known as a promising technique for achieving this goal. Engineering of enzyme suitable genes in the desired amount of features that a wide pH profile, high thermo stability, Ca^{2+} independently raw starch degrading ability, high starch concentration, activity, protease resistance, catabolite insensitive to

repression, is integrated with a high production capacity (Sivaramakrishn et al., 2006). See the table 1.1 which shows properties of some amylases from different sources. To improve α-amylase properties, both genetic manipulation and media optimization have been used, which is suitable for specific industrial application or yielding a large amount of enzymes in the culture. However, the discovery of new bacterial strains that produce α- amylases with special properties remains as a very important way of advancing the field (Hmidet et al., 2010).

Table 1.1. Shows properties of some amylases from different sources.

Source	pH optimal/ stability	Temperature optimal/ stability	Mw (kDa)	Inhibitors	Stabilizers	Reference
***Geobjnhacillusk austrophilus* PW11**	7	90°C/40°C - 100°C	-	Hg^{2+} Cd^{2+}	Mn^{2+}, Co^{2+} and Fe^{2+} and EDTA	Sharma et al., 2015.
***Zunongwangiapr ofunda* MCCC 1A01486**	7/8–9	35° C/0 °C-60 °C	66	Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{3+} , SDS and EDTA	Sr^{2+}, Fe^{3+}, Mg^{2+}, Ba^{2+}, NH^{4+}, K^{+} NaCl and $CaCl_2$	Qin et al., 2014
***Bacillus* sp. Ferdowsicous**	4.5/3.5 – 7	70 °C /≤75 °C for 45 min.	53	Hg^{2+}, Zn^{2+} and EDTA	Ba^{2+},Fe^{2+}. Na^{+}, Mg^{2+}, K^{+}, Ca^{2+}, PMSF, Triton X-100 and b-mercaptoethanol	Asoodeh et al., 2010
***Bacillus* sp.YX-1**	5.0/ 4.5-11.0	45 °C/ 30–70 °C	56	-	-	Liu and Xu, 2008
***Bacillus* sp. K-12**	6-8 /4.5-10.5	42 °C /20-55 °C	-	$MnSO^4$, $ZnSO^4$ and EDTA	starch	Kiran et al. 2005
B. subtilis	6.5/≤7.	50 °C/≤ 50 °C	48	Hg^{2+} ,Fe^{3+}, Al^{3+}	Mn^{2+}, Co_2	Marco et al. 1996
***Bacillus* sp. IMD 434**	6/4-9	65 °C/40 °C (1 h)	69.2	*N*-Bromosuccinimide, *p*-hydroxymercuribenzoicacid	Cysteine, DTT	Hamilton et al. 1999
***Bacillus* sp. US 100**	5.6/4.5- 8	82 C/90-95 C	-	-	Starch, Ca^{2+}	Ali et al. 1999
Eiseniafoetida	5.5 /7–9	50 °C /50–60 °C.	60	Cu^{2+}, Fe^{2+}, and Hg^{2}	Ca^{2+}, Mg^{2+} and Mn^{2}	Ueda et al. 2008.
Aspergillusniveus	5–5.5/2 h 4–9.5	65°C/4 h at 60°C	77	Ag^{+} and Fe^{+2}	NaCl	Da Silva et a. 2009
***Thermococcuspr ofundus* DT5432**	5.5-6/5.9-9.8	80 °C/80 °C (3 h), 90 °C (15 min)	42	Iodoacetic acid, N-bromosuccinic acid, SDS, guanidine hydrochloride	Ca^{2+}	Chung et al. 1995

Extensive potentials of amylase to be used in a broad range of industries have placed greater stress on researchers to search for more efficient amylase production ,the most notably species uses are , *B. amyloliquefaciens, B. subtilis, B. stearothermophilus, B. coagulans,* and *B. licheniformis* (Dash et al., 2015). Also fungal sources are isolating, mostly in *Aspergillus* sp. and to only one species of *Penicillium, P. brunneum*. (Afzal-Javan et al., 2013). A great deal of work has been done on the α-amylase genes, cloning in different microbes, mainly in *Escherichia coli* or *Saccharomyces cerevisiae* (Mobini-Dehkordi et al., 2011).

1.3. Cloning of the α-Amylase Gene

DNA cloning is the Technique concerned with opening incredible chances to study or identify the genes involved in all known biological process (Herfindal and Gourley, 2000). Recombinant DNA technologies involve isolating a target gene, connecting it with a carrier, transforming it into another organism and use that organism to propagate the gene product. This process is also called 'cloning' (Reed, 2012). Gene cloning, studies at the molecular level of proteins, to produce a wide range of protein and protein engineering. The α-amylase gene cloning, characteristics, high level production are made for enzyme engineering and expression (Pandey et al., 2000). Expression of a cloned wild-type enzyme in a safe and easily adaptable host by a proper vector has been widely exploited for many enzyme types, but there are some national reports indicating problems encountered during the cloning. Several restrictions-mediated cloning methods have been carried out successfully for the cloning of PCR products, but they all suffer from extensive enzymatic treatment of inserts and vectors (Ozturk et al., 2013).

Restriction enzymes are necessary tools in Gene cloning because they can recognize a specific DNA sequence and cleave the DNA in specific phosphodiester bonds on both strands (Trun and Trempy, 2003). This cleavage can produce 'sticky ends' or 'blunt ends' depending upon the specific restriction enzyme. There are three main classes of restriction enzymes, indicated I, II and III. Type II is the most used type in the gene technology, it is specific and cleaves the DNA within the recognition sequence itself, Type I and III cleave at the random sites the recognition sequence is unspecific. (Nelson and Cox, 2002). The plasmid vector and the DNA fragments to be

cloned are cut by the same restriction enzymes, the DNA fragments are inserted into the plasmid vector to produce a circular recombinant DNA molecule. DNA ligase catalyzing the formation phosphodiester bond between the 3'hydroxyl of one nucleotide and 5'phosphate of another (Sambrook and Russell, 2001). In order to clone a gene, the vector transports the gene into a host cell. There are many different kinds of vectors and most of them are isolated from larger plasmids that naturally occurring in bacterial cells (Alberts et al., 2002). Generally, a cloning vector contains three elements: a cloning site allows a gene to be inserted into the vector or removed from it, antibiotic resistance gene and an origin of replication to allow the plasmid to be replicated in the host cell (Sambrook and Russell, 2001). Gene cloning and PCR play important role in biology because both techniques can provide a pure sample of an individual gene, separated from all the other genes in the cell. (Brown, 2010). The choice of a host–vector system as a set should be the consideration for the purpose of each target expression (Reed ,2012). Fungal and bacterial α-amylase gene from various sources, cloned into a suitable host organism using appropriate vectors (Sivaramakrishn et al., 2006). See Table 2 which shows some of the cloned α-amylase. To be able to take up foreign DNA, the bacteria cells need to be made competent, this is often achieved by treating them with divalent captions under cold conditions.

Table 1.2. Cloning α-amylase genes from different sources (Sivaramakrishn et al 2006).

Gene source	Recombinant host	Vector
Aspergillus kawachii IFO 4308	*S. cerevisiae*	pYcDE1
Bacillus amyloliquefaciens	*E. coli*	pETAM (derived from pKK233-2 & pET21d)
Halothermothrix orenii	*E. coli*	pBluescript SK+
Alteromonas haloplanktis	*E. coli*	pUC12
Lipomyces starkeyi	*E. coli*	pGEM-T
Lipomyces kononenkoae	*S. cerevisiae*	YIp5
Thermococcus hydrothermalis	*E. coli*	pBluescript II KS-

For transformation of *E. coli* with plasmid the DNA needs assistance to move across cell membranes and to reach the site where it can be expressed and replicated. The plasmids can be introduced by electroporation or by chemical (Sambrook and Russell, 2001). The recombinant DNA is capable of independent replication in a host cell. The host cells are transformed by recombinant DNA which is grown in culture and as the bacterium grows, when the host cell divides, copies of the recombinant DNA introduced are passed on to the progeny cells and further vector replication takes place. After a large number of cell divisions, a colony, or identical clone, of host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule, the gene carried by the recombinant molecule is now said to be cloned. (Brown, 2010). See figure 1.1.Show molecular gene cloning. The major advantage of gene cloning is to produce a relatively large quantity of proteins. To manipulate biological pathways and modify the gene by manipulating the protein structure and function (Wong, 2006).

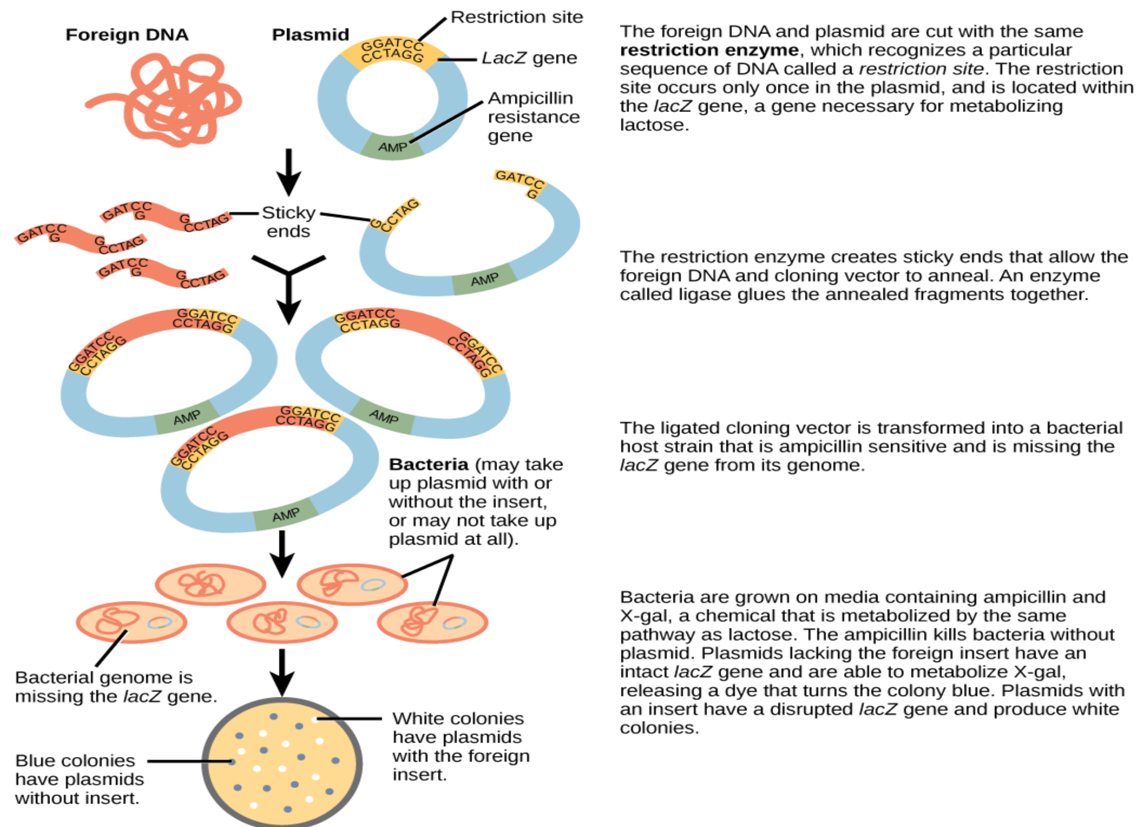

Figure 1.1. This diagram shows the steps involved in molecular cloning.

(Source:https://www.boundless.com/biology/textbooks/boundless-biology textbook/ Biotechnology-and-genomics-17/biotechnology-119/molecular-and-cellular-cloning-477-11698/).

1.4. Industrial Application of α-Amylase

Starch is the second most important source of carbon and energy, therefore, a worldwide interest has been engrossed to produce valuable products by using this economic carbon source in food processing industry (Roy et al., 2013). For commercial purposes, α-amylases are mainly derived from the genus *Bacillus* (Hmidet et al., 2010). The starch industrial processing is normally started with α-amylases (α-1,4-glucanohydrolase). Most of the starch-converting enzymes belong to the α-amylase family or family 13 glycosyl hydrolases (GH) (Miguel et al., 2013). Industrial enzyme can be used in synergistic action to increase the yield of the desired product. This process is more economical for multi-step industrial processes to reduce cost and time (Kubrak, 2010). They have diverse applications in a variety of industries such as food, fermentation, textile, paper, detergent and sugar industries, environmental pollutant remediation, conversion of starch to desired substrates by many microorganisms, infiltration of waste contains starch and production, biochemical material with the help of starch substrate, the spectrum of amylase application has expanded into many other fields, such as clinical studies, medicine and analytical chemistry (Pandey et al., 2000).

1.4.1. **Detergent Industry**

Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (Souza, 2010). Amylases can provide an environmentally friendly solution, substituting other chemicals that generate streams with high pollutant loads on wastewaters and making easier to remove stain and starchy soils (Martínez-Gallegos et al., 2014). Amylase which shows optimum activity in alkaline pH is used in laundry detergent formulations (Roy et al., 2013). In addition, enzyme stabilizes the bleaching agent and preserves effectiveness of the bleach in laundry detergent bar composition (Tiwari et al.,). However, most α-amylases are unstable in the presence of chelating reagents because their activity and stability are regulated by calcium ion binding to a common Ca^{2+}binding site in the proximity of the active site (Tamamura et al., 2014). But recently, found a novel thermostablealkalophilic α-amylase (AmyL), in *Bacillus* sp.AAH-31 with high stability in the presence of chelating regents and detergents (Xie et al., 2014).

1.4.2. **Textile Industry**

In the textile industries are using amylases to hydrolyze and solubilize the starch, which then wash out of the cloth to provide rigidity to prevent breaking of the warp thread during the weaving process. Fabrics are sized with starch. Starch is a very attractive size, because it is cheap, easily available, and it can be removed quite easily. Amylase from *Bacillus* strain was employed in textile industries for quite a long time. α-amylase is used as desizing agent for removing starch from the gray cloth before its further processing in whitening and colouring (Tiwari et al.; Souza, 2010).

1.4.3. **In Bread Making & anti-staling**

The α- and β-amylases have different but complementary functions during the bread making process. During the dough stage α-amylases break down damaged starch particles into low molecular weight dextrins, while endogenous β-amylase converts these oligosaccharides into maltose, microorganisms used this sugar in fermentation processes. The addition of α-amylase to the dough, it generates additional sugar in the dough, which improves the taste, crust color and toasting qualities of the bread. On the other hand, it increases the rate of fermentation and reduces the viscosity of dough, which results in improvements in the volume and texture of the product. In addition, maltogenic α-amylase is used in the baking industry as an anti-staling agent because of its ability to reduce the retro gradation of amylopectin and they improve the softness retention of baked goods, increasing the shelf life of these products (Miguel et al., 2013; Miao et al., 2014; Mobini-Dehkordi and Javan, 2012). The positive effects increase with the amylase dose rate, there is an optimum dose level. Since the stickiness of the dough also increases, leading, unworkable dough at higher amylase dose levels (Whitehurst et al., 2010).

1.4.4. **Medical**

α-amylase is used as a target for the design of drugs to be used in certain diseases such as diabetes mellitus, hyperlipidaemia, tooth decay (Mariano da Silva., 2014). Determination of α-amylase activity in human serum and urine is widely used in clinical laboratories, for the diagnosis of pancreatic diseases (Lan Tran et al., 2014).

1.4.5. **Production of Sweeteners from Starch**

The major utilization of α-amylase is in the production of dextrose/glucose, maltose and high fructose syrups. These sweeteners from corn Starch are increasingly replacing the traditional cane sugar all over the world. (Ahmad et al., 2015).

1.4.6. **Paper Industry**

α-amylase is also very useful in the pulp and paper industry as it reduces the viscosity of starch that is used for sizing and coating the paper instead of expensive chemically modified starches, The coating treatment enhances the stiffness and strength in the surface of paper, which improves the quality and erasebility (Souza, 2010; Tiwari et al.; Mobini-Dehkordi et al., 2012).

1.4.7. **Alcohol Industry**

Fermentable sugars are produced with the help of α-amylase as a result of conversion of the starch. Starches such as cereals (for example, potatoes) the majority of biological and chemical reactions for the production of ethyl alcohol is necessary for the production of large chemical having an important role (Hussain et al., 2013).

1.4.8. **Feed Industry**

Feed industry in the use of α-amylase is stated with increased body weight gain and feed conversion ratio. The digestibility of carbohydrates is also increased; starch is hydrolysed to glucose and fructose polymers (Sidkey et al., 2011).

1.5. α- Amylase

α-Amylase (EC 3.2.1.1) are endoenzymes which are able to cleavage α-1,4-glycosidic bonds in the inner part (endo-) of the amylose or amylopectin chain (Miguel et al., 2013). The α-amylases are calcium metalloenzymes, which require calcium ions for their activity (SINGH et al., 2011). The end products of α-amylase action is an α-configuration and varying lengths, large amounts of maltose, maltotriose, glucose, and oligosaccharides (α-limit dextrins) with the α-1,6 linkage constitute the hydrolysis products (Schaechter, 2009).

Catalytic mechanism consists of three steps showing in (Fig.1). First step is the protonation of the proton donor to the glycosyl oxygen Glu261. This is followed by a nucleophilic direct attack on the carbon 1 of the sugar residue in the -1 subset by the catalytic nucleophile (Asp231). In the next step, the aglycon part of the substrate leaves, a water molecule is activated, presumably by the now deprotonated Glu261. Water molecule hydrolyses the covalent bond between the oxygen of the nucleophile and the C1 of the sugar residue, thus completing the catalytic cycle. (Nielsen and Borchert, 2000).

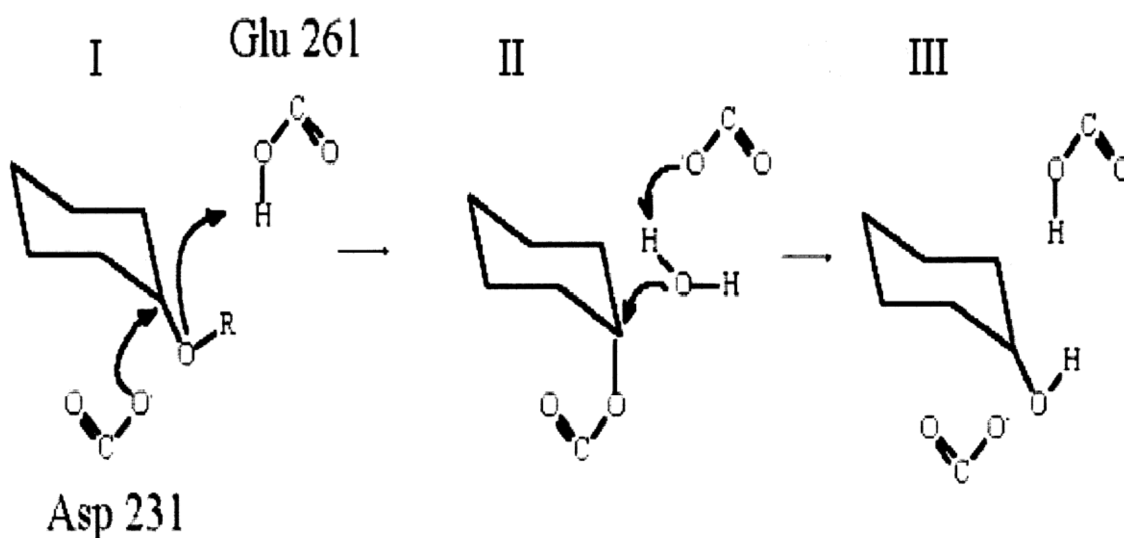

Figure1.2. Catalytic mechanism of retaining glycosyl hydrolases. (I) Protonation of the glycosidic oxygen and attack on the glucose C1 by D231. Departure of the reducing end of the substrate. (II) Activation of a water molecule, cleavage of C1-D231 covalent bonds. (III) Regeneration of the initial protonation states.

This enzyme of this group has been purified and characterized from a wide range of organisms for example, in human physiology; both the saliva and pancreatic amylases are α-Amylases. Also found in plants (adequately), fungi (*ascomycetes* and *B asidiomycetes*) and bacteria *Bacillus* (SINGH et al., 2011). α-amylases are classified into glycoside hydrolase families (GHs) 13, 57, and 119, with most of these enzymes found in the largest family GH 13, containing more than 30 kinds of glycoside hydrolyses and glycosyltransferases, such as α-amylase, α-glucosidase (EC 3.2.1.20), cyclodextringlucanotransferase (EC2.4.1.19), and branching enzyme (EC2.4.1.18) (Tamamura et al., 2014). On the other hand, debranching enzymes, such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), grouped as well in the GH13 family -

hydrolyse α-(1,6) -bonds removing the side-chains from amylopectin (Miguel et al., 2013). Most of them have maximum activity at 30–37 ^{o}C at a neutral pH; also, some exhibit maximum activity at pHs as low as 3 or as high as 10 and at temperatures more than 100 ^{o}C. Enzymes from *Aspergillus niger, A. oryzae, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus circulans, Bacillus subtilis,* and *Bacillus stearothermophilus* are of special importance from the standpoint of both basic research and industrial application. (Schaechter, 2009).

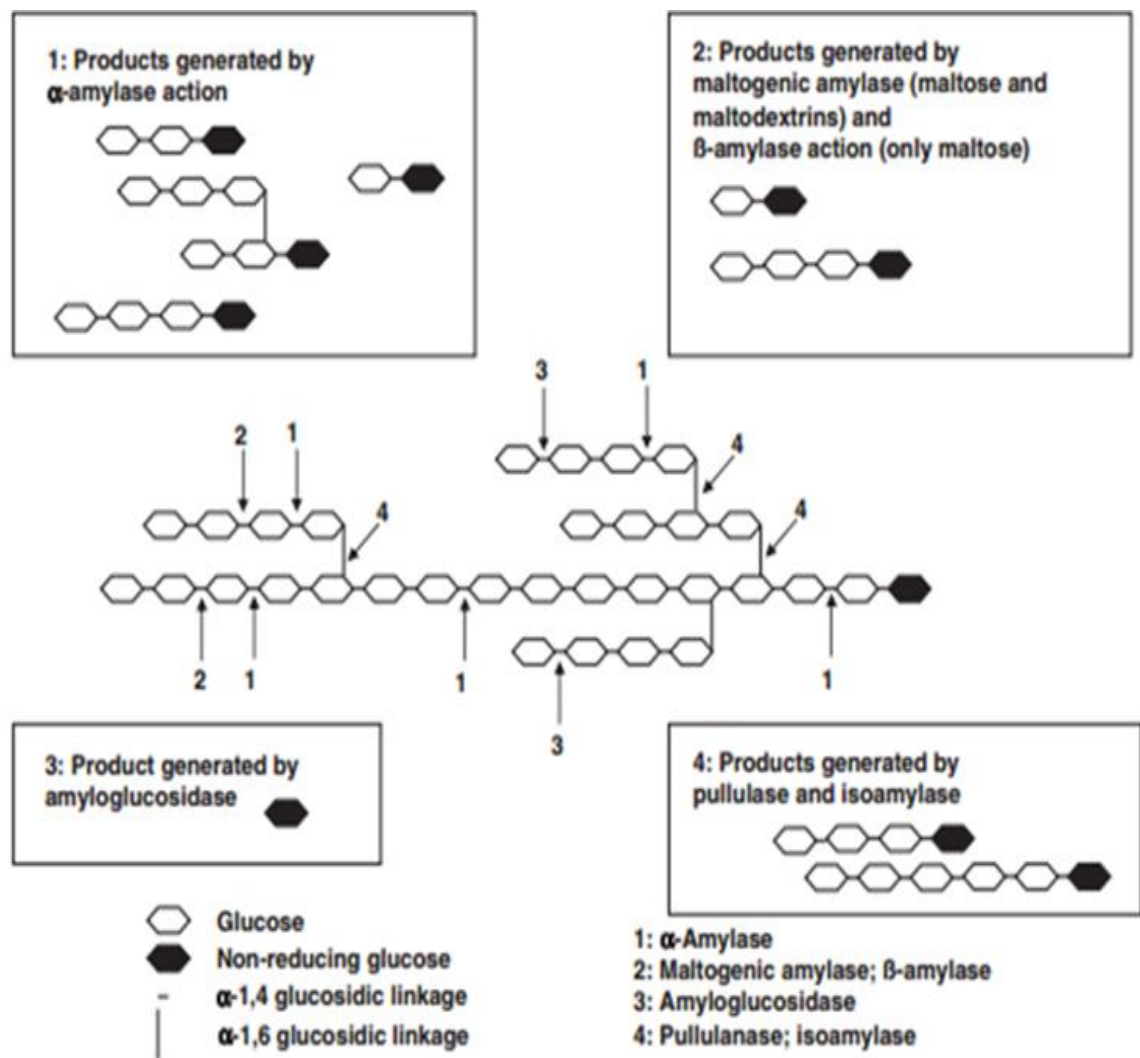

Figure 1.3. Attack sites and breakdown products for various starch degrading enzyme (Whitehurst and Van Oort., 2010).

The aim of the thesis: α-amylase in recent years is widely used in large scale production of microorganisms. Although low yield production of natural producer microorganism producing this enzyme uses recombinant DNA techniques that they are possible. Therefore, in this paper using *Bacillus circulans* ATCC 61, it can produce an α-amylase, but the amount of the enzyme is low. We increased amount of this enzyme by cloning and expressing its gene, will permit study of these interesting characteristics and properties of amylase which is produced by transformed bacterium.

2. PREVIOUS STUDY

Okamoto et al. (2015), Production of itaconic acid by *Escherichia coli* expressing recombinant α-amylase, soluble starch was used as sole carbon source. Constructed plasmid pGV3 for express α-amylase in *E. coli*, and its derivatives pGV3-BAA and pGV3-SBA harbouring the respective amyA genes from, *Bacillus amyloliquefaciens* NBRC 15535^T and *Streptococcus bovis* NRIC 1535. The recombinant α-amylase were observed, showing greater activity from *S. Bovis* NRIC 1535 at 28 ^oC, That is the optimal temperature for the production of itaconic acid, but α-amylase from *B. amyloliquefaciens* displayed no observable activity at this temperature. Also, *B. amyloliquefaciens* produces a thermostable α-amylase with the temperature optimum in the range of 50^oC – 70 ^oC. As a result, it has been concluded that SBA is more suitable for hydrolysis of starch to produce itaconic acid by using *E. coli*. Under pH-stat conditions after 69 h cultivation of *E. Coli* cells expressing SBA produced 0.15 g/L itaconic acid, used 1% starch. Actually, *E. coli* cells expressed SBA has similar growth rates when grown in the presence of 1% glucose or starch, as a result of the expression of an active α-amylase that enabled utilization of starch to produce itaconic acid in *E. coli*.

Celińska et al. (2015), The main objective of this present work was cloned α-amylase (Amy1) gene from rice weevil (*Sitophilus oryzae*), the major rice pest and expressed in yeast species *Yarrowia lipolytica* Po1g strain. The recombinant α-amylase activity in the culture medium was observed after only 29 h of culturing in 5-L bioreactors., The production of the recombinant α-amylase secreted into the culture medium reached the maximum value of (81 U/L) activity units per litter. Through simple purification procedure of ammonium sulphate precipitation and affinity chromatography, it was possible to purify the enzyme to apparent homogeneity (25-fold purification factor, at 5 % yield). The optimal conditions for the α-amylase activity were pH 5.0 and a temperature of 40 °C. The α-amylase studied here did not show any obligate requirement for $Ca2^+$ ions.

Adrio and Demain (2014), Microbial enzymes are applied in various fields, including pulp and paper, leather, textiles and detergents, pharmaceuticals, chemical, food and beverages, animal feed, biofuels, and personal care, among other things.

Today improved Novozymes is the largest player in the industry. Metagenomics and genomics, are being used to discover new microbial enzymes whose catalytic properties can be improved by different strategies depend on rational, semi-rational and random directed evolution. Most recombinant industrial enzyme forms are produced in bacteria and fungi.

Fincan et al. (2014), This study reports the optimum conditions for the production, purification and characterization of extracellular thermostable α-amylase from the newly isolated strain *Anoxybacillus flavithermus*. The gram-positive, spore forming, motile, moderately thermophilic bacteria were found to be a strain of *A. Flavithermus* analysed by 16S rRNA comparison. The molecular weight of α-amylase was 60 kDa, as estimated by (SDS-PAGE).

Xuefeng et al. (2014), Extracted Enteromorpha polysaccharides (EP) from green algae have displayed a wide variety of biological activities. On the other hand, their high molecular weight leads to a high viscosity and low solubility. To solve this problem, screening bacteria from the surface of Enteromorpha, and an *Alteromonas macleodii* strain B7 possessing (EP) degradation activity in culture media. The amylase gene (amySTU) was cloned from *A. macleodii* B7 into *Escherichia coli*, resulting in the recombinant enzyme in high level expression of a cold-adapted α- amylase that can degrade EP; however, detected optimal enzyme activity at 40°C. This enzyme is halotolerant and cold-adapted. Furthermore, its high activity and stability in the presence of organic solvents, For that reason, the *A. macleodii* strain B7 and its α-amylase can be useful in practical applications in biotechnological processes and in starch processing.

Kumagai et al. (2013), In the present study, they isolated two α-amylases (EC 3.2.1.1) isozymes HdAmy58 and HdAmy82 from the digestive fluid of *Haliotis discus hannai*, with estimated molecular masses 58 kDa and 82 kDa, respectively. Additionally, they cloned the cDNAs encoding each enzyme and studied the difference in the primary structures between the two enzymes. The amino-acid sequences of 511 and 694 residues for HdAmy58 and HdAmy82, respectively, Optimal temperatures for HdAmy58 and HdAmy82 at around 30 °C while their optimal pHs at 6.7 and 6.1, respectively. Both enzymes in the same way degraded starch, glycogen, as well as

maltooligosaccharides more than maltotriose producing maltose and maltotriose as the main degradation products. But, the activity toward maltotetraose was noticeably higher in HdAmy82 than HdAmy58. The putative catalytic domains of HdAmy58 located in the 17–511th amino-acid regions, while in HdAmy82 located at 19–500th, and they displayed approximately 50% amino-acid identity to each other. These sequences also showed 62–99% amino-acid identity to the catalytic domains of recognized α-amylases that belong to GH-family 13. The difference in the molecular masses between HdAmy58 and HdAmy82 was attributed to the extension of approximately 190 residues in the C-terminus of HdAmy82. This extended region presented 41–63% amino-acid similarity with the ancillary domains of several α-amylases previously described.

Kumar et al. (2013), In this study purification, characterization and optimizing the medium for production of thermostable α-amylase from *Bacillus laterosporus* was discussed. For optimization purposes, they used Box-Behnken design (BBD) of response surface methodology (RSM) to four medium components (starch, yeast extract, peptone and NaCl). Optimum values of starch, yeast extract, peptone and NaCl were predicted at 2.44%, 0.58%, 2.34% and 0.11%, respectively, with maximum enzyme activity of 4.838 U/ml. For Enzyme purification studies were used ammonium sulphate precipitation and size exclusion chromatography (SEC). Maximum purification was obtained by SEC step and achieved high purification fold of 4.71. The maximum enzyme activity was observed in optimal conditions of temperature 60 ^{o}C and pH 7. In addition, Presence of coliseum ions and EDTA does not effect on enzyme activity, where as reduced activity of the enzyme was observed in presence of Mg^{2+}, SDS and b-mercaptoethanol.

Gurumurthy et al. (2012), This study completed the molecular characterization of a novel hyperthermostable α-amylase for industrial application. This enzyme was produced by a bacterium *Geobacillus* sp. which was isolated from geothermal spring water. Identification by biochemical tests and 16S rRNA gene sequencing. The characteristics of this bacterium showed of thermotolerant alkali-resistant. A purified preparation of amylase was obtained using Sephadex G-150 gel filtration chromatography and a DEAE-cellulose column. This purified preparation enzyme is a novel α-amylase its optimum activity at a very high temperature of 90 °C and pH 8.0. However, it can stable up to 90°C only for 10 min. The effect of EDTA and Zn^{2+}on

enzyme activity was shown maximum inhibitory activity. While, Ca^{2+}, Fe^{2+}, $Mg^{2+}$and Cu^{2+} were did not effect on the activity of the purified enzyme.

Chai et al. (2012), In this study, they report the isolation of two α-amylase genes (ASKA and ADTA) from two *Anoxybacillus* strains SK3-4 and DT3-1. They were cloned and expressed in *Escherichia coli*. That is required for wide industrial applications. The genes consist of 1,518 bp long and encode 506 amino acids. Both sequences are 98% similar but are different from other known α-amylases. To purify both enzymes using an α-CD–Sepharose column. In the presence of calcium both enzymes were highly stable. While, in the absence of calcium, they remained stable at 60°C for at least 48 h and stable in the wide range of pH 6–10. Both of the *Anoxybacillus* α-amylases exhibited similar end product profiles, could produce high levels of maltose and possess atypical protein sequences compared with other α-amylases.

Wang et al. (2012), Biofuels, ethanol is a clean fuel, play an important role in successfully solving the problem of the approaching oil shortage, In this study, they tried to use signal sequences to help amylase secretion which is a major key enzyme necessary for starch hydrolysis, for this purpose they fused two secretion signal zmo130 and zmo331 native *Zymomonas mobilis* strain at the N terminal of α-amylase from *Bacillus subtilis* and transformed into 5 different strains of *Z. mobilis* separately. zmo130 was found to direct the extracellular secretion of significant levels of active α-amylase, while zmo331 could not. Fermentation experiments showed that the recombinant *Z. mobilis* CICC 10225(p130A) exhibited the highest level production of ethanol, but another recombinant *Z. mobilis* ATCC 31821(p130A) took the shortest fermentation time, it showed the second highest level of ethanol yield. The recombined strains in our study could be an important target for the following genetic engineering of previous amylase in order to hydrolyze starch completely.

Yamaguchi et al. (2011), Here, they report further purification and characterization of α-amylase (KVA) from halophilic bacterium, *Kocuria varians* and molecular cloning of the *kva* gene. They have noticed at least six different forms of α-amylase secreted by *K. varians* into the culture medium. They inferred amino acid sequence of *kva* gene and biochemical characterizations of purified KVA protein shown

that the KVA comprises pre-pro-type precursor form of α-amylase catalytic domain, followed by the tandem repeats, moreover, which display great similarity to each other and to the starch binding domain of other α-amylases. The noticed six forms were most likely derived from various processing of the protein product. Recombinant KVA protein was well expressed in *Escherichia coli* as a fusion protein and was purified after cleavage from the fusion partner by affinity chromatography. Under such conditions of high salt concentration, The greatly acidic amino acid composition of the KVA and the highly negative electrostatic repulsion surface map of the modeled structure powerfully submitted its halophilic nature. Actually, this halophilic KVA presented a novel salt and time dependent thermal reversibility of activity from heat denaturation. Also, proteinaceous α-amylase inhibitor from *Streptomyces nitrosporeus* was inhibited KVA activity, which had been associated to inhibit only animal α-amylases. In addition to KVA with putative starch binding domain regions was found to digest raw starch.

Gangadharan et al. (2010), In the present work they describe the amplification of the α-amylase gene of *Bacillus amyloliquefaciens* ATCC 23842, cloned and overexpressed in *Escherichia coli* BL21 cells. By using ion exchange and gel filtration chromatography, they purify the recombinant enzyme. The ability to digest raw starch of the purified enzyme was characterized by observing the hydrolysis and adsorption rate on a variety of raw starches. The digestion of raw starch was studied via scanning electron microscopy, which showed an effective rate of hydrolysis. However, its kinetic properties were studied.

Reyes-Sosa et al. (2010), Here, they describe the functional characterization of a novel α-amylase (amy1) from the mesophilic cyanobacterium *Nostoc* sp. PCC 7119is first demonstrated, to the best of our information, of a purified amylase from a cyanobacterial source. The amy1gene cloned, and then overexpressed in *Escherichia coli* cells. The recombinant protein is about 56.7-kDa monomer, which has been purified by affinity chromatography. The Amy1 protein breaks down mostly starch, it is also able to cleave glycogen and dextrin, and shows no activity against Xylan or pullulan. Therefore the enzyme cannot powerfully attack the maltodextrins with degrees of polymerization lower that of maltooctaose. Maltotriose, maltose, and maltotetraose are the main products of the enzymatic reaction with starch as substrate. This enzyme displays its substrate specificity, calcium-dependent, and maximum activity at pH

between 6.5 - 7.5, and 31°C. The primary sequence analysis, kinetic and physico-chemical characterization and compared with other known α-amylases, Make these features open the door to more studies on the physiological role and industrial applications of such as a previously unexplored group of cyanobacterial α- amylases.

Chakraborty et al. (2009), In the present investigation, they have reported novel α-amylase enzyme from marine *Streptomyces* sp. D1. 45 ^{o}C is the optimum temperature for enzyme production and activity was observed and enzyme retained approximately 50% of its activity at 85 ^{o}C. Enzyme retained good activity in presence of commercially available detergent and oxidizing agents. The partially purified enzyme exhibited specific activity of 113.64 U/mg protein that resembles to 2.8-fold purification and molecular mass of the enzyme was found to be 66 kDa. The reported enzyme may have tremendous application for detergent and pharmaceutical industry.

Tao et al. (2008), In here, they reported an extracellular α-amylase from a marine bacterium *Pseudoalteromonas* sp. MY-1. Cloning and expression of this gene (amyA) in *E. coli*, then characterization of the purified recombinant α-amylase was studied. It comprised an open-reading-frame about (2 kb) and encoded protein for 669 a.a with the molecular mass of approximately 73 kDa on SDS-PAGE. The complete amino acid sequence of amyA gene displayed (86% identity) to the α-amylase form *Pseudoalteromonas haloplanktis*. Maximum activity of the enzyme is at pH 7.0 and 40ºC. The enzyme hydrolyzed soluble starch and some malt oligosaccharides to numerous oligosaccharides; in addition, maltose was the common product from different substrates.

Konsula and Liakopoulou-Kyriakides (2004), The purpose of the present work is to describe the production and characterization of extracellular thermostable α-amylase from *Bacillus subtilis*, which was isolated from fresh sheep's milk. In a medium containing low starch concentrations the maximum amylase production was obtained at 40 ^{o}C. The enzyme showed maximal activity at optimum temperature 135°C and pH 6.5 andi n the presence of either calcium or starch enzyme thermostability was enhanced. To study the hydrolysis of various starches this α-amylase was used to compare the degree of hydrolysis detected in the cell-free supernatant or the ammonium sulphate resultant crude enzyme preparation was used as α-amylase source. In this way

partial amylase purification is avoided and it decreases the cost of the hydrolysis. When the reaction temperature increase to 70 ◦C, all substrates displayed higher hydrolysis rates. Potato starch hydrolysis resulted in a greater yield of reducing sugars than all the other starches tested. Soluble and rice starch took, respectively, the second and third position regarding reducing sugars liberation, however the α-amylase studied displayed slightly lower affinity for corn starch and oat starch.

Jeang et al. (2002), The rsda gene of *Cytophaga* sp. Was cloned, sequenced, and expressed in *Escherichia coli*. The predicted protein product contained 519 amino acids comparison with genes of other starch-degrading enzymes revealed high identity to α-amylases from certain *Bacillus* species. When *E. coli* was cultured larger quantity of the raw-starch-digesting amylase (RSDA) was produced at lower temperatures. Cloning and expressing the *rsda* gene of the *Cytophaga* sp. In *E. coli* may well simplify further study of the association between the structure and function of this RSDA; transferring the *rsda* gene of a soil bacterium to normally harmless microorganisms, such as *B.subtilis* or *Lactobacillus* sp. To find new applications of the gene product in the food and feed industry.

Steyn et al. (1995), The yeast *Lipomyces kononenkoae (Lk)* secretes a highly active raw starch-degrading α-amylase (aAmy) that hydrolyses both the α-1,4 and α-1,6 bond present in raw starch, in this study clone and characterize the *LKA1* gene encoding this αAmy. The nucleotide sequence of the cDNA fragment was determined we cannot use *Lk* in existing industrial fermentations because of its slow growth rate, low ethanol tolerance, catabolite repression, poorly characterized genetics and lack of (Generally Regarded As Safe) GRAS status, *LKA1* was expressed in *Saccharomyces cerevisiae (Sc)* under the control of the phosphoglycerate kinase *(PGK1)* promoter and Northern blot analysis indicated the presence of a single 2.3-kb transcript. The 28-aa signal peptide of the LKA1 protein when expressed in *Sc* directed its secretion into the medium. A genetically engineered strain of *Sc* secreting amylase could be useful in producing drinkable alcohol, fuel ethanol, single-cell protein and maltose syrup.

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Used Lab Instruments

- Refrigerated centrifuge (SIGMA 2K15)
- Water bath (Memmert)
- Thermal Cycler (Techne)
- pH Meter (METTLER TOLEDO MP220)
- Vortex (STUART SCINTIFIC)
- Microwave (Heidolph MR Hei-standard)
- Micro-centrifuge (E.S 6)
- Electrophoresis (Bıo RAD)
- Micropipette (Isolab)
- Balance (GEC AVERY)
- Spectrophotometer (VARIAN)
- UV Translumentor (UVP DUAL-INTENSTY)
- Incubator Thermo (SCIENTIFIC HERAEUS)
- Shaker Incubator (WiseCubeWisd)
- Safety cabinets Hood (TEISTAR AV-100)

3.1.2. Media preparation

Powder dissolved in distill water allow to soak for 10 min and swirl to dissolve, the components of the liquid medium are the same as those of the agar medium without agar. This solution was autoclaved at temperature 121 ^{o}C and pressure 2-atm pressure for 15 min, and then kept at 70 ^{o}C in a water bath. 25 ml of the solution was poured into a petri dish (agar medium) or flask (liquid medium) and the plate were cooled down to room temperature for about 1 hour. After the gelatine, the Petri dish was overturned and

the agar medium was incubating overnight at 37 ^{o}C. Then it was observed and contamination dish was rejected, the Petri dish was sealed with its cover by plastic tape to prevent the agar medium from drying, store pure dish 4 ^{o}C.

3.1.3. LB (Luria Bertani)

25 g LB powder (LAB) dissolved in 1 L of distilled water.

Table 3.1. LB Media (pH 7.5)

Components	Concentrations
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distil water	1000 ml
Autoclave	

3.1.4. LA (Luria Bertani Agar)

25 g LB powder (LAB) and 15 g Agar (Oxide) dissolved in 1 L of distilled water.

Table 3.2. LA Media (pH 7.5)

Components	Concentrations
Bactotrypton	10 g
Yeast extract	5 g
NaCl	10 g
Agar	20 g
Distil water	1000 mL
Autoclave	

3.1.5. LB Ampicillin

Table 3.3. LB Ampicillin (pH 7.5)

Components	Concentrations
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distil water	1000 ml
Autoclave Allow the medium to cool before adding ampicillin	
Ampicillin(100 µg/mL)	1000

3.1.6. LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal may be spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

3.1.7. SOC (Super Optimal Catabolite Repression) Media

Table 3.4. SOC Media

Components	Concentration
distilled water	900 mL
Bactotrypton	20 g
`Yeast extract	5 g
NaCl (5 M)	2 mL
KCl (1 M)	2.5ml
Aqua dest	975 ml
2 M $MgCl_2$	10 mL
1 M Glucose	20 ml
1 L completed and autoclaved.	

3.1.8. Buffers and Solutions

- ✓ **Sodium chloride solution stock (0.5 M)**

  1.46 g NaCl dissolved in 50 ml distilled water.

- ✓ **Tris 1M (MERCK) PH 7.5**

  7.88 g tris dissolved in 50 ml distilled water.

- ✓ **NaOH solution 1M stock**

  2g NaOH dissolved in 50 ml distilled water.

- ✓ **$NaClO_4$**

  1.75 mg / ml NaClO4 was prepared under the stove.

- ✓ **10% SDS (MARCK)**

  SDS 10 g, Distilled water 90 ml. It was prepared by providing dissolution rate.

- **IPTG stock solution (0.1M)**

  IPTG 1.2g, Add water to final volume 50ml. store at 4°C.

- **Lysozyme (4 mg / ml), (Fluka)**

  Add 40 mg to 10 ml of sterile distilled water. Store at 4 °C.

- **X-Gal (2ml)**

  100mg 5-bromo-4-chloro-3-indolyl-β-d-galactoside. Dissolved in 2 ml N,N´-dimethyl-formamide. Cover with aluminum foil and stored at –20 °C.

- **RNase (10 mg / ml)**

  100 mg RNase and 10 ml of sterile distilled water was prepared. Dissolved in 100 °C for 15 min maintaining the DNase activity was inhibited. Stored at -20 °C.

- **Chloroform-isoamyl alcohol (24: 1)**

  Chloroform and isoamyl alcohol 24: 1 ratio was prepared under the hood, and stored at 4 °C.

- **Resuspend Solution**

  0.2 M NaCl (MERCK), 1 mM EDTA (Merck) pH 8.0, Taking 20 ml NaCl from (stock 0,5 M) and 0.5 ml EDTA from (stock 0.1 M). Add sterile distilled water to final volume 50 ml.

- **10X TBE Buffer (Tris / Borate / EDTA) solution**

  108 g Tris-base, (Merck), 55 g boric acid, 40 ml 0.5M EDTA (Merck) pH 8.0. Distilled water was added to complete up to 1 litter.

- **1X TBE Buffer**

  10XTA 100 ml, 900 ml of distilled water

- **TE Buffer (adjust pH=8.0)**

  10 mM Tris/HCl pH 7.6, 1 mM EDTA (Merck) pH 8.0 calculating the amount required for the wanted volume to be prepared previously were prepared from autoclaved Stock solution.

3.1.9. Primer, Vectors And Bacterial Strains

Table 3.5. Primer list of used (Manufactured by Sentromer DNA teknologi)

Primer	Nucleotide Sequence	Tm	Expected size PCR product
27F	5'-GAGTTTGATCCTGGCTCA-3'	53.7 °C	(1400) bp
1385R	5'-CGGTGTGTACAAGGCCC-3'	57.6 °C	

Table 3.6. Vectors and Bacterial Strains

Vectors/strains	**Specificity**	**Manufacture**
pGEM®-T Easy Vector	Cloning vector, ampicillin resistance	Promega
JM109	High Efficiency Competent Cells	Promega
Bacillus circulans ATCC 61	gene encoding α-amylase enzyme	Microbiologist inc.

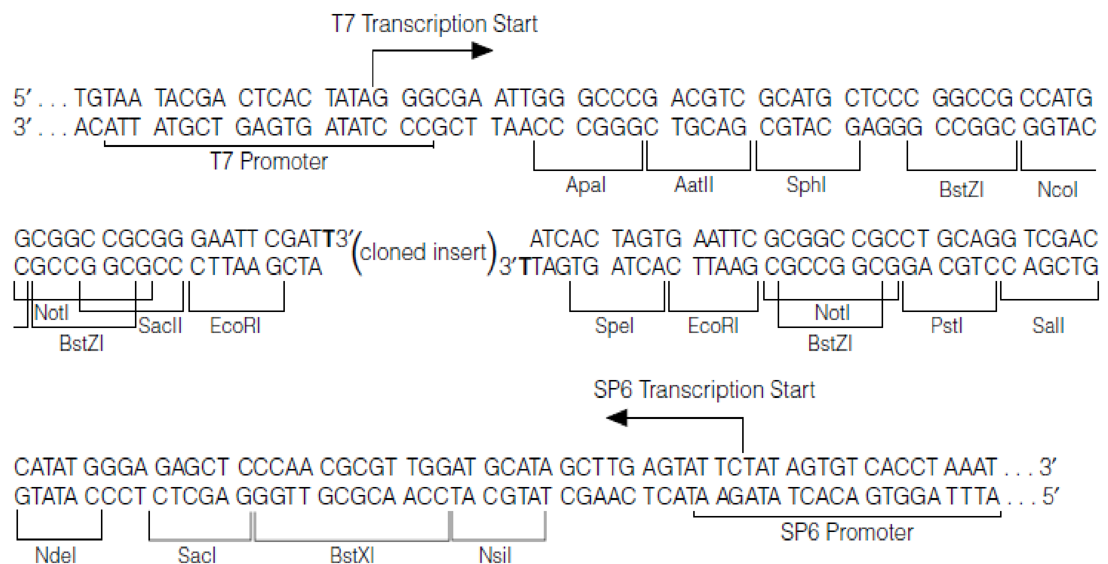

Figure 3.1. The promoter and multiple cloning sequence of the pGEM®-T Easy Vector. The top strand shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase (promega).

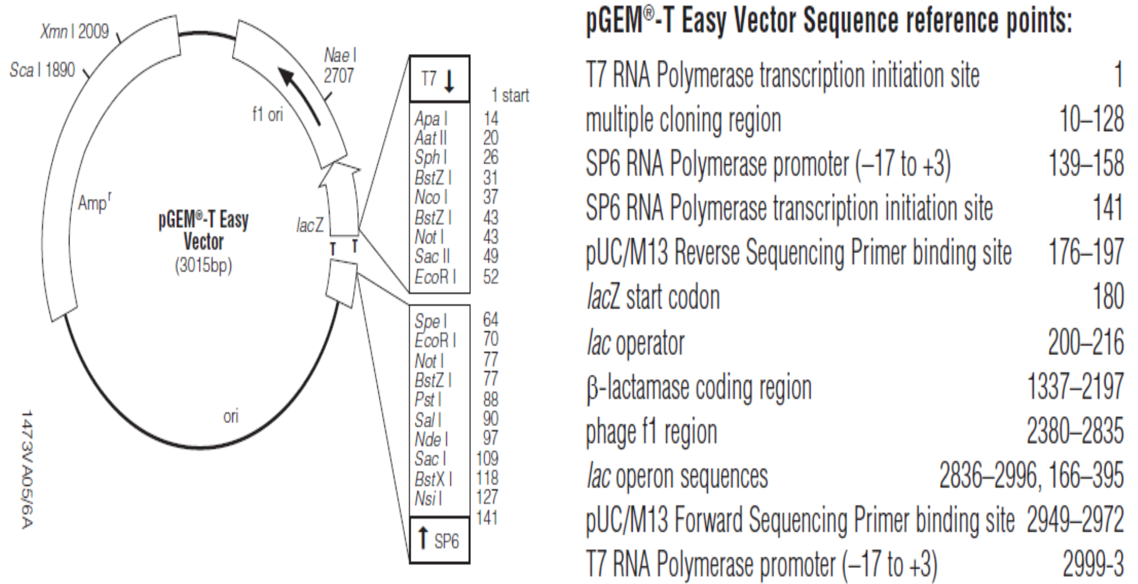

Figure 3.2. Sketch showing the pGEM®-T Easy Vector Sequences, Multi-Cloning Sites and Circle Maps (promega).

3.1.10. DNA Marker (Fermentas)

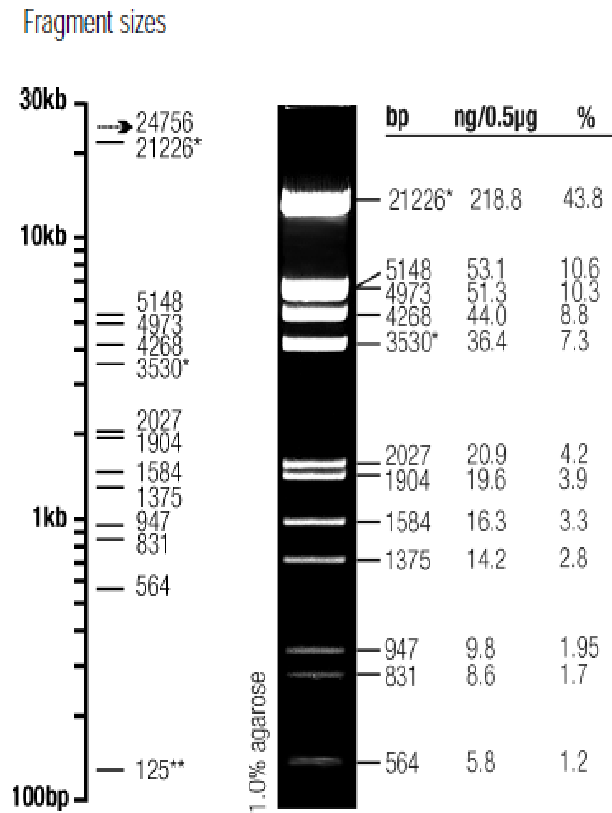

Figure 3.3. Sketch of the (Lambda DNA/EcoRI+HindIII marker, 3).

3.2. METHODS

3.2.1. Extraction of Genomic DNA from Bacteria.

We used marmur (1961), protocol for extraction of genomic DNA from *B. circulans.*

DNA extraction protocols;

1. Incubate the cells into 50 ml LB and grow overnight at 37 ^{0}C (16 hour)
2. Spin the cells down 6000 RPM, for 15 min
3. Resuspend the cells in 2.5 ml (0,2M NaCl 1mM EDTA pH 8,2
4. Add 0.3 ml lysozyme (4 mg/ml) and incubate 30 min at 37 C
5. Add 0.2 ml 10% SDS and incubate 15 min at 45°C
6. Transfer to an etched glass tube and add 0.2 NaClO4 (1.75 g/ml) and 5 ml isochloroform. Seal properly with parafilm and turn the tubes for 30 min in the turning machine.
7. Centrifuge in a table top centrifuge (the old one in Lasses lab) at speed 3900 RPM for 30 min until phase separation .
8. Take out the upper phase (aqueous phase) to a new tube. Gently add 2,5 ml 99% ice cold ethanol
9. Wrap up DNA on a bent glass pipette by spinning it between the phases
10. Wash the DNA with 70% Ethanol and let dry
11. Dissolve in 1ml 1XTE buffer, Store in refrigerator 4 C

3.2.2. Genomic DNA Concentration Measurement

The apparatus was calibrated to measure the DNA concentration, we used (VARIAN spectrophotometer), DNA absorbs light maximal at 260 nm, so DNA is measured at a wavelength of 260 nm.

If the A260/A280ratio located between (1.8 and 2.0) it means pure DNA sample.A280 nm is usually used as indicator for protein contamination, meanwhile tyrosine residues powerfully absorb at this wavelength.

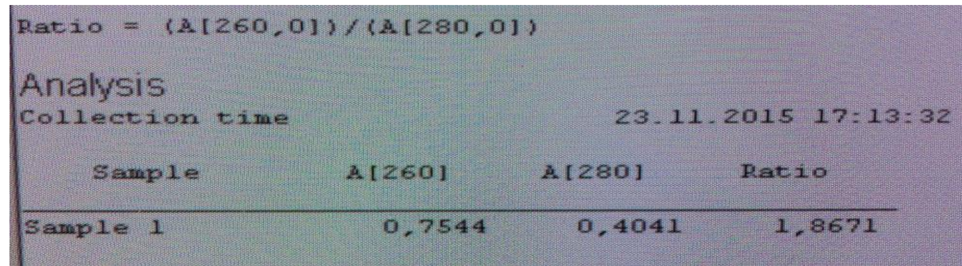

Figure 3.4. showed genomic DNA concentration measurement.

3.2.3. PCR (Polymerase Chain Reaction)

The PCR has greatly changed the way of cloning to make it more efficient and precise. Amplified small amount of DNA into a large amount of DNA in a very short time, the desired gene segments can be isolated efficiently. In order to use PCR, DNA segments as primers must be designed and used to bind to the genome DNA, because it is the only segment between two primers that can be amplified. This genome DNA is then denatured at a high temperature to serve as a template strands. Then the temperature is decreased to allow the primers to base pair to their complementary sequences on the template. DNA polymerases are used to elongate or amplify the corresponding DNA sequences exactly; it uses dNTP as building blocks of the new strands through many denaturing–annealing cycles, resulting in a product: the desired gene. It can then be isolated and joined to a vector for expression.

In 1983 Karl Mullis invented PCR and 10 years later he won a Nobel Prize for developing this new technology (Nelson and Cox, 2002).

The following formula is used to calculate Tm values of primers.

$$Tm = 2(A+T) + 4(G+C)$$

Primers for the PCR reaction and genomic DNA were prepared by diluting;

1:10 Forward primer 10 µl and 90 µl of sterile distilled water

1:10 Reverse primer 10 µl and 90 µl of sterile distilled water

Genomic DNA was 1: 100 ratio of 1 µl of DNA and 99 µl sterile distilled water.

Table 3.7. Standard PCR reaction

Component	Amount
10X PCR Buffer without Mg Cl2 (Sigma)	5 µl
Magnisiom chloride solution (Sigma)	1 µl
Deoxynucleotide mix, 10 Mm (Sigma)	1.5 µl
Forward primer	2 µl
Revers primer	2 µl
Genomic DNA	5 µl
Taq DNA polymerase 5 u/µl (Fermentas)	0.5 µl
Sterile distilled water	33 µl

Thermocycling

Thermal cycling program was developed for amplification of the amylase gene is shown in table 2.8.

Table 3.8. PCR program

STEP	TEMP	TIME
• Initial Denaturation	94°C	4 min
• Denaturation • Primer annealing • Elongation 29 Cycles	94°C 50°C 68°C	15 Sec 30 Sec 2.15 min
• Final Extension	72°C	10 - 7 min
• Hold	4-10°C	

3.2.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method used to determine the molecular weight of the DNA fragment based on the molecular size and rate of migration under the influence of an electric field. DNA fragment moves to the positively charged because nucleic acids are negatively charged, shorter DNA molecules will migrate faster than longer, visualized the band by staining the DNA with EtBr which is mutagenic substance. Also it adds loading buffers to the DNA sample to visualize DNA and sediment it in the gel wells.

In order to determine the molecular weight of the genomic DNA first well added 2 µl of 6X Loading dye (sigma) and 5 µl of DNA ladder, a mixture of the known size of DNA fragments (see Figure of DNA marker). The second well was added 1 µl genomic DNA and 2 µl of 6X Loading dye. However, to determine the size of PCR products using the same procedure, the second well is negative control and add 4 µl of PCR product to the second well instead using genomic sample, also for DNA purification added 45 µl of PCR product and 5 µl Loading dye for (large well) second and third well.

When samples loaded into the wells and closed the lid of the electrophoresis box, then applied the current to running the gel. 5 volts for 1 min 40 volts for 5 min, then 90 volts (usually 30 minutes to 1 hour). Flow was stopped after advancing enough, visualized under UV light comparing DNA band with ladder, in addition, the required band was cut out and a Quick Gel Extraction was completed (see Gel purification step).

Agarose gel preparation

0.8% agarose gel was made by mixing 0,8 g agarose (Gellyphor) with 100 ml of 1X TBE buffer. The mixture was heated in a microwave oven until transparent and solution become started to boil. Then left the gel solution to cool (to about 50 °C) after the gel solution cooled 5 µl of ethidium bromide EtBr (Amresco) was added, and mixed gently. Poured the gel slowly into a gel tray, set the comb at one side of the gel, and removed any bubbles in the solution. Wait until the gel was solidified. Then removed the comb the wells are formed, solidify gel in the tray, and was soaked into a 1 X TBE buffer-filled tank. The gel was placed with the wells facing the electrode that provide the negative current.

3.2.5. Gel Purification Steps: we used Quick Gel Extraction Kit (Invitrogen)

1. Determine the empty weight of Eppendorf using a scale sensitive, then excise a minimal area of gel that contain required DNA fragments of interest transferred to Eppendorf, and weight again for determining the weight of the gel slice containing the DNA fragment.
2. A gel determined weight and 3 volumes of Gel Solubilization Buffer (L3) were added to 1 volume of gel (1 mg ≈1 µl).
3. Incubate The tube at 50°C water bath for 10 minutes. To mix and ensure gel dissolve invert the tube every 3 minutes.
4. The mixture from step 3 over was transferred into Quick Gel Extraction Column, 800 µl of dissolved gel to each column.

   Note: column capacity is 850 µL.
5. To bind DNA. Centrifuge the column at 12,000 rpm for 1 min. Discard the fluid accumulated in the collection tube.
6. To remove all traces of agarose 500 µl 'Wash Buffer' (W1) which containing ethanol was added to the column and centrifuged again 12,000 rpm for 1 minute. Discard the Fluid accumulated in the collection tube.
7. Centrifuge the column to Remove Ethanol completely at maximum speed for 1–2 minutes.
8. Elute. The column was transferred to the sterile 1.5 ml recovery Eppendorf Tube. Add 50 µl Elution Buffer (E5) to the centre of of the membrane in the column. Incubate the tube at room temperature for 1 minute.
9. Collect. Centrifuge the tube at 12,000 rpm for 1 minute.
10. Store. The column was discarded the elution tube contains the purified PCR products. Store at 4°C for immediate use or at −20°C for long-term storage.

3.2.6. DNA sequence analyses.

After purified PCR product we send it for sequence analyses.

The nucleotide sequence of > Alpha amylase gene:

ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCTTG
CTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATG
GTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTG
AACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGT
GGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGA
CAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACAT
TAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTA
ACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAA
AGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATT
GGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTT
CAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTA
TGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGT
ATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAAATTTTCTT
TTTTGCGGGATTGGGTTAATCATGTC

```
Cloning vector pAMY-em1 cat, amyL and ermAM genes
Sequence ID: emb|AJ243541.1|  Length: 5019  Number of Matches: 1

Range 1: 2517 to 3452 GenBank  Graphics          Next Match  Previous Match
Score            Expect   Identities      Gaps        Strand
1729 bits(936)   0.0      936/936(100%)   0/936(0%)   Plus/Plus

Query  1     AGGGAAAAAACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2517  AGGGAAAAAACGGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGC  2576

Query  61    GCGCTGGAAAACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2577  GCGCTGGAAAACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTGCCGCTT  2636

Query  121   CATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2637  CATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTG  2696

Query  181   AACGGTACGGTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGAT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2697  AACGGTACGGTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCATGAT  2756

Query  241   ACACAGCCGGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2757  ACACAGCCGGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTAC  2816

Query  301   GCTTTTATTCTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2817  GCTTTTATTCTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGGGATATGTACGGG  2876

Query  361   ACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2877  ACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATTGAACCGATCTTA  2936

Query  421   AAAGCGAGAAAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2937  AAAGCGAGAAAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATT  2996

Query  481   GTCGGCTGGACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2997  GTCGGCTGGACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATA  3056

Query  541   ACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3057  ACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACA  3116

Query  601   TGGCATGACATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3117  TGGCATGACATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGA  3176

Query  661   GAGTTTCACGTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGATAGAAGAGCAGAGAG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3177  GAGTTTCACGTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGATAGAAGAGCAGAGAG  3236

Query  721   GACGGATTTCCTGAAGGAAATCCGttttttttattttgcccgtcttataaatttctttgat  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3237  GACGGATTTCCTGAAGGAAATCCGTTTTTTTTATTTTGCCCGTCTTATAAATTTCTTTGAT  3296

Query  781   tacattttataattaattttaaCAAAGTGTCATCAGCCCTCAGGAAGGACTTGCTGACAG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3297  TACATTTTATAATTAATTTTAACAAAGTGTCATCAGCCCTCAGGAAGGACTTGCTGACAG  3356

Query  841   TTTGAATCGCATAGGTAAGGCGGGGATGAAATGGCAACGTTATCTGATGTAGCAAAGAAA  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3357  TTTGAATCGCATAGGTAAGGCGGGGATGAAATGGCAACGTTATCTGATGTAGCAAAGAAA  3416
```

Figure 3.5. Blast analysis results, sequence displays that similarity of the amylase gene as we obtained.

3.2.7. Cloning of the PCR product into pGEM®-T Easy Vectors.

This vector is specifically designed for cloning of PCR products; it is pre-cut by EcoR V in the near central of a multiple cloning site, Also at both ends single 3´-terminal thymidine was added, which provide linearized vectors. The T-overhangs at the insertion site greatly improve the efficiency of ligation of single A-tailing PCR product, by certain thermostable DNA polymerases single deoxyadenosine generated independently on the template at the ends of the PCR products (Promega).

In this study *Taq* polymerase was used for PCR, which does not completely have sufficient proof reading capacity forming, 1/1000bp nucleotide mismatch error.

The DNA polymerase of *E. coli* competent cell has a much stringent proof reading and during the bacterial growth the plasmid is copied with no additional errors. The correct sequence can be identified by sequencing when a single colony is transformed into a pGEM®-T easy holding the insert of interest gene, it is used for plasmid isolation (Didriksen, 2010).

3.2.8. DNA Ligation

DNA ligases is the enzyme used for ligate DNA fragment by making a phosphodiester bond (covalent bond) between the free 3'OH and the free 5'phosphate ends of nucleic acid, this enzyme extracted from bacteriophage T4 called T4 DNA ligase (Sambrook and Russell, 2001). For this process ATP, which occurs in three different stages, is required.

I. The adenyl group from ATP is covalently attached to ligase and released inorganic phosphate.

II. Then, the adenyl group from ligase is transferred to the 5' phosphate of the DNA in the nick.

III. Finally, when the 3'OH end in the nick DNA attacks the activated 5'phosphate formed phosphodiester bond, and AMP is released (Trun and Trempy, 2003).

Ligation protocol:

1. Briefly centrifuge the pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.
2. Ligation reaction was set up as described below. Use 0.5ml microcentrifuge tube known to have low DNA-binding capacity. Vortex the 2X Rapid Ligation Buffer vigorously before each use.
3. The reaction was mixed gently by pipetting and incubated overnight at 4°C for the maximum number of transformation.

Table 3.9. Ligation reactions set up.

Reaction component	Positive control	Standard reaction		
		1	2	3
2X rapid ligation buffer, T4 DNA ligase	5 µl	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50 ng)	1 µl	1 µl	1 µl	1 µl
PCR product (DNA insert)	-	1 µl	2 µl	3 µl
Control insert DNA	2µl	-	-	-
T4 DNA ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl	1 µl
nuclease-free water to a final volume of 20 µl	11 µl	12 µl	11 µl	10 µl
Total volume	20 µl	20 µl	20 µl	20 µl

3.2.9. Transformation

Introduction of a plasmid into a competent cell called Transformation. JM109 High Efficiency Competent cell used in this study, this cell is chemically treated to allow across plasmid to the cell membrane. Plasmid vectors are capable of replicating the insert, when the plasmid vector replicated in the bacteria. Also the plasmid carries an antibiotic resistant gene which makes only the bacteria that carry the plasmid let to grow.

Protocol of Transformation

1. Frozen JM109 High Efficiency Competent Cells were removed from storage freezer and thawed on ice bath, Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.

2. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom.

3. Add 2µl of each ligation reaction to a sterile a 1.5ml low DNA binding capacity micro centrifuge tube on ice. Another tube on ice with 0.1ng uncut plasmid was set up for determination of the transformation efficiency of the competent cells.

4. Carefully transfer 50µl of cells into each tube prepared in Step 3 (Also for determination of transformation efficiency used 100µl of cells).

5. Gently flick the tubes to mix and the tube was incubated on ice for 20 minutes.

6. The cells were heat shocked for 45–50 seconds in a water bath without shaking at exactly 42°C.

7. Then, Directly return the tubes to ice for 2 minutes.

8. Add 950µl SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid.

9. The mixture was incubated at 37°C with shaking ~150rpm for 1.5 hours.

10. After incubation, at least three colonies were selected and each colony was inoculated into 3 ml of LB broth medium containing ampicillin (50 µg /ml). Then each tube was incubated at 37°C until reached with a density of 1.5-5.0 A_{600} units per ml.

11.Arterwards, plasmid DNA was extracted from each transformants using GeneEluet Plasmid Miniprep Kit (Sigma) and analyzed by restriction digestion.

3.2.10. Plasmid Isolation

GeneEluet Plasmid Miniprep Kit (Sigma) Isolation;

1. Add 40 mL of reconstituted and prechilled Lysis Reagent (see Preparation Instructions) to 400 mL of overnight culture in a 2-mL Collection Tube (provided). Mix briefly (3–5 seconds) by rapid inversion, vortexing or pipetting up and down. Incubate at room temperature for 2 minutes. Culture will typically become clear after 2 minutes.
2. Insert a Gen Elute Miniprep Binding Column into a 2-mL Collection Tube (provided). Add 500 mL of Column Preparation Solution to each column and spin for 10 seconds. Decant the flow-through and insert the column back into the Collection Tube for subsequent use.
3. Add 400 mL of Binding Solution to the lysate, cap the tube, and mix thoroughly by inverting at least 15 times. Do not vortex. Pour the mixture or pipette approximately 780 mL of the mixture to a pre-washed Binding Column seated in a 2-mL Collection Tube and spin for 20 seconds. Decant the flow-through.
4. Add 700 mL of diluted Wash Solution to each column and spin for 20 seconds. Decant the flow-through. Add another 200 mL of diluted Wash Solution to each column and spin for 30 seconds to wash and dry the filter. Carefully remove the column from centrifuge after the drying step to avoid splashing the flow-through liquid onto the dried column. If the flow-through liquid does contact the dried column, re-centrifuge the column for 20 seconds before proceeding to the elution step.
5. Transfer the Binding Column to a clean 2-mL Collection Tube (provided). Add 40 mL of Elution Solution (or water if desired) directly to the surface of the filter and spin for 30 seconds to elute. Plasmid DNA is now present in the eluate and ready for immediate use or storage at –20 °C.

3.2.11. Screening of Clones by PCR, Restriction Enzyme Digestion and Sequence analysis

This technique was used to screen for containing of a *lac* case gene insert into the pGEMT Easy vector. Colony PCR was performed with the insert-specific forward and reverse PCR primer. Ten colonies were chosen from the cloning experiment for analysis. Colony preparation for PCR was as the following: The transformation plates were examined and ten well-isolated colonies were picked, and then transferred to 3 ml sterile LB broth containing appropriate ampicillin for overnight culture and miniprep. Each colony to be screened was labelled. When the colonies incubated at least 3 hours, 10 µl of culture from each tube was taken for performing colony PCR. 10 µl of grown culture from each colony was diluted in 90µl sterile water, respectively. Each suspension was boiled for 5 min at 95°C in a thermal cycler. 2 µl of the boiled culture was used each amplification as a template. Positive clones with a suitable size DNA fragment insert were screened by PCR. A master PCR reaction protocol was; 2 µl of µl of bacterial cell suspension was added to a 25 µl reaction containing 4 µl dNTPs (10µM) (Fermentas), 1.5 µl (0.5 µM) forward (F2) and reverse primers (R2), 0.5 µl *Taq* DNA polymerase (Fermentas), and 10µl 10X *Taq* DNA polymerase buffer with Mg_{++} (Fermentas), 32.5 µl PCR graded H_2O. PCR conditions were as follows: 1 cycle of 94ºC for 2 min for initial denaturation; 30 cycles of 98ºC for 10 sec, 58ºC for 10 sec and 72ºC for 50 sec; then a final extention at 72ºC for 10 min before storage at 4ºC. Amplification products (10 µl) were analyzed on a 1% agarose gel. Positive clones with insert α-amylase gene were stored at -70ºC in 50 % glycerol. As an important note here, the amount of bacteria needed for PCR screening is usually very small. Too many bacteria in the mix will inhibit the PCR reaction.

In addition to screen transformants by PCR, a plasmid miniprep followed by restriction digestion was performed. Well-isolated colonies were picked from a plate and transferred to LB broth culture medium containing the appropriate antibiotic for selection. All cultures were incubated overnight with shaking (~250 rpm). The plasmid isolation experiment was performed using a GeneEluet Plasmid Miniprep Kit (Sigma). Once the DNA was purified, a portion of plasmid was screened by restriction digestion. 1 µg of plasmid was used in our digest. EcoRI were used for screening the orientation of the insert.

The following components were assembled to a 1,5 mL Eppendorf tube:

Restriction Enzyme Buffer 1 µl

Plasmid DNA (1.275 ng/µl) 1 µl

EcoRI1 µl

Sterile ddH_2O 7 µl

Final Volume 10 µl

The reaction mixture was incubated 1hour at 37ºC. Then, all tubes were analyzed on 1% agarose gel and positive clones were sequenced in SENTROMER.

4. RESULT

The genomic DNA isolated from *Bacillus circulans* ATCC 61 was quantified spectrophotometrically by absorbance at 260 nm and 280 nm. The concentration of genomic DNA of *Bacillus circulans* ATCC 61 according to the value of 260 nm absorbance. This total DNA solution had a concentration of 2 μg/ μL and OD 260/ 280 =1.86. This demonstrates that the total DNA extracted was high quality to perform the subsequent analysis, See figure 3.4., The molecular weight of total genomic DNA from *Bacillus circulans* ATCC 61 about 23 kDa, was estimated by agarose gel electrophoresis. This genomic DNA was used as a template to amplify the α- amylase gene by PCR with *Taq* DNA Polymerase. The α- amylase gene fragment synthesized by the polymerase chain reaction (PCR) was analyzed by agarose gel electrophoresis. Figure 4.1.a. Shows only one band that belongs to α- amylase gene and was obtained from total DNA isolated from *Bacillus circulans* ATCC 61. The expected size of the PCR product was 1400 bp, on the agarose gel.

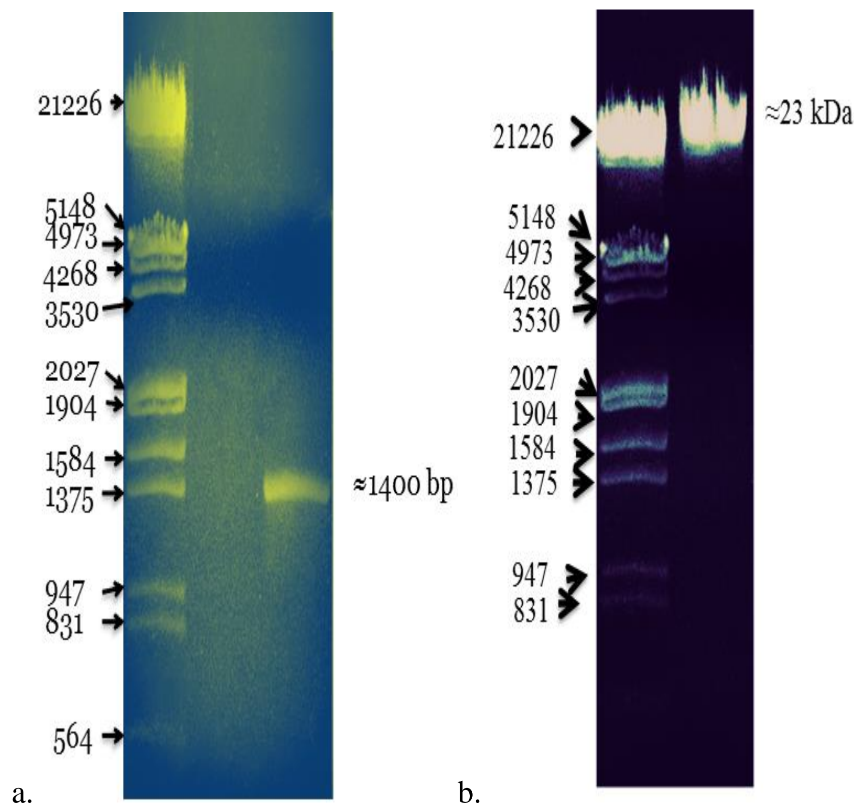

Figure 4.1. a. Show PCR product size. 1 well Marker (Lambda DNA/EcoRI+HindIII ladder), 3 well show PCR product. **b.** Show total genomic DNA of *Bacillus circulans* ATCC 61.

The α- amylase insert DNA (1400 bp) lies between the EcoRI and HindIII restriction site. α- amylase was cloned into the pGEMT-easy vector using standard cloning techniques in a ligation reaction and transformed the ligation mixture into competent cells of *E. coli* JM109. Ligation products were transformed into *E. coli* host cell, and then incubated at 37ºC for the overnight period. The next day, ten transformants were chosen and mini-preparations of plasmids encoding the α- amylase were done for screening positive clones that contain the proper orientation of insert DNA by PCR using the F2 and R2 primers as depicted in Figure 4.2.

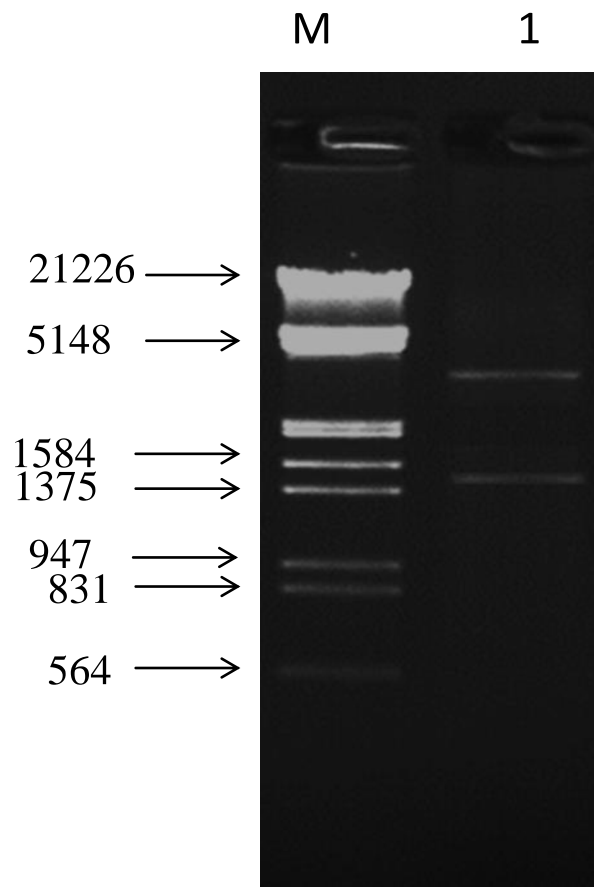

Figure 4.2. Positive clone after digestion with EcoRI.

Blast results showed the complete amino acid sequence of this gene displayed that the amylase gene is (100% identity) to the previously defined amyL gene. See figure 3.5. showed Blast analysis results. This result showed that we cloned true α-amylase gene.

5. DISCUSSION

In this study, we preferred *Bacillus circulans* ATCC 61 because it contains an α-amylase enzyme was not characterized before. Also, these bacteria can produce a α-amylase in nature, but the amount of the enzyme is low. We increased the amount of this enzyme by cloning technique, the α- amylase gene in *Bacillus circulans* ATCC 61 was amplified by PCR and by used *Taq* DNA polymerase, single deoxyadenosine is added to the 3' ends of the PCR fragments. This can be exploited by cloning the PCR product into linearized T vector, pGEMT-easy vector fitted with single overhanging 3' deoxythymidine residues on each of its 3'termini. PCR fragments that carry unpaired deoxyadenosin residues at their 3'termini can easily be cloned into vectors with single unpaired deoxythymidine residues at its 3'termini, The recombinant DNA vector is then transformed into *E.coli* JM109 competent cell. Within the host cell the vector multiplies, producing numerous identical copies.

This is the first record in the literature in which α- amylase enzyme from *Bacillus circulans* ATCC 61 was isolated and cloned.

In the study of Chen et al. successfully described cloning and expression of α-amylase gene from *Bacillus subtilis*WB800. Cloned α- amylase encoding gene amy1 into pMD18-T vector and amplified in *E. coli* DH5 α. Reconstructed Shuttle vector pP43MNX to create vector pP43X in *B. subtilis* WB800 for heterologous expression of the α-amylase, the amy1 gene. Sequencing showed that it consisted of 1545 bp (Chen et al., 2015). Also, In study of Serin , she was successfully, cloned and purified α-amylase from *Bacillus subtilis* ATCC 6051, the α-amylase gen was amplified by PCR and inserted to pDG148 plasmid vector. Both purified PCR product and pDG148 cloning vector plasmid cut with restriction enzymes HindIII and SphI (Serin, 2014). Another study Asoodeh et al. in the present work, α-amylase encoding gene from *Bacillus* sp. DR90 was isolated then cloning (Asoodeh et al., 2014). Gene encoding IAM, BsIam was identified from genomic DNA sequence with inverse PCRs. The open reading frame of the BsIam gene was 2,655 base pairs long (Li et al., 2013).The aim of the current projectof Rabbani et al. was to isolate and express randomly mutagenise in the *Bacillus subtilis* 168 *α*-amylase genes using error-prone PCR (EP-PCR) technique. Tow primers, BS168F: 5'-GTGTCAAGAATGTTTGC-3 and BS168R:

3'GTTTTGTTAAAAGATG-5', to amplify the amylase gene, currently using the following cycle in EP-PCR method: denaturation at 94◦C for 30 Sec, annealing at 40◦C for 2 min, and extension at 72◦C for 2 min in 30 cycles that were followed with 72◦C for 2 min as a post cycle. The host *E. coli* strains, XL1-Bluewas used for plasmid construction(Rabbani et al., 2011). In another study,(Lin et al. 2009), investigated the characteristics of extracellular α-amylase from *Bacillus cereus* B905 and B904 strains were studied and the amylase genes were cloned. The structural genes of amy905 and amy904 were cloned successfully using (PCR) and expressed in Escherichia coli. The amy904 genes consist of 1362 bp and protein molecular weights about 55 kDa while amy905 gene 1761 bp long and protein molecular weights were about 68 kDa.

In future we will continue to express and characterize our enzyme and will develop our enzyme with protein engineering techniques. Also, we will use this enzyme in order to develop some industrial biotechnology. We suggest, clone and assemble this gene, as we originally planned.

6. REFERENCES

Adrio, J. L., &Demain, A. L. (2014). Microbial enzymes: Tools for biotechnological processes. *Biomolecules, 4*(1), 117-139.

Afzal-Javan, F., &Mobini-Dehkordi, M. (2013). Amplification, Sequencing and Cloning of Iranian Native Bacillus subtilis Alpha-amylase Gene in Saccharomyces cerevisiae. *Jundishapur Journal of Microbiology*, *6*(8).

Ahmad, N., Mehboob, S., & Rashid, N. (2015). Starch-processing enzymes–emphasis on thermostable 4-α-glucanotransferases. *Biologia*, *70*(6), 709-725.

Alberts, B., A. Johnson, et al. (2002). Molecular Biology of the cell. New York, Garland Science.

Ali, M. B., Mezghani, M., &Bejar, S. (1999). A thermostable α-amylase producing maltohexaose from a new isolated Bacillus sp. US100: study of activity and molecular cloning of the corresponding gene. *Enzyme and microbial technology, 24*(8), 584-589.

Amylase Research Society of Japan. *Handbook of amylases and related enzymes: their sources, isolation methods, properties and applications*. Pergamon, 1988.

Asoodeh, A., Chamani, J., &Lagzian, M. (2010). A novel thermostable, acidophilic α-amylase from a new thermophilic "Bacillus sp. Ferdowsicous" isolated from Ferdows hot mineral spring in Iran: Purification and biochemical characterization. *International journal of biological macromolecules, 46*(3), 289-297.

Asoodeh, A., Emtenani, S., Emtenani, S., Jalal, R., &Housaindokht, M. R. (2014). Molecular cloning and biochemical characterization of a thermoacidophilic, organic-solvent tolerant α-amylase from a Bacillus strain in Escherichia coli. Journal of Molecular Catalysis B: Enzymatic, 99, 114-120.

Bauer, B. U. (1996). Preparation of homochiral cyanohydrins using an enzyme-membrane-reactor. University of Twente.

Brown, T. (2010). Gene cloning and DNA analysis: an introduction. John Wiley & Sons.

Celińska, E., Białas, W., Borkowska, M., &Grajek, W. (2015). Cloning, expression, and purification of insect (Sitophilusoryzae) alpha-amylase, able to digest granular starch, in Yarrowialipolytica host. *Applied microbiology and biotechnology*, 99(6), 2727-2739.

Chai, Y. Y., Rahman, R. N. Z. R. A., Illias, R. M., &Goh, K. M. (2012). Cloning and characterization of two new thermostable and alkalitolerant α-amylases from the Anoxybacillus species that produce high levels of maltose. *Journal of industrial microbiology & biotechnology, 39*(5), 731-741.

Chakraborty, S., Khopade, A., Kokare, C., Mahadik, K., &Chopade, B. (2009). Isolation and characterization of novel α-amylase from marine Streptomyces sp. D1. *Journal of Molecular Catalysis B: Enzymatic, 58*(1), 17-23.

Chen, J., Chen, X., Dai, J., Xie, G., Yan, L., Lu, L., & Chen, J. (2015). Cloning, enhanced expression and characterization of an α-amylase gene from a wild strain in B. subtilis WB800. *International journal of biological macromolecules,80*, 200-207.

Chung, Y. C., Kobayashi, T., Kanai, H., Akiba, T., &Kudo, T. (1995). Purification and Properties of Extracellular Amylase from the Hyperthermophilic Archaeon Thermococcus profundus DT5432. *Applied and environmental microbiology, 61*(4), 1502-1506.

Da Silva, T. M., Maller, A., de Lima Damásio, A. R., Michelin, M., Ward, R. J., Hirata, I. Y., ... & de Polizeli, M. L. T. (2009). Properties of a purified thermostableglucoamylase from Aspergillusniveus. *Journal of industrial microbiology & biotechnology, 36*(12), 1439-1446.

Dash, B. K., Rahman, M. M., &Sarker, P. K. (2015). Molecular Identification of a Newly Isolated Bacillus subtilis BI19 and Optimization of Production Conditions for Enhanced Production of Extracellular Amylase. *BioMed Research International*, *2015*.

Didriksen, A. (2010). Molecular biology and physiology of isolated chloroplasts from the algae Vaucheria.

Ehrlich, H. L., & Newman, D. K. (Eds.). (2008). Geomicrobiology. CRC press.

Fincan, S. A., Enez, B., Özdemir, S., &Bekler, F. M. (2014). Purification and characterization of thermostable α-amylase from thermophilicAnoxybacillusflavithermus. *Carbohydrate polymers*, 102, 144-150.

Gangadharan, D., Ramachandran, P., Paramasamy, G., Pandey, A., &Nampoothiri, K. M. (2010). Molecular cloning, overexpression and characterization of the raw-starch-digesting α-amylase of Bacillus amyloliquefaciens. *Biologia, 65*(3), 392-398.

Gurumurthy, D. M., &Neelagund, S. E. (2012). Molecular characterization of industrially viable extreme thermostable novel alpha-amylase of Geobacillus sp. Iso5 isolated from geothermal spring. *J. Pure Appl. Microbiol, 6*, 1759-1773.

Hamilton, L. M., Kelly, C. T., & Fogarty, W. M. (1999). Purification and properties of the raw starch-degrading α-amylase of Bacillus sp. IMD 434.*Biotechnology Letters, 21*(2), 111-115.

Herfindal, E. T. and D. R. Gourley (2000). Textbook of therapeutics: drug and disease management. USA, Lippincott Williams & Wilkins.

Hmidet, N., Maalej, H., Haddar, A., &Nasri, M. (2010). A novel α-amylase from Bacillus mojavensis A21: purification and biochemical characterization. *Applied biochemistry and biotechnology*, *162*(4), 1018-1030.

http://www.encyclopedia.com/topic/Enzymes.aspx

Hussain, I., Siddique, F., Mahmood, M. S., & Ahmed, S. I. (2013). A review of the microbiological aspect of α-amylase production. *International Journal of Agriculture and Biology*, *15*(5), 1029-1034.

Jeang, C. L., Chen, L. S., Chen, M. Y., &Shiau, R. J. (2002). Cloning of a gene encoding raw-starch-digesting amylase from a Cytophaga sp. and its expression in Escherichia coli. *Applied and environmental microbiology, 68*(7), 3651-3654.

Kiran, Ö., Çömlekçioğlu, U., &Arikan, B. (2005). Effects of carbon sources and various chemicals on the production of a novel amylase from a thermophilic Bacillus sp. K-12. *Turkish Journal of Biology, 29*(2), 99-103.

Konsula, Z., &Liakopoulou-Kyriakides, M. (2004). Hydrolysis of starches by the action of an α-amylase from Bacillus subtilis. *Process Biochemistry, 39*(11), 1745-1749.

Kubrak, O. I., Storey, J. M., Storey, K. B., &Lushchak, V. I. (2010). Production and properties of α-amylase from Bacillus sp. BKL20. *Canadian journal of microbiology*, *56*(4), 279-288.

Kumagai, Y., Satoh, T., Inoue, A., &Ojima, T. (2013). Enzymatic properties and primary structures of two α-amylase isozymes from the Pacific abalone Haliotis discus hannai. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 164*(2), 80-88.

Kumar, N. M., Karthikeyan, S., &Jayaraman, G. (2013). Thermostable alpha-amylase enzyme production from Bacillus laterosporus: Statistical optimization, purification and characterization. *Biocatalysis and Agricultural Biotechnology, 2*(1), 38-44.

Kumari, A., Singh, K., & M Kayastha, A. (2012). α-Amylase: General Properties, Mechanism and Biotechnological Applications-A Review. *Current Biotechnology*, *1*(1), 98-107.

Lan Tran, P., Lee, J.S., Park, K.H. 2014. Experimental evidence for a 9-binding subsite of *Bacillus licheniformis*thermostable α-amylase. ***FEBS Letters***. 588: 620-624.

Li, Y., Niu, D., Zhang, L., Wang, Z., & Shi, G. (2013). Purification, characterization and cloning of a thermotolerantisoamylase produced from Bacillus sp. CICIM 304. *Journal of industrial microbiology & biotechnology, 40*(5), 437-446.

Lin-lin, Z., Qi, W., Rong-xi, L., Yong-jun, W., &Ru-hong, M. (2009). Analysis, gene cloning and expression of two α-amylases from Bacillus cereus. *Chinese Journal of Agricultural Biotechnology, 6*(02), 135-140.

Liu, X. D., &Xu, Y. (2008). A novel raw starch digesting α-amylase from a newly isolated Bacillus sp. YX-1: purification and characterization. *Bioresource Technology, 99*(10), 4315-4320.

Lonsane BK, Ramesh MV. Production of bacterial thermostable a-amylase by solid state fermentation: a potential tool for achieving economy in enzyme production and starch hydrolysis.In: *Advances in applied microbiology, vol. 35*. San Diego: California Academic Press, 1990:1_56

Marco, J. L., Bataus, L. A., Valencia, F. F., Ulhoa, C. J., Astolfi-Filho, S., & Felix, C. R. (1996). Purification and characterization of a truncatedBacillussubtilis α-amylase produced byEscherichia coli. *Applied microbiology and biotechnology, 44*(6), 746-752.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. Journal of molecular biology, 3(2), 208-IN1.

Martínez-Gallegos, J. F., Jurado-Alameda, E., Carrasquilla-Carmona, J. L., Jiménez-Pérez, J. L., & Romero-Pareja, P. M. (2014). Characterization of the ozone effect over an α-amylase from Bacillus licheniformis. *Biochemical Engineering Journal*, *85*, 119-124.

Miao, M., Xiong, S., Ye, F., Jiang, B., Cui, S. W., & Zhang, T. (2014). Development of maize starch with a slow digestion property using maltogenic α-amylase. *Carbohydrate polymers*, *103*, 164-169.

Miguel, Â. S. M., Lobo, B. W. P., da Costa Figueiredo, É. V., Dellamora-Ortiz, G. M., & Martins-Meyer, T. S. (2013). *Enzymes in bakery: current and future trends*. INTECH Open Access Publisher.

Mobini-Dehkordi, M. Afzaljavan, F. Saffar, B. 2011. Identifying of native bacillus strain producing alpha amylase and clonig of alpha amylase gene in E.coli, *First national conference biological sciences*. P:787-789.

Nelson, D. L. and M. M. Cox (2002). Lehninger Principles of Biochemistry. New York, Worth.

Nielsen, J. E., &Borchert, T. V. (2000). Protein engineering of bacterial α-amylases. *Biochimica et BiophysicaActa (BBA)-Protein Structure and Molecular Enzymology, 1543*(2), 253-274.

Nishizawa, M., Ozawa, F., &Hishinuma, F. (1987). Molecular cloning of an amylase gene of Bacillus circulans. *DNA*, *6*(3), 255-265.

Ohdan, K., Kuriki, T., Kaneko, H., Shimada, J., Takada, T., Fujimoto, Z., ... & Okada, S. (1999). Characteristics of two forms of α-amylases and structural implication. *Applied and environmental microbiology*, *65*(10), 4652-4658.

Okamoto, S., Chin, T., Nagata, K., Takahashi, T., Ohara, H., &Aso, Y. (2015). Production of itaconic acid in Escherichia coli expressing recombinant α-amylase using starch as substrate. *Journal of bioscience and bioengineering, 119*(5), 548-553.

Ozturk, M. T., Akbulut, N., Ozturk, S. I., & Gumusel, F. (2013). Ligase-independent cloning of amylase gene from a local Bacillus subtilis isolate and biochemical characterization of the purified enzyme. Applied biochemistry and biotechnology, 171(2), 263-278.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R. 2000. Advances in microbial amylases (review article). ***Biotechnology and Applied Biochemistry,*** 31: 135-152.

Planchot, V., Colonna, P., Gallant, D. J., &Bouchet, B. (1995). Extensive degradation of native starch granules by alpha-amylase from Aspergillusfumigatus. *Journal of Cereal Science, 21*(2), 163-171.

Qian, M., Haser, R., &Payan, F. (1995). Carbohydrate binding sites in a pancreatic α-amylase-substrate complex, derived from X-ray structure analysis at 2.1 Å resolution. *Protein Science*, *4*(4), 747-755.

Qin, Y., Huang, Z., Liu, Z. 2014. A novel cold-active and salt-tolerant α-amylase from marine bacterium Zunongwangiaprofunda: molecular cloning, heterologous expression and biochemical characterization. Extremophiles, 18: 271-281.

Rabbani, M., MirMohammadSadeghi, H., Moazen, F., Rahimi, M., &Salehi, G. (2011). Cloning and Expression of Randomly Mutated Bacillus subtilis**α**-Amylase Genes in HB101. *Biotechnology research international, 2011.*

Reed, G. (Ed.). (2012). Enzymes in food processing. Elsevier.

Reyes-Sosa, F. M., Molina-Heredia, F. P., & Miguel, A. (2010). A novel α-amylase from the cyanobacteriumNostoc sp. PCC 7119. *Applied microbiology and biotechnology, 86*(1), 131-141.

Roy, J. K., Borah, A., Mahanta, C. L., & Mukherjee, A. K. (2013). Cloning and overexpression of raw starch digesting α-amylase gene from Bacillus subtilis strain AS01a in Escherichia coli and application of the purified recombinant α-amylase (AmyBS-I) in raw starch digestion and baking industry. *Journal of Molecular Catalysis B: Enzymatic*, *97*, 118-129.

Salis, A., Monduzzi, M., Solinas, V., Polaina, J., &MacCabe, A. P. (2007). Industrial Enzymes: Structure Function and Applications.

Sambrook, J. and D. W. Russell (2001). Molecular cloning. A laboratory manual. New York, Cold Spring Harbour Laboratory Press.

Schaechter, Moseli Encyclopedia of microbiology. Academic Press, 2009**.**

Segel, I. H. (1975). Enzyme kinetics (Vol. 360). Wiley, New York.

Serin, B. (2014). Cloning and Characterization of α-amylase from Bacillus subtilis ATCC 6051

Sharma, P., Gupta, S., Sourirajan, A., &Dev, K. (2015). Characterization of Extracellular Thermophilic Amylase from Geobacillus sp. Isolated from Tattapani Hot Spring of Himachal Pradesh, India. *Current Biotechnology, 4*(2), 202-209.

Sidkey, N.M., Maha, A., Reham, B., Reham, S., Ghadeer, B. 2011. Purification and characterization of α-amylase from a newly isolated *Aspergillusflavus* F2Mbb. ***International Research Journal of Microbiology,*** 2: 96-103.

Singh, R. (2007). Development of N-heterocyclic Carbenes as Organic Catalysts and Efficient Ligands in Palladium Mediated Transformations. ProQuest.

Singh, S., Sharma, V., Soni, M. L., & Das, S. (2011). Biotechnological applications of industrially important amylase enzyme. *International Journal of Pharma and Bio Sciences, 2*, 486-496.

Sivaramakrishnan, S., Gangadharan, D., Madhavan, K., Soccol, C.R., Pandey, A. 2006. α-Amylases from microbial sources an overview on recent developments. Food Technology and Biotechnology, 44: 173-184.

Souza, P. M. D. (2010). Application of microbial α-amylase in industry-A review. *Brazilian Journal of Microbiology, 41*(4), 850-861.

Steyn, A. J., Marmur, J., & Pretorius, I. S. (1995). Cloning, sequence analysis and expression in yeasts of a cDNA containing a Lipomyces kononenkoae α-amylase-encoding gene. *Gene, 166*(1), 65-71.

Sumner, J. B., & Somers, G. F. (2014). Chemistry and methods of enzymes. Academic Press.

Tamamura, N., Saburi, W., Mukai, A., Morimoto, N., Takehana, T., Koike, S., ... & Mori, H. (2014). Enhancement of hydrolytic activity of thermophilicalkalophilic α-amylase from Bacillus sp. AAH-31 through optimization of amino acid residues surrounding the substrate binding site. *Biochemical Engineering Journal, 86*, 8-15.

Tao, X., Jang, M. S., Kim, K. S., Yu, Z., & Lee, Y. C. (2008). Molecular cloning, expression and characterization of alpha-amylase gene from a marine bacterium Pseudoalteromonas sp. MY-1. *Indian J BiochemBiophys, 45*(5), 305-309.

Tiwari, S. P., Srivastava, R., Singh, C. S., Shukla, K., Singh, R. K., Singh, P., ... & Sharma, R. AMYLASES: AN OVERVIEW WITH SPECIAL REFERENCE TO ALPHA AMYLASE.

Trun, N. and J. Trempy, Eds. (2003). Fundamental bacterial genetics. USA, Blackwell.

Ueda, M., Asano, T., Nakazawa, M., Miyatake, K., & Inouye, K. (2008). Purification and characterization of novel raw-starch-digesting and cold-adapted α-amylases from Eiseniafoetida. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 150*(1), 125-130.

Van Der Maarel, M. J., Van Der Veen, B., Uitdehaag, J. C., Leemhuis, H., &Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of biotechnology, 94*(2), 137-155.

Wang, G. J., Wang, Z. S., Zhang, Y. W., & Zhang, Y. Z. (2012). Cloning and expression of amyE gene from Bacillus subtilis in Zymomonasmobilis and direct production of ethanol from soluble starch. *Biotechnology and Bioprocess Engineering, 17*(4), 780-786.

Whitehurst, R. J., & Van Oort, M. (Eds.). (2010). *Enzymes in food technology (Vol. 388)*. Chichester: Wiley-Blackwell.

Wong, D.W.S., 2006. The ABC’s of Gene Cloning. Springer. 227. USA.

Xie, F., Quan, S., Liu, D., Ma, H., Li, F., Zhou, F., & Chen, G. (2014). Purification and characterization of a novel α-amylase from a newly isolated Bacillus methylotrophicus strain P11-2. *Process Biochemistry, 49*(1), 47-53.

Xuefeng, H., Bokun, L., Ganji, R., Zhibiao, Z., Yan, L., &Zhong, H. (2014). Gene Cloning and Characterization of an α-Amylase from Alteromonasmacleodii B7 for Enteromorpha Polysaccharide Degradation. *Journal of microbiology and biotechnology, 24*(2), 254-263.

Yamaguchi, R., Tokunaga, H., Ishibashi, M., Arakawa, T., & Tokunaga, M. (2011). Salt-dependent thermo-reversible α-amylase: cloning and characterization of halophilic α-amylase from moderately halophilic bacterium, Kocuriavarians. *Applied microbiology and biotechnology, 89*(3), 673-684.

CURRICULUM VITAE

Full Name: Aram Jalil TAWFIQ

Contact Information

Address: Iraq/ Kurdistan Region/ Sulaimany-Takya

E-mail: aramjalil@gmail.com

Phone: +9647706646171

Personal data:

Date of Birth : 25/9/1987

Place of Birth: Kirkuk

Nationality : Iraqi-kurdish

Gender : Male

Marital Status : Single

Education:

- 2015-2016, **MSc**. Institute of Natural and Applied Sciences, Dicle University, Dep/ Biology.
- 2012-2013, **BSc.** College of Science, Salahaddin University, Dep/Biology.

MSc. Research:

✓ Cloning and Characterization of α-amylase from *Bacillus circulans* ATCC 61.

Language skills:

Languages	Speaking	Writing	Reading	Understanding
Kurdish	Native	Native	Native	Native
English	Good	Very good	Very good	Very good
Arabic	Good	Good	Very good	Very good
Turkish	Fair	Fair	Fair	Good