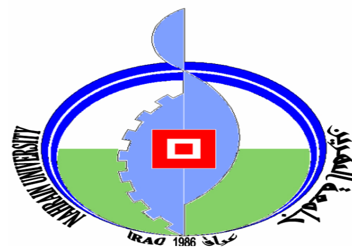
Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al- Nahrain University
College of Science
Department of Biotechnology



Extraction, partial purification and Characterization of Isoamylase produced by Locally isolated *Psuedomonas*

A Thesis
Submitted to the College of Science of Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Hanin Saadun Abdul-Wahab

B. Sc. Biotechnology (2005)

Al-Nahrain University

October 2008 Thul- Quda 1429

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Acknowledgments

First of all praise to Allah the lord of the universe, peace be upon Mohammed the messenger of Allah and upon his Relatives and Companions. First and foremost my thanks must go to my supervisor ***Dr. Hameed Majeed*** *for his guides and great support throughout my study. I specially appreciate his warm and gentle approach to my supervision, as well as his immense patience in all his dealings with me during my time at the laboratory. The knowledgment I gained from him and academic and non- academic matters have been invaluable and will definitely be beneficial to my future career.*

I would like to thank my family; I am very grateful for their unreserved love, to which have been sources of inspiration and moral support.

It is a pleasure to thank my collegues especially Miss. Shaymaa Al-Rajehi, Miss. Asmaa Ali, Miss. Randa Mohammed, Miss. Israa Abd and to whom that I did not mentioned.

Hanin

.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Supervisor Certification

I, certify that this thesis was achieved under my supervision at the Department of Biotechnology, College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

Signature:
Supervisor: **Dr. Hameed M. Jasim**
Scientific Degree: Assistant Professor
Date:

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:

Name: **Dr. Kadhim Mohammad Ibrahim**

Scientific degree: Professor

Title: Head of Biotechnology Department

Date:

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:
Name: **ABDUL WAHID B. AL-SHAIBANI**
Scientific Name: Professor
Date:

(Chairmen)

Signature:
Name: **Ghazi M. Aziz**
Scientific Degree: Assistant Professor
Date:

(Member)

Signature:
Name: **Shaymaa Al- Rajehi**
Scientific Degree: Lecturer
Date:

(Member)

Signature:
Name: **Hameed M. Jasim**
Scientific Degree: Assistant Professor
Date:

(Member/ Supervisor)

I hereby certify upon the decision of the examining committee

Signature:
Name: **Dr. Laith Abdul Aziz Al-Ani**
Scientific Degree: Assistant Professor
Title: Dean of College of Science
Date:

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Summary

Eighty soil and sewage samples were collected from different environmental locations to isolate isoamylase producing *Pseudomonas*. From these samples, 100 isolates were obtained, among them 40 were identified as *Pseudomonas* sp. according to the morphological characteristics and biochemical tests. These isolates were screened to examine their ability in isoamylase production. Results showed that all these isolates were producers of isoamylase. An isolate (*Pseudomonas* sp. H3) was the most efficient in producing isoamylase when the activity of the crude enzyme in the culture was 0.394 U/ml.

Optimum conditions for isoamylase production by *Pseudomonas* sp. H3 were studied. These conditions include the optimum; pH, growth temperature, type and concentration of carbon source, type and concentration of nitrogen source type and concentration of phosphate source. Results showed that the maximum activity for crude enzyme was (0.70 U/mg protein) which gained when the bacterial isolate was grown in a production medium containing ; 1 % maltose, 0.4 % peptone, 0.4% K_2HPO_4, pH 6.0, in a shaker incubator at 150 rpm at and incubation at 35ºC for 24 hr.

Enzyme produced under the optimum conditions was purified by precipitation with ammonium sulphate (60% saturation), followed by dialysis and purification by gel filtration using Sephacryl G-300. Enzyme activity of the partially purified enzyme was 1.853 U/ml. Results of enzyme characterization showed that optimum pH for the activity and stability were 5.0 and 5.5, respectively, while optimum temperature for the activity and stability was 35ºC.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

List of Contents

Lists of Contents……………………………………………………….I- VIII
Lists of Tables…………………………………………………………...VI
Lists of Figure ....III- IX
Lists of Abbreviaitions…………………………………………………X

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1: Introduction………………………………………………………………1

1.2: Literature Review………………………………………………………3

1.2.1: Genus *Pseudomonas*………………………………………………….3

1.2.2: Amylases…………………………………………………………...4

1.2.3: Sources of amylases production………………………………………6

1.2.3.1: Animal and Plant Sources…………………………………………..6

1.2.3. 2: Microbial Source_.._6

1.2.4: Amylases are classified into the following categories

1.2.4.1: – amylase…………………………………………………………8

1.2.4.2: - amylase………………………………………………………....10

1.2.4.3: Glucoamylases……………………………………………………......11

1.2.4.4: Debranching Enzymes……………………………………………….11

1.2.5: Isoamylases…………………………………………………………12

1.2.6: Application of Isoamylase……………………………………………...14

1.2.7: Starch……………………………………………………………………..16

1.2.8: The structure of Isoamylas ……………………………………………21

1.3: Branching Enzyme…………………………………………………… 23

1.4: Factors Effect on Isoamylase Production……………….…………………..23

1.4.1: Effect of pH……………………………………….……………………....23

1.4.2: Effect of Carbon Source……………………………………...………24

1.4.3: Effect of Nitrogen Source……………………………...…………….25

1.4.4: Effect of Temperature……….…………………...……..………...25

1.4.5: Calcium Ions……...…………………………………………………26

CHAPTER TWO: MATERIALS AND METHODS

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.1: Materials……………………………………………………………27
2.1.1: Equipment and Apparatus……………………………………………...27
2.1.2: Chemicals…………………………………………………………….....28
2.1.3: Culture media…………………………………………………………..29
2.1.3.1: Nutrient Broth ………..………………………………………………..29
2.1.3.2: Nutrient Agar....…...……………………………………….…………...29
2.1.3.3: Starch- Nutrient Agar Medium.………….…………...………...…29
2.1.3.4: Production medium of Isoamylase…………….………………………..29
2.1.3.5: Cetrimide agar medium…………………………………………….30
2.1.3.6: King A medium……………..……………………….…………30
2.1.3.7: King B medium…………………………………………….………...31
2.1.3.8: Gelatin medium……………………………………………………...32
2.1.4: Solutions……………..…………………………………………………32
2.1.4.1: Lugal's Solution………………………………………………………32
2.1.4: Bovine Serum Albumin Solution ……..……………….……………………32
2.1.4: Starch Solution……………...……………………………………………….33
2.1.5: Buffers…………………………………………………………………….33
2.1.5.1: phosphate buffer…………………………………………………...33
2.1.6: Dyes………………………………………………………………………33
2.1.6.1: Commassie Brilliant blue G-250…………………………………….33
2.1.6.2: Gram's Stain……………………………………………………….33
2.1.7: Reagents……………………………………………………………….34
2.1.7.1: Catalase reagent ……………………………………………….34
2.1.7.2: Oxidase reagent …………...34
2.2: Methods……….……………………………………………………34
2.2.1: Sterilization methods………………………..………………………34
2.2.1.1: Moist heat sterilization……………………………………………34
2.2.1.2: Dry heat sterilization……..……………………………………….34
2.2.2: Sample Collection……………………………………………………..34

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.2.3: Sample Preparation……….……………………………………...35
2.2.4: Isolation of *Pseudomonas* sp……………………………….…..35
2.2.5: Maintenance of *Pseudomonas* spp…………………………….….35
2.2.5: Short term storage…………………………………………….…..35
2.2.5: Medium term storage……………..……………………………....35
2.2.6: Measurement of bacterial growth……………………….……….36
2.2.7: Identification of Local isolates……………………………....….36
2.2.7.1: Morphological, cultural and physiological characteristics……..…..36
2.2.7.2: Miccroscopical Characteristics……………………………….36
2.2.7.3: Biochemical tests……………………………………………...36

- Catalase Test…………………………………………….………...36
- Oxidase Test……………………………………….…………....37
- Growth on Cetrimide Agar……………………………………...37
- Growth on King A………………………………………………37
- Growth on King B…………………………….………………...37
- Gelatin hydrolysis test…………………………………………..37

2.2.8: Assay of Isoamylase………………………...……………………...38
2.2.9: Determination of Protein Concentration …………………………......38
2.2.10: Determination of Optimum Conditions for Isoamylase Production...39
2.2.10.1: Carbon Sources………………....……………………………….39
2.2.10.2: Concentrations of Carbon Sources…..…...…………………….…….40
2.2.10.3: Nitrogen Source…………………...……………………….……….40
2.2.10.4: Concentrations of Nitrogen Source……………………………….40
2.2.10.5: Phosphate source………………………………………...……..40
2.2.10.6: Concentrations of phosphate………………...…………………..……41
2.2.10.7: Effect of pH……………………..……………………………….41
2.2.10.8: Effect of temperature…………………………………………….41
2.2.11: Isolation and Purification of Isoamylase……..……………………...41
2.2.11.1: Extraction of enzyme……………………………………….…..41

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.2.11.1: Ammonium Sulphate Precipitation...…………….…………....42
2.2.11.2: Precipitation with Ammonium Sulphate…………….……….…...42
2.2.11.3: Dialysis of crude Enzyme…………………………………….42
2.2.11.4: Gel Filtration…...………………………………………………42
2.2.12: Determination of Optimal pH for Isoamylase Activity and Stability…………………………………………………………………...43
2.2.12.1: Effect of pH on Isoamylase Activity…………………….………..43
2.2.12.2: Effect of pH on Isoamylase Stability…………………….………..43
2.2.12.3: Effect of Temperature on Isoamylase Activity……….…………..44
2.2.12.4: Effect of Temperature on Isoamylase Stability….………….…….44
2.2.12.5: Effect of Sodium Azide on isoamylase activity …………………44

CHAPTER THREE: RESULTS AND DISCUSSION

3.1: Isolation of *Pseudomonas* species……………………………………..46
3.2: Identification of bacterial isolates..………………………………….…46
3.3: Screening of the locally isolated Pseudomonas sp. for isoamylase production……………………………………………………………………...48
3.4: Optimal conditions for production of Isoamylase……..……………….49
3.4.1: Effect of carbon source……………………………………………......49
3.4.2: Effect of Carbon Source Concentration ……………………………...50
3.4.3 Effect of Nitrogen Source ……………………………………….…52
3.4.4: Effect of Nitrogen Source Concentration …………………………….53
3.4.5: Effect of Phosphate Source ……………………………………………..54
3.4.6: Effect of pH……. …………………………………………………….57
3.4.7: Effect of Temperature……………………………………………...58
3.5: Isoamylase Purification……………………………………………....59
3.5.1:ammonium sulphate Precipitation…………………………………….…59
3.5.2: Purification of isoamylase by gel filtration ………………………….…60
3.6: Isoamylase Characterization……………………………………...62
3.6.1: Effect of pH on isoamylase Activity……………………………….....62

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

3.6.2: Effect of pH on isoamylase Stability……………………………….64

3.6.3: Effect of Temperature on isoamylase Activity……………………...65

3.6.4: Effect of Temperature on isoamylase Stability……………………...66

3.6.5: Effect of Inhibitors on Isoamylase Activity ………………………...68

CONCLUSIONS AND RECOMMENDATIONS

Conclusions……………………………………………………………...69

Recommendations ………………………………………………………70

References………………………...71

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Lists of Tables

Table No.	Title	Page No.
(1-1)	Activities of Glycosyl hydrolase family 13 enzymes	9
(1-2)	Different amylases enzyme and their sources	12
(3-1)	Local isolates from different environmental Samples	45
(3-2)	Morphological and biochemical characteristics of the locally isolated *Pseudomonas* sp.	46
(3-3)	Activity of isoamylase production from *Pseudomonas* isolates	47
(3-4)	Steps of isoamylase purification from locally isolate *Pseudomonas* sp. H3.	61

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Lists of Figures

Figure No.	Title	Page No.
(1-1)	Chemical structure of Amylose	16
(1-2)	Chemical structure of Amylopectin	17
(1-3)	Formation of Amylose	18
(1-4)	Formation of Amylopectin	19
(1-5)	The structure of Isoamylase	22
(2-1)	Standard Curve for Quantitative Estimation of Protein	39
(3-1)	Effect of different carbon source on Isoamylase Production by *Pseudomonas* sp. H3 after incubation in a shaker incubator (150 rpm) at 28ºC for 24 hr.	49
(3-2)	Effect of different carbon concentrations on isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator (150rpm) at 28ºC for 24 hr.	50
(3-3)	Effect of different nitrogen source on isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator (150 rpm) at 28ºCfor 24 hr.	52

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

(3-4)	Effect of different nitrogen concentrations on isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator(150rpm) at 28ºC for 24 hr.	53
(3-5)	Effect of different phosphate source on production of isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator (150 rpm) at 28ºC for 24 hr.	54
(3-6)	Effect of different concentrations of K_2HPO_4 on isoamylase production by *pseudomonas* sp.H3 after incubation in a shaker incubator (150 rpm) at 28°C for 24 hr.	55
(3-7)	Effect of pH values on isoamylase production by *Pseudomonas* sp. H3 after incubation in a shaker incubator with (150 rpm) at28°C for 24hr.	56
(3-8)	Effect of incubation temperature isoamylase production by *Pseudomonas* sp. H3 after incubation in a shaker incubator (150 rpm) at 28°C for 24 hr.	58
(3-9)	Purification of isoamylase from locally isolate *Pseudomonas* sp. H3.by gel filtration chromatography using Sephacryl G-300.	60
(3-10)	Effect of pH on the activity of partially purified isoamylase produced by *Pseudomonas* Sp. H3.	62
(3-11)	Effect of pH on the stability of partially purified isoamylase produced by *Pseudomonas*. Sp. H3.	63
(3-12)	Effect of temperature on activity of partially purified isoamylase produced by *Pseudomonas* sp.H3.	65

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

(3-13)	Effect of temperature on stability of partially purified isoamylase produced by *Pseudomonas* sp. H**3**	66
(3-14)	Effect of sodium azide on isoamylase activity produced by *Pseudomonas* sp.H3.	67

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Chapter One
Introduction &
Literature Review

This document was created using

SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

1: INTRODUCTION AND LITERATURE REVIEW

1.1: Introduction

Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Gupta *et al.,* 2003).These enzymes are universally distributed throughout the animal, plant and microbial kingdoms. Although Pandy *et al*., (2000) had indicated that isoamylase from fungal and bacterial sources have dominant application in industrial sectors, but bacterial amylases are used more frequently than those of fungal origin in industry (Reedy *et al*., 2003). Microbial isoamylases (E. C. 3.2.1.68) are extracellular amylases that hydrolysis - 1, 6 linkages in amylopectin, and glycogen but not pullulan that hydrolyzed by pullulanase (Harada *et al*., 1968) .Isoamylases differ from pullulanase in their substrate specifity and in their amino acids sequence (Lee and Whelan, 1971 and Myers, 2000).

Isoamylases can be used in industrial production of high maltose syrup, high fructose syrup and amylose, and also used in combination with other starch hydrolyzing enzymes, such as, with cyclodextrin glucanotransferase (CGTase), malto- oligotrehalose synthase and malto- oligo trehalose trehalohydrolase in the production of disaccharides trehalose from liquefied starch (Randleman, 1997). They also useful in the production of resistant starch (Shi *et al*., 2006)

Isoamylase was isolated from numerous bacteria such as *Pseudomonas amylodermosa*, which has the highest producing ability, *Bacillus amyloliquefaciens*, *Escherichia coli* and *Flavobacterium* sp. (Harada, 1948).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Aims of This Study:

1. Isolation and identification of isoamylase producing *Pseudomonas* sp. from different environmental locations.
2. Investigating the ability of the bacterial isolates in production of isoamylase.
3. Determination of the optimal conditions for isoamylase production.
4. Selecting the most efficient *Pseudomonas* isolate in isoamylase production and gained optimum activity and stability for temperature and pH.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

1.2: Literature Review

1.2.1: Genus *Pseudomonas*

Pseudomonas are motile by one or several polar flagella (rarely non motile), Gram- negative, aerobic that utilize glucose, , characterized by straight or slight curved rod but not helical, pili forming, catalase and oxidase are positive, growth occurs from 4°C to 42°C and were chemoorganotrophic (Holt *et al*., 1994).

Members of this genus are classified into five groups based on rRNA homology. These bacteria are clinically important because they are resisting to most antibiotics and they are capable of surviving in severe conditions. They also produce a similar layer that is resisting to phagocytosis. The genus *Pseudomonas* is often encountered in hospital and clinical work because it is a major cause of hospital acquired (nosocomal) infections. Its main targets are immunocompromised individuals, burns victims, and individuals on respirators or with indwelling catheters. Additionally, these pathogens colonize the lung of cystic fibrosis patients, increasing the mortality rate of individuals with the disease. Infection can occurs at many sites and can lead to urinary tract infections, sepsis, pneumonia, pharyngitis, and a lot of other problems (Stolp and GudKari, 1984). Species of this genus like *P. fragi*, *P. putida*, and *P. Fluorescence* produces insoluble pigments like Pyoveridine, which is a yellow pigment.

The genus *pseudomonas* included two pigmentation groups as genetic characters, which are so- called fluorescent and non-fluorescent subgroups. Some species of *Pseudomonas* are plant pathogens like *P.putida*, other are opportunistic pathogenic for human and animals like *P.aeruginosa*, they are also play an important role in spoilage of food, dairy product, meat, poultry and eggs. If spoilage develops after storage under refrigerated condition, *Pseudomonas* usually prevails (Brooks *et al*., 1995).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

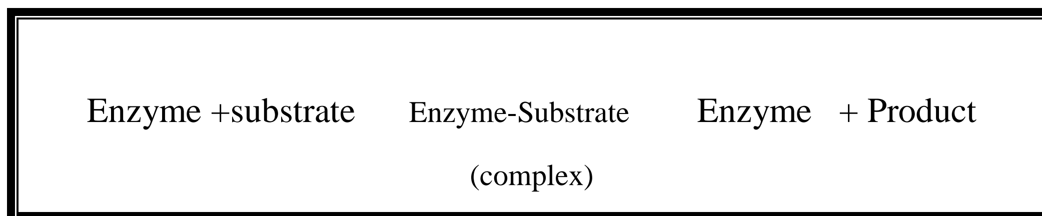
Pseudomonas species characterized by its ability to produce many enzymes like (esterase, alginase, colaginase, elastase and protease) which support these bacteria to infect body tissues (Ding *et al*., 1985; Linker and Evans, 1984; Holder and Neely, 1989; Marne and Vandel, 1998).

Genetics of *Pseudomonas* species have attracted increasing attention from bacterial genetics. The main reasons are their widespread occurrence, their biological properties and simplicity of conditions required for cultivation in laboratories (Stolp and Gudkari , 1981). Of all *Pseudomonas* species far, the best known from the genetically point of view, is *Pseudomonas aerogenosa* of which strain PAO has been the most extensively studied.

1.2.2: **Amylases**

In 1816, Kichhoff was used wheat extract to hydrolyze starch. In 1831 another scientist called Leuchs discovered a substrate in human saliva capable of hydrolyzing starch, this substance was called ptyalin but later Payen and Persoz in 1833 designated it as Diastase. Beijernek in 1895 was the first to call enzymes that hydrolyze starch as amylases (Bernfeld, 1951).

Enzymes are catalysts, that they accelerate reaction rate. Enzymes act by binding to reaction molecules (substrate) to form an enzyme- substrate complex. The site of attachment and the surrounding parts of the enzyme that stress the substrate bonds constitute the enzyme active site.



The reaction is complete when the product forms and the enzyme is released in its original condition.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Enzymes are proteins made of long chains of unique sequence of amino acids that form complex shapes. Although, cells contain many enzymes, each kind of enzyme has a precise structure and function, and each enzyme catalysts a specific reaction. This specifity results from an enzyme's surface is unique and usually couples with only one kind of substrate. Any structural changes will denature the enzyme's effectiveness by altering the active site and slowing down the reaction rate. Therefore, the rate of an enzymatic reaction depends on conditions in it's the immediate environment. These conditions affect the shape of the enzyme and modify the active site and its substrate.

Enzymes are very important in food industry (Reed, 1975). At 19th century, enzymes have used for malt extract fermentation and in 1941 in North America for sweet syrup production instead of wheat flour.

Amylases are enzymes which attack and hydrolyze glycosidic bond in starch, glycogen, and their polysaccharides derivatives. Therefore, the amylase enzymes are called starch hydrolyzing enzyme (Fogarty, 1983).

They can be divided into two categories, endoamylases and exoamylases. Endoamylase catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non reducing end successively resulting in short products (Itkor *et al.,* 1989; Gupta *et al*., 2003). These enzymes are widely distributed in plants, animals and microorganism (Whitaker, 1972; Norman, 1979; Fogary and Kelly, 1980).

1.2.3: Sources of amylases production

Amylases produce from different sources, human, animals, plants and microorganisms like (fungi, yeasts and bacteria). These considered as the main source for amylases production which they use in industrial applications (Pandey *et al*., 2000). Amylases formed into the cell (intracellular) and then release to out (extra cellular) (Priest, 1977).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

1.2.3.1: Animal and Plant Sources

Amylases find in plant kingdom in cereals such as wheat, malt, white corn (Shrma *et al*., 2000, Nonak *et al.,* 2003). Also find in some fruits and juice such as apple juice (Liliana *et al*., 2002). -amylase found in large quantities in plant roots such as Japanese radish (*Rephnus sativus*) (Aibara *et al*., 1978).

While the animal sources amylases found in algae (Sen and Ckakrabarty, 1987) and studied in pancreatic, intestine and muscles of pigs (Zolt waska, 2001) and also find in small quantity in pancrice of cows and sheep (Daxlally, 1983) and crab (Metapogrpsus messor) (Sather, 1969).

1.2.3. 2: Microbial Source

Bacteria, fungi and molds are the main sources for amylase production. They produce from thermophilic actinomyctes like, *Thermoactinomycetes, Thermonospora* these amylases have different usages in industrial application. *Streptomyces* also release amylase as well as degradation enzymes (Ben *et al*., 1999).

An aerobic bacteria, thermophilic and spore forming also produce amylase (isoamylase, Pullalanase, -amylase) such a *Clostridium thermosaccharolyticum*, *Clostridium thermosulforcgenes*, and *Clostridium thermohyrosulforicum* (Fogarty and Kelly, 1980; Madi and Antranikian 1989).

-amylase produce from photosynthetic, thermophilic bacteria such as, *Chloroflexus aurantiacus* by break down maltotriose or maltotertrose (Ratana khanokchai *et al*., 1992).

Eukaryotes also produce amylase such as yeasts, *Saccharomyces cervisiae* and *Endomyces fibuligera* (Reddy and Basappa, 1996). Fungi produce high quantities and important industrially amylases such as *Aspergillus niger* which produces the six types of amylase (One *et al*., 1988).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

The amylase enzyme produced from *Aspergillus oryzae* has named Taka-amylase (Toda *et al*., 1982).A complete molecular model of Taka enzyme consisting of 478 amino acid residues was built with the aid of amino acid sequence data. *A. ustus* also produces -amylase, amyloglucosidase at high temperature and it uses in degradation of ragi in flour (*Eleusine coracana*) and in wheat (*Triticum vulgare*), to produce ragi and lactic acid (Shamala and Sreekantiah, 1987).

Many species of *Bacillus* like *B. amyloliquefaciens*, *B. stearothermophlius* and *B. licheniformis* produce thermophilic amylases that tolerate high temperature may reach to 90ºC, and these amylases have many industrial applications such as food industries, detergents etc.(Cornelis *et al*., 1982;Pretorius *et al*., 1986).

1.2.4: Amylases are classified into the following categories:

1.2.4.1: -amylases

E.C.3.2.1.1, -1, 4 glucan-4-glucanhydrolylase.

These enzymes can be found in plant, animal and in microorganisms (Fogarty, 1983).

A classification system for glycosyl hydrolyses, based on sequence similarity, and has led to the definition that most of the starch hydrolyzing enzymes belongs to the -amylase family or family 13 glycosyl hydrolyses based on amino acid sequence homology according to the classification of (Herrissat, (1991) as shown in table (1-1). These enzymes are endo- split enzymes, which randomly attack the 1, 4 glycosidic bonds in amylose, amylopectin, and glycogen to produce dextrin, maltose, and glucose (Howling, 1989; Piest, 1992).

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Although many have indicated that this enzyme is incapable of attacking the – 1, 6 glycosidic bonds (Fogarty and Kelly, 1980) but many studies showed that some -amylases are capable of hydrolyzing the – 1, 6 glycosidic bond. For example the – amylase produced by *Thermoactinomycetes volgaris* and *Streptococcus bovis* (Tonozuka *et al*., 1993).

Many studies have reported that the mode of action of this enzyme on amylose consists of two steps: in the first step, Maltose is produced while the second step leads to the production of glucose and maltose as products.

During its action on amylopectin, this enzyme produces glucose and maltose in addition to branched chain of -limited dextrin (Walker and Whelan, 1960; Kulp, 1979).

Table (1-1): Activities of Glycosyl hydrolase family 13 enzymes (Reddy *et al*., 2003)

Enzyme	Main Substrate
Amylosucrase	Sucrose
Sucrose phosphorylase	Sucrose
Glucan debranching enzyme	Starch, glycogen
Cyclomaltodextrin glycossyltransferase	Starch
Amylomaltase	Starch, glycogen
Maltopentaoe-forming -amylase	Starch
-amylase	Starch
Oligo-1,6- glycosidase	1,6- - D- glucosidic Linkages in some oligosaccharides
- glucosidase	Starch

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Amylopullulanase	Pullulan
Cyclomaltodextrinase	Linear and cyclomaltodextrin
Isopullulanase	Pullulan
Isoamylse	Amylopectin
Maltotetraose- forming - amylase	Starch
Glucodextranase	Starch
Trehalose-6- phosphate hydrolase	Trehalose
Maltohexaose of forming -amylase	Starch
Maltogenic amylase	Starch
Neopullulanase	Pullulan
Malto-oligosyl trehalase hydrolase	Trehalose
Malto-oligosyl trehalase synthase	Maltose

1.2.4.2: -amylase enzymes

E.C.3.2.1.1, -1, 4- glucan maltohydrolase.

Widely distributed enzymes among plant kingdom members and especially in malted cereals (e.g. Barely and Wheat), Soya bean, and potato (Howling, 1989).

These enzymes had been isolated from different microorganisms such as *Bacillus megateruim, Clostridium* spp., *Bacillus polymyxa* and *Streptomyces* spp. (Shinke *et al*., 1974; Friedberg and Rhodes, 1986; Takekawa *et al*., 1991; priest, 1993).

- Amylases are exo- splitting amylase, which attacks the non-reducing ends in the chains of amylose, amylopectin and glycogen successively resulting in -maltose as final products (Robyt and Whelan, 1968; Kulp, 1979; Fogarty and Kelly, 1980).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

During the hydrolysis of amylopectin and glycogen, this enzyme produces, in addition to -maltose, large quantities of -limit dextrin linked together through -1, 6 glycosidic bonds. This could be attributed to the inability of this enzyme to attack the -1, 6 glucosidic bonds (Whitaker, 1972; Fogarty, 1983; Howling, 1989; Takasaki, 1989; Priest, 1993).

1.2.4.3: Glucoamylases

E.C.3.2.1.3 - 1,4glucan glucohydrolase. Also called amyloglucosidase, Fungi are the main source of this enzyme and it is produced mainly by *Aspergillus* spp., *Rhizopus* spp., and *Corticium rolfsii* (Fogarty, 1983; Sakashi *et al*., 1986; Priest, 1992). These enzymes attack successively the -1, 4 glucosidic bonds in amylose, amylopectin and glycogen to produce -glucose residues from their non-reducing ends. These enzymes can also hydrolyze -1, 3 glycosidic bond but at slower rate than that of -1, 4 glycosidic bond. (Fogarty, 1983; Tonozuka *et al*., 1993).

1.2.4.4: Debranching Enzymes

E.C.3.2.1.6 Glycogen 60 glucanohydrolase.

Enzymes of this group have the ability to attack the -1, 6 glycosidic bonds in amylopectin, glycogen and pullun.

This group consists of two main enzymes: - the isoamylase and pullulanase (also called R-enzyme).The former one acts on amylopectin and glycogen while the later one acts on amylopectin and pullulan.

These enzymes are considered very important tools for studying the conformation of carbohydrate for being highly specific in breaking the branched -1, 6 glycosidic bonds (Kulp, 1979), as in table (1-2), which shows amylases and their different sources.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Table (1-2): Different amylases enzyme and their sources (Harada *et al*., 1984).

Enzyme	Source
-amylase	*Bacillus acidocaldaruis, Bacillus amyloliguefacies, B Bacillus substilis, Aspergillus niger, Aspergillus expansum, Clostridium acetobutylicum. Rhizopus javanicus*
-amylase	*Bacillus cereus, Bacillus substilis*
Amylodglucosidase	*Pencillium spp. Sacchromyces diastaticum, Rhizopus niveus, Aspergillus niger, Aspergillus oryzae.*
Debranching enzyme	*Klebsiela pneumonia, Bacillus endopullulyticus, Pseudomonas spp.*

Isoamylases are distinct from pullulanase in their substrate preferences. They are most active on amylopectin, glycogen and -limit dextrin substrates while they are inactive on pullulan. Pullulanases are active on pullulan while they are inactive on amylopectin (James *et al*., 1999). Both families of debranching enzyme can also be distinguished at the level of their protein sequences (Myers *et al*., 2000).

1.2.5: Isoamylase

According to the Enzyme Commission of the international Union of Biochemistry and Molecular Biology, isoamylase is classified as follows (IUBMB, 2007):

Accepted name: isoamylase

Reaction: hydrolysis of (1-6)- -D-glucosidic branch linkages in Glycogen, amylopectin and their -limit dextrin.

Systematic name: glycogen -1, 6- glucanohydrolase

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Other name(s): debranching enzyme

EC: 3.2.1.68

Isoamylase are extra cellular enzymes that catalyze the hydrolysis of the -1, 6 linkages of amylopectin and glycogen. They are known to be present in yeast (Maruo and Kobayashi, 1951; Gunja *et al*., 1961) and in higher plants (Hobson *et al*., 1951).

Isoamylase was initially identified and described by Harada *et al*., (1968). Its physico-chemical and molecular characteristics were subsequently elucidated and described in several publications (Yokobayashi *et al*., 1970; Yokobayashi *et al*., 1973; Kitagawa *et al*., 1975; Kainuma, *et al*., 1978; Katsuya *et al*., 1998).

The enzyme consists of 750 amino acid residues and has a molecular mass of about 83kDa. The three- dimensional structure of isoamylase was elucidated by the X-ray structure analysis (Katsuya *et al*., 1998). The *pseudomonas* gene *pmi* (also referred to as ISO) encoding isoamylase was cloned and sequenced (Amemura *et al*., 1998; Chen *et al*., 1990).

The enzyme can be used in industrial production of high maltose syrup, high fructose syrup and Amylose. If starch is hydrolyzed by - amylase and - amylase together with isoamylase, a product containing (95–99) % maltose can be generated.

The first bacterial debranching enzyme found to hydrolyze the inter-chain linkages in both amylopectin and glycogen was isolated from a strain "*Aerobacter aerugenosa*" (*Klebsiells pneumonia*) (Abdullah *et al*., 1966). The enzyme turned out to be a pullulanase which differs from isoamylase in its substrate specify (Lee and Whelan, 1971).

Many microorganisms produce isoamylase (Harada, 1984). Among such microorganisms, *Pseudomonas amylodermosa* has the highest producing capability (Harada.T, K. Yokobayashi, and A. Misaki, 1968), *Escherichia coli* (Jeanningro *et al*., 1975), *Bacillus amyloliquefaciens* (Urlaub and W ber

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

1975), and *Flavobacterium* sp. (Sato and Park, 1980). Normally the method for purification isoamylase is varied with the source of the enzyme (Yokobayashi *et al*.; 1975; Chang *et al*., 1986: and Swinton, 1989).

Isoamylases are debranching enzymes that hydrolyze -1, 6- linkages in linked glucan polymers. In plants, they have been shown to be required for the normal synthesis of amylopectin.

Amylopectin is a branched glucan polymer that is a major constituent of plant primary determinant of their structural and physical properties, the fine structure of amylopectin is distinct from that of glycogen in animals and bacteria in that glycogen is randomly branched, the branches are more numerous, and the chains are shorter compared with amylopectin (Gallant *et al*., 1997).

The importance of debranching enzyme activity to amylopectin synthesis was demonstrated by James *et al*., (1995), who showed that the *SU1* locus of maize encodes a starch- debranching enzyme.

1.2.6: Applications of Isoamylase

1-The isoamylase enzyme is used in the production of food ingredients from starch. Isoamylase is used in combination with other starch hydrolyzing enzymes. It hydrolyses starch to linear dextrin, which subsequently degraded to -1, 4 linked gluco-oligosaccharides by - amylase, maltose by - amylase, or glucoseamylase (Rendleman, 1997).

2- In the production of glucose syrup from starch, starch is liquefied using heat-resistant -amylase then; glucoamylase is added to convert the starch hydrolysate to glucose syrup. The addition of isoamylase from *Pseudomonas amylodermos*a results in a syrup with higher glucose content while reducing the amount of added glucoamylase (Norman, 1982).

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

3- Isoamylase also used in the production of maltose and maltitol. The enzyme is added to the liquefied starch after -amylase has been inactivated by heat treatment. At the same time, -amylase is added. The hydrolysis is carried out at an elevated temperature (50-55ºC) and pH of about 5.0 and results in the formation of maltose syrup. The syrup is purified and concentrated and then subjected to crystallization to obtain crystalline maltose. Maltose can then be converted to maltitol by the catalytic hydrogenation. Maltitol is used as a sugar substituted in the production of non-cariogenic hard candies, chewing gum, and other confectionary (Hirao *et al*., 1998).

4- Isoamylase is also used together with cyclodextrin glucanotransferase (CGTase), malto-oligosyltrehalose synthase, and malto- oligosyl trehalose trehalohydrolase in the production of disaccharides trehalose from liquefied starch. The reaction product is trehalose syrup, which is subsequently purified and concentrated. Trehalose is used in food (for example, in bakery goods, beverages, confectionery, and breakfast cereals) as a textuurizer, stabilizer, humectants, and sweetener.

5-Isoamylase can also be used in conjugation with CGTase to enhance the production of cyclodextrin from starch. Cyclodextrins are used as encapsulating agents for food additives, flavor, and vitamins (Randleman, 1997).

6-Poorly starch or non- digestible starch (resistant starch) may obtained by processing low amylase potato starch with isoamylase. After the reaction has been completed, the enzyme is inactivated by heat or by lowering the pH to about 2.0. Then, pH is increased to 6-7 and the solution is kept at room temperature for 16 hrs. During that time, the debranching short length- chain

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- 1, 4- glucans retrograde and crystallize. The obtained crystalline cake of linear chain - 1, 4- glucans is recovered by filtration or by air- dried. The final product contains about 75 % resistant starch that is not digested by pancreatic amylase at 37ºC during 120 min (Shi *et al*., 2006).

1.2.7: **Starch**

Starch is composed of two polymers amylopectin and amylose. Natural starch is usually 10-30% amylose and 70-90% amylopectin. **Amylose** molecules are linear chains of D-glucose in - 1, 4 linkages as shown in fig. (1-1).

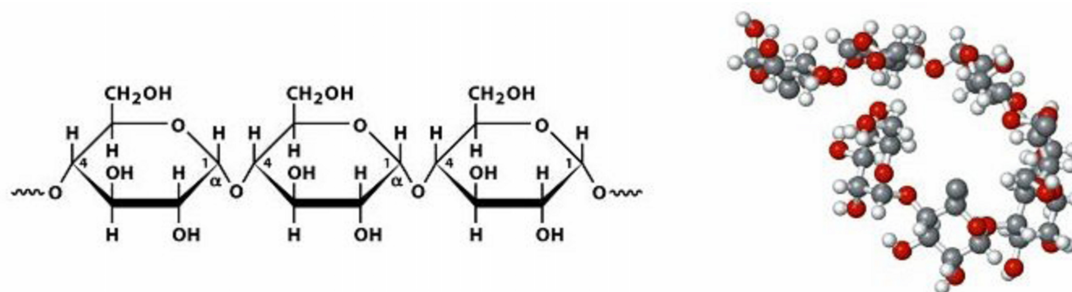


Figure (1-1): Chemical structure of amylose.

Amylopectin is a highly branched chain of glucose monomers as shown in figure (1-2). Most of the glucose linkages in amylopectin are - 1, 4 as for amylose, whereas the branches are -1, 6. Branches occur ~every 12- 30 residues (Manners, 1989).

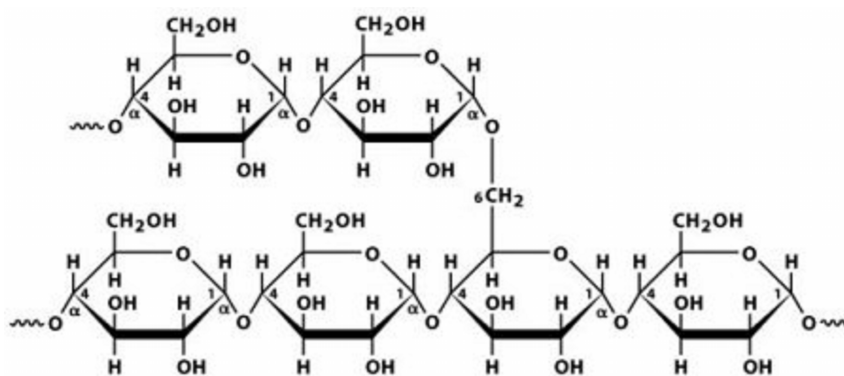


Figure (1-2): Chemical structure of Amylopectin

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Both amylose and amylopectin synthesis begins with synthesis of ADP- glucose (is established as the sole precursor for starch synthesis) from glucose -1- phosphate and ATP by ADP- glucose pyrophosphorylase (aslimiting step in the pathway), as shown in the figure (1-3), (Tasi and Nelson, 1966; Lin *et al*., 1988; Smith *et al*., 1989; Ball *et al.,* 1991; Muller- Rober *et al*., 1992; Stark *et at.,* 1992).

This enzyme is heterotetromar composed of two distinct subunits both in higher plants and in Chlmydomonas (Iglesais *et al*., 1994). In most cases ADP- glucose pyrophosphorylase is activated by 3- phosphoglycerate and inhibited by orthophosphate (Chosh and Preiss, 1966; Preiss, 1993; Peiss and Sivak, 1996). Next starch synthase catalyzes the formation of glucose of an -

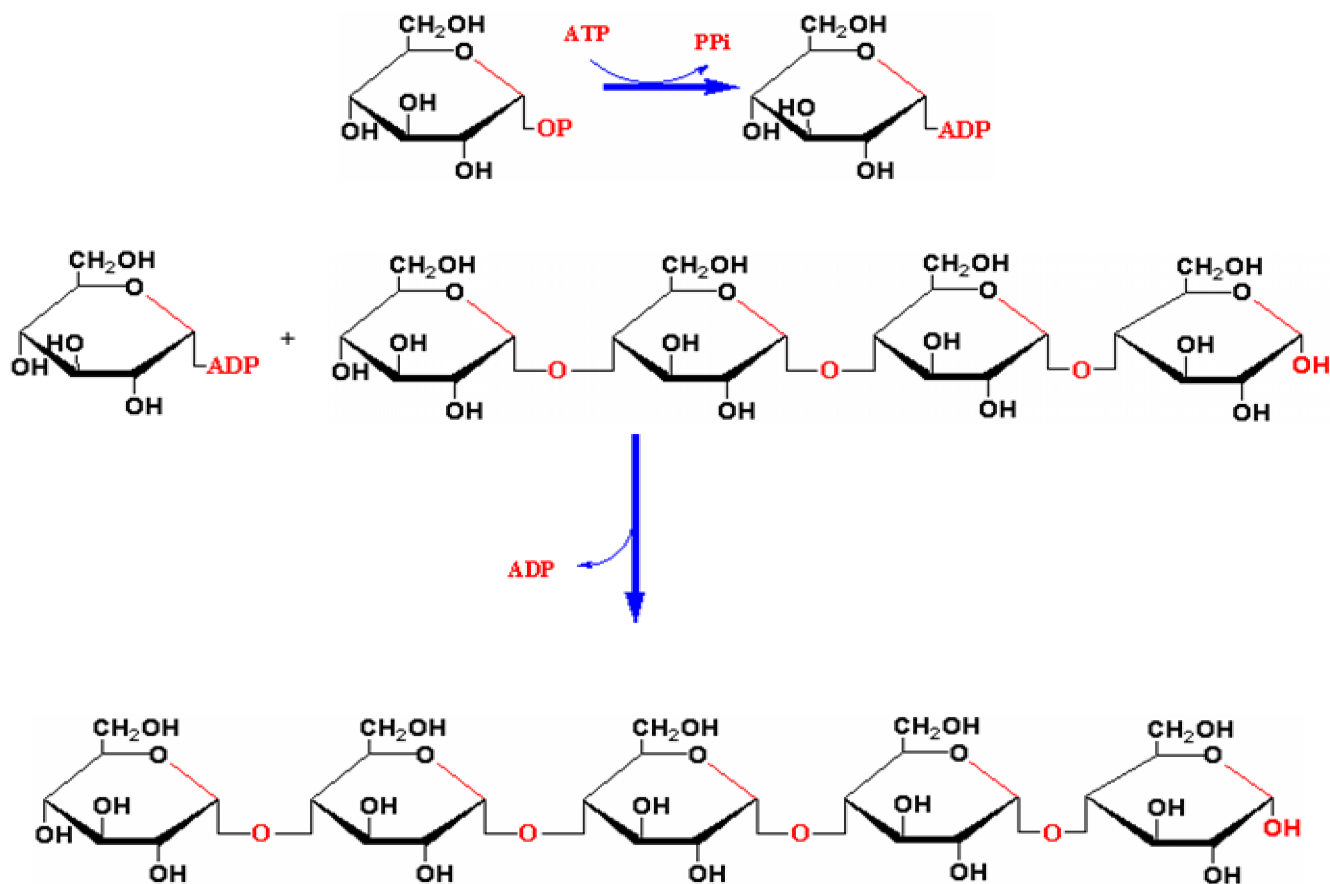


Figure (1-3): Formation of Amylose

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

1, 4 linkages between the non- reducing end of a preexisting glucose chain and the glucosyl moiety of ADP- glucose with release of ADP. Finally, the - 1,6 linkages of the branches are synthesized by starch branching enzyme that hydrolyzes an - 1,4 linkage within the chain and then forms - 1,6 linkage between the reducing end of the spliced glucan chain and another glucose residues (Smith, 1999) as shown in the figure (1-4).

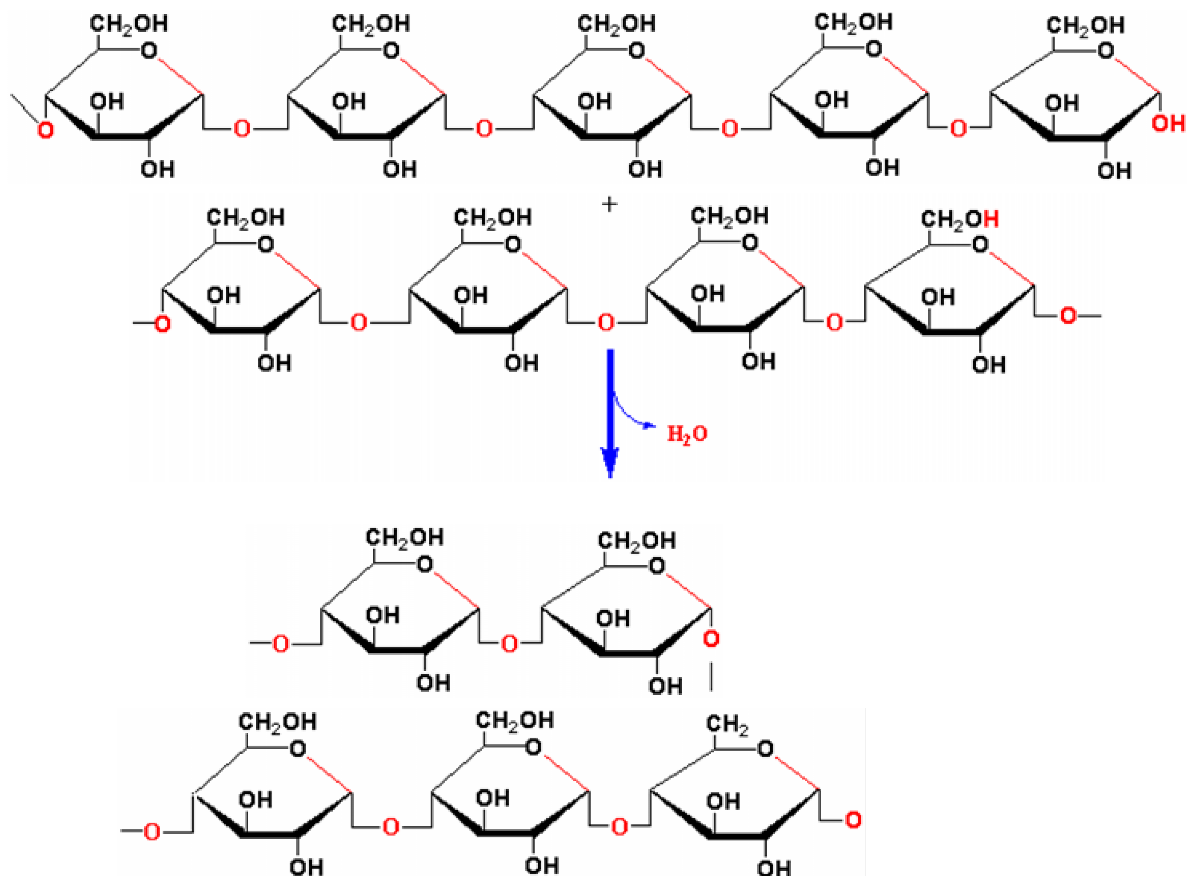


Figure (1-4): Formation of Amylopectin

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Two models have been proposed to explain the role of debranching enzymes in starch synthesis.

1- The glucan- trimming model

Suggests that debranching enzyme work on irregularly branched preamylopectin in the plastid stroma and at the edges of the starch granule (Ball *et al*., 1996; Myers *et al*., 2000). As the preamylopectin increases in size, debranching enzymes cleave the widely spaced branches and generate a regularly branched glucan structure that is “competent to crystallize”. At the outer edges of the starch granule, the glucan may not be crystalline, and the glucan chains of amylopectin there are branched by starch- branching enzymes. This extensive branching inhibits further crystallization. Debranching enzymes hydrolyze some of these - 1, 6 branches, especially those that are widely spaced, to produce more regions of crystallization- competent glucan. This model proposes that the extensive branching of preamylopectin followed by trimming of the outer glucan chains to produce regions of glucan with the competence to crystallize may explain the regular distribution of - 1,6 branch clusters in amylopectin. In debranching enzyme mutants, preamylopectin may become so branched its outer edges that further extension is prevented, limiting amylopectin synthesis and the growth of starch granules. Preamylopectin in the stroma may become so branched that crystallization is prevented altogether and soluble phytoglycogen accumulates instead.

2- The second model the water soluble polysaccharide-clearing model, was suggested by Zeeman *et al*., (1998). This model proposes that the principle substrate for debranching- enzyme during starch synthesis is branched, water soluble glucan. This glucan is synthesized in the plastid stroma by starch synthases and starch- branching enzymes, and its accumulation inhibits starch synthesis because it is an alternative, competitive sink for ADP-Glu. In

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

mutants with reduced debranching enzyme activity, branched water- soluble glucans are elaborated at the expense of amylopectin and phytoglycogen accumulates.

The difference between these two models is the nature of the glucan that is primary target of debranching enzyme activity during starch synthesis and consequently whether debranching enzymes play a direct or an indirect role in amylopectin synthesis (Hasnain *et al*., 2003).

1.2.8: The Structure of Isoamylase

The three dimensional structure of isoamylase from *Pseudomonas amylodermosa*, which hydrolyzes - 1, 6 glucosidic linkages of amylopectin and glycogen has been determined by X-ray structure analysis. The enzyme has 750 amino acids and a molecular weight of 83 KDa. The structure of isoamylase is organized into three domains: amino-terminal domain, a carboxyl- terminal domain, and a central (,) barrel catalytic domain (Baba *et al*., 1999 and Romeo *et al*., 1988).

There are two catalytic loops connecting the domains, the loop that connects the NH_2- terminal domain to the (,) barrel is 18 residues long (223- 240), and the loop joining the end of the (,) barrel to the COOH- terminal domain is 13 residues long (613-625) (Katsuya *et al*., 1998).

There is also an extra domain of approximately 60 residues, named domain B (Yoshi *et al*., 1998). This domain is present in - amylase and Cycloglucanotransferase, but not in branching enzyme while the COOH- terminal domain and NH_2 and COOH domain are similar to the NH_2 and COOH terminal domains in branching enzymes(Matsuura *et al*., 1984).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Figure (1-5): The Structure of Isoamylase

The tertiary structure of isoamylase has been determined by X-ray crystal analysis, in this figure, spirals and plate like arrows represent -helices and -strands, respectively, of the structure. The overall structure of the isoamylase molecule is similar to those of -amylases and CGTases. The active site of isoamylase is located at the center of the molecule. The -1, 6-glucosidic linkages of amylopectin or glycogen are hydrolyzed in the active site of isoamylase (Katsuya *et al*. 1998).

1.3: Branching Enzyme

Branching enzymes (1, 4-glucan: 1, 4-glucan 6- glucosyl transferase branch c; E.C. 2.4.1.18) has an important role in the determination of the structure of starch in plants and of glycogen in animals and bacteria as it catalyzes the formation of the - 1, 6 branch points in these polysaccharides.

This is achieved by cleavage of the -1, 4 glycosidic linkage, yielding a non- reducing end oligosaccharides chain, and subsequent attachment of the oligosaccharides to the -1, 6 position (Di Mauro, 1994 and Chen, 1995).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Branching enzyme belongs to the amylase family of enzymes (Bada, 1991; Romeo *et al*., 1988). Members of this group include - amylase, pullulanase/isoamylase, cyclodextrin glucsnotransferase (CGT), and branching enzymes.

X-ray structure of members of this family showed that they have a central barrel that contains the catalytic residues. This domain is similar to the barrels of other members of the family. Also, the CooH-terminal of isoamylase and amylase, while the NH2-terminal domain of BE is similar to the NH2- terminal domain in isoamylase (Matsuura *et al.,* 1984).

1.4: Factors Effecting Isoamylase Production

1.4.1: Effect of pH

The pH of the media is very crucial factor because it affects the growth of the microorganisms and their activities. It also has an effect in metabolism and enzyme synthesis (Lonsane and Ramish, 1987). The production and activity of isoamylase, like other enzymes, are greatly influenced by the pH of the media. The catalytic site of the enzyme contains ionic groups, which should be in the appropriate form to keep the shape of the catalytic site and to facilitate the binding with reactants molecules (Segal, 1975). Therefore, any deviation from the optimum range will affect the activity of isoamylase.

Bender *et al*., (1961) had showed that the medium containing (peptone, yeast extract, Kcl, $MgSO_4.7H_2O$, maltose, $FeSO_4.7H_2O$) was inoculated with *Aerobacter aerogene* at 30ºC for 20 hrs. Then it was inoculated into liquid medium which composed of $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, Kcl, $FeSO_4.7H_2O$ and liquefied starch at 30ºC and pH ranging from 5 to 7 for 48hrs. The activity of the resulting isoamylase was 138unit/ml, and when added sodium hydroxide to the liquid medium during the culture and cultured

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

under the same condition, the activity of the resulting isoamylase was 190unit/ml.

While Wallenfels *et al*., (1966) had demonstrated that the medium containing ammonium salts as nitrogen source and liquefied starch as a carbon source under the condition of ph 5-8 will produce a large amount of isoamylase.

Takahshi *et al*., (1996) had demonstrated that the optimum pH for isoamylase production from a strain of *Flavobacterium* isolated from soil was 6.0 while the maximum enzyme production was with the medium containing (3%(w/v) soluble starch, 3% Soya bean protein, 0.01% L-glutamate, 0.14% KH_2PO_4, 0.02% $MgSO_4.7H_2O$ and 0.01% $Cacl_2.2H_2O$) for 48hrs at 30℃ was pH (5.3).

1.4.2: Effect of Carbon Source

In order to enhance the microorganisms to produce the desired enzyme, the media, where the microorganism is grown, must be supplemented with appropriate carbon source. Different carbon sources can be used but each microorganism has only one or only few proper carbon sources since the production of this enzyme in many microorganisms occurs by induction (Good fellow *et al*., 1987).

Bender *et al*., (1961) had studied the effect of maltose, maltotriose and pullulan on the production of isoamylase from *Aerobacter earogenes*, and he found that the media containing maltose gave the highest level of production of this enzyme. Wallentels *et al*., (1966) showed that 5-10 different carbon sources had tested on *Aerobacter aerogenes*, liquefied starch gave the highest productivity of the isoamylase.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

1.4.3: Effect of Nitrogen Source

The nitrogen source is one of the basic elements for production of isoamylase. It is supplemented to the media in the form of inorganic salts or as organic sources of nitrogen (Kuo and Hartman, 1966).

Wallentels *et al*., (1966) had tested the effect of peptone, yeast extract and more than 0.2% of ammonium salts on the production of isoamylase, and he found that ammonium salts gave the highest productivity of isoamylase.

1.4.4: Effect of Temperature

Temperature is an important factor for growth and enzyme production. Each microorganism has a temperature for its growth and production and there might be compatibility between the optimum temperature of growth and optimum temperature for the enzyme production (Priest, 1993).

Bender *et al*., (1961) found that optimum temperature for isoamylase from *Aerobacter erogenes* is about 30ºC while the temperature for production was 40ºC. Takahashi *et al*., (1996) found that a strain of *Flavobecterium* also produce isoamylase at optimum temperature 30ºC.

1.4.5: Calcium Ions

Calcium ions are radical factors in the stability of the isoamylase enzyme. Machius et al., (1998), showed that the calcium- binding site conserved in all enzymes forms part of an unprecedented linear triadic array, with two Ca ions (Ca I &Ca II) are flanking a central Na ions (Ca- Na-Ca metal triad). In the case of bacterial isoamylase, the metal triad and an additional Ca ion (Ca II) contribute to the increased thermostability.

Isoamylase was stabilized and stimulated by Ca^{2+}, (Takahashi, 1996), the enzyme undergo reversible transition from the active to the inactive state upon removal of Ca ions with chelating reagents such as EDTA, and because

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

the distance of the Ca from the catalytic center precludes direct involvement in catalysis, it's role is assumed to be structural (Vallee *et al*., 1959).

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Chapter Two
Materials &
Methods

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2: Materials and Methods

2.1: Materials

2.1.1: Equipment and Apparatus

Table (2-1): The following equipments and apparatus were used in this study.

Apparatus	Origin
Autoclave	Karl Kolb (Germany)
Refrigerator centrifuge	Harrier (U.K)
Distillator	Gallenkamp (England)
Hot plate with magnetic stirrer	Gallenkamp
Incubator	Gallenkamp
Electrical Oven	Gallenkamp
pH-meter	Gallenkamp
Sensitive balance	Mettler (Switzerland)
Shaker incubator	Sartorius (Germany)
UV- Spectrophotometer	GFL (Germany)
Spectrophotometer	Aurora instrument Ltd. (England)
Vortex	Stuart scientific (U.K)
Water bath	Memmert (Germany)
Micropipette	Witey (Germany)
Portable centrifuge	Hermle laboratechnik (Germany)

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.1.2: Chemicals

Table (2-2): Chemicals used in this study.

Chemical	Origin
Agar, Tryptone, Starch, Sucrose, Lactose, Crystal violet, Safranin, Yeast extract	Difco (U.S.A)
Iodine crystals	Fluka (Switzerland)
Maltose, Glucose, Sodium Hydroxide, Ortho phosphoric acid, Hydrochloric acid (HCl), Ammonium sulfate, Methanol, K_2HPO_4, KH_2PO_4, Absolute ethanol)	BDH (England)
Potassium Iodine	BDH (England)
Glycerol, Peptone, Bovine serum Albumin, Sephacryl G-300	Sigma (U.S.A)

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

2.1.3: Culture Media

2.1.2.1: Nutrient Broth (Sigma USA)

This medium was prepared according to the instruction of manufacturer company.

2.1.3.2: Nutrient Agar (Sigma USA)

This medium was prepared according to the instructions of manufacturer company.

2.1.3.3: Starch – Nutrient Agar Medium

This medium was prepared by supplementing nutrient agar with 1% soluble starch and sterilized by autoclaving.

2.1.3.4: Cetrimide agar medium (Stolp and Gudkari, 1984)

This medium composed of the following components:

Component	Weight (g)
Peptone	20
$MgCl_2$	1.5
K_2SO_4	10
Cetrimide	0.3
Agar	15

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2, then volume completed to 1000 ml and sterilized by the autoclaving.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

2.1.3.5: King A medium (Starr, *et al*., 1981)

This medium is consists of the following components:

Component	Weight (g)
Peptone	20
K_2 SO_4	10
$MgCl_2$	1.4
Glycerol	10
Agar	15

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2, then volume was completed to 1000 ml and sterilized by the autoclaving.

2.1.3.6: King B medium (Starr *et al*., 1981)

This medium composed of the following components:

Component	Weight (g)
Peptone	20
$MgSO_4.7H_2O$	3.5
K_2SO_4	1.5

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Glycerol	10
Agar	15

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2, then volume was completed to 1000 ml and sterilized by the autoclaving.

2.1.3.7: **Gelatin medium** (Stolp and Gudkari, 1984)

This medium is composed of the following components:

Component	Weight (g)
Gelatin	4
KH_2PO_4	0.5
K_2HPO_4	0.5
Glucose	0.05

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2, then volume was completed to 1000 ml and sterilized by the autoclaving.

2.1.3.8: Production medium of Isoamylase (Harada *et al.*, 1968)

This medium composed of the following components:

Components	Weight (g)
Maltose	20

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Sodium glutamate	4
Diammonium hydrogen phosphate	3

These components were dissolved in 950 ml of distilled water, pH was adjusted to 6.0 then volume was completed to 1000 ml and sterilized by autoclaving.

2.1.4: Solutions

2.1.4.1: **Lugal's Solution** (Fad, 1976)

It was prepared by dissolving 10g of potassium iodine in 25ml of distilled water and 5g of iodine was then added with stirring until completely dissolved. The volume was completed in a volumetric flask to 100 ml with distilled water and kept in a dark bottle.

2.1.4.2: Bovine Serum Albumin (BSA) Solution (0.1 %)

This solution was prepared by dissolving 0.1 g of bovine serum albumin in a suitable quantity of distilled water, and then the volume was completed to 100 ml with distilled water in a volumetric flask.

2.1.4.3: Starch Solution (1 %)

This solution was prepared by dissolving 1g of soluble starch in 100 ml of distilled water.

2.1.5: Buffers

2.1.5.1: Phosphate buffer

This solution was prepared by mixing 196 ml of solution A and 804 ml of solution. Both solutions were prepared as the following:

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

Solution A, it was prepared by dissolving 9.0 g of potassium dihydrogen phosphate in 950 ml of distilled water, then volume was completed with D.W. to 1000 ml in a volumetric flask.

Solution B, it was prepared by dissolving 23.8 g of dipotassium hydrogen phosphate in 950 ml of distilled water, then volume was completed with D.W. to 1000ml in a volumetric flask.

2.1.6: Dyes

2.1.6.1: Commassie Brilliant Blue G-250 (Bradford, 1976)

This dye was prepared by dissolving 100mg of Commassie Brilliant Blue G-250 in 50 ml of 95% ethanol, then 100 ml of 85% orthophosphoric acid was added. The volume was then completed in a volumetric flask to 1000ml with distilled water.

2.1.6.2: Gram's Stain

It was prepared according to the procedure described by Simbert and Krieg (1981).

2.1.7: Reagents

2.1.7.1: Catalase reagent (Atlas, *et al*., 1995)

This reagent was prepared to be consists of 3 % hydrogen peroxide.

2.1.7.2: Oxidase reagent (1%) (Koneman, *et al*., 1992)

This reagent was prepared by dissolving 1gm of N, N, N; N-tetra-methyl-p-diamine dihydrochloride in 100 ml of distilled water and kept in a dark bottle at 4°C.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

2.2: Methods

2.2.1: Sterilization methods (Colline and Lyne, 1987)

2.2.1.1- Moist heat sterilization: Culture media were sterilized by the autoclave at 121ºC for 15min. and (15Ib/In), while carbon sources were sterilized for 5 min. under the same conditions.

2.2.1.2- Dry heat sterilization: Glasswares were sterilized in the oven at 180ºC for 2 hours.

2.2.2: Sample Collection

Samples of soil and sewage were collected from different locations in Baghdad governorate (Al-Dura, Palestine Street, Al-Jadirya, Al-Karada, Al-Togar Q.) during the period from 1/11/2007 to 30/12/2007. They were packed in labeled nylon bags, and transferred to the laboratory for analysis.

2.2.3: Sample Preparation

One gram of each sample was added to 9ml of distilled water in a sterile test tube, mixed vigorously, and let to stand for a few minutes. Serial dilutions were made, 100 µl of the suitable dilution was spread by loop on nutrient agar plates, then plates incubated at 28ºC for 24 hrs.

2.2.4: Isolation of *Pseudomonas* sp.

Colonies present on nutrient agar medium after incubation were replica-plated on cetrimide agar medium (as a selective medium for ***Pseudomonas*** sp.) and incubated at 37ºC for 24 hrs (Holt *et al*., 1994).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

2.2.5: Maintenance of *Pseudomonas* isolates

Maintenance of *Pseudomonas* spp. was conducted according to Maniatis *et al*. (1982), as the following:

2.2.5.1: Short -term storage

Bacterial isolates were maintained for few weeks on nutrient agar plates. The plates were slightly wrapped in parafilm and stored in the refrigerator at 4 ºC.

2.2.5.2: Medium -term storage

Bacterial isolates were streaked on nutrient agar slants and incubated at 28ºC for 24 hour. Isolates of bacteria were maintained on these slants for few months. Such medium was prepared in screw-capped vials containing 15-20 ml of the medium. After that, the slants were taken and wrapped with parafilm and stored at 4ºC.

2.2.6: Measurement of Bacterial growth

Growth of bacteria was monitored by measuring the optical density of nutrient broth medium after incubation for 24 hrs at 28C in spectrophotometer at 600 nm.

2.2.7: Identification of bacterial isolates

2.2.7.1: Cultural Characteristic

Shape, color, viscous growth, size and edge of the colonies were described according to Collee *et al*., 1996 on plate nutrient agar after incubation of plates at 28ºC for 24 hr.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.2.7.2: Microscopical Characteristics (Harley and Prescott, 1996)

A touch of each isolate colony was transferred and smeared on a clean slide and fixed by heat. The smear was stained crystal violet (for 1 minute) and excess stain was washed then treated with iodine for 1min.) and the washed with distilled water, then decolorized with absolute alcohol and the excess was washed, and then counterstained with safranine (for 30 sec.) and the excess was washed, then examined under light microscope

2.2.7.3: Biochemical tests

•**Catalase Test** (Maza *et al*., 1997)

This test was performed by adding few drops of hydrogen peroxide solution (3%) on a single colony of each isolate grown on nutrient agar. Production of gaseous bubbles indicates a positive result.

•**Oxidase Test (**Harrely and Prescott, 1996)

Filter paper was saturated with oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride), then single colony of each bacterial isolate was transferred onto the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive result.

•**Growth on Citrimide Agar** (Stolp and Gadkari, 1981)

This test was used as selective medium for *Pseudomonas* sp.; bacterial isolates were streaked on citrimide agar plates and incubated at 37°C for 24hr.

•**Growth on King A medium (**Cruickshank *et al*., 1975)

This test was performed to study the ability of bacterial isolates in production of pyocyanin pigment. Single colony of each isolate was streaked on kings A medium and incubated at 37ºC for 24hr.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

•**Growth on King B medium** (Cruickshank *et al*., 1975)

This test was performed to study the ability of bacterial isolates in production of fluorescin pigment. Single colony of each isolate was streaked on King B medium and incubated at 37ºC for 24hr. Then the plates were exposed to U.V. light to detect the presence of fluorescin.

•**Gelatin hydrolysis test** (Atlas *et al*., 1995)

This test was performed to demonstrate the ability of bacterial isolates in hydrolyzing gelatin (collagen).

Nutrient -gelatin tube were inoculated with bacterial isolates and incubated at 37ºC for 24-48 hr., then tubes were placed in a refrigerator for 30-60 minutes. Liquefaction of nutrient agar-gelatin media indicates a positive result.

2.2.8: Assay of Isoamylase

Assay of isoamylase activity was achieved according to Kobayashi, (1976), as follows:

The reaction mixture consisted of 5ml of 1% soluble glutinous rice starch solution, 1 ml of 0.5M acetate buffer (pH 3.5), and 1ml of enzyme solution. After incubation at 40°C for 1 hr, 1 ml of the reaction mixture was withdrawn and mixed with 1 ml of 0.01 N iodine solution. Water was then added to make a total volume of 25 ml. The increase in the optical density at 610nm was measured using a photocell (1cm wide) and the enzyme activity was determined as the following equation mentioned by Kobayashi (1967):

$$\text{Enzyme Activity (U/ml)} = \frac{\text{Ab (610) nm}}{1 \times 0.01 \times 60}$$

Ab = Absorbency

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

The enzyme unit of isoamylase was defined as the increase in absorbance up to 0.01 which is directly proportional to the enzyme concentration Takahashi, 1977).

2.2.9: Determination of Protein Concentration

Protein concentration was determined according to the method described by Bradford (1976) by using Coomassie blue G-250 and Bovin serum albumin (BSA) to draw standard curve and estimate protein in culture filtrate, as follows:

Different concentrations of BSA (20, 40, 60, 80, and 100µg/ ml) were prepared from BSA stock solution, then 20µl of each concentration was transferred to a sterile test tube in addition to 50µl of 1 N NaOH, and 1 ml of coomassie blue reagent. After mixing well and left to stand at room temperature for 5 min absorbance was measured at 595 nm, then the relationship between BSA concentrations and absorbencies was plotted as shown in figure (2-1).

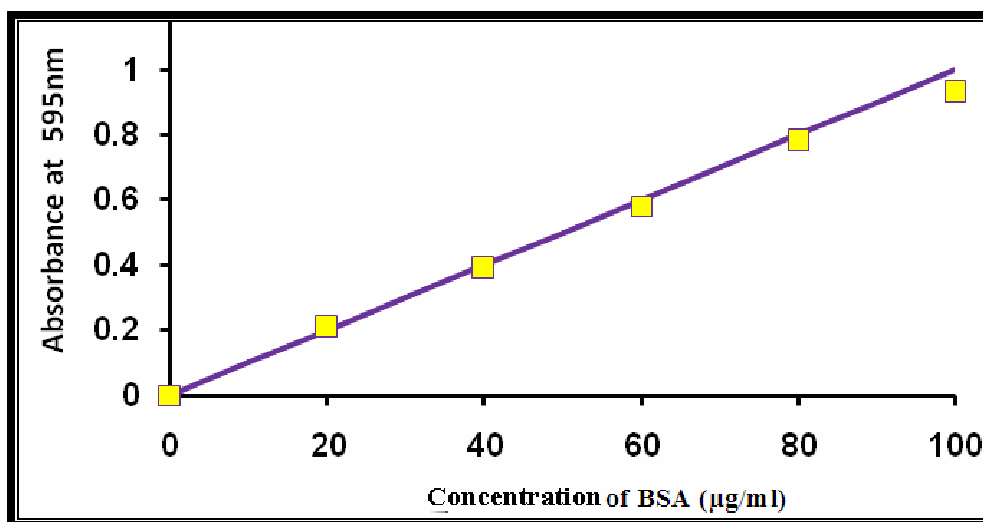


Figure (2-1): Standard curve of BSA against the absorbance at 595 nm to determine protein concentration.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.2.10: Screening of *Pseudomonas* Isolates for Isoamylase Production

In order to select the efficient isolate in isoamylase production, the ability of these isolates in enzyme production was assayed using production medium (described in2.1.3.8) by determining enzyme activity (U/ml) of the crude enzyme in culture filtrate using enzymatic assay procedure mentioned in (2.2.8). Results indicated in table (3-3) showed that two isolates were more efficient in enzyme production more than other isolates.

2.2.11: Determination of Optimum Conditions for Isoamylase Production

In order to determine the optimum conditions for isoamylase production, the selected isolate of *Pseudomonas* spp., the most efficient isolates in enzyme production, was used to determine the optimum conditions for isoamylase production, as follows:

2.2.11.1: Carbon Sources

Six carbon sources (fructose, lactose, sucrose, galactose, glucose and maltose) were used to determine the optimum for isoamylase production by the selected isolate of *Pseudomonas* sp. These carbon sources were added in a concentration of 2 % (w/v).

2.2.11.2: Concentration of Carbon Source

Different concentrations (0.5, 1, 1.5, 2, 2.5, and 3%) of maltose were used to determine the optimum in isoamylase production by the selected isolate of *Pseudomonas* spp.

2.2.11.3: Nitrogen Sources

Five nitrogen sources (tryptone, peptone, yeast extract, ammonium chloride and ammonium nitrate) were used to determine the optimum in

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

enzyme production by the selected isolate of *Pseudomonas* spp., These sources were added in a concentration of 0.4 % w/v to the production medium and incubated in a shaker incubator (150 rpm) at 28ºC for 24hrs.

2.2.11.4: Concentrations of Nitrogen Source

Different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5% w/v) of peptone were used to determine the optimum in isoamylase production by the selected isolate of *Pseudomonas* spp.

2.2.11.5: phosphate sources

In order to determine the optimum phosphate source for isoamylase production by the selected isolate of *Pseudomonas* spp, different phosphate sources (K_2HPO_4, KH_2PO_4, and Na_2HPO_4, NH_2HPO_4, and diammonium hydrogen phosphate) were used to supplement the production medium in a concentration of 0.3%.

2.2.11.6: Concentrations of phosphate source

Production medium was supplemented with different concentrations of the optimum phosphate source (0.1, 0.2, 0.3, 0.4 and 0.5%) of K_2HPO_4 determine the optimum concentration for isoamylase production isoamylase by the selected isolate of *Pseudomonas* spp. Flasks were then incubated in a shaker incubator (150rpm) at 28ºC for 24 hrs.

2.2.11.7: Effect of pH

In order to determine the optimum pH for production of isoamylase by the selected isolate of *Pseudomonas* spp., different pH values (4, 5, 6, 7 and 8) were used. Medium was incubated in a shaker incubator (150rpm) at 28ºC for 24 hrs.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.2.11.8: Effect of Temperature

Different incubation temperatures (25, 28, 30, 35 and 40°C) were used to incubate the selected isolate in a shaker incubator (150rpm) for 24 hours.

2.2.12: Isolation and Purification of Isoamylase

2.2.12.1: Extraction of enzyme

Extraction of isoamylase was achieved according to Harada *et al.* (1968), by inoculating 200 ml of the production medium with 200µl of fresh culture of the selected isolate and incubated under the optimum conditions for isoamylase production. After incubation, cultures were centrifuged at 6000 rpm for 20 min. Enzyme activity and protein concentration were then estimated in the crude filtrates.

2.2.12.2: Ammonium Sulphate Precipitation

This step was done by adding ammonium sulphate to the crude enzyme gradually with continuous mixing at saturation ratios ranging from 40%- 80%, then the solution was centrifuged at 6000 rpm for 20 min. After each addition, enzyme activity and protein were determined for each precipitation step. This procedure was applied for each precipitation ratio after dissolving in 2 ml of phosphate buffer (pH 6.0).

2.2.12.3: Dialysis of Crude Enzyme

The precipitated enzyme was further purified in dialysis tube to remove the ammonium sulphate. Dialysis tube was prepared by soaking in distilled water for 24 hr then the concentrated enzyme solution was placed in the dialysis membrane, to allow salt to pass out of the bag while enzyme was retained.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

2.2.12.3: Gel Filtration

The final step of isoamylase purification was gel filtration through Sephacryl-300. Suspension of sephacryl-300 was packed after degassing for 10 min. into the column. After setting of the column matrix size to 1.6*50 cm; it was equilibrated with phosphate buffer solution (pH 6.0). Crude enzyme obtained after the dialysis step was added to the column in a concentration of 3 mg/ml, and eluted with phosphate buffer (pH 6.0). Three ml fractions were collected in test tubes during 6 seconds. Protein concentration in each fraction was monitored spectrophotometrically at 280 nm. Fractions of the protein peaks were assayed for isoamylase activity. Fractions containing enzymatic activity were collected and stored in refrigerator for further characterization.

2.2.13: Determination of optimal pH for Isoamylase Activity and Stability

The following main characteristics of the partially purified isoamylase were determined and as following:

2.2.13.1: Effect of pH on Isoamylase Activity

Using buffer solution described in (2.4.1) which had been distributed evenly into aliquots, the pH was adjusted in each one according to the required value as described by Eliss and Morrison (1982). 0.1 ml of the purified isoamylase was added to 0.9 ml of buffer solution with different pH value ranging from 4 to 8 in a medium containing starch as substrate, then isoamylase assay was performed. Activity of isoamylase was plotted against pH value to determine the optimal pH for isoamylase activity.

2.2.13.2: Effect of pH on Isoamylase Stability

Equal volume of partially purified enzyme and buffer solution (2.4.1) with pH ranging from 4 to 8 were incubated in a water bath at 35ºC for 30

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

min. then tubes were transferred immediately into an ice bath. The enzymatic activity for each tube was determined as it described in (2.11).

The remaining activity (%) for the isoamylase was plotted against the pH value to determine the stability of the isoamylase.

2.2.13.3: Effect of Temperature on Isoamylase Activity

0.1 ml of the purified isoamylase was added to 0.9 ml of buffer solution with various temperatures (25, 30, 35, 40, 45, 50, 55 and 60˚C) in a medium containing starch as substrate, then isoamylase assay was performed. Activity of isoamylase was plotted against the temperature value to determine the optimal temperature for isoamylase activity.

2.2.13.4: Effect of Temperature on Isoamylase Stability

The partially purified enzyme was incubated in a water bath at 35°C for different times (10, 20, 30, 40, 50 and 60min.), then immediately transferred into an ice bath. The remaining activity (%) for the isoamylase was plotted against the temperature value to determine the stability of the isoamylase.

2.2.13.5: Effect of Sodium Azide on Isoamylase Activity

Equal volumes of purified enzyme were incubated in water bath with 0.05, 0.1 and 0.2 mM of sodium azide for 10min. then immediately transferred into an ice bath. Enzymatic activity was measured.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Chapter Three

Results & Discussion

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

3: Results and Discussion

3.1: Isolation of *Pseudomonas* species

One hundred bacterial isolates were obtained from different soil and sewage samples as indicated in table (3-1).

Table (3-1): Bacterial isolates from different soil and sewage samples

Source of Sample	No. of samples	No. of Isolates	Growth on Cetrimide agar
Soil	50	77	32
Sewage	30	23	8
Total	80	100	40

Among the total isolates, only 40 were able to grow on cetrimide agar plates, as indication of belonging to *Pseudomonas*.

3.2: Identification of bacteria isolates

The 40 isolates were further identified according to the morphological and biochemical characteristics. Colonies of isolates which were plated on nutrient agar showed almost morphological characteristics similar to those of *Pseudomonas* sp. such as mucoidal and smooth growth, in shapes with flat edges and elevated center, whitish or creamy in color.

Microscopical examination of each isolate showed that they were non-spore forming, gram negative and rod shape. These results were agreed with those mentioned in (Holt *et al*., 1994).

These isolates were subjected to some biochemical tests. Results in table (3-2) showed that these isolates gave positive results for oxidase,

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

catalase, growth in king A and king B medium, and growth at 42ºC. But they were unable to grow at 4ºC. These results indicated that such isolates are belonging to *Pseudomonas* sp. All these isolates were examined for their ability to produce isoamylase production.

Table (3-2): Morphological and biochemical characteristics of the locally isolated *Pseudomonas* spp.

Test	Result
Colony color	Green
Cell shape	Rod
Gram reaction	-ve
Catalase production	+ve
Oxidase production	+ve
Growth in King A	+ve
Growth in King B	+ve
Gelatin hydrolysis	+ve
Growth at 4°C	-ve
Growth at 42°C	+ve

3.3: Production of Isoamylase by *Pseudomonas* isolates:

Results indicated in table (3-3) showed that all of 40 isolates were able to produce isoamylase. Among them, isolate H3 and H21 were the most efficient isolates in enzyme production when the enzyme activities in crude filtrate of them were isolates was 0.394 U/ml and 0.292 U/ml, respectively.

This document was created using SOLID CONVERTER PDF. To remove this message, purchase the product at www.SolidDocuments.com

Table (3-3): Ability of Locally isolated *Pseudomonas* spp. to produce isoamylase

Symbol	Source	E.A.(U/ml)	Symbol	Source	E.A.(U/ml)
H1	Soil	0.011	H21	Soil	0.292
H2	Soil	0.210	H22	Soil	0.167
H3	Soil	0.394	H23	Soil	0.212
H4	Soil	0.150	H24	Soil	0.183
H5	Soil	0.225	H25	Soil	0.235
H6	Soil	0.111	H26	Soil	0.119
H7	Soil	0.234	H27	Soil	0.178
H8	Soil	0.137	H28	Soil	0.25
H9	Soil	0.206	H29	Soil	0.111
H10	Soil	0.21	H30	Soil	0.070
H11	Soil	0.213	H31	Soil	0.189
H12	Soil	0.215	H32	Soil	0.226
H13	Soil	0.020	H33	Sewage	0.026
H14	Soil	0.176	H34	Sewage	0.123
H15	Soil	0.228	H35	Sewage	0.231
H16	Soil	0.233	H36	Sewage	0.214
H17	Soil	0.1	H37	Sewage	0.001
H18	Soil	0.146	H38	Sewage	0.141
H19	Soil	0.027	H39	Sewage	0.218
H20	Soil	0.199	H40	Sewage	0.073

E. A. = Enzyme activity

From the previous results isolate H3 was selected to investigate the optimum conditions for enzyme production.

3.4: Optimal conditions for Isoamylase production

3.4.1: Effect of Type of Carbon Source

Six carbon sources (Lactose, sucrose, fructose, galactose, glucose and maltose) were used as sole sources for carbon and energy to select the favorable one for isoamylase production by the locally isolated *Pseudomonas* H3. Results mentioned in figure (3-1) showed that the maximum production of isoamylase was obtained in a production medium supplemented with

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

maltose as a sole source for carbon and energy. Enzyme activity of isoamylase in the crude filtrate was 0.672 U/ml, while the specific activity was 0.421U/mg proteins. This result agreed with Bender *et al*., 1961 and Wallenfels *et al*., 1966) who found that the optimal medium composition for isoamylase production from *Aerbacter aerogenes* was 2% of maltose as inducer for isoamylase production. While Seinouke and Nobuya, (1967) found that dextrin was as effective a maltose for isoamylase production by *Escherichia intermedia.*

On the other hand, other carbon sources gave the following enzyme activities (0.708, 0.62, 0.625, 0.592 and 0.626 U/ml), respectively and specific activities and specific activities (0.313, 0.321, 0.362, 0.3and 0.356 U/mg). In other study, Spencer- Martin and N. Van, (1977) was found that the soluble starch was the optimum carbon source for isoamylase production by *Lipomyces kononenkaoe.*

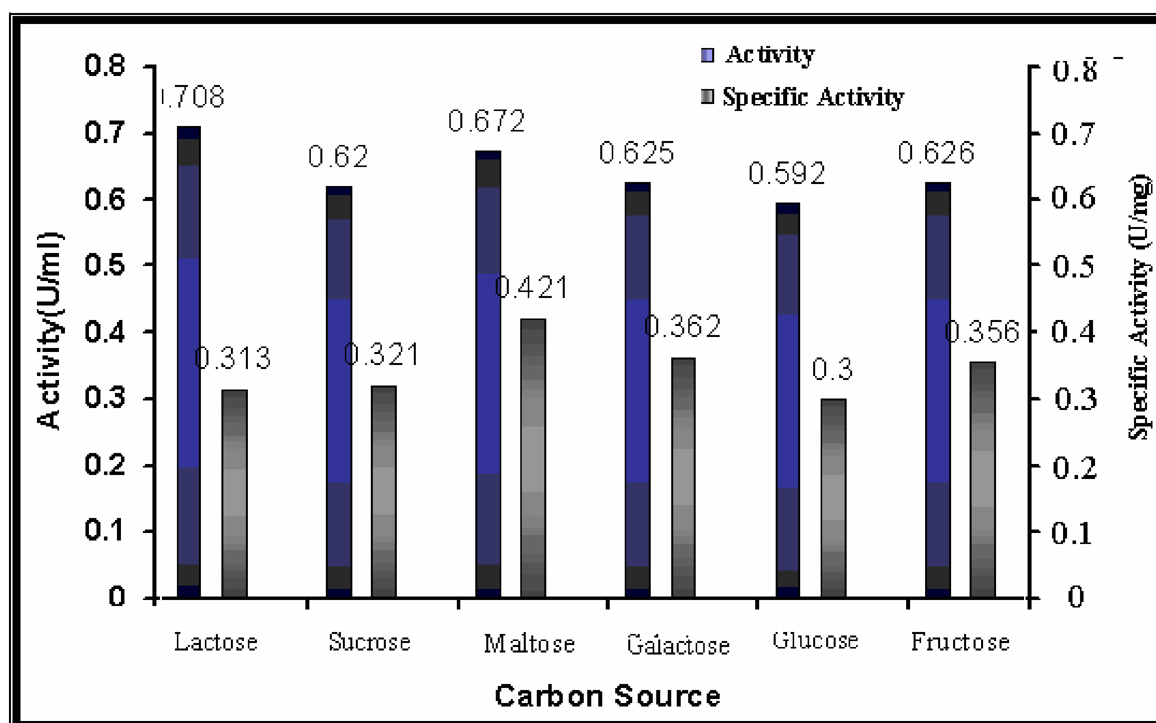


Figure (3-1): Effect of different carbon source on isoamylase production by *Pseudomonas* sp. H3 after incubation in a shaker incubator (150 rpm) at 28°C for 24 hr.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

According to this result maltose was chosen as the optimum carbon source used in the next experiments of optimization for isoamylase production.

3.4.2- Effect of Carbon Source Concentration

Different concentrations of maltose were used to determine the optimum concentration for the production of isoamylase by *Pseudomonas* H3. Results in figure (3-2) showed that the maximum production of isoamylase was obtained by using 1% of maltose in the production medium which gave the specific activity of 0.534 U/mg in the culture filtrate. This indicates that this concentration of carbon source is the best for providing the microorganism with the needed energy for growth. This result was agreed with Jer- yiing *et al.*, (1989) who found that the maximum isoamylase production was obtained when maltose was used as a sole source for carbon and energy at concentration of 1.10%. While other concentrations of maltose gave less production, and less activity as it was shown in figure (3-2).

.

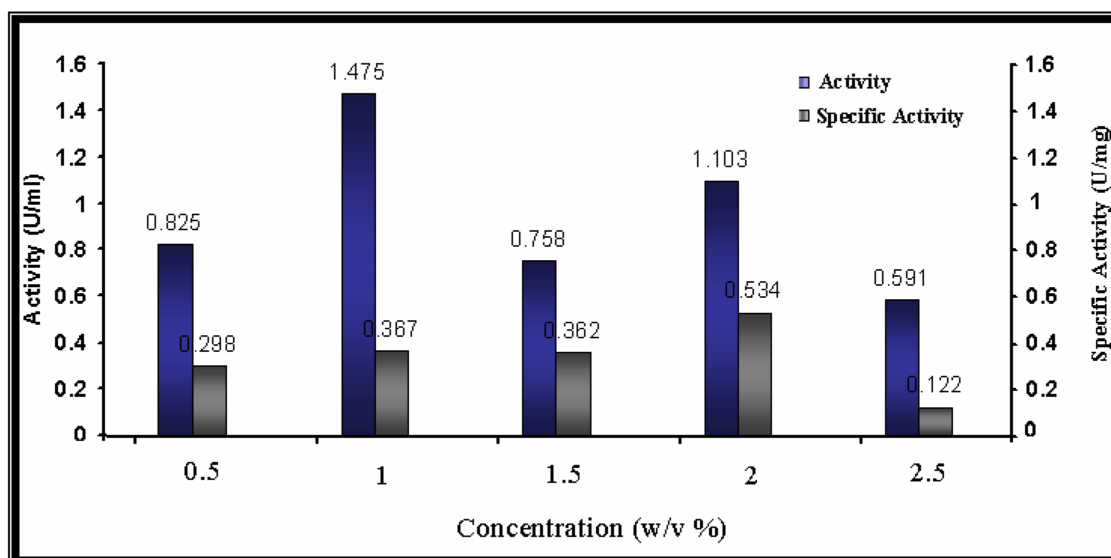


Figure (3-2): Effect of different concentrations of maltose on isoamylase production by *Pseudomonas* sp. H3 after incubation in a shaker incubator (150 rpm) at 28°C for 24 hr.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

According to these results, maltose was used in the next experiment of optimization for isoamylase production.

3.4.3- Effect of Type of Nitrogen Source

Different nitrogen sources (peptone, tryptone, yeast extract, NH_4Cl and NH_4NO_3), were added to medium to determine the optimum source for isoamylase production by the locally isolated *Pseudomonas* sp. H3. Results in figure (3-3) showed that the optimum nitrogen source for isoamylase production by the locally isolated *Pseudomonas* sp. H3 was peptone at which the specific activity of the crude enzyme was 0.552U/mg. This may be because peptone is easier to be used by microorganism than the other sources to achieve requirements for growth, cell division and production of different metabolites culture medium.

Other nitrogen sources which they are tryptone, yeast extract, NH_4Cl and NH_4No_3 gave lower production of isoamylase in culture medium. Their specific activities of the crude enzyme culture filtrate were 0.402, 0.433, 0.427 and 0.356 U/mg respectively, this is may be because these nitrogen sources are not easily utilized by microorganism therefore, these sources not provide the optimal conditions for growth propagation, and production of metabolites.

In other study; it was observed that ammonium salts in a concentration of more than 0.2% is the best for isoamylase production by *A. aerogenes* (Bender *et al*. 1961). Generally, the effect of nitrogen sources in production of the enzyme varies between different microorganisms (Martin and Demain, 1980).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

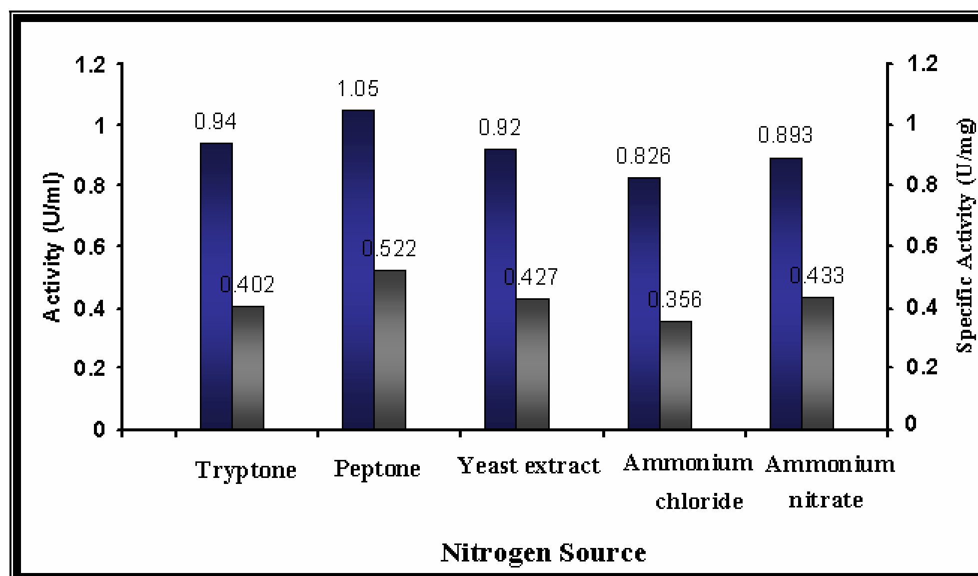


Figure (3-3): Effect of different nitrogen sources on isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator (150rpm) at 28ºC for 24hr.

According to these results, peptone was used in next experiments of optimization for isoamylase production.

3.4.4- Effect of nitrogen source concentrations

Five concentrations of the optimum nitrogen (peptone) 0.1, 0.2, 0.3, 0.4 and 0.5% were used to determine the optimum concentration for isoamylase production by the locally isolates *pseudomonas* sp H3. Results in figure (3-4) showed that the optimal concentration of peptone for enhance isoamylase production was 0.4 % which gave specific activity of enzyme in culture filtrate was 0.552 U/mg. However, the specific activity was decreased when peptone concentration was less or greater than 0.4 %.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

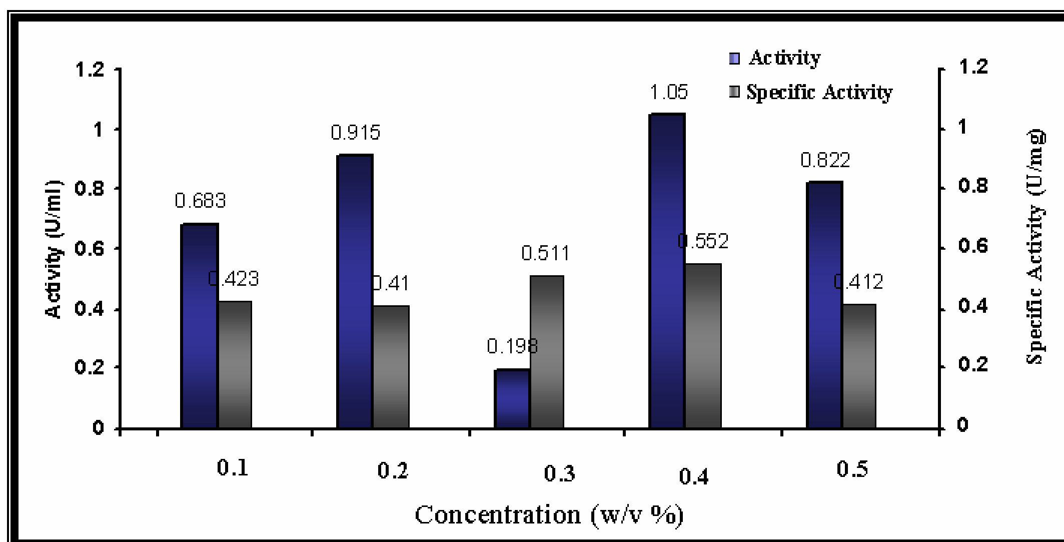


Figure (3-4): Effect of different concentrations of peptone on isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator (150rpm) at 28ºC for 24 hr.

In other study, Bender *et al.*, (1961), found that 0.8% of peptone in production medium for isoamylase production by *Escherichia intermedia* was the best in comparison with many other nitrogen sources (ammonium phosphate, soybean, corn and rice bran).

According to these results, peptone as used in a concentration of 0.4 % in the next experiments of optimization for isoamylase production.

3.4.5- : Effect of Type of Phosphate source

Different phosphate sources were also studied to determine the optimum for isoamylase production by the locally isolated *Pseudomonsp*.H3. For this purpose four types of phosphate sources (K_2HPO_4, KH_2PO_4, Na_2HPO_4 and $NH_4H_2PO_4$) were added separately, to the production medium at a concentration of 0.3 %.

Results indicated in figures (3-5) showed that the optimum phosphate source for isoamylase production at which the enzyme gave its maximum

This document was created using SOLID CONVERTER PDF. To remove this message, purchase the product at www.SolidDocuments.com

specific activity was a K_2HPO_4 when a concentration of (0.4%) of it gave an the enzyme activity 0.663 U/ml and a specific activity of the crude enzyme of 0.673 U/mg, this may be due to the effect of such concentration of phosphate source on buffering capacity which has a direct effect on the pH of the production medium.

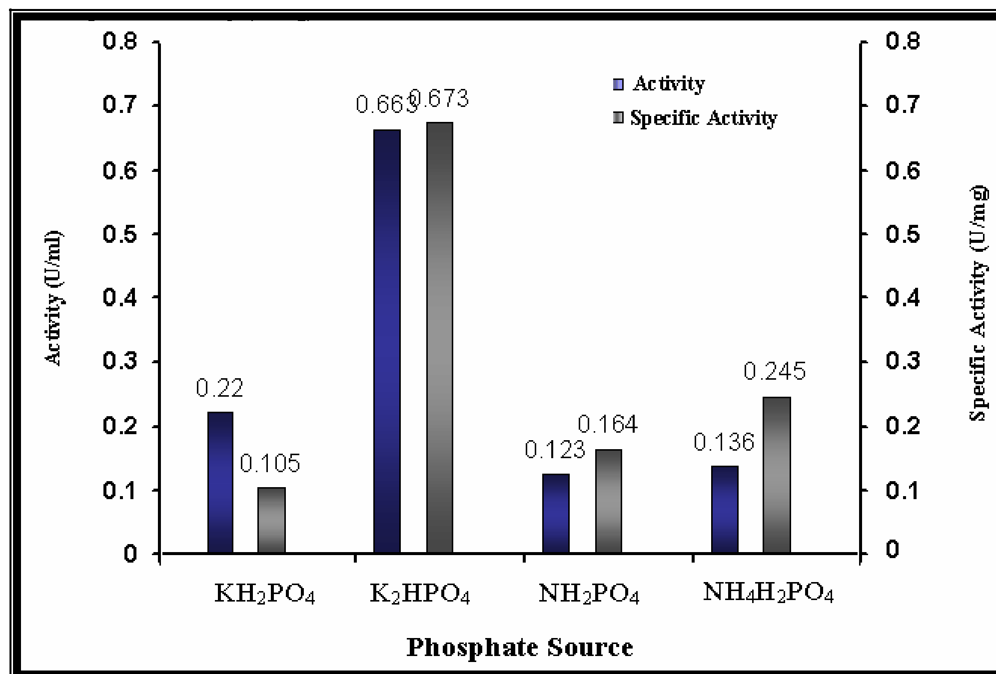


Figure (3-5): Effect of different phosphate sources on isoamylase production by *Pseudomonas* sp.H3 after incubation in a shaker incubator (150rpm) at 28°C for 24 hr.

Buffering capacity usually maintain a specific pH value which means that more molecules of the buffer components present, larger number of H and OH ions can be absorbed without changing the pH value (McKee, 1996).

Other concentrations of the phosphate sources resulted in lower specific activities as shown in figure (3-6).

Bender *et al*. 1961 and Wallenfels *et al*., (1966) found that optimum phosphate source in production medium of isoamylase from *A. aerogenes* was K_2HPO_4 at a concentration of 0.5% (w/v) and 1% from *Escherichia intermedia.* Box *et al*., (1978) mentioned that the optimum phosphate source

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

was 0.38% KH_2PO_4 for isoamylase produced by *Pseudomonas amylodermosa.*

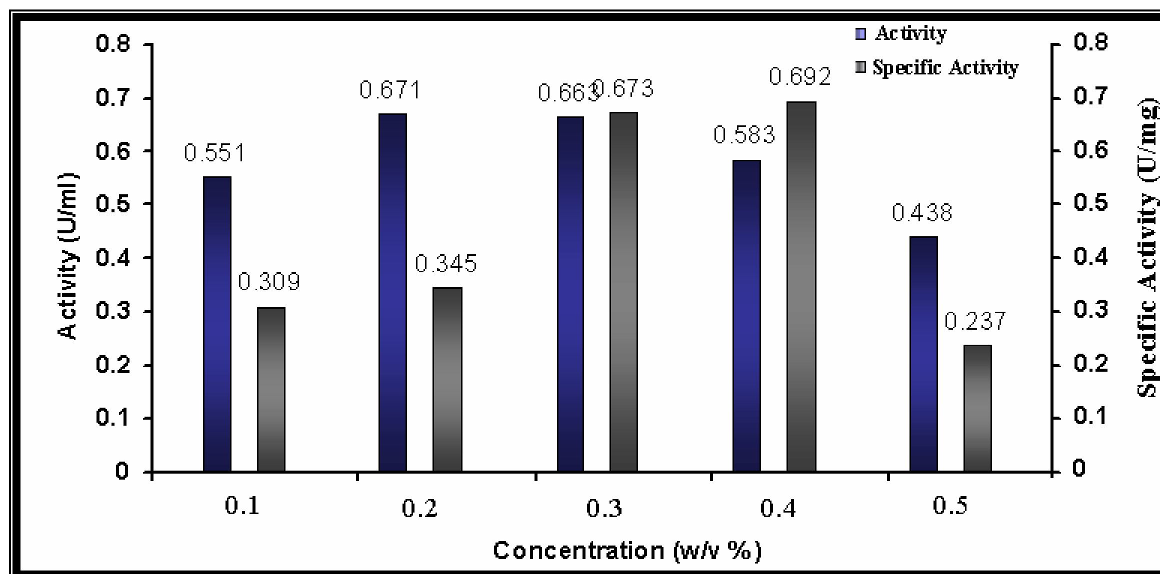


Figure (3-6): Effect of different concentrations of K_2HPO_4 on isoamylase production by *pseudomonas* sp.H3 in a shaker incubator (150 rpm) after incubation at 28°C for 24 hr.

3.4.6-Effect of pH

Different pH values were used to determine the optimum pH for production of isoamylase by *Pseudomonas* sp. H3.Results indicated in figure (3-7) showed that maximum production of the enzyme was obtained when the pH of the production medium was adjusted to 6.0, at which enzyme activity in the crude filtrate was 0.778 U/ ml while specific activity was 0.843 U/mg. This result was agreed with that obtained by Takahashi *et al*., (1996) who found that the optimum pH value for isoamylase production by *Flavobacterium odoratum* was 6.0. This may be because this pH value may achieve the optimum conditions for the bacterial growth and enzyme production.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

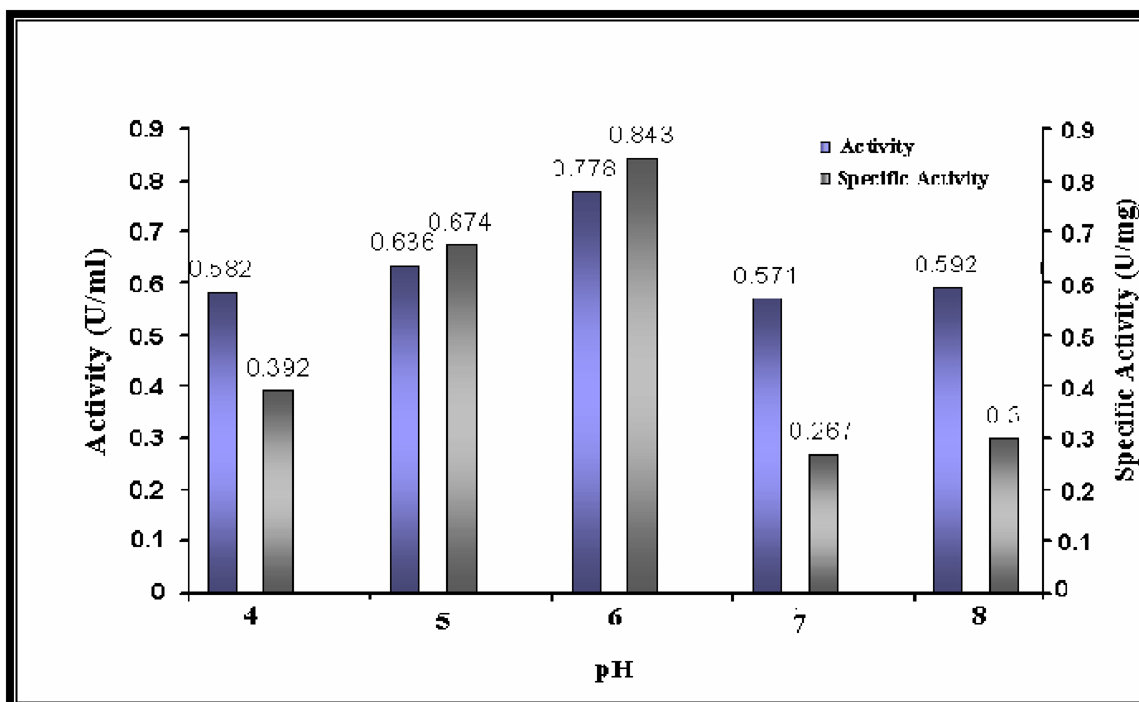


Figure (3-7): Effect of pH on isoamylase production by *pseudomonas* sp.H3 after incubation in a shaker incubator (150 rpm) at 28°C for 24 hr.

On the other hand, Castro *et al.* (1992) found that the optimum pH for extracellular isoamylase production from *Bacillus circulans* was 5.0.

The effect of pH value on bacterial growth and enzyme production is due to the following reasons, (Bull and Bushnell, 1976).

- Its effect on the properties of the culture medium including the stability of the nutrients molecules transport and ionization.
- pH values affect the stability and catalysis of the enzyme.

According to this result, the optimal pH (6.0) was used in the next experiments of optimization for isoamylase production.

3.4.7: Effect of temperature

In order to determine the optimum incubation temperature for isoamylase production by the locally isolated *Pseudomonas* sp. H3, different

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

incubation temperatures (25, 28, 30, 35 and 40°C) were used for this purpose. Result indicated in figure (3-8) showed that the maximum production of isoamylase in culture medium was obtained at 35°C at this temperature, the specific activity was 0.850 U/mg, and this may be because that this temperature was the optimum for growth and propagation of the producing microorganism. Optimum temperatures for production of the enzyme usually depend on the type of producing microorganism. In other study, Takahashi *et al*., (1996) reported that optimum temperature for isoamylase production by *F. odoratum* KU for activity were (50°Cand 45°C) in the presence of $CaCl_2$, respectively. On the other hand, Castro *et al*., (1992) showed that the optimum temperature for isoamylase production by *B. circulans* was 60°C, while Gunja *et al*., (1961) reported that 30°C was the optimum temperature for isoamylase production by *konoknenkoae Lipomyces.*

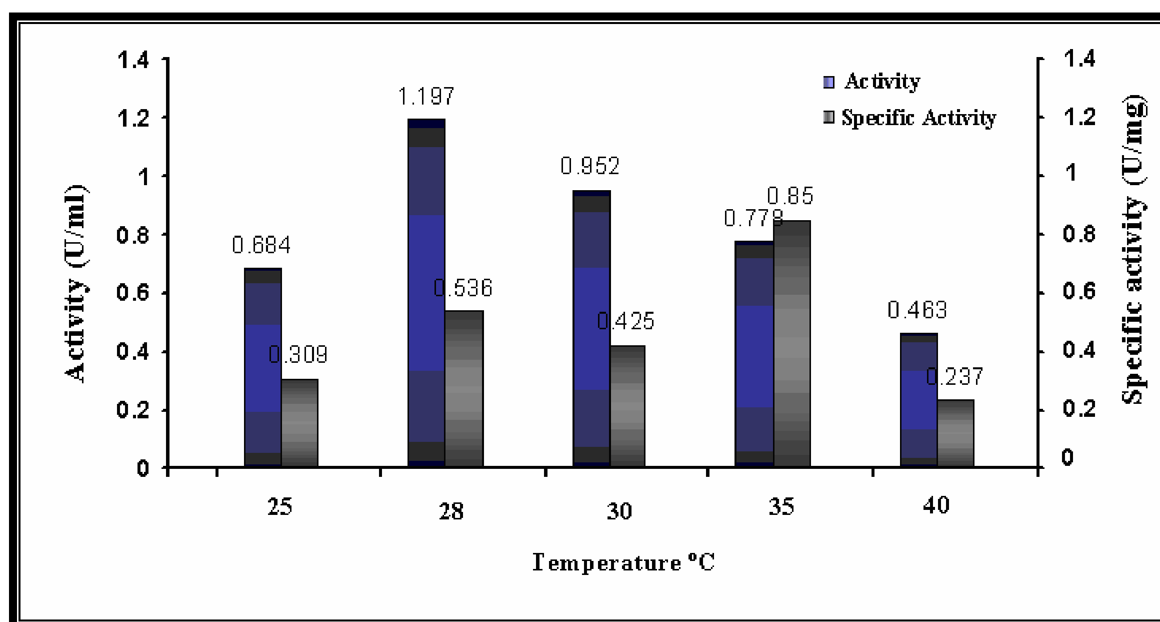


Figure (3-8): Effect of incubation temperature on isoamylase production by *Pseudomonas* sp. H3 after incubation in a shaker incubator (150 rpm) at 28°C for 24 hr.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

3.5: Isoamylase purification

Isoamylase produced by the locally isolated *Pseudomonas* sp. H3 under the optimum conditions was partially purified through a sequence of purification steps. Including ammonium sulphate precipitation, dialysis and gel filtration chromatography technique by sephacryl G-300.

3.5.1: Ammonium sulphate precipitation

In this study, ammonium sulphates were used in a gradual saturation ratio ranging between (40– 80%). It has been found that the precipitated enzyme reaches its maximal activity (1.686 U/ml) and specific activity (1.130U/mg) of protein with (6.258%) yield at the saturation of 60% of the ammonium sulphate.

Protein precipitation using ammonium sulphate depends on the salting out phenomenon. Scince ammonium sulphate has the ability to neutralize charges at the surface of the protein and to disrupt the water layer surrounding the protein, it will eventually cause a decrease in the solubility of the protein which, in turn will lead to the precipitation of the protein by the effect of salt (Englard and Seifter, 1990).

Ammonium sulphate are widely used because of its availability, high solubility and low cost. Other important feature of ammonium sulphate is that causes no damages to proteins (Volesky and Loung, 1985).

3.5.2: Purification of isoamylase by gel filtration:

The purification steps for isoamylase from *Pseudomonas* sp. H3 can be summarized in table (3-4). After being dialyzed, the enzyme extract was further purified using Sephacryl G– 300. Figure (3-9) shows the protein peak obtained from gel filtration step.

Enzyme solution was added to the column, the enzyme eluted by phosphate buffer solution (pH 6.0) and the fractions were collected

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

(3ml/fraction), the flow rate was 30 ml/1 hour. Then the absorbency for each fraction was measured at 280 nm. Enzyme activity was determined for each fraction.

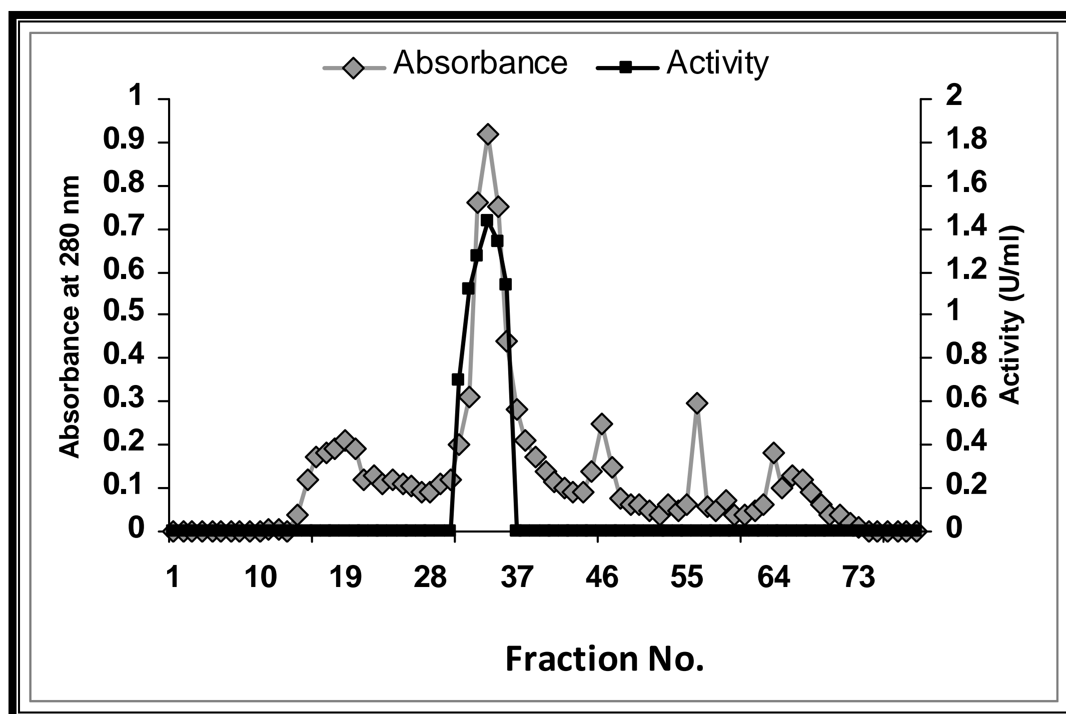


Figure (3-9): Purification of isoamylase from locally isolate *Pseudomonas* sp. H3 by gel filtration chromatography using Sephacryl G-300.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Table (3-4): Steps of isoamylase purification from locally isolate *Pseudomonas* sp. H3.

Purification steps	Volume (ml)	Enzyme Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (Unit)	Fold purification	Yield %
Sp. H3Crude enzyme	**150**	**0.898**	**1.612**	**0.557**	**134.7**	**1**	**100**
Precipitation With ammonium sulphate(60%)	**5**	**1.686**	**1.492**	**1.130**	**8.43**	**2.028**	**6.258**
Gel Filtration Sephacryl G-300 after concentration	**3**	**1.853**	**1.397**	**1.326**	**5.559**	**2.380**	**4.126**

3.6: Isoamylase Characterization

3.6.1: Effect of pH on isoamylase activity

Effect of pH on the partially purified isoamylase was studied in a pH range of (4–8) as shown in figure (3-10). Results showed that the maximum activity of isoamylase was at pH 5.5, while the activity was decreased above and below this pH value. The pH values have effect on the ionic state of enzyme by affecting on the amino acids chains which are necessary for tertiary structure of enzyme. This results in changing of ionic state of the substrate and which will be reflected on the activity of enzyme. Higher or

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

lower pH from the optimum will lead to denature the enzyme and losing its activity (Whitaker and Bernard, 1972; Segal, 1976; and Nielsen *et al*., 2001).

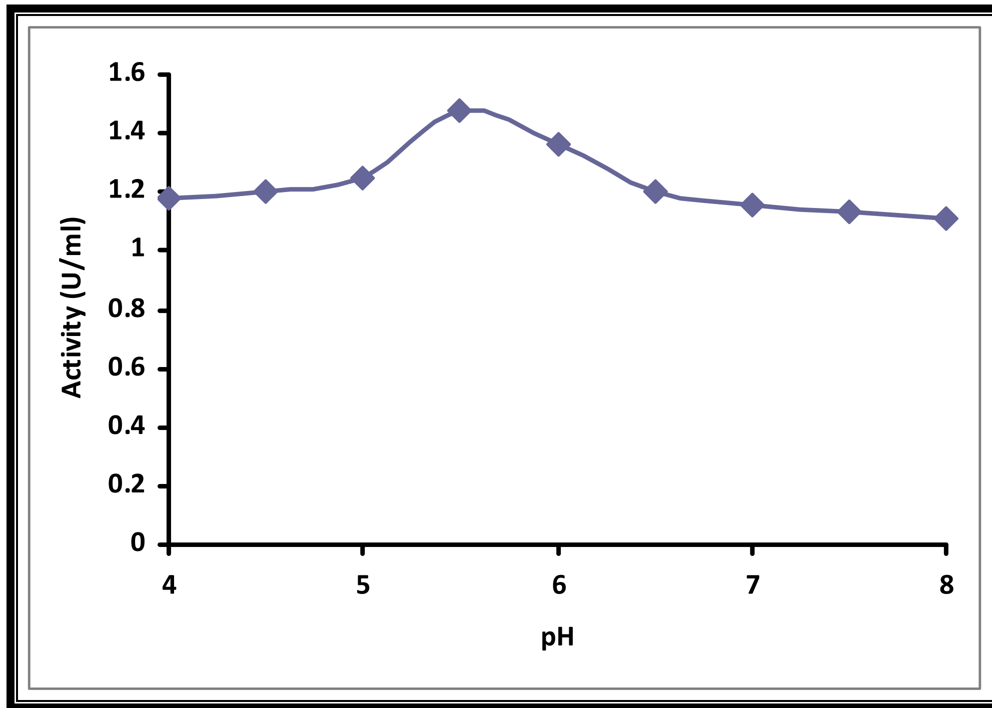


Figure (3-10): Effect of pH on the activity of partially purified isoamylase produced by *Pseudomonas* sp. H3.

Harada, *et al*., (1968) demonstrated that the optimum pH value for isoamylase activity from *Ps. amylodermosa* was 3.0 to 4.0. On the hand, Spencer- Martin *et al*., (1982) showed that the optimum pH for the activity of isoamylase from *L. kononenkoae* was 5.6.

3.6.2: Effect of pH on isoamylase stability

In order to determine the optimum pH for the isoamylase stability, the enzyme was incubated in buffer solution with pH range (4-8) at 30°C for 30min.

Results indicated in figure (3-11) showed that isoamylase has a good stability at pH range between 4.5-6.0 in which it keeps more than 90 % of its total activity.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

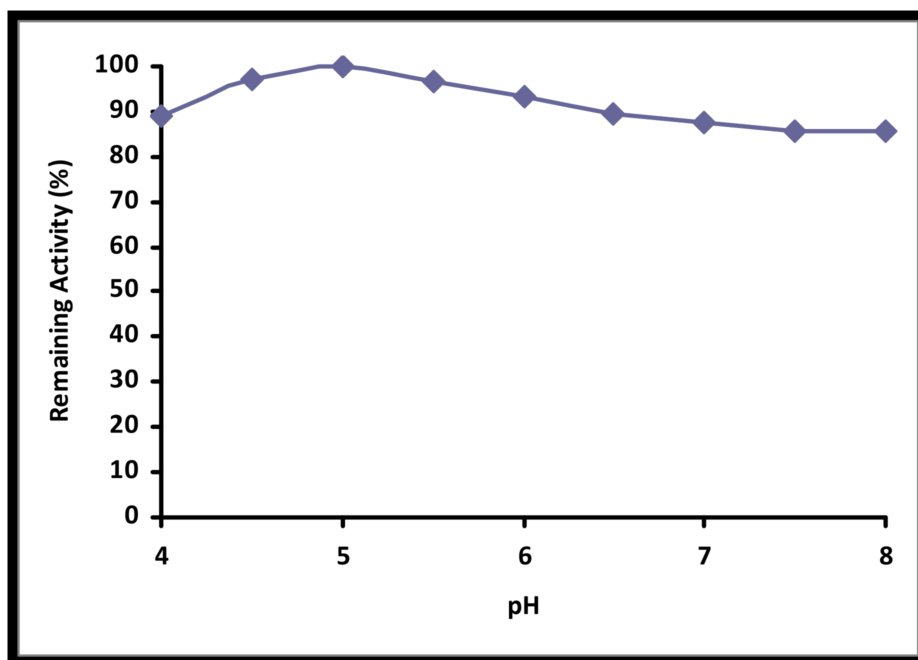


Figure (3-11): Effect of pH on the stability of partially purified isoamylase produced by *Pseudomonas*. Sp. H3.

A decrease in the stability of the enzyme was noticed at pH less than and more than the optimum pH for stability. This decrease in the isoamylase activity could be attributed to the effect of pH of the secondary, tertiary or quaternary structure of the enzyme, since enzymes subjected to irreversible denaturation in strong acid and alkaline conditions. Whitaker, (1972) had indicated that most isoamylase from *Pseudomonas* sp. have optimum pH (3.5-5.5), while Harada *et al*., (1968) demonstrated that the optimum pH value for isoamylase stability from *Ps*. *amylodermosa* was 3.0 to 6.0.

3.6.3: Effect of Temperature on isoamylase activity

Stability of partially purified enzyme was studied at different temperatures ranging from 25 to 60ºC to determine the optimum for enzyme activity. Results indicated in the figure (3-12) showed that the activity of isoamylase reaches the maximum 1.411 U/ml at 35ºC, and decreased to

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

(1.21 U/ml) at 60ºC, which may be attributed to the denaturation of the enzyme after incubation at high temperature causing structural and conformational change in whole enzyme at the structure of the protein and changing the structure of the molecule, therefore active site affecting the enzyme binding with the substrate and reaction, this result is reasonable with that obtained by Wang (1999).

The increase in enzyme activity with temperature is due to the elevating in reaction energy for the enzyme and the substrate which leads to the formation of enzyme- substrate complex and this will result in increasing the rate reaction speed (Ubran *et al*., 2001). Senosuke and Nobuya (1967) showed that the optimum temperature for isoamylase from *E. intermedia* was 47ºC.

Spencer- Martin *et al*., (1980) demonstrated that the optimum temperature for isoamylase activity from *L. kononenkoae* was 30ºC. Katsuya *et al*., (1998) showed that the optimum temperature for activity of isoamylase from *Ps. amylodermosa* was 50 ºC.

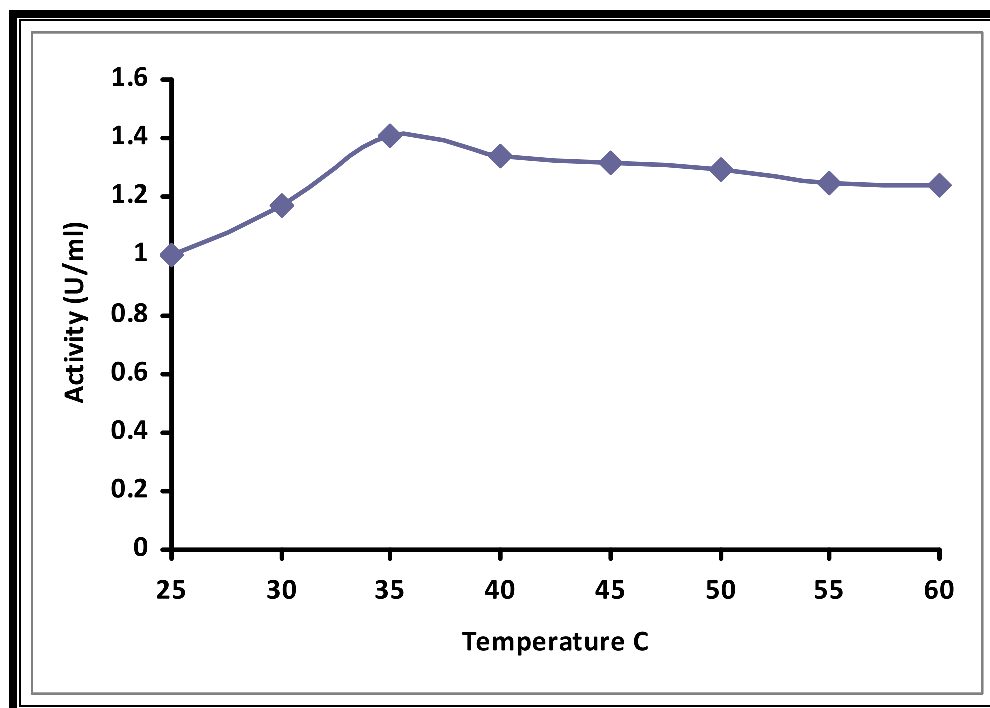


Figure (3-12): Effect of temperature on activity of partially purified isoamylase produced by *Pseudomonas* sp.H3.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

3.6.4: Effect of Temperature on isoamylase Stability

The purified isoamylase was incubated at 35ºC for different periods of time 10, 20, 30, 40, 50 and 60 min. to determine the stability of isoamylase at this temperature and the results in the figure (3- 13) showed that, the maximal function (100 %) activity was observed for 10 min. at 35 ºC more than 85 % of the activity was remained after incubation for 20- 40 min. at the same temperature, then activity decreased gradually. This decreasing of enzyme activity with the increase of temperature is obvious in most enzymes. Enzyme sensitivity differs toward temperatures; many enzymes are highly sensitive to high temperatures and lose its activity at 35ºC like catalase extracted from cows liver (Godfrey, 1983) while other enzymes still active for few minutes at 100ºC like Ribonuclease. Some enzymes features (Molecular weight complexity) have a relationship with enzyme sensitivity towards temperature. Mostly, enzymes with low molecular weights which composed of single polypeptide chain and contain disulfide bonds (s-s bonds) are more stable in high temperature than complex enzymes with high molecular weights. The environmental conditions also contribute in increasing and decreasing the enzymes sensitivity temperatures, like pH, ionic strength and the presence of other materials with the enzymes (Segal, 1976; Whitaker, 1972).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

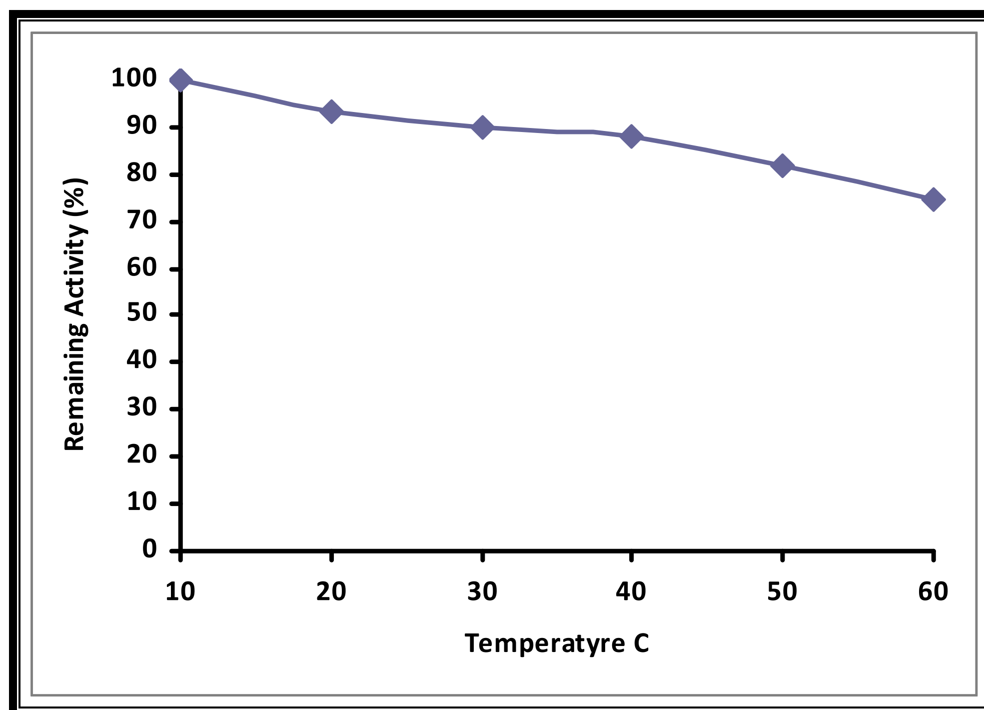


Figure (3-13): Effect of temperature on the stability of partially purified isomaylase produced by *Pseudomonas* sp. H3.

3.6.5: Effect of Inhibitors on isoamylase activity

To determine the effect of inhibitors on isoamylase activity, partially purified enzyme was incubated with different concentrations of sodium azide for 15 min. Results indicated in figure (3-14) showed that the enzyme activity was drastically inhibited by Ca+2, while, other metal ions phosphates and surfactants exhibited no significant inhibitory or accelerating effect on enzyme production (Houng *et al*., 1989, and Takahashi *et al*., 1996).Under these conditions, treatment of isoamylase with 0.05, 0.1 and 0.2 % mM sodium azide inhibit the activity to 0.523, 0.324 and 0.204 U/ ml respectively, and the remaining activity were 100%, 62% and 39% respectively.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

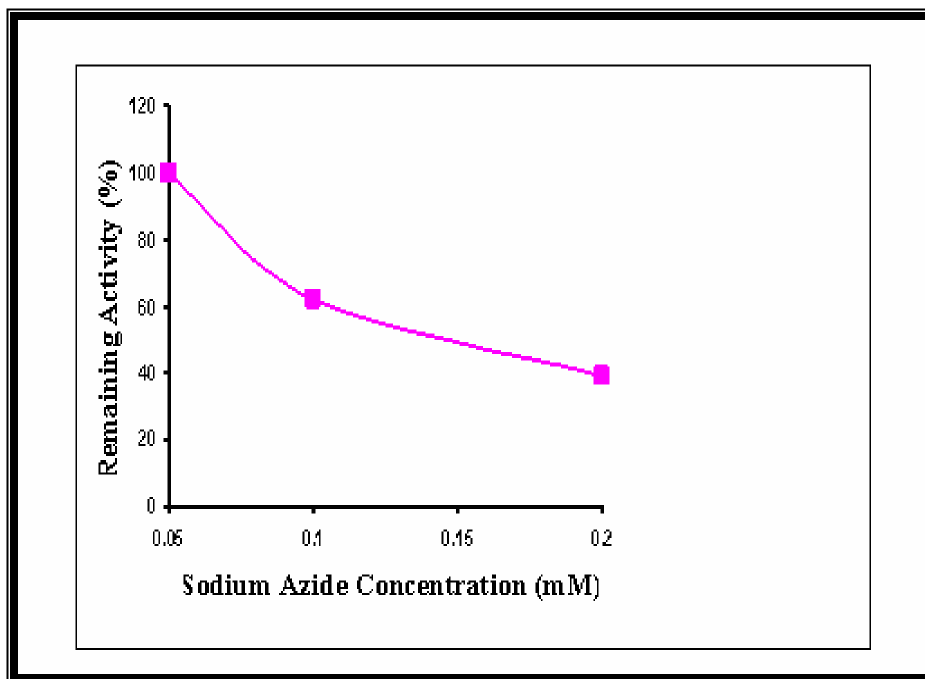


Figure (3-14): Effect of sodium azide on isoamylase activity produced by *Pseudomonas* sp. H3.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Conclusions
&
Recommendations

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Conclusions:

- Local *Pseudomonas* isolates from different soil and sewage samples were able to produce isoamylase and the best one was *Pseudomonas* sp. H3.

- Optimum conditions for isoamylase production by the locally isolated *Pseudomonas* sp. H3 involved the use of maltose (1 %), peptone (0.4 %), diapotassium hydrogen phosphate (0.4 %) at pH 6.0, and incubation at 35ºC for 24 hrs.

- Partially purified isoamylase was active at pH 5.5, temperature of 35ºC, and it was stable at pH 5.0 with 35ºC for 10 min.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

Recommendations:

- Complete identification of locally isolated *Pseudomonas* sp.
- Determination of the genetic loci responsible for isoamylase production by the locally isolated *Pseudomonas* sp. H3.
- Genetic modification of *Pseudomonas* sp. H3 by physical and chemical mutagenesis to enhance its ability in isoamylase production.
- Studying the kinetics of isoamylase produced by *Pseudomonas* sp. H3.
- Determination of the optimum conditions for isoamylase production by the locally isolated *Pseudomonas* sp. H3 using solid state fermentation.
- Fill purification of isoamylase produced by the locally isolated *Pseudomonas* sp. using different chromatographical techniques.
- Utilization of the purified enzyme in different food and industrial applications.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

References

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

- **Abdullah, M., B.J. catley, E. Y. C. Lee, J. Robyt, K. Wallenfels, and W. J. Whelan (1966)**. The mechanism of carbohydrase action. I I. Pullaulanace, an enzyme specific for the hydrolysis of -1, 6 – bonds in amylose oligosaccharides. Cereal Chem. 43: 111-118.
- **Amemura, A., Charkraborty, R., Fujita, M., Noumi, T., and Fujita M., (1988)**. Cloning and nucleotide sequence of the isoamylase gene from *Pseudomonas amylodermosa* SB-15. J. Biol. Chem. 263, 9271-9275.
- Atlas**, R. m., Parks, L.C. and Brown, A. E. (1995).** Laboratory manual of experimental microbiology.
- **Baba, T., Kimura, K., Mizuno, K., Etoh, H., Ishida, Y., Shida. O., and Arai, Y. (1991).** Biochem. Biophys. Res. Commun. 181, 87-94.
- **Ball, S., Guna, H. P., James, M., Myers, A., keeling, P., Mouille, G., Buleon, A., Colonna, P., and Preiss, (1996).** From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. Cell 86, 349-352.
- **Ben, Am.; Mezghani, M.; Bejor, S. (1999)**. A thermstabled-amylase producing maltohexose from a new isolated *Bacillus* sp. US 100: study of activity and molecular cloning of the corresponding agent. Enzyme. Mocrobiol. Technol. 24: 548- 549.
- **Bendr, H., and K. Wallenfels (1961)**. Unter- su chungen a pullulan. Spezifischer Abbau druch ein bakterielles Enzyme. Biochem. Z. 334: 79-95.
- **Bernfeld, P. (1951).** Enzymes of starch degradation and synthesis. In: Advances in Enzymology.3: 397- 428.
- **Box, K. –C., Chen. C. and Wen- H. (1978).** Optimization of cultivation composition for isoamylase production. 31: 61-64.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Bradford, M. M. (1976).** A rapid and sensitive method for the quantification of microorganism quantities of protein utilizing the principle of protein- dye binding. Anal. Biochem. 72: 248-254.
- **Brooks, G. F.; Butel, J. S; Ornston, L. N. J.; Jawetz, e; Malnick, J. S., and adelborg, E.A. (1995).** *Pseudomonas* spp. resistance to antimicrobial drugs. In medical microbiology. Jawetz, Melnick and Adelbeg (Eds.). P. 140- 142 2180- 2220. Hall international Inc. Asimon and Schuster Company U. S. A.
- **Bull, A. T. and Bushnell, M. E. (1976).** Environmental control of fungal growth. In: The filamentous fungi (eds. J. E. Smith and D. R. Berry). 2:23- 26.
- **Chen, J. H., Chen, Z. Y., Chow, T. Y., Chen, J. C., Tan, S. T., and Hsu, W. H., (1990)**. Nucleotide sequence and expression of the isoamylase gene from isoamylase- hyper producing mutanat, *Pseudomonas amylodermosa* JD 210. Biochim. Biophys. Acta 1087, 309-315.
- **Chen, Y. T., and burchell, A. (1995**) in *The Metabolic and Molecular Basis of Inherited Diseases* (scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 1, 935-965.
- **Collee, J. G.; Barrie P.; Andrew, G.; Anthong, M. and Tsimmons, F. (1996).** Test for identification of bacteria (Cha. 17), In: Mackie and MC carthey; practical medical microbiology. Churchill, Living stone, New York.
- **Colline, C. H. and Lyne, P.M. (1987).** Micribiological methods, 5th Edition. Butter Worth and Co. publisher Ltd., London.
- **Cornelis, P.; Dianeffe, C. and Willemot, K. (1982).** Cloning and Expression of *Bacillus coagulans* amylase gene in *Escherichia coli*. Mol. Gen. Genet. 186: 507- 511.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Cruickshank, R.; Duguid, j. P.; Marmion, B. P. and Swain, R. H. A. (1975).** Medical Micrbiology. In. New York.
- **DiMaura, S., and Jsujiion, S. (1994)** in Myology (Engel, A. G., and Frazini- Armstrong, C., eds) 2: 1554-1576.
- **Ding, J. Lory, S. and Tai, P.C. (1985).** Orientation and expertion of the cloned hemolysin Gene of *Pseudomomas aeruginosa* gene. 33: (312-321).
- **Eliss, K. J. and Morrison, J. F. (1982).** Buffers of constant ionic strength for studying pH dependent processes. In: Methods in enzymology. Purich, D. L. (ed.) 87, pp. 405- 410. Academic Press. New York.
- **Englard, s., and Seifter, S. (1990).** Precipitation techniques In: Methods in Enzymology (ed. Murray, E. d. and Dentscher, P.) 182: 425- 441.
- **Fad, J. A. (1976).** Bacteriological Analytical Manual. 7-49.
- **Fogarty, W.M. and Kelly, C, T. (1980).** Amylases, Amyloglucosidase and related glucanases. In: Microbial enzymes and Bio conversion (ed. Rose A.H.) Chp.3:115-170. Academic press. London.
- **Fogarty, W.M., (1983).** Microbial amylases. In: (Microbial Enzymes and Biotechnology, W.M. Fogarty, ed., (pp-192), Applied Science Publishers. London.
- **Friedberg, F. and Rhodes, C. (1986).** Cloning and characterization of - amylase gene from *Bacillus polymyxa*. J. Bacterial., (6313): 819-824.
- **Fujita, N., kubo, A., Francisco, P.B., Nakakita, M., Harada, K., minoka, N., and Nakamura, Y. (1999).** Purification,

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

characterization and cDNA structure of isoamylase from developing endosperm of rice. *Planta* 208, 283-293.

- **G. R. Castro, G. F. Garcia, and F. Siñeriz (1992)**. Extracellular isoamylase produced by *Bacillus circulans* MIR-137. J. Appl. Microbiol. 73 (6): 520-523.
- **Gallant DJ, Bouchet B, Baldwin PM**. Microscopy of starch: evidence of a new level of Carbohydrate polym. 1997; 32:177-191.
- **Ghosh, H.P., Preiss, J. (1966)** J. *Biol. Chem*. 241, 4491-4504.
- **Godfrey, T. (1983).** Comparison of key characteristics of industrial enzyme type and source. In: Industrial Enzymology (eds. Godfrey T. Riechlt, J.) 466-502.
- **Goodfellow, M.**, **Lacy, J. and Todd, C. (1987).**Numerical classification of thermophilic Streptomyces. J. Gen. Microbiol. 133: 3135- 3149.
- **Gunja, Z. H., D. J. Manners, and K. Maung (1961)**. Studies on carbohydrate metabolizing enzymes. VII. Yeast isoamylase. Biochem. J. 81: 392-398.
- **Gupta R, Gigras P, Mohapatra H, Goswami VK, and Chauhan B. (2003).** Microbial -amylase: a biotechnological perspective. Process Biochem. 38: 1599-1616.
- **Harada, T., Yokobayashi, K. and Misaki, A. (1968).** Formation of isoamylase by *pseudomonas*. Applied Microbiology 16, 1439- 1444.
- **Harada, T., Yokobayashi, K. and Misaki, A. (1968).** Formation of isoamylase by *Pseudomonas*. Applied Microbiology 16, 1439-1444.
- **Harada, W., Matsuura, Y., Kusunoki, M., Kakudo, M. (1984).** Structure and possible catalytic residues of taka- amylase a.J. Biochemistry, 95:697-703.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Harely, P. J. and Prescott, M. L. (1996).** Laboratory, exercises in microbiology WCB, Mc Grow- Hill, U. S. A.
- **Hasnain, H.; Alexandra, M.; Robert, S., and Sam Z. (2003).** Three isoforms of Isoamylase contribute Differenrt Catalytic Properties for the Debranching of Potato Glucans. The Plant Cell, 15: 133-149.
- **Henrissat B. (1991).** A classification of starch hydrolysis. International Biodeterioration, 15: 15-19.
- **Hirao, M., Hijiya, H., and Miyaka, T., (1988)**. Anhydrous crystals of maltitol and whole crystalline hydrogenated starch hydrolyzate mixture solid containing the crystals, and process for the production and uses thereof. U. S. Patent 4, 717,756.
- **Hobson, P. N., W. J. Whelan, and S. Peat (1951)**. The enzymatic synthesis and degradation of starch. XIV. R- enzyme. J. Chem. Soc., P., 1451- 1459.
- **Holder, I. A., and Needy, A.N. (1989)**. *Pseudomonas aeruginosa* catalase as virulence factor in burned host kinin cascade. Infection and Immunity p.3345-3348.
- **Holt, J. G.; krieg, N.R.; Sneath, P.H.A.; Staley, J. T. and Williams, S. T. (1994).** Bergy's manual of determinative bacteriology. 9th ed. P: 169- 172. Williams and willkins. U.S. A. Company Baltimone.
- **Houng. J Y, Chen KC, and Hsu WH. (1989).** Production of an isoamylase from *Pseudomonas amylodermosa* mutant strain JD210. Eur. J. castro. Heptol., 10 (7): 607-610.
- **Howling, D. (1989).** Mechanism of starch hydolysis. International Biodeterioration, 15: 15- 19.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Iglesias, A.A., Charng, Y.Y., Ball, S., Preiss, J. (1994)** *Planta. Physiol*. 104, 1287-1294.
- **Itkor, P., Shda, O., Tsukagoshi, N. and Udaka, S. (1989).** Screening for raw starch digestion bacteria. Agric. Biol. Chem. 53:53-60.
- **James, M. G., Robertson, D. S., and Myers, a. M. (1995**). Characterization of the maize gene sugary I, a determinant of starch composition in kernels. Plant Cell 7, 417- 429.
- **James, G.; Mary K. Beatty; A froza R. Heping Cao; Wendy W. Michael L. and Alan M. Myer (1999).** Purification and molecular genetics and characterization of Zpu 1, a pullulanase. Type Starch – Debranching enzyme from Maize. Plant physiol (119): 255-266.
- **Jeanningrose, R., n. C reuzet, C, frixon, and J. Cattaneo (1975).** A debranching enzyme in *Escherichia coli*. Biochem. Soc. Trans. 3:336-337.
- **Jer- Ying, h., Kuo- Cheng, C. and Wen- Hwei, H. (1989).** Optimization of cultivation medium composition for isoamylase production. J. Appl. Microbiol. Biotechnol. 31: 61- 64.
- **Kainuma, K., Kobayashi, S., and Harada, T., (1978**). Action of *Pseudomonas* isoamylase on various branched oligo- and poly- saccharides. Carbohydrate Res. 61, 345-357.
- **Katsuya, Y., Mezaki, Y., Kubota, M., and matsuura, Y., (1998**). Three- dimensional structure of *Pseudomonas* isoamylase at 2.2 A° resolution. J. Mol. Boil. 281, 885-897.
- **Kitagawa, H., Amemura, A., and Harada, T., (1975).** Studies on the inhibition and molecular properties of crystalline *Pseudomonas* isoamylase. Ag. Boil. Chem. 39, 989-994.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Koneman, E. W.; Allen, S. D; Janda, W. M. and Schreckenberger, D. C. (1992), c**olor plates and textbook of diagnostic microbiology. 4 th ed. J. B. Lippincott Company, Philadelphi.
- **Kulp, K. (1979).** Carbohydrase, In: (Enzyme in food processing, G. Reed, ed., pp.45-123), Academic press, New York, London.
- **Kuo, M. J. and Hartman, P.A (1966).** Isolation of amylolytic Strains of *Thermoactinomyces vulgaris* and production of thermophilic actinomyces amylases. J. Bacterio., 92 (3): 723-726.
- **Lee EYC, Whelan WJ (1971).** Glycogen and starch debranching enzymes. *In* P Boyer, ed, The Enzymes, vol. 5. Academic Press. New York. Pp: 191-234.
- **Liliana, C.; Lozeno, c. and Lozeno, J.E. (2002)**. Amylase for Apple juice processing effect of pH, heat and Ca ions. Food. Technol, Biotechnol. 40 (1): 33-38.
- **Lin, T. - P., Caspar, T., Somervill, C., Preiss, J. (1988)** *Plant physiol*. 86, 1131-1135.
- **Linker, A. and Evans, L.R. (1984).** Isolation and characterization from mucoid Strains of Pseudoomnsd aeruginosa. J. Bact. 159 (3): 958- 964**.**
- **Lonsane B. K., Ramesh, M. V., (1990).** Solid state fermentation for production of - amylase by *Bacillus megeterium* IGM. Biot. Lett. 9 (5): 323-328.
- **Machius, M., declerck, N., Huber, R. and Wiegand, G. (1998)**. Activation *Bacillus licheniformis* - amylase through a disorder-disorder transition of the substrate- binding sitemediated by a calicium- sidoium metal triad. Structure 6: 281- 292.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Madi, E. and Antranikian, G. (1989).** Identification of a starch degrading an aerobic thermophile producing thermostable - amylase and pullulanase. Appl. Microbiol. Biotechnol. 30: 422- 425.
- **Maniatis, T.; Fritch, E. F. and Sambrook, J. (1982).** Molecular Cloning. A laboratory. Cold spring Harbour. New York.
- **Marne, C. and Vandel. A. (1998).** Extracellular enzyme of fecal strains of *Pseudomonad aeruginosa*. Eur. J. Clin. Micrbiol. And Infection disease. 7 (2): 307-309.
- **Martin, J- F. and Demain, A. L. (1980).** Control of Antibiotic Biosynthesis Microbiol. Rev. 44 (2): 123- 251.
- **Matsuura, Y.**, Kusunoki, M., Harada, W. and Kakuko M. (1984). J. Biochem. (Tokyo) 95: 697-702.
- **Mauro, B. and Kobayshi, T. (1951).** Enzymatic Scission of branch links in amylopectin. Nature 167: 606-609.
- **Maza, L.M.; Pezzlo, M. T.; Baron, E.J. (1997).** Color atlas of diagnostic microbiology. Mosby- yearbook, Inc, U. S. A.
- **McKee, J. R., and McKee, T. (1996)**. Biochemistry, an Introduction. P. 121. USA.
- **Muller- Röber B. Sonnew ALD, U., Willmitzer, L. (1992).** EMBO J. 11: 1229-1238.
- **Myers, A. M, Morell M K, James SG:** recent progress toward under standing the amylopectin crystal. Plant Physiol. 2000 (122): 989-997.
- **Neilsen, J. E.;Borchert, T. V.; andVriend, G. (2001).** The determinants of - amylase pH- activity profiles. Protein Engineer. 14 (7): 505- 512.
- **Nonaka, T., Fujihashi, M.; Kita, A.; Hagihara, H.; Ozaki, K.; Ito, S. and Miki, K. (2003)**. Crystal structure of calcium free -

This document was created using SOLID CONVERTER PDF. To remove this message, purchase the product at www.SolidDocuments.com

amylase from *Bacillus* sp. strain KSM-K 38 (Amy 38) and It's sodium Ion Binding sites. J. Biol. Chem. 278 (27): 24818-24824.

- **Norman, B. B. (1979).** The application of polysaccharide degrading enzymes in the starch industry. In: Micrbial polysaccharides and polysaccharase. (eds.) Berkely, R. C. W., *et al*.,) chp.15: 339-374. Academic Press.
- **Norman, B. E., (1982).** Saccharification of starch hydrolysate. U. S. Patent 4, 335, 208.
- **One, K.; Shintani, K. and Shikata, S. (1988).** Agric. Biol. Chem. 52: 1966 (cited by Pandy *et al*., 2000).
- **Pandy, A.; Nigma, P.; Soccal, V. T.; Singh, D. and Mohan, R. (2000).** Advances in Microbial amylase. Biotechnol. Appl. Biochem. 31: 135- 152.
- **Preiss, J., Sivak, .M. N. (1996**). Photos assimilate Distribution in Plants and Crops: Source- Sink Relationships (Zamski, E., Schaffer, A. A., eds), P. 63, Marcel Dekker, New York.
- **Pretorius, I.S., Dekock, M. J.; Britz, H. J. and Lategan, P.M. (1986).** Numerical taxanomy of - amylase producing *Bacillus* species. J. Appl. Bacteriol. 60: 351-360.
- **Priest, F.G. (1977).** Bacterial. Rev.41: 711-753 (cited by Lin *et al*., 1998).
- **Priest, F.G. (1992).** Enzymes, Extracellular. In: encyclopedia of microbiology. Academic Press, Inc. 2 : 81-93.
- **Priest, F.G. (1993).** *Bacillus.* In: (Biotechnology, H-J. Rehm, and G. Reed, eds., 368-400.
- **Rantanakhano kchai, K.; Kaneko, J.; kamio, Y. and Izaki, K. (1992).** Appl. Enviromen. Microbiol. 58: 2490-2494 (cited by Pandy *et al*., 2000).

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Reddy, O. V. and Basappa, s.c. (1996).** Biotechnol. Lett. 18: 1315- 1318. (cited by Pandy *et al*., 2000).
- **Reddy, N.S.; Nimmagadda, A. and Roa, R.S. (2003).** An overview of Pandey, A., Nigma, P.; Soccal, C. R.; Soccal, V.T.; Singh, D. and Mohan, R. (2002). Advances in microbial amylase. Biotechnol. Appl. Biochem. 31; 135-152.
- **Reed, G. (1975).** Carbohydrates. In: Enzymes in Food processing, 2*, Academic press. New York.
- **Remeo, T., Kumar, A., and Preiss, J. (1988)**. Gene (*Amst*.) 70, 363- 376.
- **Rendelman, J. A. Jr., (1997)**. Enhancement of cyclodextrin production through use of debranching enzymes. Biotechnol. Appl. Biochem. 26, 51-61.
- **Robyt J.F. and W helan, W.J. (1968).** Amylase and their action on starch and it 's derivatives. In starch and it's derivatives, J.A., radley, ed., 423-497), Champan and hall, London.
- **Ruth, H.**, **Steinberger and Karen w. (1981).** Biology Laboratory manual 3rd edition.(7): 75-76.
- **Sakashi, H., kurosawa, K, and Takao, S. (1986).** Screeiong of microorganisms for raw starch saccharifying enzyme production. Agric Biol. Chem., 50 (6):1661-1664.
- **Sather, B.T. (1969**). Comp. Biochem. Biphy. 28: 371- 379. (cited by Saito, N. 1973).
- **Sato, H. H., and Y. K. Park. (1980**). Purification and characterization of extracellular isoamylase from *Flavobacterium* sp. starch stärke.32: 132-136.
- **Segal, I. H. (1975).** Enzyme kinetics. Hohn Wiley and Sons, New York, London.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Seinouke U. and Nobuya N. (1967)**. Production of an isoamylase by Escherichia inermedia. Vol. 1 (3): pp. 492- 496.
- **Sen, S., and Charkrabaty, S.L. (1987)**. Amylase from *Lactobacillus cellibiosus* D- 39 isolate from vegetable wasted: Characteristics of immobilized Enzymes and whole cell. Enzyme. Microbiol. Technol. 9: 112-116.
- **Shamala, T.R. and Sreekantiah, K.R. (1987).** Degradation of substrates by a crude enzyme preparation and utilization of the hydrates for Lactic Fermentation. Enzyme. Microbial. Technol. 9: 726-729.
- **Sharma, A.; Sharma, S. and Gupta, M.N. (2000).** Purification wheat germ amylase by precipitation protein Expression and purification. Enzyme. Microbiol. Technol. 18: 111-114.
- **Shi, Y-C., Cui, X., Birkett, A. M., and Thatcher, M. G., (2006)**. Resistant starch prepared by isoamylase debranching of low amylase starch. U. S. Patent 7, 081, 261 B 2.
- **Shinke, R., Nishira, H. and Mugibayashi, N. (1974).** Isolation of - amylase producing microorganism. Agric. Biol. Chem., 38: 665-666.
- **Smith, A. 1999**. Making Starch, Curr. Opinion in plant Biology 2: 223-229.
- **Smith, A., M., Bettey, Bedford, I.D. (1989)** *Plant physiol*. 89, 982-985.
- **Spencer- Martin, (1982).** Exracellular isoamylase produced by the yeast *Lipomyces kononenkae.* 44 (60), 1253- 1257.
- **Spencer- Martins, I. and N. Van Uden (1977)**. Yields of yeast growth on starch. Eur. J. Appl. Microbial. 4: 29- 35.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Stark, D. M.; Timmerman, K. P., Barry, G. F., preiss, J., Lishore, G. M. (1992).** Science 258: 287- 292.
- **Starr, M. P.; Stolp, H.; Traper, h. G.; balaws, A. and Schiegel, H. G. (1981**). The prokaryotes, A hardbook of Habitats, Isolation and Identification of bacteria. Mc Grow- Hill, New York, U. S .A.
- **Stolp, H. and Gudkari, D. (1981).** Non- pathogenic members of the genus *Pseudomonas*. In: The Prokaryotes (Eds. Starr, P. M.; Stolp, H.; Turner, H. G.; Balaws, A., and Schlegel, H. G. pp: 721-741. Vol. I.
- **Stolp, H. and Gudkari, D. (1984**). Non- pathogenic members of the genus *Pseudomonas*. In: The Prokaryotes (Eds. Starr, P. M.; Stolp, H.; Turner, H. G.; Balaws, A., and Schlegel, H. G.1: 721-741.
- **Takahashi, K.; ABE J-I.; Kxozuma, T.; Yoshidam M.; Nakamura, N. and Hizukuri S. (1996).** Production and purification of an isoamylase from Flavobacterium odoratum. J. Enz. And Microbial technology. 19:456-461.
- **Takasaki, Y. (1989).** Nvel maltose-producing amylase from *megaterium* G-2. Agric. Biol. Chem. 53(2): 341-347.
- **Takashi, K.; Abe J-I.; Kozuma T.; Yoshida M.; Nakamura N, and Hizukuris (1996**). Production and application of isoamylase from Flavobacterium odoratum. J. Enzy. And Micrbiol. Technology. 19: 456-461.
- **Takekawa, S., Uozumi, N, Tsukagoshi, N. and Uda, S. (1991).** Proteases involved in generation of -and -amylase from a large amylase precursor in *Bacillus polymyxa.* J. Bacterial 173 (21): 6820-6825.
- **Tasi, C.Y., Nelson, O. E. (1966)** Science 151, 341-343.

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

- **Toda, H., Kondo, K. Narita, K. (1982).** The complete sequence of Taka- amylase A. proc. Jpn. Acad. 58: 208-212.
- **Tonozuka, T., Ohtsuka, M., MOGi, S-I., Sakai, H., Ohta, T. and Sakano, Y. (1993).** Aneeophullulanse- type -amylase gene from *Thermoactinomycetes vulgaris* R-47. Bio Sci. Biotech. Biochem. 57(3): 395-401.
- **Ubran, A.; Martina, L.; thoreston, E. and Erichjaeger, K. (2001).** BsbxA and DsbC effect exracellular enzyme formation in *Pseudomonas aeruginosa*. J. bacterial. 193 (2): 587- 596.
- **Uralub, H., and G. Wöber. (1975)**. Utilization of branched - glucans in Bacillus amyloliquefaciens. Biochem. Soc. Trans.3: 1076- 1078.
- **Vallee, B., L., Stein, E.A. Summer well, W.N., and Fisher, E.H. (1959).** Metal content of - amylase of various origins. J. Biol. Chem. 234 (11): 2910- 2950.
- **Volesky, B., and Loung, L. (1985).** Microbiol Enzymes Production, purification and isolate (CRC) (critical Review in Biotechnology. 2: 199.
- **Walker, G.J. and Whelan W.W. (1960).** The mechanism of carbohydrase action. Biochem.J., 76:264-268.
- **Wallenfels, K., H. Bender, and J.R. Rached (1966)**. Pullulanase fom *aerobacter aerugenes*: production in a cell- bound state. Purification and properties of the enzyme. Biochem. Biophys. Res. Commun. 22. 254-261.
- **Wang, H. (1999).** Bioelecrochemical characteristics of cholesrol oxidase immobilized in a polyaniline film. 56: 22-30.
- **Whitaker, J.R. (1972).** Principles of Enzymology for food sciences. Marcel Dekker, Inc. New York. 8: 22-23.

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

- **Whitaker, J.R., and Bernhard, R.A. (1972).** Experiments for an introduction to Enzymology. The Wibber press Davis. 2: 12-14.
- **Yokobayashi, K., akai, H., Sugimoto. T., Hirao, M., Sugimoto, and K., Harada, T., (1973).** comparisson of the kinetic parameters of *Pseudomonas* isoamylase and *Aerobacter* pullulanase. Biochem. Biophys. Acta, 293, 197-202.
- **Yokobayashi, k., Misaki, A., Harada, T., (1970)**. Purification and properties of *pseudomonas* isoamylase. Biochem. Biophys. Acta, 212, 458-469.
- **Yoshi, Yoshihiro M., Michio K. and Yoshiki M. (1998).** Three dimensional structure of *Pseudomonas* isoamylase at 2.2 A° resolution. J. Molecular Biol. 281 (5): 33- 37.
- **Zeeman Sc, Northrop K, Smith AM, Rees T:** A starch-accumulating mutant of *Arabodopsis thaliana* deficient in a chloroplastic starch- hydrolyzing enzyme. *Plant* J. 1998, 15: 357-365.
- **Zoltwaska, K. (2001).** Purification and characterization of - amylase from intestine and muscle of Ascaris suum. 3:23-24.

This document was created using SOLID CONVERTER PDF

To remove this message, purchase the product at www.SolidDocuments.com

الإهداء

....

سيد الخلق ونور الهدى ورسول المحبة وخاتم النبيين.. (صلى الله عليه وسلم)

إلى الذين تمنيت روحي فداهم.. نور عيني ..

إلى نصفي المكمل وتوأم روحي .... التي قرنت سعادتي بسعادتها ....

إلى الذين جرت وتجري دمائهم في عروقي ....

إلى من علمني حرفا وملكني عبدا.........

اهدي ثمرة جهدي امتنانا وتقديرا

حنين

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com

بسم الله الرحمن الرحيم

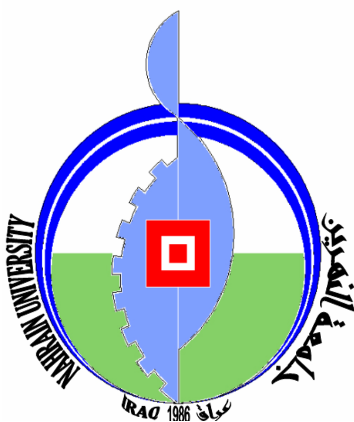
نرفع درجات من نشاء وفوق

كل ذي علم عليم

صدق الله العظيم

سورة يوسف (أية76)

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com



جمهورية العراق
وزارة التعليم العالي و البحث العلمي

لنهرين

كلية العلوم

حيائية

أستخلاص وتنقيةوتوصيف ألايسوأميليز المنتج من

محلية من بكتريا *Pseudomonas* sp.

مقدمة إلى كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

This document was created using SOLID CONVERTER PDF
To remove this message, purchase the product at www.SolidDocuments.com

ون عبد الوهاب

بكالوريوس تقانة احيائية- عة النهرين ( )

أيلول

This document was created using SOLID CONVERTER PDF To remove this message, purchase the product at www.SolidDocuments.com