الخلاصة

درست قابليه العزله المحليه *Xanthomonas campestris* H6 على انتاج انزيم الالفا اميليز و قد تبين ان هذه العزله لها قابليه على انتاج انزيم الالفا اميليز الذي يفرز خارجيا.

بعدها درست الظروف المثلى لانتاج انزيم الالفا اميليز من هذه البكتريا وتم الحصول على اعلى انتاجيه للانزيم في وسط PM1 الحاوي على ٣,٥ % كلوكوز ، ٢ % مل بيبتون ، ٢,٥ ٢ % K_2HPO_4 ، عند اس هيدروجيني ٧ الحضن و بدرجه حراره ٣٠ م.

اوضحت النتائج التي تم الحصول عليها ان انزيم الالفا اميليز المنتج من بكتريا *Xanthomonas campestris* H6 هو من الانزيمات التي يتم افرازها بصوره اساسيه و انه لا يتأثر بعملية التثبيط بواسطه الكلوكوز مثل الكثير من انواع الاميليزات .

تم تنقية الانزيم في البدايه بعملية الترسيب بواسطة املاح كبريتات الامونيوم حيث كانت الفعاليه النوعيه ١٧,٥٦ وحده / مل و بعدد مرات تنقيه ١,٩ . بعدها نقي الانزيم بواسطة الترشيح الهلامي على عمود السيفاروز CL-6B حيث كانت عدد مرات التنقيه ٤,٢ بينما كانت الحصيله الانزيميه ٢١,٦٩% .

اختبرت نقاوة الانزيم و ذلك باجراء الترحيل الكهرباي في هلام متعدد الاكريل امايد في ظروف غير ماسخه للبروتين و اظهرت النتائج ظهور حزمه واحده فقط للبروتين مما يشير الى ان خطوات التنقيه كانت ناجحه .

و اظهرت النتائج ان الوزن الجزيئي للانزيم هو ١٤٠٠٠ دالتون. و ان الاس اهيدروجيني الامثل لفعالية الانزيم هو ٧ و ٦ حيث احتفظ الانزيم بكامل فعاليته عندما تم حضنه في هذه القيم ينما احتفظ الانزيم باكثر من ٩٠% من فعاليته عند اس هيدروجيني ٧,٥.

تم دراسة الثبات الحراري للانزيم حيث اظهرت النائج ان الانزيم احتفظ بكامل فعاليته عند درجات حراره ٣٠، ٣٥، ٤٠م و احتفظ الانزيم بحوالي ٩٥% من فعاليته عند درجة ٤٥م.

و عند التحري عن فعالية الانزيم بوجود العوامل الكلابيه ا وضحت النتائج عدم وجود أي تغير ملحوظ في فعالية الانزيم عند حظنه مع EDTA مما يدل على ان هذا الانزيم هو من الانزيمات اللافلزيه و التي لا تتأثر بوجود العوامل الكلابيه.

تنقيه و توصيف انزيم الالفا اميليز المنتج من العزله المحليه
Xanthomonas capmestris H6

رسالة مقدمة
الى كلية العلوم – جامعة النهرين
وهي جزء من متطلبات نيل درجة الماجستير علوم
في التقانة الاحيائيه

من قبل
يعرب راجي الحصونه

ربيع الاخر
١٤٢٥

1. Introduction & Literature Review

1-1 Introduction

The bioconversion of raw plant material into commercially important chemicals has become a stimulating field of biotechnology (Verhasselt *et. al*., 1989). Despite the fact, that metabolic pathways leading to such kind of bioconversion have been largely revealed, but almost nothing is known about the genes involved in the regulation of their expression. Moreover, if one wants to use plant raw materials like cellulose or starch in fermentation process, the secretion of the required hydrolyzing enzyme need to be studied.

One of the bacterial strains which have a major role in bioconvreston of the raw plant material is *Xanthomonas campestris*. *Xanthomonas campestris* is Gram negative bacteria which infect cruciferous plant causing black root disease. This bacterium have gained its importance from its ability to produce the xanthan polysaccharide which has many applications in industry, and a range of the extracellular enzyme that have a prime role bioconversion process of plant raw material into industrially useful materials(Hu *et. al.,* 1995)

One of these enzymes which are produced by *Xanthomonas campestris* is α – amylase enzyme that has a major role in the bioconversion of starch into smaller subunits in addition to its role in *Xanthomonas campestris* pathogenicity (Dow and Daniels 1994).

Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Windish and Mhtre 1965). Gupta *et. al*., (2003) have shown that the alpha amylase family comprises a group of enzymes with a variety of different specificity that all act on one type of substrate being glucose residues linked through α- 1,6 , α- 1,4 and α- 1,1 glycosidic bonds.

These enzymes are universally distributed throughout the animal, plant and microbial kingdoms. Although Pandy *et. al.,* (2000) had indicated that enzymes 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 (Reddy *et. al*., 2003).

Even though there are different microbial sources exist for the efficient production of this enzyme, but only a few selected strains of fungi and bacteria meet the criteria for commercial production (Reddy *et. al*., 2003). Since the thermostable alpha amylase of *Bacillus licheniforms* was first employed in starch liquefaction process in early of the seventieth, the α-amylase has been introduced into various industries and the spectrum of amylase application has widened in many other fields, such as medical, molecular and as well as the wide spread application in starch liquefaction and food industry (Shaw and Ou – Lee 1984; Pandy *et. al*., 2000).

In addition to its industrial importance, α – amylase has agricultural importance being considered as one of the positive plant pathogenicity factor in phytopathogenic bacteria (Kari and John 1988) and Nein *et. al.*, (1992) have used this enzyme as a module to study enzyme synthesis and transportation through plasma membrane in Gram-negative bacteria.

So due to the commercial and industrial importance of α – amylase , this study was aimed to:

- Determination of the optimum conditions for the α – amylase produced by the local isolate *Xanthomonas campestris*.
- Partial purification of the α – amylase produced by *Xanthomonas campestris.*
- Characterization of some of properties of the partially purified α – amylase.

1-2 Literature Review:

1-2-1 Genus *Xanthomonas:*

The first reported observation of phytopathogenic bacterium that nowadays would be referred to the genus *Xanthomonas* was made in 1881 by Wakker in case of yellow disease of hyacinths.(Mortimer, 1981). Elliot, (1930) was the first to classify members of this genus into independent group.

Xanthomonas are gram negative and obligately aerobic plant pathogens which are the causal agents of disease of at least 124 monocot and 268 dicot plant hosts (Leyns *et. al.,* 1984). Cells of *Xanthomonas* are straight bacilli of about 0.7 – 1.8 µm in length and 0.4 – 0.7 in width. Cells are usually found as singular or pairs and they are rarely found in chains. Optimum temperature is 25 – 30 °C and colonies are yellow in color, oxidase negative and catalase positive, GC content is about 65 – 71%(Bergy's manual 1994).

This bacterium is well known to produce an exopolysacchride, the xanthan gum , the xanthan pigment seems to be unique to the genus *Xanthomonas* and unlike those produced by other yellow pigmented bacteria (Star and Stephan, 1964), and a number of extracellular enzymes which find a variety of application in oil drilling, agriculture, and food industry (Sanford and Baird, 1983).

Synthesis of the extracellular enzymes and xanthan is activated by products of a cluster of at least five genes, designated *rpf* (regulation of pathogenicity factors) (Tang *et. al.,* 1990).

Being producer of xanthan pigment and being a wide spread plant pathogens, this bacteria has been, and still, the focus of many scientist and researchers. Xanthan polysaccharides which because of its physical properties and lack of toxicity to man and animals have a wide range of

uses in industry .They are gelling agents, emulsifiers, stabilizing agents and plasticizers particularly in food industry(Sanford and Baird 1983). Plant disease caused by *Xanthomonas* species occur the world over. Representatives of practically all major groups of higher plants suffer from one or more such disease, which range in economic importance from highly significant to negligible cultivars (Umemura and Kawano, 1983).

1-2-2 Xanthomonas campestris

Xanthomonas campestris is a phytopathogenic bacteria which is casual agent of black rot of crucifer plants, resulting in heavy loss in agriculture worldwide (Onsando, 1992). This species consists of more than 140 pathovars, which can be differentiated on the basis of the host plants they infect (Verneire *et. al.*, 1993). A distinctive feature of most of these pathovars is that they exhibit high degrees of host specificity in infection and members of the *Xanthomonas* sp. exhibit the highest level by far of both host range and race specificity. Although the species itself has a very wide host range that includes at least 68 plant families and more than 240 genera, any given strain is limited to a very narrow range of hosts, often a group of genera within one plant family or a group of species within one genus. This makes *Xanthomonas* an ideal species for the study of plant / microbe interactions (Asha and Dean, 2003: Hayward, 1993).

This bacterium is characterized by its ability to produce xanthan pigmented and different extracellular enzymes like α- amylase, polygalacturonate, lyase, protease and endoglucanase (Nein *et. al*., 1992; Dow and Daniels, 1994). This ability is attributed to the possession of a mechanism that help the organism secrete the enzymes through the periplasmic space to the outer membrane (Hu *et. al.*, 1995). Synthesis of

extracellular enzymes in *Xanthomonas campestris* pathovar *campestris* (Xcc) is subject to co-ordinate regulation by a cluster of genes called *rpf* (for *r*egulation of *p*athogenicity *f*actors). These genes are located within a 21 kb region of the chromosome of *Xanthomonas campestris*.(Dow, *et al.,* 2000). These extracellular enzymes play a major role in the pathogenicity of this species and they contribute collectively to pathogenesis because these enzymes are cell wall degrading enzymes which enable the *Xanthomonas* sp. to penetrate the xylem of susceptible plant. Dow *et. al.,* (2000) showed that mutation in the genes responsible for the synthesis of the extracellular enzymes had resulted in reduced pathogenicity. Nein *et. al.,* (1992) have found that pleiotropic mutant of *Xanthomonas campestris* showed reduced pathogenicity and accumulation of polygalacturonate, lyase, α- amylase and endoglucanase in the periplasm. This clearly demonstrated that the reduced pathogenicity observed with these mutants was due to the defect in exporting enzymes across the outer membrane.

Another feature of this species is that unlike *Rhizobium, Agrobacterium, Pseudomonas, Ralstonia* and *Erwinia*, members of the genus *Xanthomonas* are always plant-associated (although not always pathogenic) and none are free-living or soil-borne.

The biochemistry and genetics of *Xanthomonas campestris* are largely unknown. Methods for plasmid transformation by electroporation (Wang and Tseng, 1992) and conjugal transfer of plasmids (Daniels *et. al.,* 1984) have just been recently developed for gene cloning in this species, and a transducing phage has been characterized (Weiss *et. al.,* 1994). However, these techniques have not been extended to other genetic studies of this species, and no genetic map has yet been reported.

Therefore, as a first effort to characterize and study the chromosome and due to unavailability of a genetic map the chromosome, recently many researchers have constructed a physical map for these bacteria. Tseng *et. al.*, (1999) were able to construct a physical map and they found that the chromosome of *Xanthomonas campestris* is about 4.8 Mb in length. De Silva *et. al*., (2002) have shown that the chromosome is about 5Mb in length. Other studies have indicated that the chromosome of *Xanthomonas campestris* is 5Mb nucleotides, which encodes for about 4129 proteins (Widjaja *et. al*., 1999).

1-2-3 The Amylase Family

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

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

They can be divided into two categories, endoamylases and exoamylases. Endoamylases 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 microorganisms (Whitaker, 1972; Norman, 1979; Fogary and Kelly, 1980). They can be divided into the following main groups:

1-2-3-1 α-amylases

E.C.3.2.1.1, α-1, 4-glucan-4-glucanhydrolase

These enzymes can be found in plant, animal and they are the most abundant starch-hydrolyzing enzyme in microorganisms (Fogarty, 1983).

A classification system for glycosyl hydrolyses, based on sequence similarity, has led to the definition that most of the starch hydrolyzing enzymes belong to the α-amylase family or family 13 glycosyl hydrolases based on amino acid sequence homology according to the classification of Henrissat, (1991). Table (1) shows known activities of Glycosyle hydrolase family 13 enzymes.

These enzymes are endo-split enzyme, which randomly attack the α-1, 4 gycosidic bonds in amylose, amylopectin, and glycogen to produce dextrin, maltose, and glucose (Howling, 1989; Piest, 1992).

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

Many researches had 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 α-limit dextrin (Walker and Whelan, 1960; Kulp, 1979).

Table 1 Known activities of Glycosyle hydrolase family 13 enzymes(Reddy, 2003).

Enzyme	Main Substrate
Amylosucrase	Sucrose
Sucrose phosphorylase	Sucrose
Glucan branching enzyme	Starch, glycogen
Cyclomaltodextrin glycosyltransferase	Starch
Amylomaltase	Starch, glycogen
Maltopentaoe-forming α-amylase	Starch
α- amylase	Starch
Oligo-1,6-glucosidasse	1,6-α-D-glucosidic linkages in some oligosaccharids
α- glucosidase	Starch
Amylopullulanase	Pullulan
Cyclomaltodextrinase	Linear and cyclomaltodextrin
Isopullulanase	Pullulan
Isoamylse	Amylopectin
Maltotetraose-forming α-amylase	Starch
Glucodextranase	Starch
Trehalose-6-phophate hydrolase	Trehalose
Maltohexaose0forming α-amylase	Starch
Maltogenic amylase	Starch
Neopullulanase	Pullulan
Malto-oligosyl trehalase hydrolase	Trehalose
Malto-oligosyl trehalose synthase	Maltose

1-2-3-2 β-amylases enzyme:-

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

Widely distributed enzyme 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).

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

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

1-2-3-3 Glucoamylases:-

E.C. 3.2.1.3 α-1, 4-glucan 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; Sakaki *et. al,* 1986; Priest, 1992).

This enzyme attack successively the α-1,4 glycosidic bonds in amylose, amylopectin and glycogen to produce β-glucose residues from their non-reducing ends. These enzyme can also hydrolyze α-1,3 glycosidic bond

but at slower rate than that of α-1,4 glycosidic bond. (Fogarty, 1983; Tonozuka *et. al.,* 1993).

1-2-3-4 Debranching enzymes:-

E.C. 3.1.2.6 Glycogen 60glucanohydrloase

Enzymes of this group have the ability to attack the α-1,6 glycoside bond in amylopetin, glycogen and pullun.

Mauro and Kobayshi (1951) was the first to isolate these enzyme from yeast. This group consists of two main enzymes:-the isoamylase and pullulanase. The former one acts on glycogen and amylopectin 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 α-1,6 glycosidic bonds (Kulp, 1979).

Table 3 shows amylases and their different sources.

Table 2 show the different amylases enzyme and their sources (Harada *et. al*., 1984)

Enzyme	Source
α-amylase	*Bacillus acidocaldaruis* *Bacillus amyloliguefaciens* *Clostridium acet.obutylicum* *Aspergillus candidus* *Aspergillus niger* *Penicillium expansum* *Mucor michei* *Rhizopus javanicus*
β- amylase	*Bacillus cereus* *Bacillus subtilits*
Amyloglucosidase	*Aspergillus niger* *Aspergillus oryzae* *Pencillium* sp. *Rhizopus niveus* *Endomycopsis capsularis* *Saccharomyces diastaticus*
Debranching enzyme	*Klebsiela pneumonia* *Bacillus endopullulyticus* *Pseudomonas* spp.

1-2-4 Factors Affecting a- Amylase Production:-

1-2-4-1 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 propar carbon sources since the production of this enzyme in many microorganisms occurs by induction (Goodfellow *et. al.*, 1987).

Musaeva (1966) had tested the effect of starch, glycerin, manitol and dextrin on the production of α- amylase, and he found that media containing 6% starch gave the highest level of production of this enzyme.

Other researchers showed that maltose could be the main stimuli in production of the α- amylase of *Streptomyces* spp. and *Nocardia* spp. (Mordarski *et. al.*, 1970; Mordarski *et. al.*, 1972). In another experiment, it was found that production of α- amylase could only be stimulated in presence of either maltose or starch (Wojskaowicz, 1977). Sinha and Chandra (1981) used starch as a carbon source for production of the enzyme from *S. rimosus*.

Fairbrain *et. al.*, (1986) had studied the effect of different carbon source on α- amylase production by *S. limosus*. They had used glucose, lactose, galactose, sucrose, maltose, xylose, malt extract, soluble starch and corn starch and each source was used independently in the media. It have been shown that the highest productivity of α- amylase obtained from maltose and malt extract being used as carbon source while Teodoro and Martines (2000) showed that glucose has a repression effect on the production of amylase enzyme by *Bacillus* sp..

Other researchers have indicated that starch as the suitable carbon source for production of alpha amylase in many different bacteria. Starch is the appropriate carbon source in *Thermoactinomyces viridis, Proteus vulgaris* and in *Bacillus* sp. TS-24 (Upton and Fogarty, 1977; Lin *et. al.*, 1998).

1-2-4-2 Effect Of Nitrogen Source:-

The nitrogen source is one of the basic elements for production of α-amylase. It is supplemented to the media in the form of inorganic salts or as organic sources of nitrogen. (Kuo and Hartman, 1966). The effect of the nitrogen source varies from one microorganism to another. For example, Upton and Fogary (1977) had found, among eight different nitrogen sources tested on *T. viridis*, peptone gave the highest productivity of the α- amylase . While Obi and Odibo (1984 a, b) found that ammonium sulphate is the best source for the production from *Thermoactinomyces* spp.

As a general case, most of the microorganisms prefer the nitrogen source in the form of ammonium salts and some of them are incapable of utilizing the nitrogen sources in the form of nitrate ions (Priest, 1993).

1-2-4-3 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 α- amylase, 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 α- amylase.

It has been found that the optimum pH for alpha amylase production is near the neutrality point or slightly deviated from it. In addition it has been found in some researches that the highest productivity of the α-amylase enzyme from *Bacillus megaterium* at pH 7.

However, different microorganism uses different pH values for the optimum production of α- amylase enzyme. For example, the optimum pH of *T. vulgaris* is ranging 6.5 – 7.5 (Kuo and Hartman, 1966) while the optimum pH of *Thermus* sp. Ranging from 5.5 -6.5 (Shaw *et. al.,* 1995).

Heinen and Heinen (1972) had demonstrated that the medium constitution (carbon source, nitrogen source, salts, metal ions ...etc.) can play major role in keeping the pH value within the convenient limit for growth and activity of alpha amylase – producing microorganisms. In this context, Raimbult and Alzad (1980) used urea as a nitrogen source to reduce the acidity of the media while Beckord *et. al.,* (1945) used calcium salts to optimize the media for production of alpha amylase by *Bacillus subtilis.*

1-2-4-4 Temperature:-

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

The optimum temperature of the α- amylase varies according to the source of the enzyme, Ghosh and Chamdra (1980) found that the optimum temperature of the α- amylase from *Bacillus aqiarius* is about

38 ˚C, while Bahri and Ward (1990) used the 50 ˚C for production of alpha amylase from *S. thermoviolaceus*.

1-2-4-5 Calcium Ions:-

Calcium ions are radical factor in the stability of the α-amylase enzyme. Machius *et. al.*, (1998). showed that the calcium-binding sit conserved in all α-amylase enzymes forms part of an unprecedented linear triadic array, with two ca ions (CaI and CaII) flanking a central Na ion (Ca – Na – Ca metal triad). In the case of bacterial α-amylases, the metal triad and an additional Ca ion (CaIII) contribute to the increased thermostability.

Moreover, α-amylases undergo reversible transition from the active to the inactive state upon removal of Ca ions with chelating reagents such as EDTA, and because 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).

1-2-5 a- Amylase Synthesis and Transport:-

In Gram negative bacteria, the extracellular proteins have two lipid bilayers to cross, in contrast to the proteins targeted in to the periplasm or the outer membrane. α-amylase is synthesized as a preprotein precursor composed of a typical signal peptide, Mature α-amylase and a C-terminal propeptide. The latter part is removed by a nonspecific protease shortly after secretion in the extracellular medium (Feller *et. al.*, 1998).

Christoph (1998) and Claverie *et. al.*, (2003) had indicated that α-amylase enzymes in Gram negative bacteria is secreted using pathway II of protein secretion pathways which is the primary pathway for the secretion of extracellular degradative enzymes by gram-negative bacteria.

Type II secretion pathway involves a separate step of transport across the inner membrane prior to transport across the cell envelope. A distinctive feature of this pathway is that the protein being transported will be exported to the periplasm via the secretion system. This pathway is called *sec*- dependant pathway (Pugsley, 1993).

A signature of type II dependant protein export is the presence of a short (about 30 amino acids), mainly hydrophobic amino-terminal signal sequence in the exported protein. The signal sequence aids the protein export and is cleaved off by a periplasmic signal peptidase when the exported protein reaches the periplasm (Feller *et. al.,* 1998). This propeptide has no intramolecular chaperone function as the recombinant α- amylases produced with or without the propeptide have identical structural, kinetic and ion binding properties. However, in the absence of the propeptide, the enzyme accumulates in the periplasmic space of *E. coli* cells, whereas the full-length precursor is recovered in the culture medium. This has lead to the proposal that the propeptide is a secretion helper (Feller *et. al.,* 1998, Tutino *et. al.,* 2002).

In type II secretion pathways, transport across the outer membrane requires an additional set of inner and outer membrane proteins. Pugsley (1993) have shown that in the case of pullulanase secreted by *Klebsiella oxytoca*, the best-studied example of type II secretion, 14 additional secretion factors, which are encoded by a continuous gene cluster, are necessary and sufficient for secretion. At least seven of these proteins are located in the cytoplasmic membrane, while PulS and PulD are outer membrane proteins.

In *Xanthomonas campestris*, Nein *et. al*., (1992) showed that secretion of the α- amylase , and other extracelllular enzymes, to the out side can not be completed only with the presence of protein of 83.5 kDa. This

signal peptidase protein, which is called is called xpsD protein, is an outer membrane protein with part of it exposed to the cell surface and it is responsible for the processing of the C – terminal propeptide of the α-amylase. Mutation in the ORF of this protein had lead the accumulation of the enzyme , together with other enzymes, in the cytoplasm of the bacteria.

Other examples of type II secreted protein include the *out* pathway of *Erwinia* spp. for the secretion of pectic enzymes and cellulases, and secreation of elastase, exotoxin A, phospholipase C by *Pseudomonas aeruginosa*, and also the secreation of amylase and protease by *Aeromonas hydrophila*. (Hobbs and Mattick, 1993*).*

1-2-6 Mode of Action Of α- Amylase:-

Tester *et. al*., (2004), have demonstrated that digestion of starch is effected by hydrolyzing enzymes in a complex process which depends on many factors; these include the botanical origin of starch, whether the starch is amorphous or crystalline, the source of enzymes, substrate and enzyme concentration, temperature and time, as well as the presence of other substances in the multi - component matrix in which starch occurs naturally, e.g. cereal grains. Native starch is digested (i.e. hydrolyzed) slowly compared with processed (gelatinized) starch whose crystalinity has been lost and where the accessibility of substrate to enzymes is greater and not restricted.

α- amylase is one of the endo-splitting enzymes which attacks α- 1, 4 glycosidic bonds in amylase, amylopectin and glycogen from the inside. This type of enzyme exerts its effect in a random and disorganized fashion to produce glucose, maltose and dextrin and for this reason this type of enzyme is called "liquefying enzymes" (Fogarty and Kelly, 1979).

The mode of action of α- amylase when acting on amylose consists of two stages. In the first stage, maltose and maltotriose are produced while in the second stage; glucose and maltose are produced as final products. During its action on amylopectin, glucose, maltose, and branched chain of α- limit dextrin are produced (Fogarty and Kelly, 1980).

Koshland in 1953 was the first to suggest a mechanism for the hydrolysis of starch by the effect of amylase enzyme. Different amylases have different mode of action on starch (Skov *et. al.*, 2001).

The a-amylase reaction mechanism is a general acid catalysis, similar to all of the glycoside hydrolases and the chemical groups that participate in the hydrolysis reaction composed of positively charged amidazole groups and a negatively charged carboxylic group. The reaction is initiated by simultaneous protonation of the glycosidic bond by a proton donor and a nucleophilic attack on the anomeric carbon of the glucose moiety. This leads to the covalently linked substrate - enzyme intermediate. The intermediate can react with either water or with another saccharide molecule (Sinnot, 1990; Skov *et. al.*, 2001).

1-2-7 Amylase Application:-

Amylases are among the most important enzymes and are of great significance in present-day biotechnology (Pandy *et. al.*, 2000).

These enzymes constitute a class of industrial enzymes having approximately 25% of the enzymes market and they are the first enzyme to be exploited by genetic engineering techniques (Sindhu *et. al.*, 1997; Rao *et. al.*, 1998). Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (Pandey *et. al.*, 2000).

Although both bacterial and fungal amylases are abundant, fungal amylase are more frequently used than their bacterial counterpart in industry. These enzymes have many application in industry. For example it used as additive in detergent, in desizing of textile and proper formation of dextrin in baking. Other researches showed that thermostable amylases are used for liquefying starch and to produce the high fructose corn syrup (Norman, 1979; Shawn and Pan, 1989).

In medicine, α- amylase together with many other microbial enzymes such as protease or lipase can be orally supplemented for their ability to offer effective digestive support that work synergistically with, or as alternatives to the animal derived enzymes in case of disorders (Brad, 1997).

Satoh *et. al.,* (1993) were able to make use the α- amylase enzyme in new field. They were able to use α- amylase gene, cloned from *Streptococcus bovis*, in the construction of new efficient cloning vector to be used in *lactobacillus delbrueckii*, which is known to be extremely difficult to be transformed.

When using in industry, an important criteria should be put in consideration which the thermostability. The thermostability of this enzyme must be matched to the application. For example, thermostabile alpha amylase should be used for liquefaction of starch at high temperature and the thermolabile enzyme should be used in baking. (Shaw *et al.,* 1995).

1-2-8 Purification Of a Amylases:-

Enzyme application in pharmaceutical and clinical sectors requires high-purity amylases. Thus, it was very important to develop economic processes for their purification to obtain chemically pure enzymes with maximum specific activity. Several methods have been made possible for purification and characterization of α- amylase, from both mesophilic and thermophilic bacteria, and these methods include ammonium sulphate precipitation, dialysis, ion exchange chromatography, gel filtration and different solvents like acetone (Pandey *et. al.*, 2000). Purity of the amylase obtained depends on the method of purification.

Mastui *et. al.*, (1977) used the following steps to purify the amylase enzyme: ammonium sulphate precipitation followed by ion exchange chromatography of DEAE-Cellulose and DEAE – Sephadex A 50 consecutively. Then they used gel filtration (Sephadex 75) twice. By doing this they were able to get a 1764.7 fold purified enzyme with 2.4% recovery. Nein *et. al*., (1992) were able to purify the α- amylase from *Xanthomonas campestris* using ammonium sulphate precipitation.

Uguru *et. al*., (1997) described a thermostable extracellular *a*-amylase from *B. subtilis*, which was purified 24-fold to a specific activity of 2200 units/mg per liter. Bolton *et. al*., (1997) purified α- amylase to homogeneity using a combination of ammonium sulphate precipitation, ion-exchange chromatography and gel filtration.

An extracellular thermostable α- amylase produced by *B. licheniformis* was purified by two-phase separation in a poly ethylene glycol system followed by gel filtration and ion-exchange chromatography (Ivanova *et. al.*,1993).

Katoh and Kohei, (1997) purified an α- amylase, produced by recombinant cells, by specific elution from anti-peptide antibodies. Ilori

et. al., (1997) purified extracellular α- amylase from *Micrococcus luteus* and *Lactobacillus brevis*. Purification was by 28- and 70-fold by gel filtration and ion exchange chromatography, respectively.

Shih and Labbe (1995) used a sequence of steps in order to get a purified α- amylase from *clostridium perfringens*. The supernatant fluid was concentrated by dialysis against polyethylene glycol chips. Concentrated fluid was then dialyzed against Tris-HCl buffer and then applied to a DEAE-Sephacel column. Active fractions from the DEAE-Sephacel column were dialyzed against sodium phosphate buffer ultrafiltration.

Finally the concentrated material was applied to a Sephacryl S-100. The amylase obtained was 24.4 fold purified with 11.3 recovery.

Although there are only a very few reports describing the purification of *a*-amylase from yeasts, a raw starch - digestive thermostable *a*-amylase from yeast *Cryptococcus* sp. was purified in just one step by using an cyclodextrin– Sepharose 6B column (Iefuji *et. al.*,1996).

Recently, Teotia and Gupta., (2002) were able to develop a new protocol for the purification of α- amylase enzyme. They found that α-amylase from various sources was found to bind alginate in free solution. The alginate–enzyme complex could be precipitated with Ca. The enzyme activity could be recovered by dissolving the precipitate in 1 M maltose and precipitating alginate alone by addition of Ca. According to this method they were able to purify the wheat germ amylase Based upon these observations, α- amylase from wheat germ was purified with 68-fold purification and 72% recovery.

1-2-9. Global Production Of Amylases:-

Aunstrup (1977) had estimated that the overall global production of the amylase enzymes in 1977 have reached 610 tones of pure enzyme. This enzyme comprise about:

* 300 tones of Glugoamylase (Amyloglucosidase).
* 300 tones of bacterial amylase.
* 10 tones of fungal amylase.

Most of these amounts had been used in the processes of starch liquefaction.

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3. Results & Discussion

3.1 Ability of *Xanthomonas campestris* H6 in α – amylase Production:

In order to test the ability of *Xanthomonas campestris* H6 (Xc H6) in α – amylase production, Xc H6 was allowed to grow on nutrient agar plates supplemented with 1% starch for 18 hours at 30 ˚C. After the flooding the plate with lugal's solution, a clear zone developed around the colonies of Xc H6,as shown in figure 3, and this result indicates the ability of the Xc H6 to produce the extracellular α – amylase (fig. 3).

After detection of α – amylase production, Xc H6 was grow in PM1 medium to estimate the activity of α – amylase in crude culture and results have revealed that the specific activity was 8.09 U/mg protein while the enzyme activity in crude extract was 12.93 U/ml and protein concentration in culture filtrate was 1.2 mg/ml.

3.2 Determination Of The Optimum Condition for α – Amylase Production:

3.2.1 Effect of pH Medium:

Optimum pH represents one of the important parameters which effect enzyme activity. It was found that most enzymes remain active within a wide or narrow range of pH. However most of enzymes appear to be active at the optimum pH of the microorganism itself (Reed, 1975).

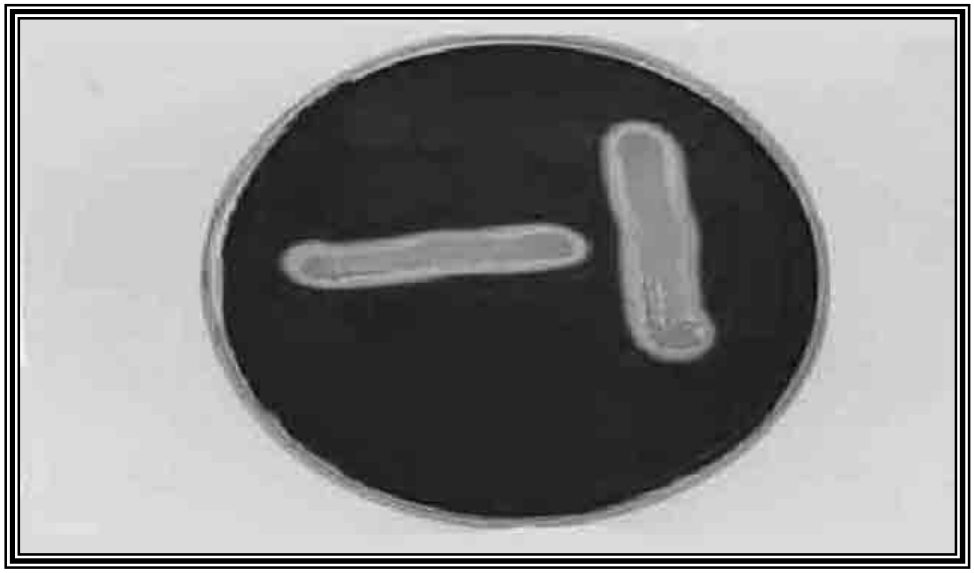

Figure (3): hydrolysis of starch by Xc H6 on PM1 media.

To investigate the initial medium pH effect on α – amylase production, Xc H6 was grown on PM1 medium with different pH values (6, 6.5, 7, 7.5, and 8).

Results in figure (4) showed that, the highest α – amylase production was obtained from Xc H6 when the pH of PM1 medium was equal to 7, and in this case the enzyme activity was 8.31 U/ml while the specific activity was 10.33 U/mg protein. The specific activity of the enzyme was decreased when Xc H6 allowed when the medium pH was less or more than the optimum pH.

Other studies also showed that pH7 is the optimum medium pH for α – amylase from *Streptomyces. hygroscopicus (*Hidaka *et. al*., 1978) and from *Bacillus stearothermophilus. (*Al-Safar 2000).

The fundamental characteristics of extracellular amylases are expected to reflect the pH and temperature of the environment in which they are grown and that means the optimum pH for the α – amylase and pH of the growth are the same (McTigue,, *et. al.*, 1964). Nevertheless many researchers have indicated that the pH of growth and pH of α – amylase production is not necessarily compatible (Volesky and Loung, 1985). The effect of pH can attributed to two factors, the first factor is its ability to affect the characteristics of the medium including nutrient solubility and transportation and thus it affects the nutrient availability to the growing microorganism while the second factor is the effect of the pH on enzyme ionizable group and thus affects the stability (Bull and Bushnel, 1997).

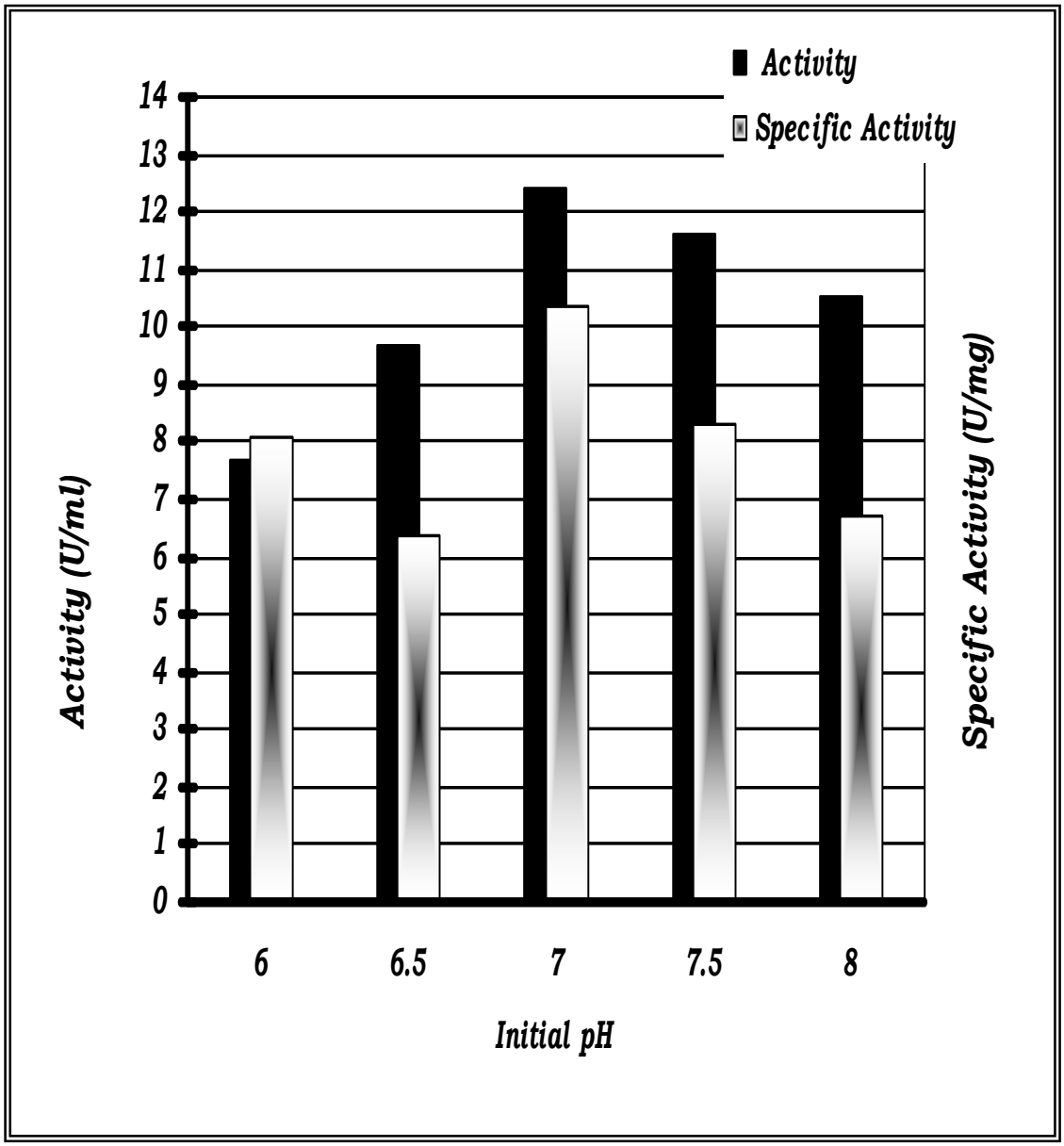

Fig. (4): Effect of medium pH on the ability of *Xanthomonas campestris* H6 in α – amylase production. Cultures were grown at 30 ˚C in shaker incubator for 24 hours.

3.2.2 Effect Of Nitrogen Source:

The amylase synthesis by several microorganisms has been correlated to the presence or absence of various amino acids and complex nitrogenous sources in the culture medium (Hewitt. and Solomons, 1969).

To determine the optimum nitrogen source for production of α-amylase by *Xanthomonas campestris* H6 in PM1 medium, six nitrogen srouces were examined

Results in fig. (5) showed that PM1 medium supplemented with peptone as a sole source of nitrogen, gave the highest values of specific activity of about 10.409 U/mg protein, enzyme activity of 12.49 U/ml while the protein concentration was 1.2 mg. PM1 medium supplemented with yeast extract resulted in a specific activity of 9.25 U/mg, activity of 11.10 U/ml and protein concentration of 1.2 mg/ml in crude filtrate. The lowest level of α – amylase was obtained from PM1 medium supplemented with $NH_4(SO_4)_2$ as sole nitrogen source. Although Priest, (1993) and other researchers had indicated that casein is very suitable nitrogen source for the production of α – amylase but results in fig (5) shows that low specific activity was gained from using casein as a sole source of nitrogen for the α – amylase production by *Xanthomonas compestris* H6.

Figure (6) showed that the optimum concentration of peptone for the production of α – amylase in PM1 medium was at 2 and 2.5 g/100 ml respectively and there was a significant decrease in the specific activity of the enzyme when concentration of 4 g/100 ml was used.

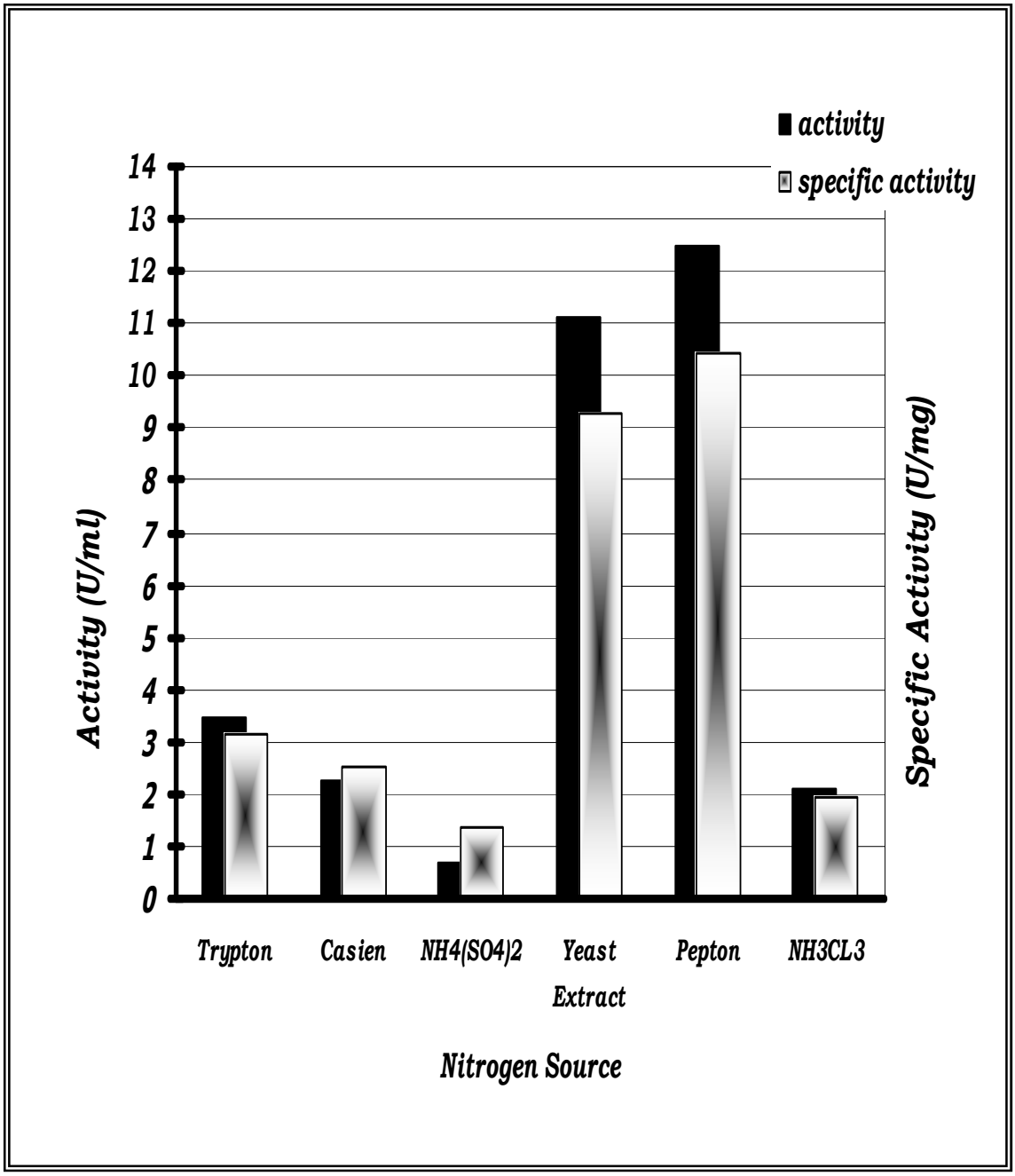

Fig. (5): Effect of different nitrogen sources on *Xanthomonas campestris* ability to produce α – amylase enzyme in PM1 medium. Cultures were grown at 30 ˚C in shaker incubator for 24 hours.

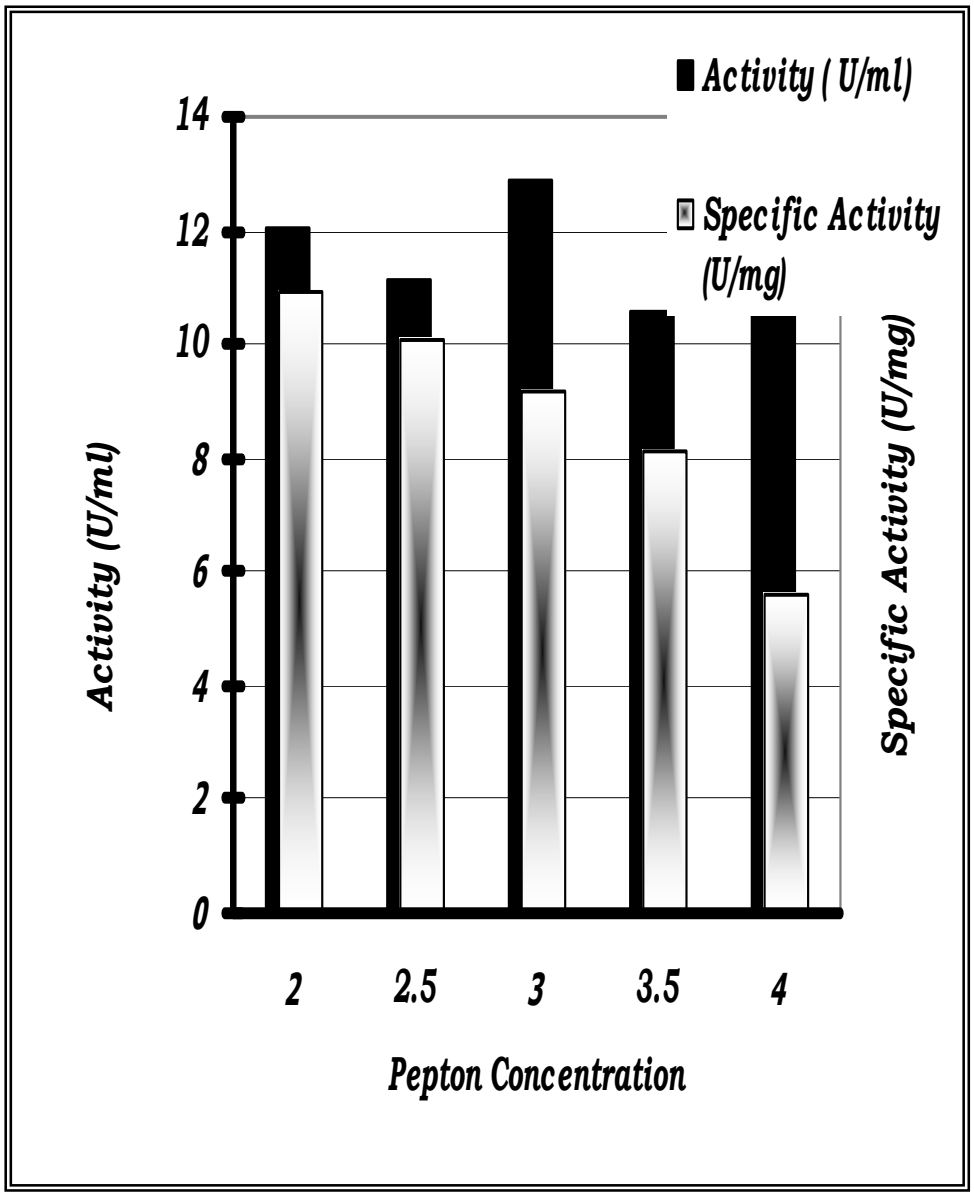

Fig. (6): Effect of peptone concentration on *Xanthomonas campestris* ability to produce α – amylase enzyme in PM1 medium. Cultures were grown at 30 ˚C in shaker incubator for 24 hours

Hizukuri *et. al.,* (1994) have found that the highest productivity of α – amylase was obtained when yeast extract and peptone were used as sources of nitrogen for the production of periplasmic α – amylase from *Xanthomonas campestris* strain K – 11151. Other studies have also shown that peptone was the proper nitrogen source for the α – amylase production. Hsu *et. al.,* (1998) have found that the maximum α – amylase activity was obtained in a medium containing peptone and soluble starch as a nitrogen and carbon sources. Martinez and Teodoro, (2000) have found that, the addition of yeast extract or peptone to the liquid medium shortened the lag period and increased both the dry weight of the cell and the enzyme synthesis. Therefore, the result suggests that yeast extract and peptone is favoured for the growth and synthesis of amylase by the some organism. In another study Martinez and Santos (2003) have shown that peptone was the optimum nitrogen source for maximum amylase synthesis in *Bacillus* sp., Peptone is considered as one of the best nitrogen sources because it's a mixture of amino acids, polypeptides and minerals while yeast extract is a source of growth factor and vitamins.

3.2.3 Effect Of Carbon Source:

Levels of the α – amylase in crude culture supernatants varied greatly in response to the sole carbon source used for growth of the organism. Since the secreted enzyme was highly stable, enzyme synthesis but not enzyme turnover is likely to play a major role in mediating the synthesis of extracellular enzyme (Blum *et. al.,* 1996).

In order to determine the proper carbon source to be used in PM 1 medium for the induction of α – amylase production by *Xanthomonas*

campestis H6, five different carbon sources have been tested, Maltose, Glucose, Starch, Lactose and Sucrose were used for this purpose.

Result shown in fig. (7) showed that using glucose as a sole source of carbon in PM1 medium induced the highest level of α – amylase production by Xc H6. in this case the specific activity was 12.36 U /mg protein while activity and protein concentration were 18.71 U/ml and 1.4 mg/ml respectively.

From figure (7), it can be noted that the specific activity of α – amylase in the crude filtrate was 9.04 U/mg protein when starch was used as a sole carbon source. Although starch is supposed to be the prefect carbon source for α – amylase production, but this might be due to the complexity of starch structure to be utilized be microorganism for production of different proteins and growth factors when compared with the simplicity of glucose as a sole carbon source for this purpose. Presence of maltose and lactose in the media had resulted in specific activity of 8.06 and 7.41 respectively and the lowest specific activity was obtained when sucrose was used as a sole source of carbon.

From the above results, it could be concluded that the α – amylase produced by Xc H6 is independent of starch and it's responsive to the availability of many different carbon sources. And since the higher specific activity was obtained when glucose was used as a carbon source then it's possible to conclude that this enzyme is constitutively produced by Xc H6 and it's, in contrast to many other α – amylases, not subjected to catabolite repression by the presence of glucose in the medium.

Other studies have indicated that α – amylase could be constitutively induced by glucose or many other carbon sources. Salva and Moraes, (1989) studied the effects of different carbon sources on α – amylase production.

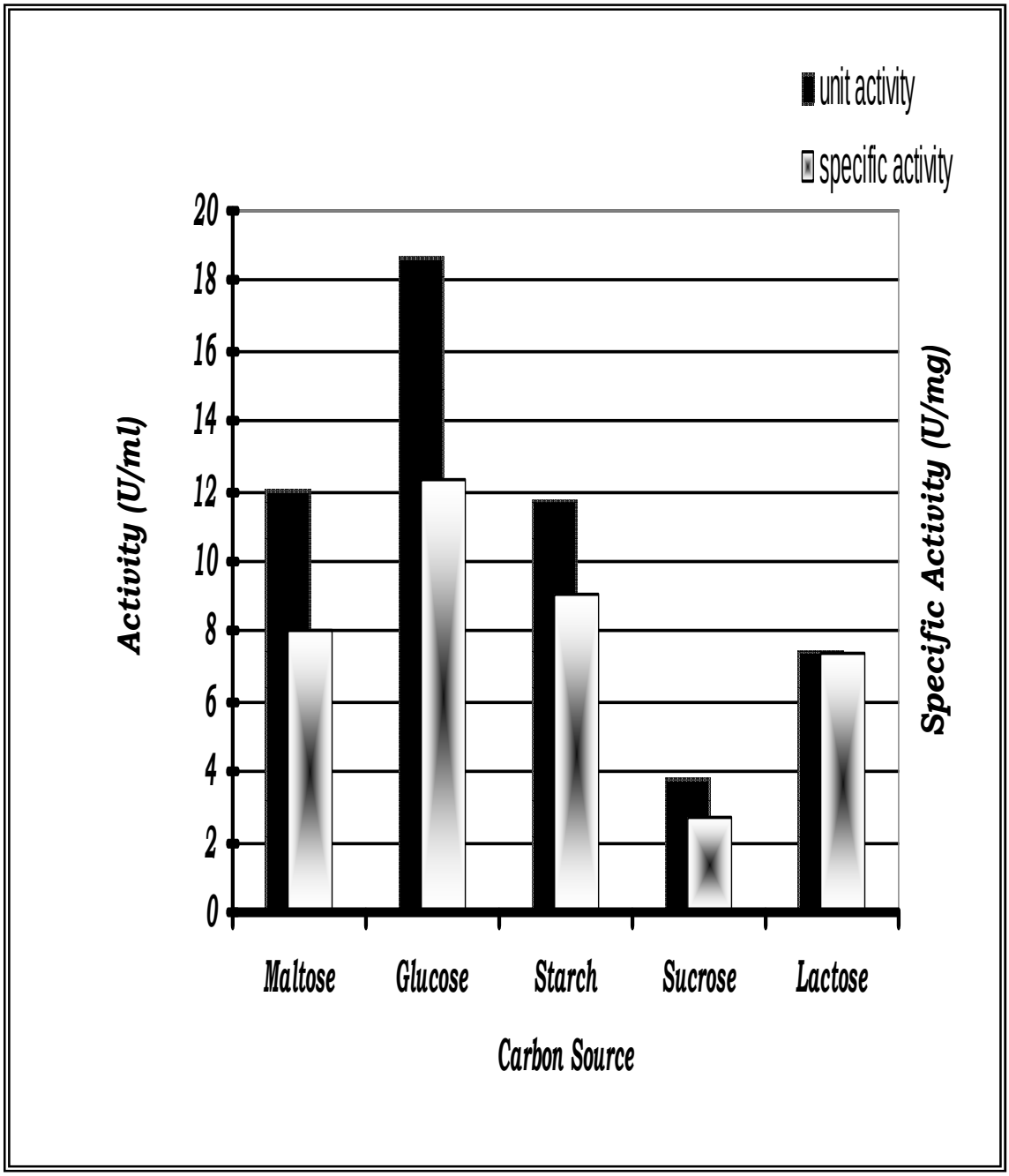

Fig. (7):Effect of different carbon sources on *Xanthomonas campestris* ability to produce α – amylase enzyme in PM1 medium. . Cultures were grown at 30 ˚C in shaker incubator for 24 hours

And they found that glucose was able to induce the enzyme whereas lactose, dextran and soluble starch were found to be suitable for enzyme production, the highest enzyme yield was obtained when glucose was used (Pandy *et. al.*,2000).

Chung *et. al.*, (1995) have found that the addition of glucose to the media have no catabolite repression effect on the α – amylase produced by *Thermococcus profundus.*

In *Staphylococcus mutans* ,Simpson and Russel, (1998) have found that starch had no effect of α – amylase while the addition of maltose increased the amylase activity two to three fold and glucose had no catabolite repression on amylase productivity. Nahas and Waldemarin (2002) have found that glucose have the ability to induce the synthesis of α – amylase in *Aspergillus ochraceus*. Attia and Ali (1974) showed that glucose was very effective inducers of α – amylase in *Aspergillus. Awamori.* Many other researches shown that glucose is one of the good inducer for the α – amylase in many different microorganisms.

After determination the optimum carbon source for Xc H6 in production of α – amylase, gradual concentrations of glucose was added separately to PM1 medium (2, 2.5, 3, 3.5, 4 and 4.5 %) to examine the optimum glucose concentration for α – amylase production by Xc H6 .Results in fig. (8) showed that the ability of Xc H6 in production of α – amylase was raised when PM1 medium was supplemented with glucose concentration of 3.5%, this concentration resulted in specific activity of in 12.10 U/mg protein (protein concentration was 1.3 mg/ml) while the enzyme activity was 15.7 U/ml. So it was considered as best glucose concentration for α – amylase production by Xc H6 in the next optimization experiments.

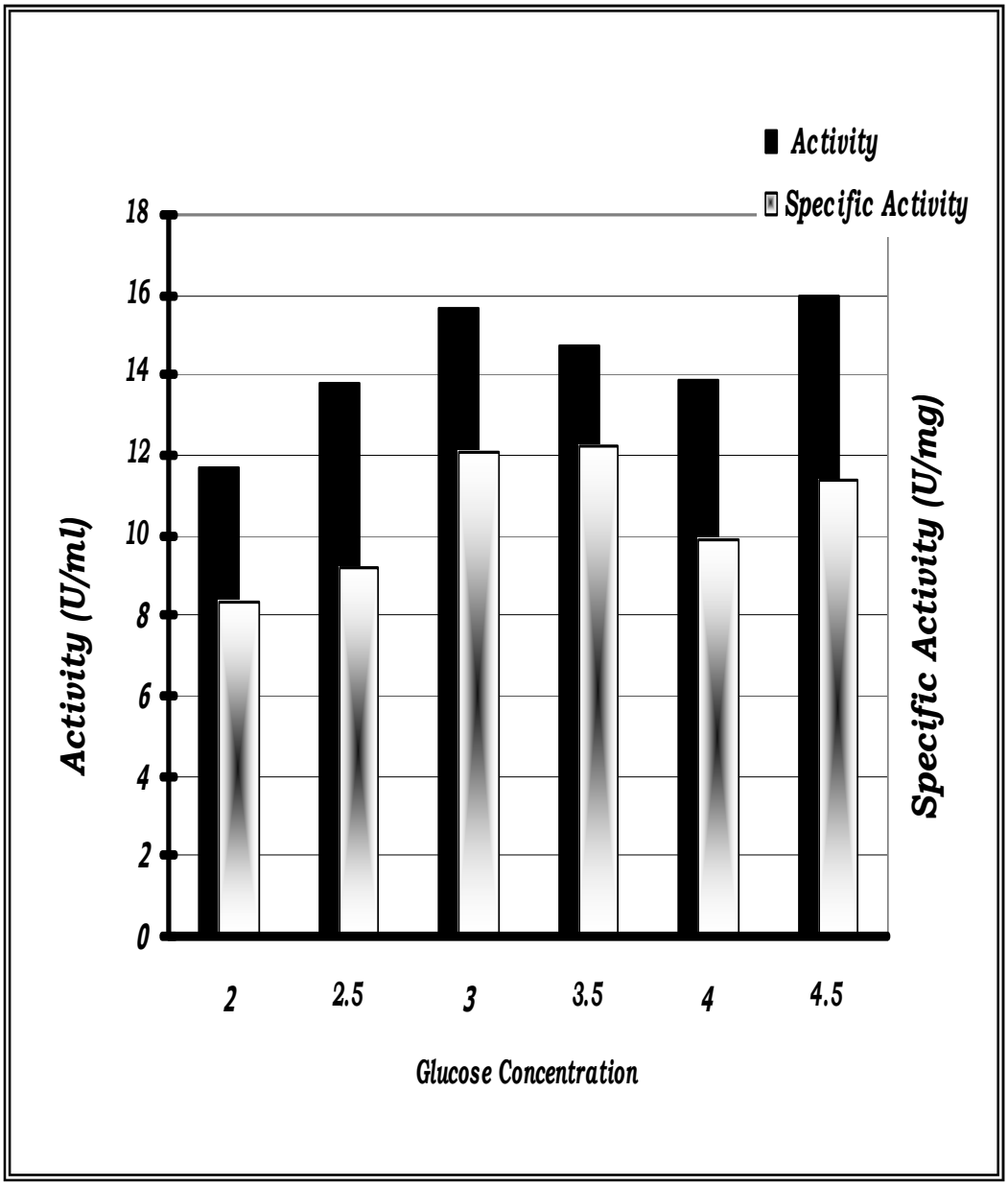

Fig. (8): Effect of glucose concentration on *Xanthomonas campestris* ability to produce α – amylase enzyme in PM1 medium. . Cultures were grown at 30 ˚C in shaker incubator for 24 hours

3.2.4 Effect of Phosphate Source:

In order to determine the optimum phosphate source and its concentration for the α – amylase production by Xc H6, dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were tested in PM1 medium with different concentration 2, 2.5, 3, 3.5, 4 g/100ml for both of them.

Results in fig. (9) showed that the optimum concentration of K_2HPO_4 for the α – amylase production was 2 gm /100 ml, and under this condition the specific activity was 12.93 U/mg while the activity was 15.52 U/ml activity (protein concentration in the crude filtrate was 0.5 mg/ml). These results demonstrate that that there is a constant decrease in the α – amylase productivity as the concentration of K_2HPO_4 increases.

Results in fig. (10) demonstrated that KH_2PO_4 had a better capability in increasing α – amylase productivity by Xc H6. The highest productivity was gained from using 2.5 g of KH_2PO_4 as a phosphate source which resulted in specific activity of 13.75 U/mg, protein concentration was 1.2 mg/ml and enzyme activity was 16.50 U/ml.

Results (fig. 9 and 10) indicated that KH_2PO_4 (2.5%) was the optimum phosphate source for α – amylase production by Xc H6 because it gained the highest productivity.

3.2.5 Effect Of Temperature:

Temperature is considered as a crucial and effective factor in the growth of microorganism and their metabolism and in order to investigate the role of temperature on the production and activity of the α – amylase. *X.*

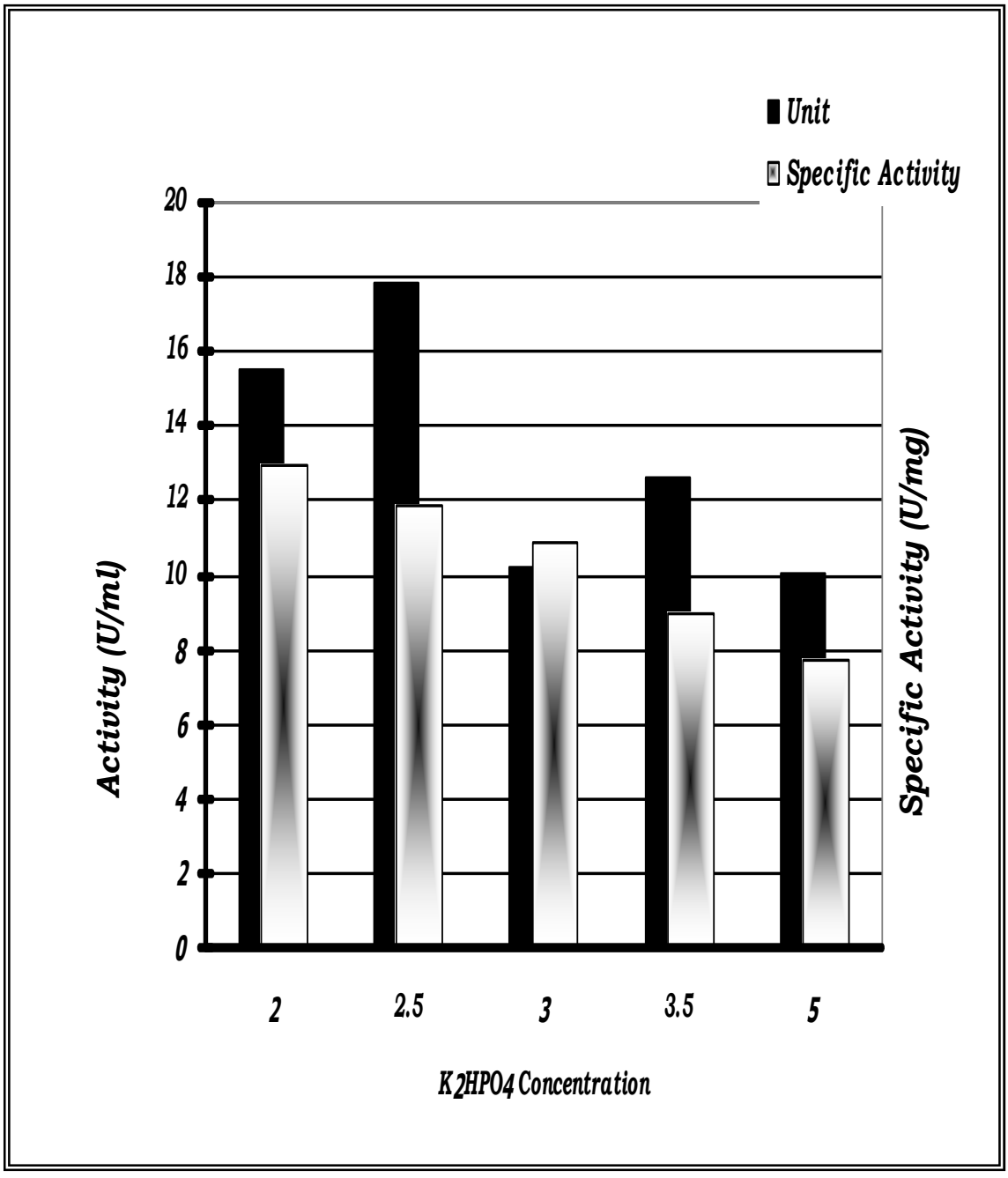

fig. (9) Effect of different concentration of K_2HPO_4 on *Xanthomonas campestris* H6 on α – amylase production. Cultures were grown at 30 ˚C in shaker incubator for 24 hours.

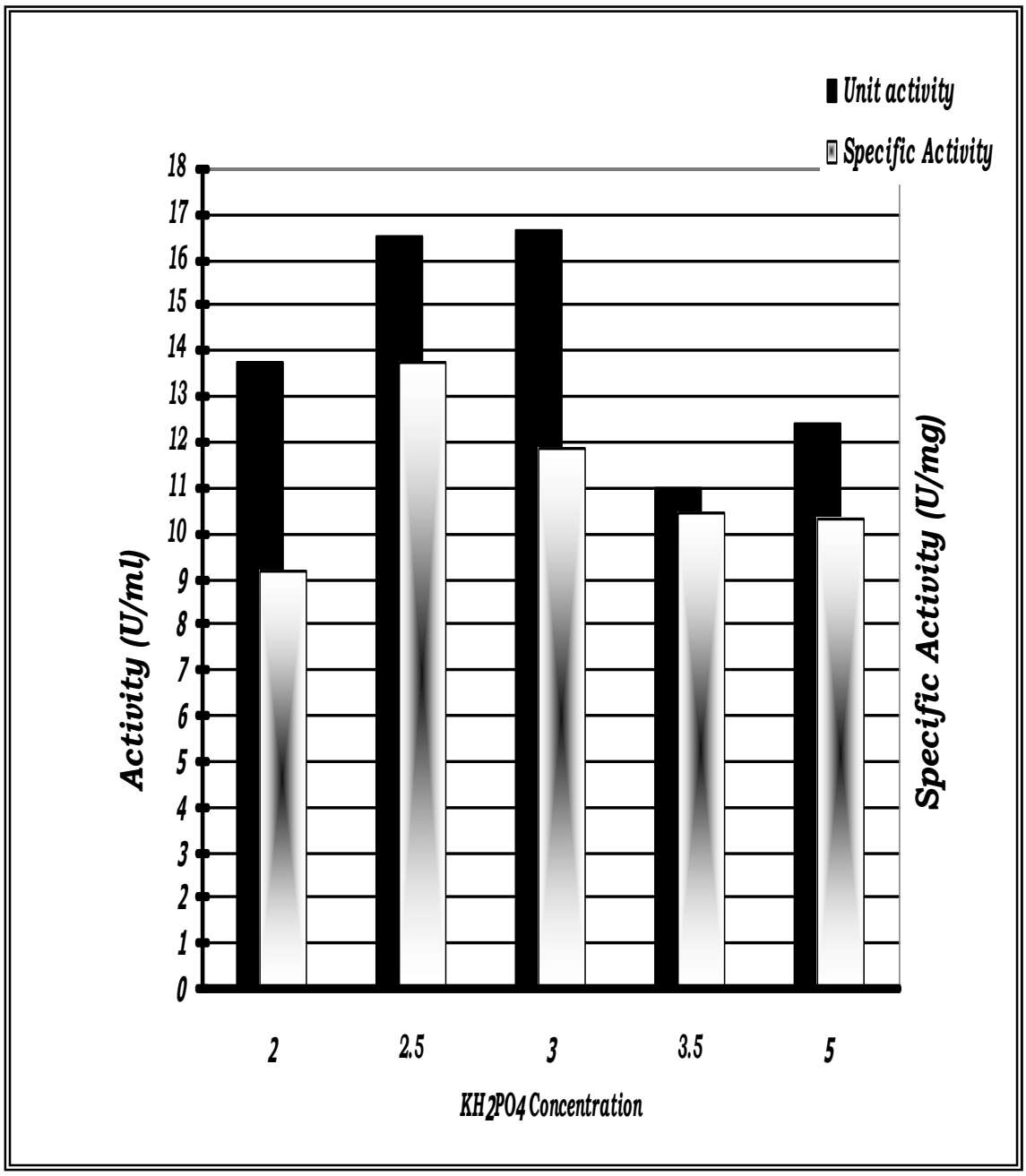

fig. (10) Effect of different concentration of KH_2PO_4 on *Xanthomonas campestris* H6 on α – amylase production. Cultures were grown at 30 ˚C in shaker incubator for 24 hours

campestris H6 was incubated at different temperature (20, 25, 30 35 and 40 ˚C).

Results depicted in fig (11) showed that that the utmost level of α – amylase productivity was at 30 ˚C with specific activity 13.54 U/mg, activity 13.76 U/ml and protein concentration 1.09 mg/ml and there was a significant and obvious decrease at 35 ˚C and 40 ˚C which resulted in specific activity of 6.185 U/mg and 3.73 U/mg respectively.

These results demonstrate that the α – amylase is produced in its maximum level at the optimum temperature for the growth of Xc H6 and this can be explained by the ability of this organism to grow properly and use the medium nutrients efficiently at this temperature, which in turn can result in better production of constitutive enzymes and other vital products including α – amylase enzyme.

The low specific activity that was obtained at 35 ˚C and 40 ˚C can be attributed to the inability of Xc H6 to grow properly at these temperatures and thus affect its ability to produce different constitutive enzymes such as α – amylase.

Generally the decay of enzymatic activity within the decreasing or increasing temperatures is attributed to thermal effect on either the growth of the microorganism or the velocity of the enzymatic reaction inside the cells, or on both of them, reflecting the properties of the enzyme and its environments (Cornish-Bowden, 1979). At low temperature, the decrease in the enzymatic activity is caused by the unsuitability of these temperatures for the growth of the organism where growth is slow down and metabolism is delayed, while at high temperature the water evaporates and affects growth and other factor related to the growth such as oxygen concentration and speed of enzymatic activity inside the cells (Tengerdy, 1985).

Below optimum and up to optimum temperature, like all enzymes, amylase catalyses a (bio)chemical reaction. A fundamental of chemical thermodynamics is that all reaction rates will increase as the temperature increases. With a typical amylase, therefore as temperature increases to the optimum, the rate at which an enzyme catalyses the breakdown of starch increases. The reason for this is as the temperature of reaction increases to the optimum, the rate at which the enzyme and the starch substrate molecule collide is increased also (the collision theory). Another reason is that, amylase enzyme has an optimum shape or flexibility and will hold this ideal shape at the optimum temperature.

More over enzymes that do well at very high temperatures tend to have a more rigid, inflexible structure that may be held together by covalent linkages, known as disulfide bridges, between different regions of the structure (Internet 1).

Many research have used the 30 ˚C as the optimum temperature for the α – amylase production from different microorganisms. For example Fairbarn *et. Al.*, (1986) have used this temperature to obtain α – amylase from *Streptomyces* and Sinha and Chandra, (1981) have used 30 ˚C with 4 days incubation as optimum condition for α – amylase production from *S. rimosus* but it should be noted that α – amylase can be produced at different temperatures from different microorganisms.

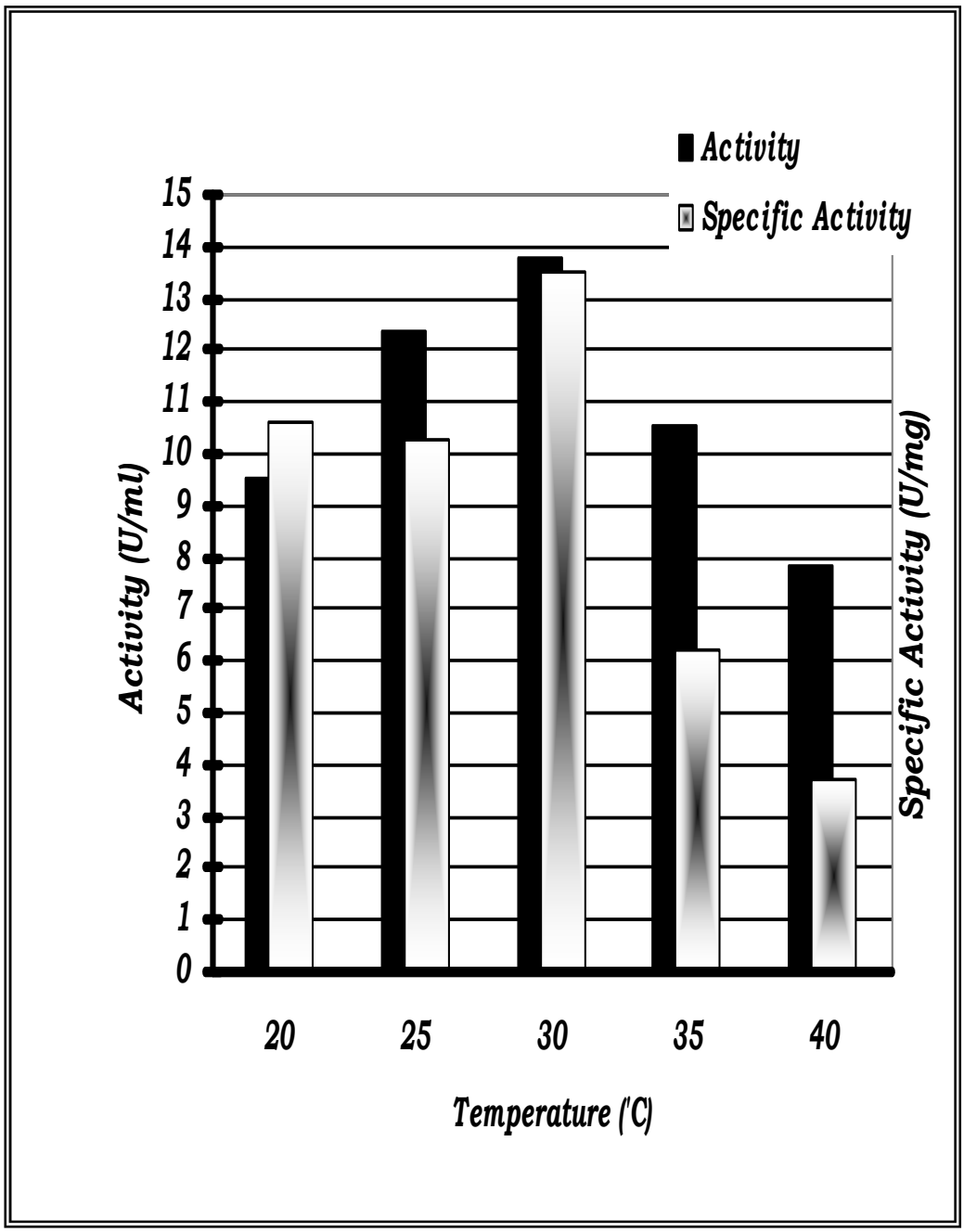

Fig. (11) Effect of Temperatures on *Xanthomonas campestris* H6 ability to produce α – amylase. Cultures were grown at 30 ˚C in shaker incubator for 24 hours

3.3 Alpha Amylase Purification

In order to further characterize the α – amylase produced by Xc H6, a sequence of purification steps have been conducted and these steps includes ammonium sulphate precipitation, dialysis and gel filtration using sepharose CL – 6B.

3.3.1 Ammonium Sulphate Precipitation:

The first step in α – amylase purification was precipitation with ammonium sulphate at different concentration 20, 40, 60 and 80%. The aim of precipitation is to remove water and obtain a partially purified enzyme.

Results showed that the lowest activity of α- amylase was at saturation ratio 0 – 20% which resulted, after dialysis step, in a specific activity of 1.5 U/mg protein. An increase in the specific activity was notable as the saturation ratio was increased to 40, 60, and 80 % which resulted, after dialysis step, in 4.97, 9.64, and 17.56 U/mg. The specific activity was then decreased at 80 – 100% saturation concentration giving 6.76 U/mg.

From the above result it can be noted that most of the α- amylase specific activity present in the precipitant formed at the saturation ration 80% and it represents about 76.25 % of the total specific activity (table 3).

Protein precipitation using ammonium sulphate depends on the salting out phenomenon. Since 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 it causes no damages to proteins (Volesky and Loung, 1985).

After being precipitated, the enzyme extract was dialyzed against Potassium – Phosphate buffer 0.005M – CaCl 0.025 M for three continuous days with three successive changes.

3.3.2 Gel Filtration:

The purification steps for α – amylase from Xc H6 cell extract can be summarized in (table3). These steps comprise concentration using ammonium sulphate precipitation, dialysis and gel filtration using sepharose CL – 6B. The concentration step appears to be very active in removing some components of the cell extract and giving a partially purified enzyme with a 1.9 fold purification and 76.25% yield.

After being dialyzed, the enzyme extract was further purified using sepharose CL – 6B (pharmacia). Fig. (12) shows the protein peaks obtained from gel filtration step using sepharose CL – 6B and it reflects the presence of two proteins.

Estimation of the α – amylase activity in all eluted fractions showed that the first peak contains no α – amylase activity while α – amylase activity was found to be concentrated in the second peak. The fractions of the second peak were then all collected and lypholized and after determining α – amylase activity, it had been found that the partially purified enzyme had demonstrated a specific activity of 41.14 U/mg protein with 4.5 purification fold and 21.69 % over all yields.

Table (7)

Purification steps of α – amylase

Produced by *X. campestris* H6

Purification Step	Vol. (ml)	Activity (U/ml)	Prot. Conce. (mg/ml)	Specific Activity (U/mg)	Total Act.(Units)	Purif. Fold	Yeild (%)
Crude Extract	**250**	**12.5**	**1.4**	**8.99**	**3147.5**	**1**	**100**
Amm. Sulphate Preciptat.	**50**	**48**	**3.7**	**17.5**	**2400**	**1.9**	**76.2**
Gel Filtration (Sepharose CL – 6B)	**20**	**34.1**	**0.8**	**41.1**	**683**	**4.5**	**21.6**

Sepharose CL – 6B was used in the final purification step for its characteristic, for being more rigid cross linked agaroses, easily prepared and can be used several times after activation (Veralag, 1989).

Different scientists have used different methods for the purification of α – amylase from different organisms. Hizukuri *et. al.*, (1994) have used ammonium precipitation followed by ion exchange chromatography using carboxymethyl – toyopearl 650M to purify the periplasmic α – amylase from *Xanthomonas campestris*. Shaw *et. at*., (1995) had obtained a highly purified α – amylase from *Thermus* sp. by affinity absorption on starch granules. Kobayashi *et. al*., have purified the α – amylase of *Thermococcus profundus* to homogeneity by ammonium sulfate precipitation, DEAE-Toyopearl

chromatography, and gel filtration on Superdex 200HR. Kochhar and Dua, (1990) purified the α – amylase from *Bacillus amyloliquefaciens* by the

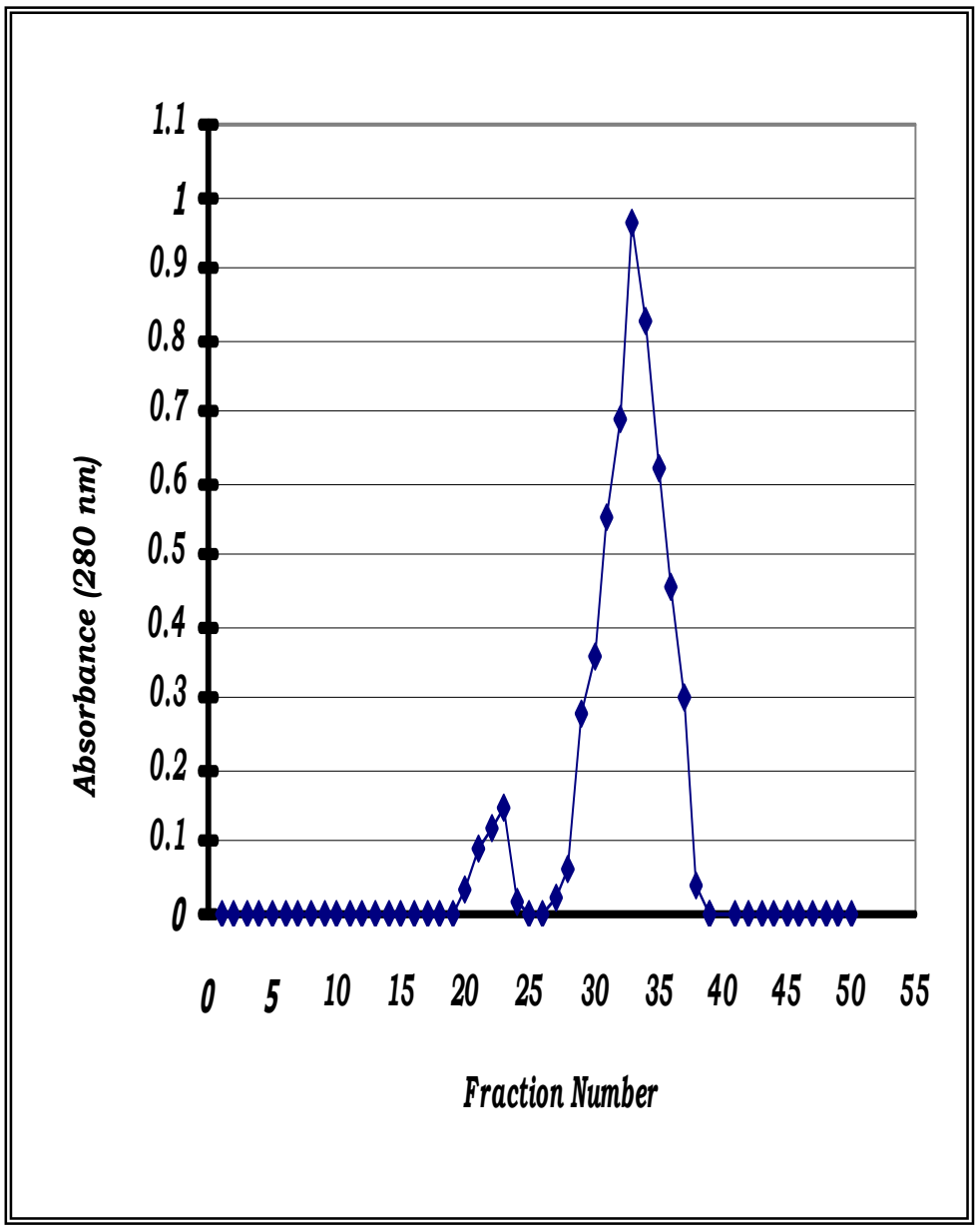

Fig. (12):Elution of α – amylase enzyme on sepharose CL – 6B. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

following steps: thermal treatment at 80 ˚C for 30 min. followed by ion exchange on DEAE – Cellulose column and finally by gel filtration using Biogel P – 100 to produce 100 purification fold with 62% over all recovery. Alpha amylase of *Streptococcus mutans* was purified by treatment the cell extract with 30% KOH and thermal treatment at 90 ˚C, centrifugation and two precipitations with equal volume of 95% ethanol (Simpson and Russel, 1995).

3.4 Test Of α – Amylase Purity:

The purity of the partially purified α – amylase was determined by polyacrylamide gel electrophoresis under non denatured conditions. The partially purified enzyme preparation showed only single protein band on the gel after staining with commassie brilliant blue G – 250, indicating the probability of to reaching the apparent homogeneity of purification (fig. 13).

The location of the protein band on the gel could be as a result of the low molecular weight of the protein, since the electrophoresis mobility of proteins in the gel essentially depends on the molecular weight of the protein in addition to its net charge (Blackshear, 1984).

Fig.(13): Polyacrylamide gel electrophoresis pattern of α – amylase of Xc H6

3.5 Molecular Weight Determination:

Gel filtration technique was used for determining the molecular weight of α – amylase produced and partially purified from Xc H6. For this purpose , several standard proteins with know molecular weight were used like bovine serum albumin (BSA) (67000 Dalton), lysozyme (14000 Dalton), trypsine (55000 Dalton) and pepsin (34700 Dalton). Solution of each of these biomolecules were transferred separately to the gel filtration chromatography column and run throughout sepharose CL – 6B matrix of the column. To calculate the elution volume of each of them, blue dextran – 2000 was used to determine void volume of the column. Figures 14, 15, 16, 17 and 18 showed the elution curve of each of these biomolecules. A relationship between the ratio of elution volume to the void volume (V_e / V_o) and molecular weight of these standard proteins were plotted (fig. 19) to estimate α – amylase molecular weight. And it was found that the α – amylase molecular weight was about 14.000 Dalton.

Molecular weight determination using gel filtration have been used through out many studies to find out the molecular weight of α – amylase enzyme. Pfueller and Elliott, (1969) have used gel filtration to estimate the Mwt. of α – amylase from *Bacillus stearothermophilus* and found it to be 53000 Dalton . shimazaki *et al.,* (1984) have found that Mwt. of α – amylase form *Schizophyllum commune* is about 66000 Dalton.

α – amylase from Bacillus sp. was estimated to be around 15500 using osmotic pressure method.

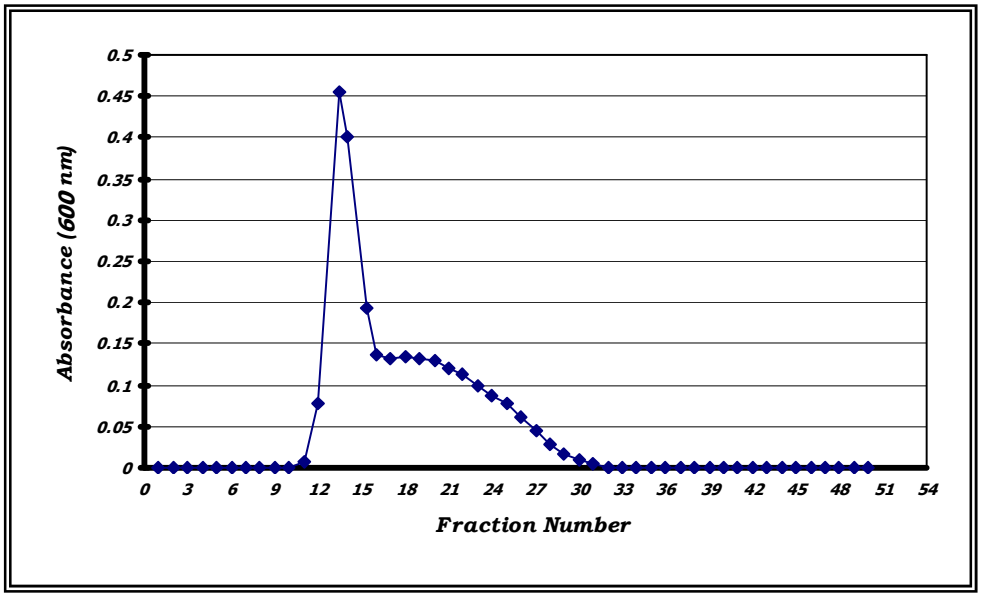

Fig. (14) Elution of Blue dextran on sepharose CL – 6B. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

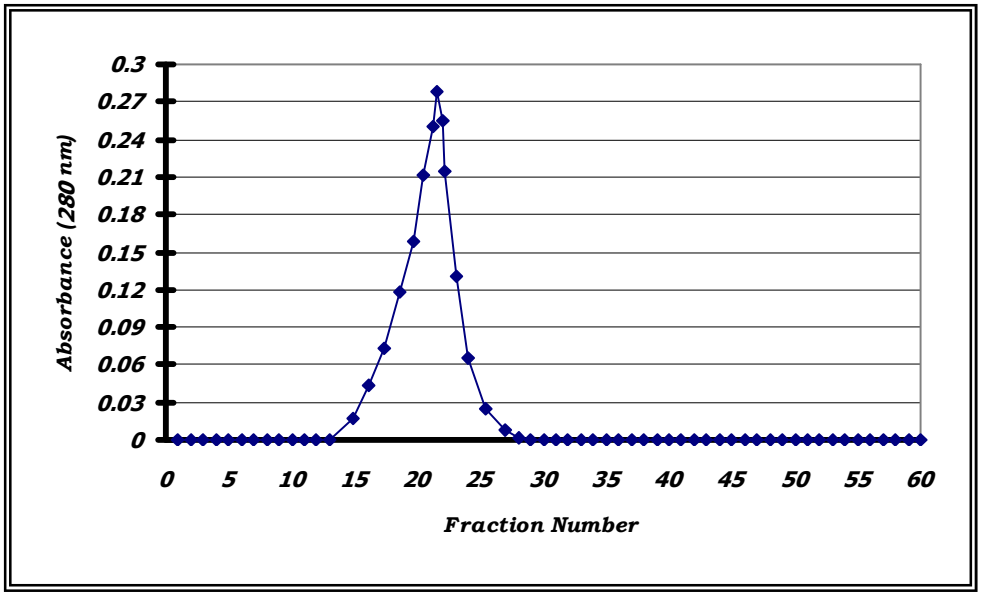

Fig. (15) Elution of BSA on sepharose CL – 6B. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

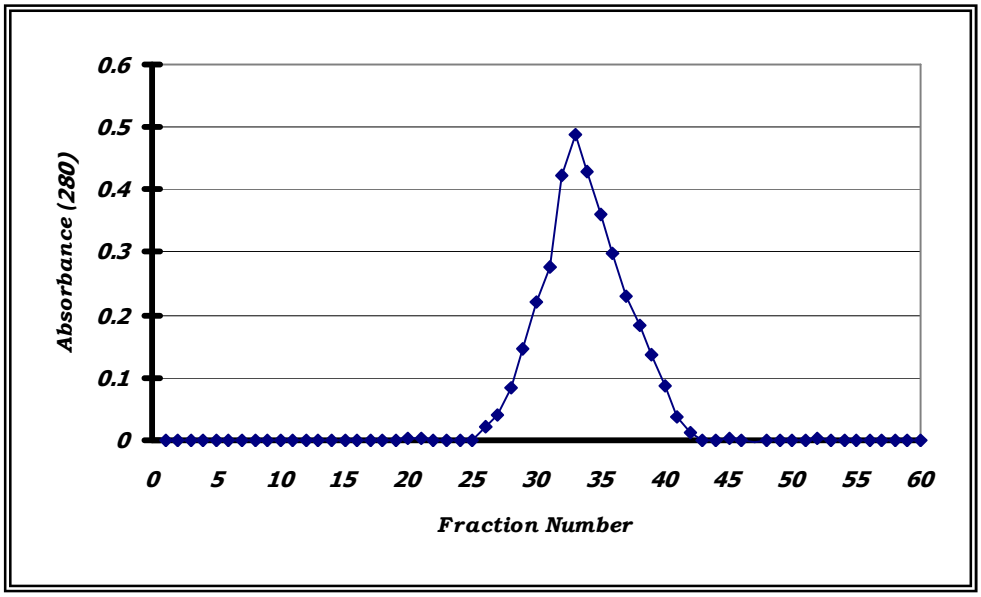

Fig. (16) Elution of Lysozyme on sepharose CL – 6B. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

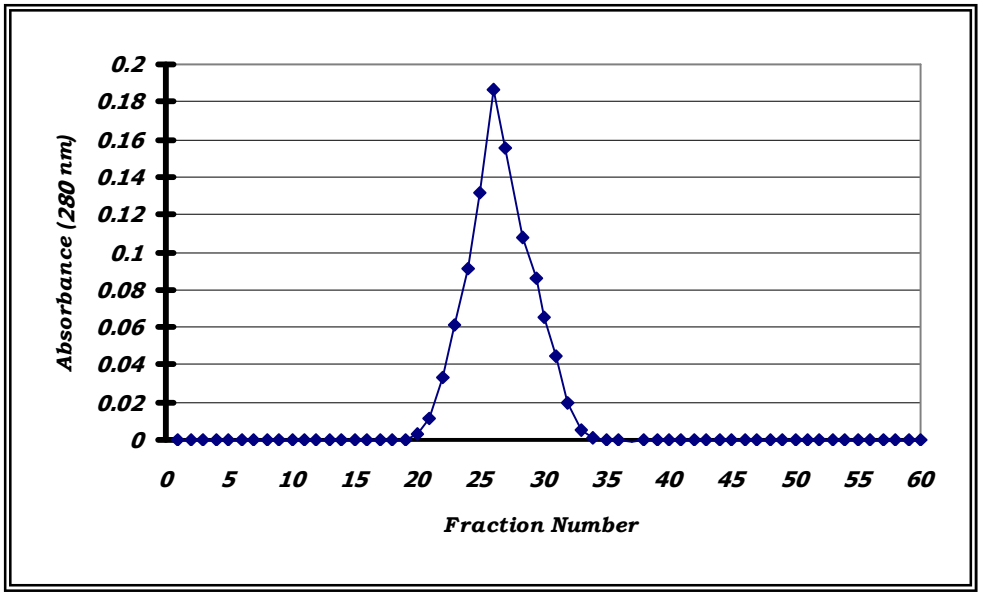

Fig. (17) Elution of Pepsin on sepharose CL – 6B. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

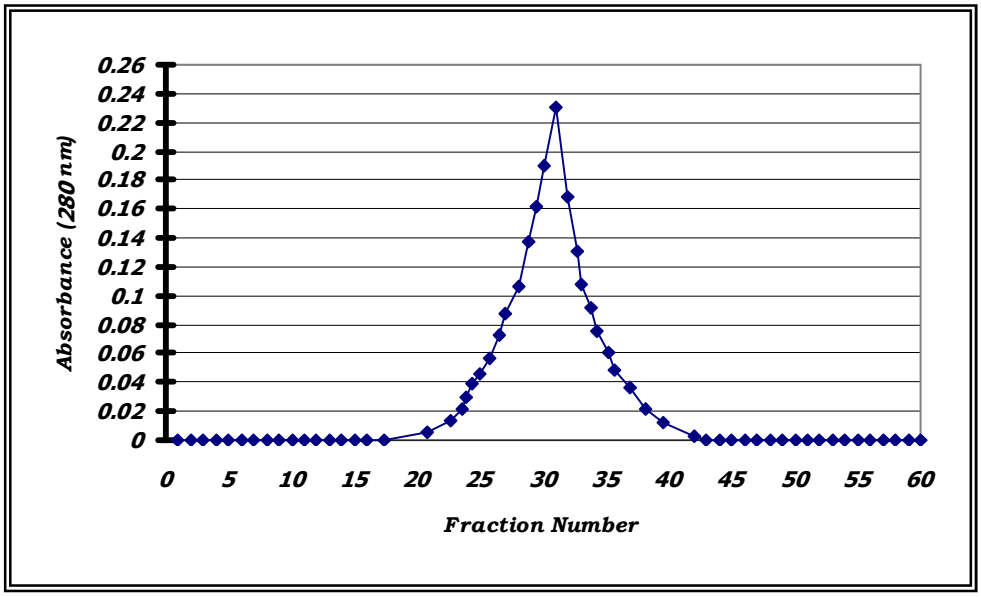

Fig. (18) Elution of Trypsin on sepharose CL – 6B. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

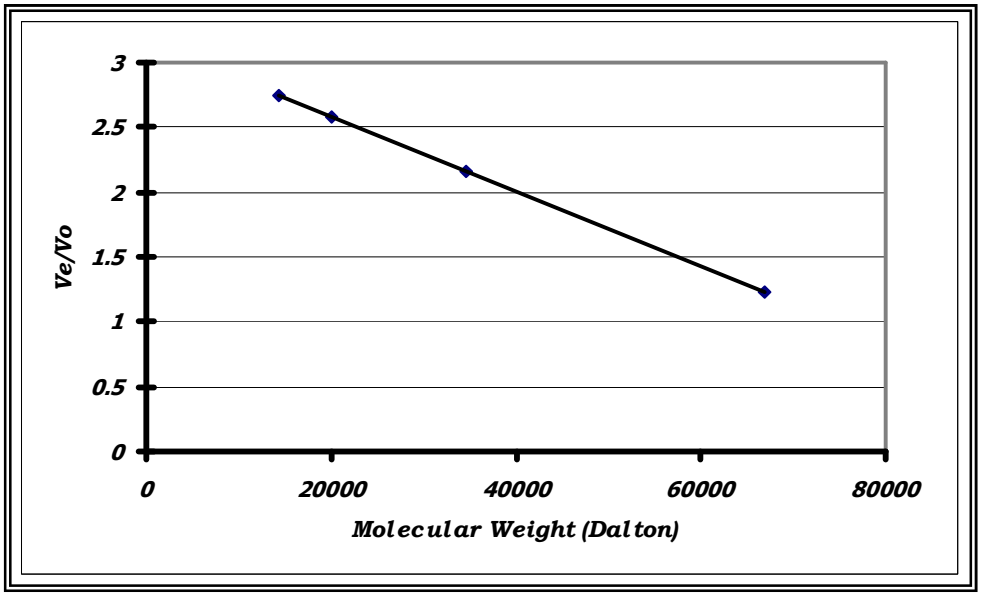

fig. (19): Molecular Weight of standard protein. Flow rate was 3ml / min. and fraction volume was 3ml / tube.

3.6 Alpha Amylase Characterization:

3.6.1 Effect of pH on α – Amylase Activity:

The effect of pH on the partially purified Xc H6 α – amylase was studied in a pH range of 5 – 8 as shown in fig. (20). A wide range of pH 7 – 8 was observed to be suitable for the activity of α – amylase but there was a decrease in the activity at pH 5 – 6.5. However the highest activity of enzyme was obtained at pH 7.5 where the enzyme was at the highest level of activity.

From the above mentioned results, it can be concluded the enzymatic activity at neutral or slightly basic values was higher than values of acidic or slightly acidic values. The obvious decrease in the activity could be as result of effect of pH on enzyme activity by affecting the critical group at active site of the enzyme and preventing these groups to be in the correct ionization state for the reaction to be completed.

3.6.2 Effect of pH on α – Amylase Stability:

In order to determine the optimum pH for the α – amylase stability, the enzyme was incubated in buffer solution with pH range (5 – 8) at 30 ˚C for 30 min.

Results in fig. (21) show that α – amylase has a good stability at pH range 6.5 – 7.5 in which it exhibited more than it 90% of its total activity. A decrease in the stability of the enzyme was noticed at pH 6 and 8 while there was a significant and sharp decline in the stability of the enzyme at pH 5 and 5.5 where the enzymatic activity was less than 40% of the total activity.

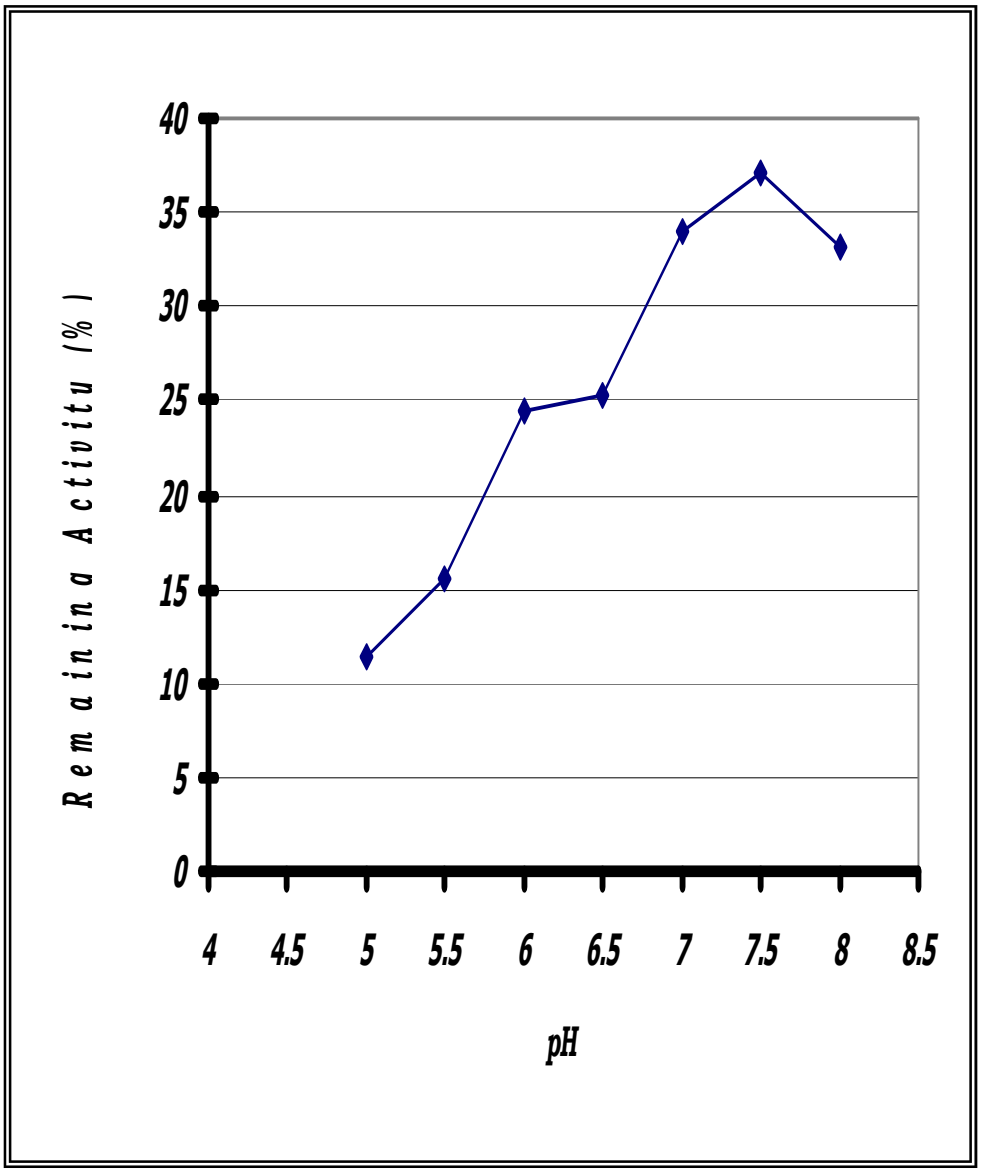

Fig. (20) Effect of pH on α – amylase activity of *Xanthomonas campestris* H6.

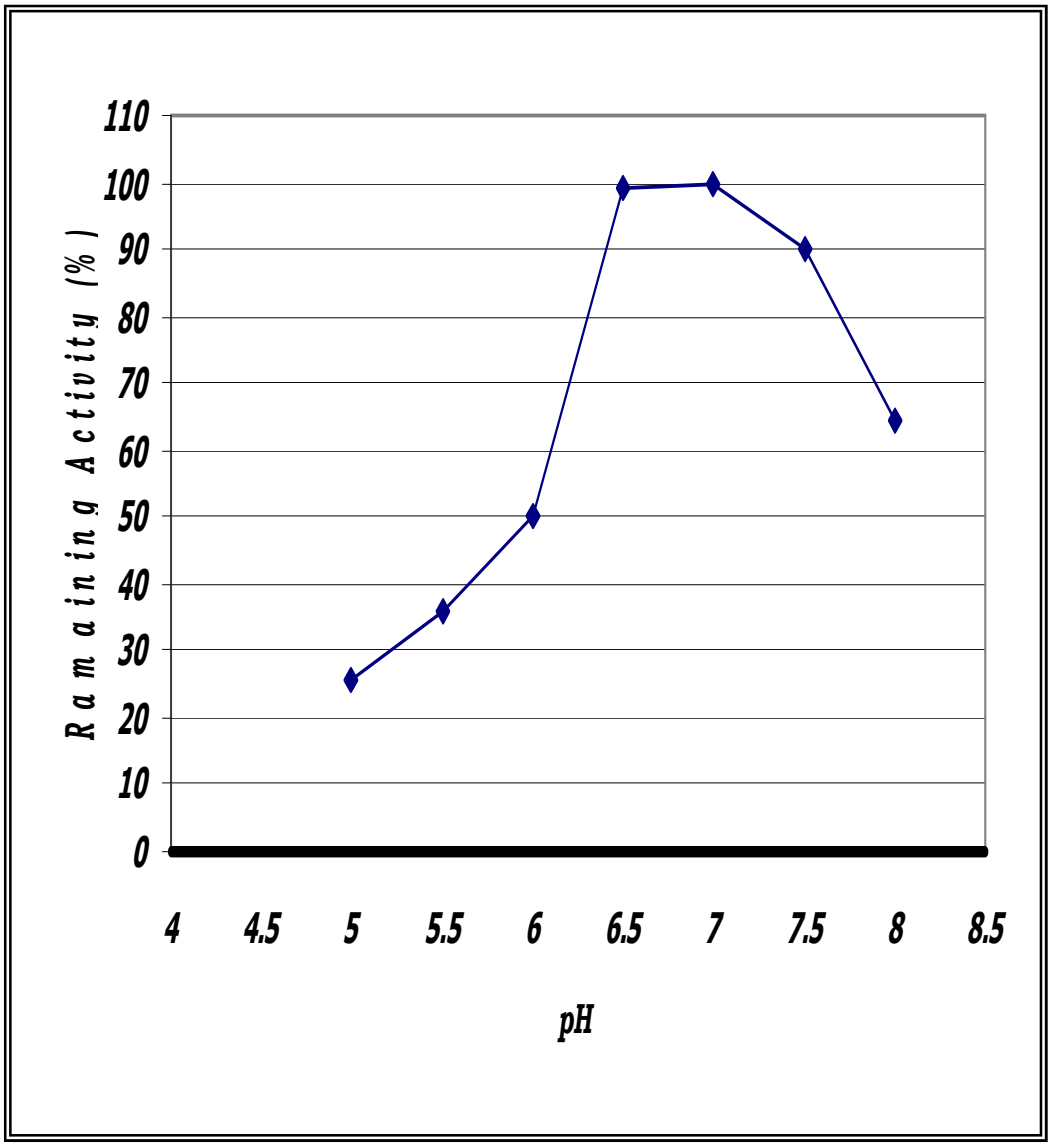

Fig. (21) Effect of pH on α – amylase stability of *Xanthomonas campestris* H6.

This decrease in the α – amylase activity could be attributed to the effect of pH on the secondary, tertiary or quaternary structure of the enzyme since Whitaker (1972) had indicated that, most enzymes subjected to irreversible denaturation in strong acid and alkaline conditions.

Hsu *et. al.,* (1998) have found that purified α – amylase had optimal pH 9.0 while the α – amylase produced by *Bacillus subtilis* had optimum pH 6.0. By comparison, α – amylase produced by Xc H6 is less stable than α – amylase from *Thermus* sp.and *Bacillus subtilis* which were found to be stable at pH from 4 – 10 on standing for 2 hr. at 37 ˚C and pH 5.0 – 10 at below 70 ˚C respectively.

3.6.3 Thermal stability Of α – Amylase:

To investigate the stability of the partially purified α – amylase on standing different temperature degrees, the enzyme was incubated at different temperatures ranging from 30 ˚C to 60 ˚C for 15 min.

Fig. (22) revealed that the enzyme has retained its complete activity at 40 ˚C with slight increase in the activity at 45 ˚C where the enzyme had kept about 94 % of its total activity and about 82 % at 50˚C. A decline in the activity of the enzyme was observed at temperatures above 50 ˚C where the enzyme had kept 62 % at 55˚C and only 49% of its activity when it was incubated at 60˚C. At temperatures just above optima, there may be a situation where the enzyme is in a sort of equilibrium where it temporarily loses some of its structure and then regains it to work again. At higher temperatures, the three – dimensional structure of the protein destabilizes and might be disrupted.

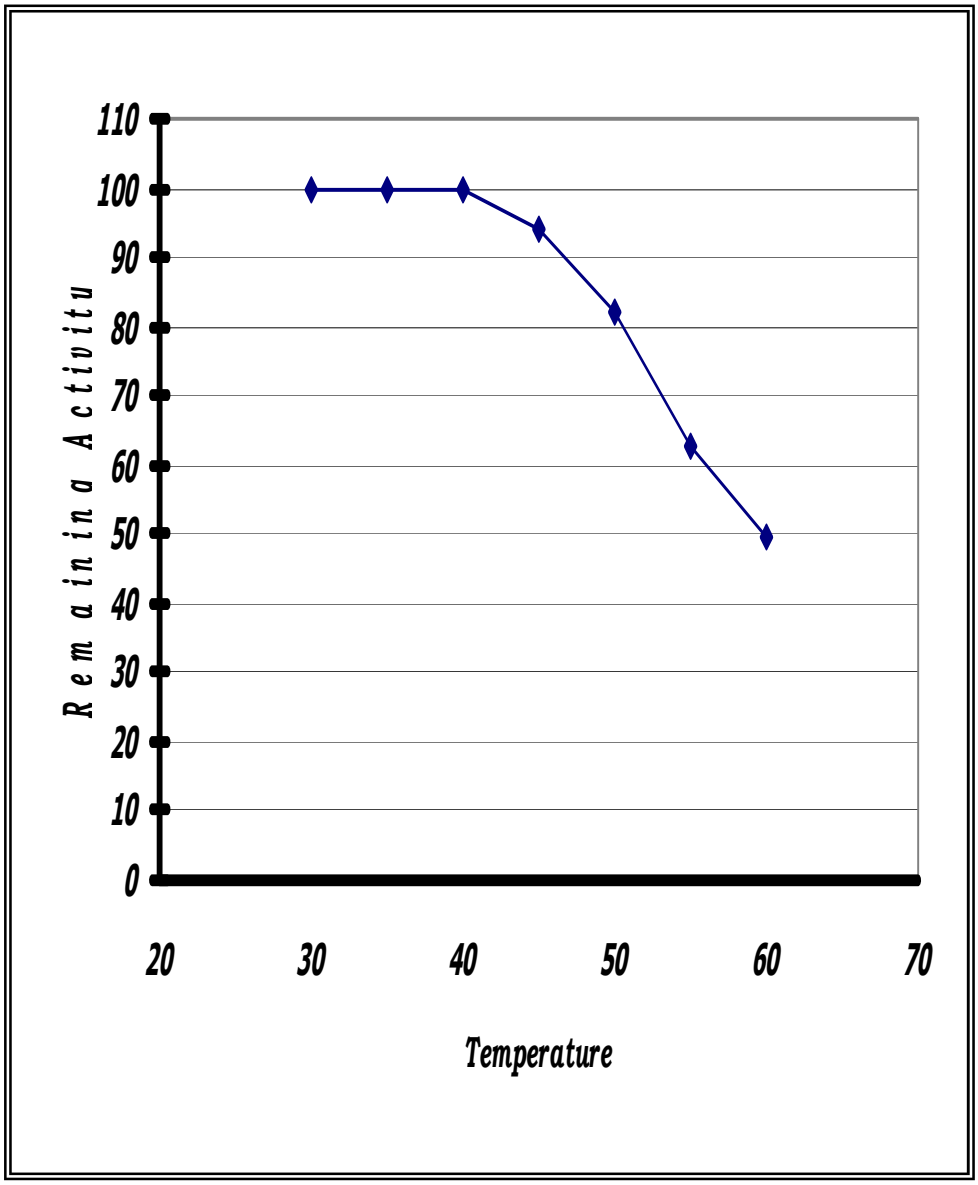

Fig. (22) Effect of temperature on α – amylase stability of *Xanthomonas campestris* H6.

Next, the partially purified α – amylase was incubated at 40 ˚C for different periods of time 10, 20, 30, 40, 50, and 60 min. to investigate the thermal stability of the enzyme at this temperature and fig. (23) shows that maximum α – amylase activity was obtained when enzyme incubated at 40 ˚C and 98% of the activity was retained at 50 ˚C. At 60 ˚C more than 85% of the activity was kept. These results are agreeable to results obtained by Hizukuri *et. al.,* (1994) whom were found that α – amylase from *Xanthomonas campestris* is stable until 55 ˚C

Other studies have found α – amylase from different sources has different optimum temperatures according to the source microorganism and its environment. Shin and Labbe, (1995) have found that purified α – amylase from *Clostridium perfringens* was inactivated between 35 and 40 ˚C. While Shaw *et. al.,* (1998) demonstrated that α – amylase of *Thermus* sp. is stable over 50 ˚C with optimum temperature 70 ˚C.

3.6.4 Effect of EDTA On α – Amylase Activity:

Vallee *et. al*., (1959) and many other studies have stated that α – amylase is a metalloenzyme which depends on the presence of Ca ions for its activity and since EDTA has the ability to remove Ca, so activity of α – amylase produced by many microorganism is supposed to be suppressed by EDTA.

To investigate weather α – amylase is metalloenzyme or not, α – amylase enzyme produced by Xc H6 was incubated with EDTA at different concentration, 0, 1, 3, and 5 mM.

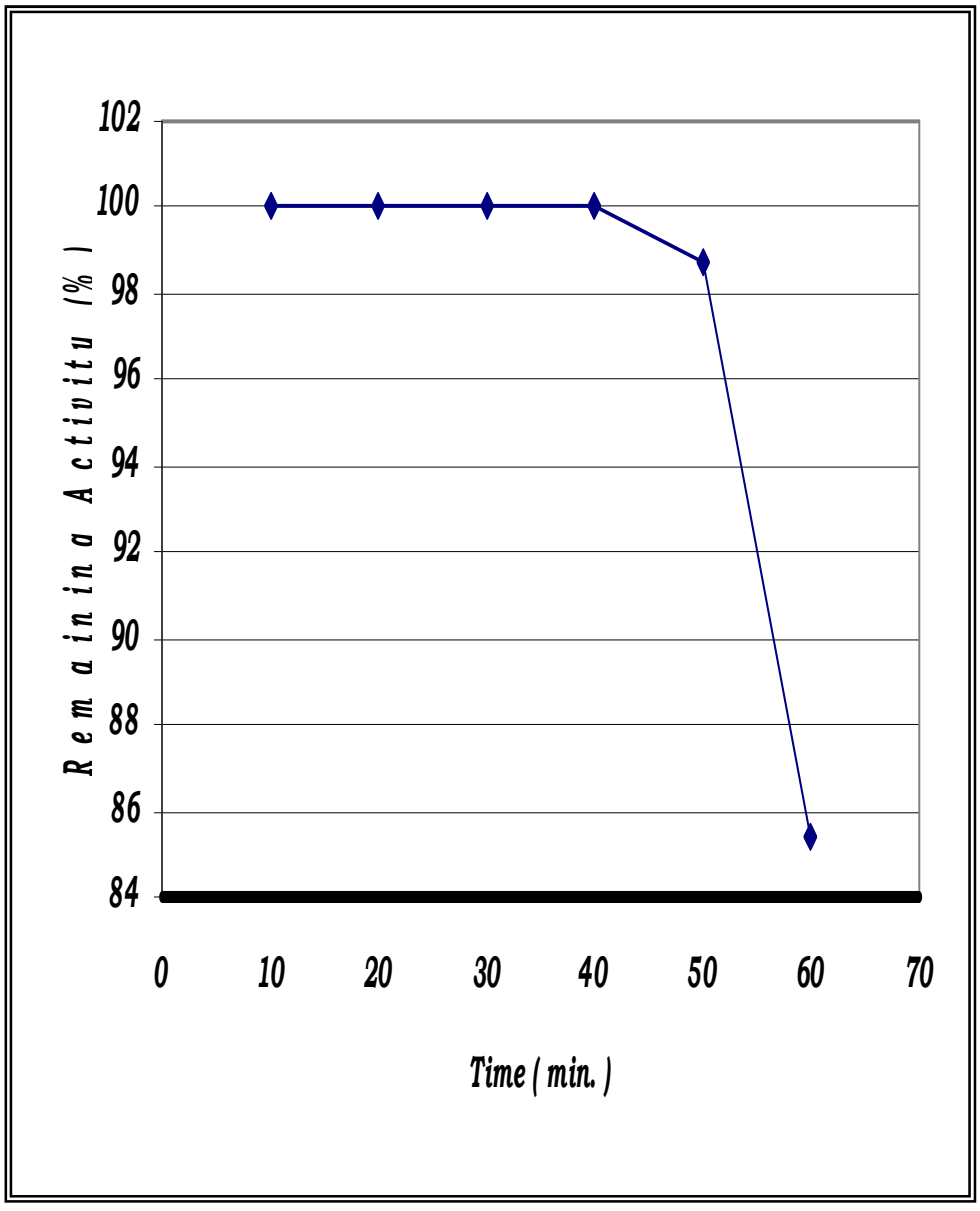

Fig. (23) Stability of α – amylase on 40 ºC at different periods of time (10, 20, 30, 40, 50, 60 min)

Fig. (24) shows the results of incubation of α – amylase with different concentration of EDTA and these results demonstrate that α – amylase had retained its activity during incubation with EDTA.

The above results indicate that α – amylase produced by Xc H6 is non metalloenzyme and its activity is not affected by the presence or absence of Ca ions in medium.

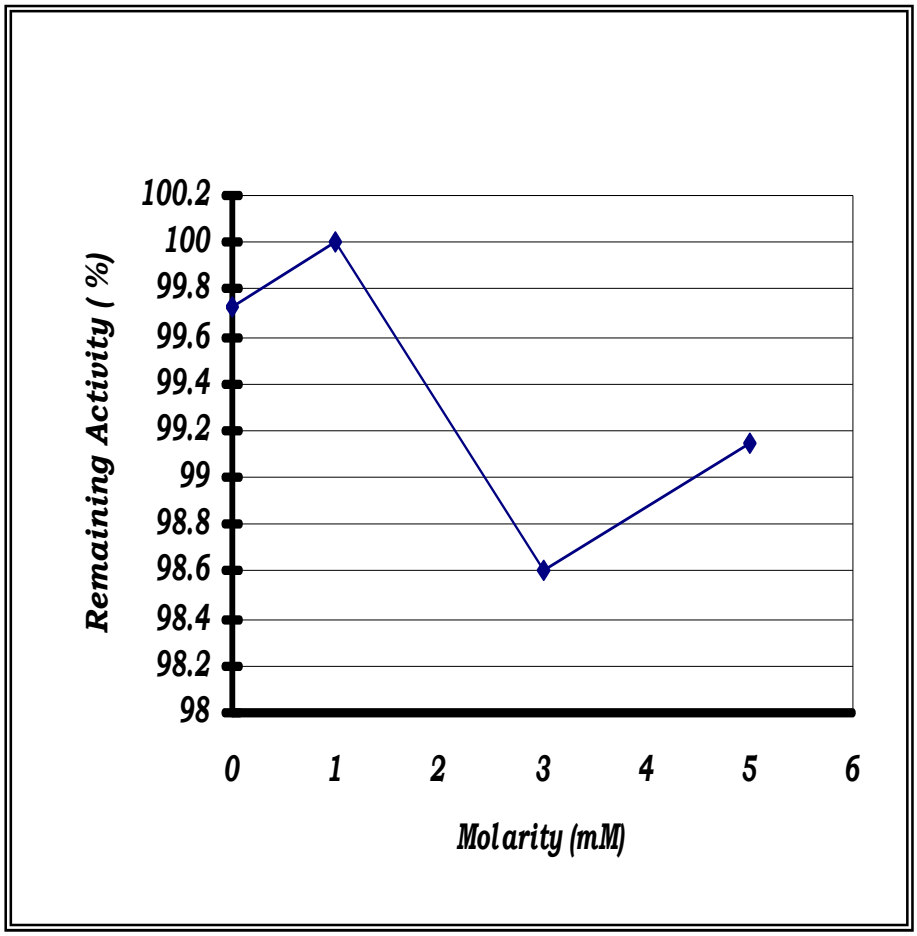

Fig. (24): Effect of EDTA on α – amylase produced by *Xanthomonas campestris* H6.

2. Material & Methods

2.1 Instruments and Chemicals:

2.1.1 Instruments:

The instruments used in this study are listed in the table (3).

Table 3: instruments used in this study.

Instrument	Source
Autoclave	Harayma (Japan)
Shaker Incubator	Gallenkmp. (Japan)
Magnetic Stirrer	Ikamaga RET (Japan)
Oven	Galenkamp (England)
Vortex	Rota Mixer Hati (USA)
Balance	Sartorius (Germany)
Sensitive Balance	Sartorius (Germany)
pH meter	Metler Toledo (England)
Vacuum Pump	Schu Co. Inc. (England)
UV – Visible spectrophotometer	Aurora Instruments Ltd (England
Centrifuge	Heraeus (England)
Chromatography Column	Pharmaci Fine Chemicals (Sweden)
Water Bath	Atom (England)

2.1.2 Chemicals:

Chemicals used in this study are listed in the following table

Table 4: Chemicals used in this study.

Company	Chemicals
Difco (USA)	Yeast Extract, Agar, Trypton, Starch, Sucrose, Lactose, casein, Acrylamide.
Fluka (Switzerland)	Iodine crystals, Sodium potassium tartarate, 3,5-Dinitro Salicylic Acid (DNSA), Riboflavin, $MgCl_2$.
BDH (England)	MgSO4.7H2O, NaCl, Maltose, Glucose, Sodium Hydroxide, Maltose,85% orthophosphoric acid, $NH_4(SO_4)_2$, NH_3Cl_3, Hydrochloric acid (HCl), Glycine, Ammonium Persulfate, Ammonium sulphate, perchloric acid, methanol , Tri – Chloroacetic Acid , acetic acid, phosphoric acid , NH_4Cl, , $CaCl_2$.
Hopkin and Williams LTD	Potassium Iodide, $FeSO_4.7H_2O$, K_2HPO_4.
Sigma (USA)	Nutrient Broth, Nutrient Agar, Glycerol, Malt Extract, Pepton, Bovine Serum Albumin, Tris-Base, Bisacrylamide, Bromophenol Blue, Tetra Methyl Ethylene Diamine (TEMED).
Pharmacia Fine Chemical (Sweden)	Sepharose CL – 6L

2.2 Culture Media:

2.2.1 Nutrient Broth:

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

2.2.2 Nutrient Agar:

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

2.2.3 Starch - Nutrient Agar Medium:

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

2.2.4 PM 1 Medium (Lee *et. al.*, 1989):

This medium composed of the following compounds (%):

Glucose	3
$MgSO_4.7H_2O$	0.3
Pepton	2.2
KH_2PO_4	3

pH was adjusted to 7.0 and sterilized by autoclaving at 121 °C for 15 min.

2.3 Buffers and Reagents:

2.3.1 Lugal's solution (Fad, 1976):

It was prepared by dissolving 10 g of potassium iodide in 25 ml of distilled water; 5 g of iodine was then added with stirring until completely

dissolved. The volume was then completed in a volumetric flask to 100 ml with distilled water and kept in a dark bottle.

2.3.2 Phosphate buffer:

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

2.3.3 Soluble - Starch Solution (0.5 %):

This solution prepared by dissolving 0.5 g of soluble starch in 25 ml of phosphate buffer (pH 7), the mixture was then heated on hot plate to boiling point until starch was completely dissolved, then it was let to cool at room temperature and the volume was completed to 100 ml in a volumetric flask with phosphate buffer.

2.3.4 3,5- Di NitroSalicylic Acid Solution (DNSA):

This solution was prepared according to Whitaker and Bernhard, (1972) by dissolving 1 g of DNSA in 50 ml of distilled water. Then 20 ml of 2 M sodium hydroxide solution was added followed by adding 30 g of sodium – potassium tartarate gradually until it was completely dissolved. The volume was completed to 100 ml in volumetric flask with distilled water and kept in dark bottle.

2.3.5 Maltose Stock Solution:

This solution was prepared by dissolving 0.2 g of maltose in 20 ml of distilled water, and then volume was brought up to 100 ml in a volumetric flask.

2.3.6 Bovine Serum Albumin Stock Solution:

Protein Bovine Serum Albumin (BSA) stock solution was prepared by dissolving 10 mg of BSA in10 ml of 0.05 M phosphate buffer (pH 7).

2.3.7 Resolving Gel Buffer Solution:

This solution was prepared by dissolving 36.3g of Tris-Base in 50 ml of 1M of HCl. The pH was adjusted to 8.8 and the volume was then completed to 100 ml.

2.3.8 Stacking Gel Buffer Solution (0.5M):

This solution was prepared by dissolving 6g of Tris – Base in distilled water and the pH was then adjusted to 6.8 using 1M HCl. The volume was then completed to 100 ml in volumetric flask.

2.3.9 Reservoir Buffer Solution:

This solution was prepared by dissolving 3g of Tris – Base with 14.4 g of glycine in 800 ml of distilled water. pH was adjusted to 8.3 and the volume was then completed to 1000 ml.

2.3.10 Acrylamide–Bisacrylamide Buffer Solution (30%):

This solution was prepared by dissolving 30 g of acrylamide and 0.8 bisacrylamide in 80 ml of distilled water then the volume was completed to 100 ml and kept in dark bottle.

2.3.11 Ammonium Persulfate Solution (10%):

This solution was prepared instantly by dissolving 1g of ammonium persulfate in 10 ml of distilled water.

2.3.12 Riboflavin Buffer Solution (0.004%):

This solution was prepared by dissolving 4 mg of riboflavin in 100 ml of distilled water and kept in dark bottle.

2.3.13 Sample Buffer:

This solution was prepared by dissolving 0.04g of sucrose in 0.2 ml of the purified enzyme. 50 µl of bromophenol blue was then added, as an indicator in the electrophoresis medium.

2.3.14 Fixing Solution:

This solution composed of 40% methanol and 10% Trichloroacetic Acid (TCA).

2.3.15 Destaining Solution:

This solution Composed of 40 % methanol and 10% acetic acid.

2.3.16 Tris – Acetate – Phosphate Buffer (Ellis and Morrison, 1982):

This solution was prepared by mixing Tris – HCl, acetic acid and phosphoric acid with a final concentration of 0.2 M. the pH was adjusted according to the needed values.

2.3.17 EDTA Stock Solution (1M):

It was prepared as a stock solution with final concentration of 1 M then 1, 3 and 5 mM were prepared from this stock solution.

2.4 Dyes:

2.4.1 Bromophenol Blue (0.25%)(Atlas *et.al.,* 1995):

This dye was prepared by dissolving 0.25 g of bromophenol blue in 100 ml of distilled water. Mixed until completely dissolved.

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

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

2.5 Bacterial Strain:

Bacterial strain *Xanthomonas campestris* H6 used in this study was obtained from the department of biotechnology, college of science, Al-Nahrain University (Al-Delaimi, 2000).

2.6 Methodology:

2.6.1 Growth of Bacterial strain:

Xanthomonas campestris H6 was allowed to grow on nutrient broth. Aeration of the liquid culture was achieved by incubating the flasks in

shaker incubator (150 rpm) at 30 °C for about 18 hr. on both liquid and solid media.

2.6.2 Maintenance of *Xanthomonas campestris* H6:

Maintenance of *Xanthomonas campestris* H6 was conducted according to Maniatis *et. al.* (1982), and as the following:

1. Short term storage:

Colonies of strain H6 was maintained for a periods few weeks on the surface of agar medium, then slantes were tightly wrapped in parafilm and stored at 4 °C.

2. Meduim term Storage:

Strain was maintained in stab cultures for long period of few months. Such cultures were prepared in small screw – capped vials containing 2 - 3 ml of nutrient agar medium. The media were inoculated using a sterile, straight platinum wire that is dipped into a dense liquid culture of bacteria and then stabbed deep in to the agar medium, after proper incubation, the cap was wrapped tightly in parafilm to prevent desiccation of the medium and kept at 4 °C.

3. Long Term Storage:

Long term storage was achieved by adding 1.5 ml of sterilized glycerol to an exponential growth culture of strain H6 in a screw-cupped bottle with a final volume of 10 ml and stored at -20 °C .

2.6.3 Starch Hydrolysis test:

Strain H6 was inoculated into the plate of starch – nutrient agar plates (2.2.3) and incubated at 30 °C for 24 hr. after incubation the plate was flooded with lugal's solution (2.3.1). The development clear zone around the colonies indicates a positive result (Atlas *et.al.*, 1995).

2.6.4 α - Amylase Assay:

α – amylase activity was assayed by measuring the reducing sugars released during the reaction, using starch as a substrate, according to Miller, (1959).

Enzyme activity (unit / ml) is defined as the amount of enzyme which produces one micromole (μmole) in a minute under the estimation condition. While specific activity expresses the units activity per each milligram (mg) of a protein. Alpha amylase activity was determined according to Miller (1959).

2.6.4.1 Maltose Standard Curve:

Maltose standard curve was prepared and as following:

1- The following volumes of maltose standard solution were prepared from maltose stock solution (2.3.5) in tests tubes (triplicate for each volume). Then proper volumes of distilled water were added and as table 4:

2- One ml of DNSA (2.3.4) was added to each tube.

3- Tubes were boiled in water bath for 5 minutes.

4- Tubes were cooled using tap water.

5- Ten ml of distilled water was added to each tube.

6- Absorbency at 540 nm was then measured. Tube number one was used as a blank.

7- A standard curve was plotted between the concentration of maltose and the correspondent absorbance at 540 nm.

table 4: preparation of maltose standard curve.

Tube No.	Maltose Sol (ml)	Distilled Water(ml)	Maltose (mg/ml)
1	0	1	0
2	0.1	0.9	0.2
3	0.3	0.7	0.6
4	0.5	0.5	1.0
5	0.7	0.3	1.4
6	0.8	0.2	1.6
7	1.0	0	2

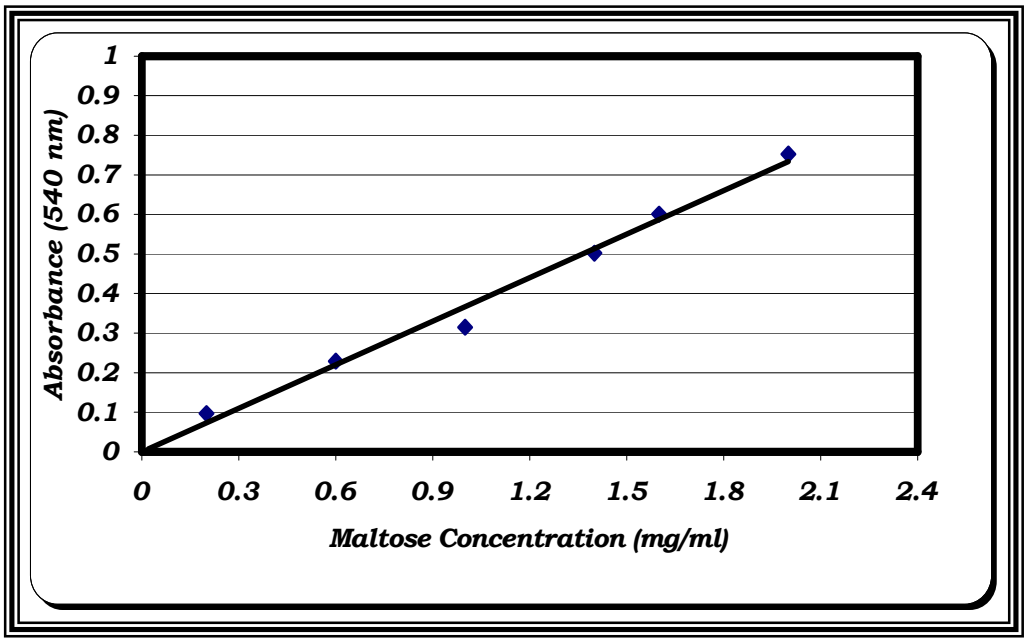

Fig. (1)Maltose Standard Curve

2.6.4.2 Alpha Amylase Activity Determination:

1. A mixture of enzyme and the substrate was prepared by adding 0.1 ml of the crude enzyme to 0.9 ml of the starch solution (2.3.3) in tests tubes. Tubes were incubated in water bath at 30 °C for 10 minutes.
2. 1 ml of DNSA (2.3.4) was then added to the mixture to stop the reaction. Tubes were then incubated at boiling temperature in a water bath for 5 minutes.
3. Tubes allowed cooling and then 10 ml of distilled water was added to each tube.
4. Optical density at 540 nm was then measured.

2.2.5 Determination of Protein Concentration:

Protein concentration determination was performed as originally described by Bradford (1976). And as following:

1. Several dilution of standard protein (BSA) were prepared from BSA stock solution (2.3.6) in the same buffer and according to volumes in table 5:
2. Then 1 ml of commassie brilliant blue G-250 (2.4.2) was added to each dilution and left to stand for 2 min. at room temperature.
3. The absorbance at 595 nm was measured: the blank was prepared from 0.1 ml of the buffer and 1 ml of the dye reagent.
4. A standard curve was plotted between the amounts of protein corresponding absorbance of the standard protein. The protein

concentrations of unknown samples were calculated from the standard curve (fig. 2).

Table (5): preparation of BSA standard curve

BSA conce. (µg/ml)	Buffer (µl)	Protein amount (µg/ml)	Total Volume
2	98	2	0.1
4	96	4	0.1
6	94	6	0.1
10	90	10	0.1
14	86	14	0.1
16	84	16	0.1
20	80	20	0.1

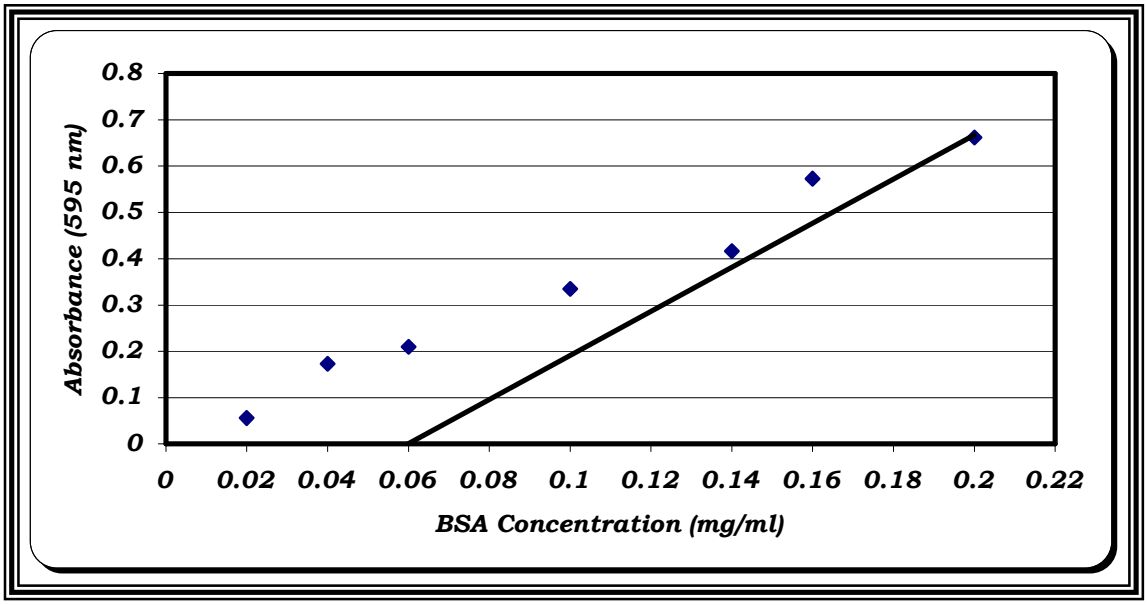

Fig. (2): Bovine Serum Albumin Standard Curve.

2.6.5 Optimal Condition for Alpha Amylase Production:

Several factors were studied in order to determine the optimal conditions for α- amylase production from *Xanthomonas campestris* H6 and as follows:

2.6.5.1 Determination of the Optimal Carbon Source:

PM1 media supplemented with different carbon sources (maltose, glucose, sucrose, starch and lactose) at concentration of 3 g/100 ml were inoculated with 100 µl of exponentially growth culture (O.D.= 0.6) of *Xanthomonas campestris* strain H6 and incubated for overnight at 30 °C with 150 rpm shaking to determine the optimum carbon source for the α-amylase production enzyme.

2.6.5.2 Determination Of The Optimal Concentration of The Carbon Source:

Different concentrations of the best carbon source for the production of the α – amylase was tested (2, 2.5, 3, 3.5, 4 %) to determine the optimal concentration of best carbon source for the α – amylase production.

2.6.5.3 Determination of the Optimal Nitrogen Source:

In order to determine the optimal nitrogen source for the production of the α- amylase enzyme, different nitrogen sources was tested in a concentration of 2.2 %. These sources are: peptone, casein, ammonium sulphate, yeast extract, trypton and ammonium chloride.

2.6.5.4 Determination Of The Optimal Concentration of Best Nitrogen Source:

PM1 medium was supplemented with different concentration of the best nitrogen source (2, 2.5, 3, 3.5, 4 %) to determine the optimal concentration of the best nitrogen source for the of α- amylase production.

2.6.5.5 Determination Of The Optimal Temperature For α- Amylase Production:

PM1 media inoculated with *Xanthomonas campestris* H6 was incubated at different temperatures (20, 25, 30, 35 and 40 °C) to investigate the optimum temperature for the of α- amylase production.

2.6.5.6 Determination Of The Optimal pH For α-Amylase Production:

PM1 media with different pH values (6, 6.5, 7, 7.5 and 8) were inoculated with *Xanthomonas campestris* H6 and then incubated at 30 °C a shaker incubator (rpm 150)to determine the optimal pH value that is suitable for the α- amylase production.

2.6.5.7 Determination Of The Optimal Phosphate Source For α-Amylase Production:

in order to determine the optimum phosphate source for α- amylase production, (KH_2PO_4 and K_2HPO_4 were supplemented to PM1 media separately at different concentration (2, 2.5, 3, 3.5 and 5 %).

2.6.6 α – Amylase Purification:

Crude filtrate was obtained by allowing the bacteria to grow in PM1 media under the optimized conditions. Then purification steps were performed in order to obtain the α – Amylase in its pure form and to further study some of the enzyme characteristics and as following:

2.6.6.1 Ammonium Sulphate Precipitation Step:

Crude filtrate, obtained by allowing the bacteria to grow in PM1 media under optimized conditions, was concentrated using ammonium sulphate with a saturation ratio of 60 – 80 %.

2.6.6.2 Dialysis:

Supernatant obtained from the above mentioned step was redissolved in distilled water and dialyzed against Phosphate – Potassium Buffer for three successive days with three substitutions with in three days.

2.6.6.3 Gel Filtration:

The final step of α- amylase purification was gel filtration through sepharose CL – 6B according to Salin and Brown-Peterson(1993) with slight modifications.

Sepharose CL - 6B suspension was packed after degassing for 10 min. into the column. After settling of the column matrix, matrix size was 2 * 14 cm, it was equilibrated with potassium phosphate buffer solution (pH 7) containing 0.4M NaCl. Extract obtained after the dialysis step was added to the column and equilibrated with the phosphate buffer (pH 7). Three ml fractions were collected in test tubes. Protein concentration in each fraction was monitored spectrophotometrically at 280 nm, fractions of the protein peaks were assayed for α-amylase activity. fractions

containing enzymatic activity were collected, lyophilized and stored at 5 °C for further characterization.

2.6.7 Poly acrylamide gel Electrophoresis under nondenatured conditions:

In order to test the purity of the α- amylase obtained from the gel filtration step, protein poly acrylamide gel electrophoresis was performed for the partially purified enzyme according to Blackshear, (1984).

Separating gel was prepared by mixing 7.5 ml of acrylamide-bisacrylamide (2.3.10), 17.5 ml of distilled water and 3.75 ml of resolving gel buffer(2.3.7), the solution was degassed for 10 min. using a vacuum pump, then 1.5 ml of ammonium persulphate and 15 µl of TEMED were added to the degassed solution and mixed gently. Using Pasteur pipette, the separating gel was transferred to Polyacrylamide gel electrophoresis (PAGE) tubes, using another pipette, the top of the gel was covered with isobutyl alcohol. The gel was then allowed to polymerize for 1 hour at room temperature.

Stacking gel was prepared by adding 2.5 ml of acrylamide – bisacrylamide solution, 5 ml of stacking gel buffer (2.3.8), 10 ml of distilled water and 2.5 ml of riboflavin solution (2.3.12). The solution was then degassed for 10 min. using a vacuum pump. 15 µl of TEMED solution was then added and mixed gently. The stacking gel was then transferred slowly over the separating gel to polymerize for about 1 hour at 25°C .

The PAGE tubes were submerged in the reservoir buffer (2.3.9) and 100 µl of the sample buffer (2.3.13) was loaded on the gel surface. The power supply was connected and run at 2.5 mA tube of constant current until the traking dye enter the separating gel, and then the current was increased to

6 mA. The total run time for the gel was about 4 hours. Then, polyacrylamide gels were removed from the PAGE tubes and placed separately in test tubes. Gels were immersed in fixing solution (2.3.14) for 1 hour, fixing solution was then poured off and the gels were immersed in the staining solution (Coommassie Brilliant Blue G-200) for 3 hours. Then staining solution was poured off and the gel was immersed with destaining solution (2.3.15) to remove the unbound stain.

The destaining process continued until blue bands of protein were obtained. Gels were stored in test tube 7% acetic acid.

2.6.8 Enzyme Characterization Assays:

Some of the main characteristics of the purified α-amylase were determined and as following:

2.6.8.1 α – Amylase Molecular Weight Determination:

Molecular weight of the partially purified enzyme was determined by gel filtration chromatorgraphy method, through sepharose CL-6B using standard proteins as a molecular markers like lysozyme (1400 Dalton) , Trypsine (20000 Dalton) pepsin (34700), bovine serum albumin (67000) and blue dextran (2×10^6) which was used to determine column void volume (V_0).

Gel filtration for the sample and each of the standard protein was performed as described in (2.6.6.3) and the elution volume (V_e) for each protein was estimated.

Then, the relationship between the ratio of elution volume of each protein to the void volume of blue dextran elution volume (V_e /V◦) and the logarithm of the molecular weight of each standard proteins to obtain the standard curve which it was used then to estimate the molecular

weight of the α – Amylase enzyme according to Laue and Rhodes, (1990).

2.6.8.2 Effect of pH on α- Amylase activity:

Using buffer solution described in (2.3.16) 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 α-amylase was added to 3 ml of buffer solution with different pH values ranging from 4 to 8 containing starch as a substrate and α-amylase assay was then performed.

The activity of α-amylase was plotted against the pH values to determine the optimal pH for α-amylase activity.

2.6.8.3 Effects of pH on α- Amylase Stability:

Equal volumes of purified enzyme and buffer solution (2.3.17) with pH range from 4 to 8 were incubated in a water bath at 30 °C for 30 min. then tubes were transferred immediately into an ice bath. The enzymatic activity for each tube was determined as it was described in (2.4.6).

The remaining activity (%) for the α-amylase was plotted against the pH values to determine the stability of the α-amylase.

2.6.8.4 Thermal Stability for α- Amylase:

Test tubes containing the partially purified α-amylase were incubated in water bath for 15 min. at various temperatures (30, 35, 40, 45, 50, 55 and 60 °C) then immediately transferred into an ice bath.

Enzymatic activity was then measured and the remaining activity (%) was then plotted against the temperature.

2.6.8.5 Thermal Stability for α-Amylase at 40 °C:

The partially purified enzyme was incubated in a water bath at 40 °C for different times (10, 20, 30, 40, 50 and 60 min.) and immediately transferred into an ice water bath.

Enzymatic activity for the α-amylase was then determined and the remaining activity was then plotted against the time.

2.6.8.6 EDTA Effect on α- Amylase Activity:

Equal volumes of purified enzyme were incubated in water bath with 0, 1, 3 and 5 mM of EDTA in Tris – Hcl (pH 7.4) at 35 °C for 10 min. then immediately transferred into an ice bath. Then the enzymatic activity was measured.

Conclusions:

- PM1 medium containing glucose 3.5 %, peptone 2 %, K_2HPO_4 2.5 %, pH 7 and incubation at 30 ºC was very appropriate conditions for the extracelluar α – amylase enzyme production from *Xanthomonas campestris* H6.
- Purification protocols using ammonium sulphate precipitation followed by gel filtration using Sepharose CL – 6B was successful in purifying the extracelluar α – amylase to apparent homogeneity.
- α – amylase produced *Xanthomonas campestris* H6 was more active at neutrality or slightly basic conditions more than acidic or slightly acidic conditions.
- α – amylase *Xanthomonas campestris* H6 has a molecular weight of 14000 Daltons.
- Partial purified extracelluar α – amylase has a narrow range of thermostability and the activity is decreased if the enzyme was incubated at temperature above 40 ºC.
- The α – amylase produced by *Xanthomonas campestris* strain H6 is a non – metalloenzyme and its activity is not affected by the presence of the chelating agents like EDTA.

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Recommendations:

- Elucidation of the molecular and pathological role of extracelluar α – amylase in the pathogenesis of *Xanthomonas compestris.*
- Further study to investigate the mechanism of action of extracelluar α – amylase produced by *Xanthomonas compestris.*
- Further study to investigate the production of of extracelluar α – amylase produced by *Xanthomonas compestris* using fermenters for large scale production.
- Enhance the ability of *Xanthomonas campestris* H6 in production of α – amylase by physical, chemical and molecular mutagenesis.
- Study of the genetics of *Xanthomonas compestris* H6 in addition to the role of genetic elements responsible for α – amylase production.

References:

Aunstrup, K. (1979). Production, isolation, and economics of extracellular enzymes. Appl. Biochemistry & Bioengineering *2*: 27−69.

Asha M. B. and Dean W. (2003). Pathogen profile: *Xanthomonas citri*: breaking the surface. Molecular plant patholology *4* (3): 141–157.

Atlas, R.M., Parks, L.C. and Brown, A.E. (1995). Laboratory manual of experimental microbiology. Moby – Year Book Inc., Wesline Industrial Drive, St. Louis, Missouri.

Attia, R.M. and Ali S.A. (1974). Utilization of agricultural wastes by *Aspergillus awamori* for the production of glucoamilase. Rev. Microbiol. *5*: 81-84.

Bahri, S.M. and Ward, J.M. (1990). Cloning and expression of an α-amylase gene from *Streptomyces themoviolaceus* CUB 74. J. Gen. Microbio., *136*: 8110818.

Beckord, L.D.; Kneens, E. and Lewis, K.H. (1945). Bacterial amylases production on wheat bran. Ind. Eng. Chem., *37*: 692-696.

Bernfeld, P. (1951). Enzymes of starch degradation and synthesis. In: Advances in Enzymology. *3*: 379-428.

Bergy's Manual of Determinative Bacteriology (1994). Williams and Wilkins, Baltimore. England.

Blackshear, P.J. (1984). Systems for polyacrylamide gel electrophoresis. In: Methods In Enzymology. (ed. Jakoby W.B.) *104*: 237 – 256. Academic Press, New York.

Blum, R., Feick P., Puype M., Vandekerckhove J., Klengel R., Nastainczyk W., and Schulz I.(1996). Tmp21 and p24A, two type I proteins enriched in pancreatic microsomal membranes, are members of a protein family involved in vesicular trafficking. J. Biol. Chem. *271*: 17183-17189

Bolton, D. J.; Kelly, C. T. and Fogarty, W. M. (1997). Purification and characterization of the α-amylase of *Bacillus flavothermus*. Enz. Microb. Technol., *20*, 340-343.

Brad R. (1997). Unique Features and Application of Non – Animal Derived Enzymes**.** Clinical Nutrition Insight *5*(10): 1 – 4.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein – dye binding. Anal. Biochem. *72*: 248 – 254.

Bull, A. I. and Bushnell, M. E. (1976). Environmental control of fungal growth. In: The filamentous fungi. Smith, J. E. and Berry, D. E. (eds.). *2*:1 – 26, Edward Arnold, London.

Christoph J. H. (1998). Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants Microbiol. And Mol. Biol. Rev. *62*(2): 379–433.

Chung, Y. C., T. Kobayashi, H. Kanai, T. Akiba, and T. Kudo. (1995). Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. Appl. Environ. Microbiol. **61:**1502-1506.

Claverie P., Viganob C., Ruysschaertb J. M., Gerday C. and Feller G. (2003). The precursor of a psychrophilic a-amylase: structural characterization and insights into cold adaptation. Biochimica et Biophysica Acta *1649*: 119–122.

Cornish – Bowden, A. (1979). Fundamentals of enzyme kinetics. London, Butterworth.

Da Silva A.C., Ferro J.A., Reinach F.C., Farah C.S., Furlan L.R., Quaggio R.B., Monteiro C.B., Van M.A., Almeida N.F., Alves L.M., do Amaral A.M., Bertolini M.C., Camargo L.E., Camarotte G., Cannavan F., Cardozo J., Chambergo F., Ciapina L.P., Cicarelli R.M., Coutinho L.L., Cursino J.R., El-Dorry H., Faria J.B., Ferreira A.J. (2002). Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature. *417*(6887):459-63.

Daniels, M. J., C. E. Barber, P. C. Turner, M. K. Sawczyc, B. W. Byrde, and A. H. Fielding. (1984). Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. *3***:** 3323–3328.

Dow, J. M. & Daniels, M. J. (1994). Pathogenicity determinants and global regulation of pathogenicity in *Xanthomonas campestris* pv. *campestris*. In Molecular and Cellular Mechanisms in Bacterial Pathogenesis of Plants and Animals, pp. 29-41. Edited by J. L. Dangl. Berlin : Springer.

Dow, J. M., Feng, J.-X., Barber, C. E., Tang, J.-L., Daniels, M. J. (2000). Novel genes involved in the regulation of pathogenicity factor production within the rpf gene cluster of *Xanthomonas campestris*. Microbiology *146*: 885-891.

Elliot, C. (1930). Manual of bacterial plant pathogen. Baltimore : Williams and Wilkins.

Ellis, K.J. and Morrison, J.F. (1982). Buffers of constant ionic strength for studying pH dependant process. In: Methods in enzymology. Purch D.L. (ed). *87*: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. pp.7-49.

Fairbairn, D. A., Priest, F. G. and Stark, J. R. (1986). Extracellular amylase synthesis by *Streptomyces limosus*. Enzyme Microb. Technol., *8*:89 – 92.

Feller G., S. D'Amico, A.M. Benotmane, F. Joly, J. Van Beeumen, C. Gerday(1998). Characterization of the C-terminal propeptide involved in bacterial wall spanning of alpha-amylase from the psychrophile *Alteromonas haloplanctis*. J. Biol. Chem. *273*: 12109– 12115.

Fogarty, W.M., (1983). Microbial amylases. In:(Microbial Enzymes and Biotechnology, W.M. Fogarty, ed., pp.1-92), Applied Science Publishers. London.

Fogarty W.M. and Kelly C.T. (1979). Developments in microbial extracellular enzymes. In: Wiseman A, editor. Topics in enzyme and fermentation biotechnology *3*: 45-108.

Fogarty, W. M. and Kelly, C.T.(1980). Amylases, Amyloglucosidase and related glucanases. In: Microbial enzymes and Bioconversion (ed. Rose A.H.) Chp. 3: 115- 170. Academic Press. London.

Friedberg, F. and Rhodes, C. (1986). Cloning and characterization of Beta – amylase gene from *Bacillus polymyxa*. J.Bacteriol., *163*(3): 819-824.

Ghosh, S.B. and Chandra A.K. (1980). Production of thermostable α-amylase by *Bacillus apiarius* CBML 152. In: Advances in Biotechnology (ed. Moo-young. M) vol. III: 313-321, Pergarmon Press. New York.

Goodfellow,M., Lacy, J. and Todd, C. (1987). Numerical classification of thermophilic *Streptomyces*. J. Gen. Microbiol., *133*: 3135-3149.

Gupta R, Gigras P, Mohapatra H, Goswami VK, and Chauhan B (2003). Microbial α-amylases: a biotechnological perspective. Process Biochem. *38*: 1599-1616.

Harada, W., Matsuura, Y., Kusunoki, M., Kakudo, M. (1984). Structure and possible catalytic residues of taka-amylase a. J. Biochemistry, *95:* 697- 703.

Hayward, A.C. (1993). The hosts of *Xanthomonas*. In *Xanthomonas* (Swings, J.G. and Civerolo, E.L., eds), pp. 1–17. London: Chapman & Hall.

Heinen, U.J. and Heinen, W. (1972). Characterization and properties of a caldoactive bacterium producing extracellular enzymes and two related strains. Arch. Microbiol. *89*: 1-23.

Henrissat B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. *280*: 309-316.

Hewitt, C.J. and Solomons, G.L(1996). The production of a-amylase (E.C.3.2.1.1.) by *Bacillus amyloliquefacien*s, in a complex and a totally defined synthetic culture mediumJ. Ind. Microbiol. *17*: 96-99.

Hidaka, H., adachi, T., Yshida, K. and Niwa, T. (1978). Studies on the amylase from *Strptomyces hygroscopius* SF-1084. J Jap. Socc. Starch Sci., *25*(2): 148 - 154.

Hizukuri S., Abe J., Onitsuka N., Nankano T., Shibata Y., and Entani E.(1994). Purification and Characterization of Periplasmic a-Amylase from *Xanthomonas campestris* K-1 1 151. J. Bacteroil. *176*(12):3584-3588.

Hobbs, M., and Mattick J. S. (1993). Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and proteinsecretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. *10:* 233–243.

Howling, D. (1989). Mechanism of starch hydrolysis. International Biodeterioration, *15*: 15-19.

Hsu, W. H., Long L. L. Chyau C. C. (1998). Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol. Appl. Biochem. *28*: 61– 68.

Hu, N. T., Hung M. N., Liao C.T. and Lin M.H. (1995). Subcellular location of xpsl a protein required for extracellular secretion by *Xanthomonas campestris pv campestris*. Microbiology *141*: 1395-1406.

Ilori, M. O., Amund O. O, (1997). Purification and properties of an alpha-amylase produced by a cassava-fermenting strain of *Micrococcus luteus*. Folia Microbiologica *42*(5): 445-449.

Iefuji, H., M. Chino, M. Kato, and Y. Iimura. (1996). Raw-starch-digesting and thermostable -amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing. Biochem. J. *318*:989-996.

Internet: www.enzyme.co.uk

Itkor, P., Shda, O., Tsukagoshi, N. and Udaka, S. (1989). Screening for raw starch digesting bacteria. Agric. Biol. Chem. *53*: 53-60.

Ivanova, V., E. Dobreva, E. Emanuilova (1993). Purification and characterization of thermostable alpha-amylase from *Bacillus licheniformis*. J. Biotechnol., *28*, 277-289.

Kari S. G. and John M. P.(1988): Molecular Cloning, Characterization, and Nucleotide Sequence of an Extracellular Amylase Gene from Aeromonas hydrophila. J. Bacteriol. *62*: 1325-1332.

Katoh, M. T. and Kohei M. (1997). Purification of alpha-amylase by Specific Elution from Anti-Peptide Antibodies. Appl. Microbiol. Biotechnol., *47*: 521-524.

Kochhar, S. and Dua, R.D. (1990). Thermostable liquefying α – amylase from *Bacillus amyloliquefaciens*. Biot. Lett. *12*(5):303 – 396.

Kulp, K. (1979). Carbohydrates, 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 amylotic strains of *Thermoactinomyces vulgaris* and production of thermopholic actinomyces amylases J. Bacterio., *92*(3): 723-726.

Laue, T.M. and Rhodes, D.G. (1990). Determination of Size, Molecular Weight and Presence of Subunits. Methods in Enzymology *182*:566-587.

Lee P. K.; Chang, H. N. and Kim, B. H. (1989). Xanthan production by *Xanthomonas campestris* in continuous fermentation. Biotech. Lett. *11*(8):573 – 578.

Leyns, F., DeCleene M., Swings J., and Deley J. (1984). The host range of the genus *Xanthomonas*. Bot. Rev. *50***:** 308–356.

Lin L. L., Chyau C. C. and Hsu W. H. (1998). Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol. Appl. Biochem. *28:* 61–68.

Lonsane B.K, Ramesh, M.V.,. (1990). Solid state fermentation for production of α- amylase by *Bacilluls megaterium* IGM. Biot. Lett. *9*(5): 323-328.

Machius, M., declerck, N., Huber, R. and Wiegand, G.(1998). Activation *Bacillus licheniformis* α - amylase through a disorder – disordertransition of the substrate – binding site mediated by a calcium – sodium metal triad. Structure *6*: 281 – 292.

Maniatis, T.; Fritch, E.F. and Sambrook, J. (1982). Molecular Cloning. A laboratory Manual. Cold Spring Harbour Laboratory. Cold Spring Harbour. New York.

Matsui I, Yoneda S, Ishikawa K, Miyairi S, Fukui S, Umeyama H, Honda K. (1994). Roles of the aromatic residues conserved in the active

center of Saccharomycopsis alpha-amylase for transglycosylation and hydrolysis activity. Biochemistry. *33*(2):451-8.

Martins M. L and Santos E.O.(2003). Effect of the medium composition on formation of amylase by *Bacillus sp*. Braz. arch. biol. technol. *46*(1):57 – 61.

Martins M. L. and Teodoro C. E.(2000). Cutlure condition for the production of thermostable amylase by *Bacillus* sp. Braz. J. Microbiol. *31*(4):982-302.

Mauro, B. and Kobayshi, T. (1951). Enzymatic scission of branch links in amylopectin. Nature *167*: 606 – 609.

McTigue, M.A., Kelly, C.T., Fogarty, W.M. and Doyle, E.M. (1994). Production studies on the alkaline amylases of three alkalophilic *Bacillus* spp. Biotechnology Letters,*16*: 569-574.

Miller, G.L.(1959). Use of Dinitro Salicylic Acid reagent for determination of reducing sugar. Anal. Chem. *31*(3): 426 – 428.

Mordarski, H., Wieczorek, J., Jaworska, B. and Mordarski, M. (1972). Starch hydrolysis in *Nocardia* and allied taxa. Acta Microbiologica Polonica, Series A *21*: 27- 32.

Mordarski, M. Wieczorek, J. and Jaworska, B. (1970). On the condition of amylase production by actinomycetes. Archivum Immunolgia et Therapiae Exermimentalis, *18*: 375-381.

Mortimer P. (1981). The genus *Xanthomonas* pp 743 – 763 In: Mortimer P. Starr, Heinz Stolp , Hans G, Truper Albert Balows Hans G, Schlegel, The Procaryotes: a hand book of habitat, isolation and identification of bacteria.Springer-verlag New York.

Musaeva, T.I. (1966). Effect of inorganic nitrogen on the synthesis of amylolytic enzymes in *Aspergillus awarmori* cultures. Uzbeksk. Bio. Zh., *10(*2): 12-14.

Nahas E. and Waldemarin M. (2002). Control of amylase production and growth characteristics of *Aspergillus ochraceus*. Microbiologia *44*(1): 5 – 10.

Nein T., Huang M. N., Tsai H. F., Yang B. U., Chow T. Y. and Tseng Y. H.(1992). Cloning, characterization and nucleotide sequence of the gene for secreted α- amylase from *Xanthomonas campestris* pv.*campestris*. J. Gen. Microbiol. *138*: 1647-1655.

Norman, B.B. (1979). The application of polysaccharide degrading enzymes in the strch industy. In: Microbial polysaccharides and polysaccharase. (eds. Berkely, R.C.W., et al.,)Chp.15:339-374.Academic Press.

Obi, S.K.C. and Odibo F.J.C. (1984a). Some properties of a highly thermostable α- amylase from a *Thermoactinomyces* sp. Can. J. Microbiol., *30*: 780-785.

Obi, S.K.C. and Odibo F.J.C. (1984b). Partial purification and characterization of a thermostable *actinomycetes* β- amylase. Appl. Environ. Microbiol., *42*(3): 571-575.

Onsando, J. M. (1992). Black rot of crucifers. In Plant Diseases of International Importance. II : Diseases of Vegetable and Oil Seed Crops, pp. 243±252. Edited by H. S. Chaube, J. Kumar, A. N. Mukhopadhyay & U. S. Singh. Englewood Cliffs, NJ: Prentice Hall.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, and Mohan R (2000). Advances in microbial amylases. Biotechnol. Appl. Biochem. *31*: 135-52.

Pfueller, S. L. and Elliott, W. H. (1969). The extracellular α – amylase of *Bacillus stearthermophillus*. J. Biol. Chem. *244*(1): 48 – 54.

Priest, F.G. (1992). Enzymes, Extracellular. In: encyclopedia of microbiology. Academic Press, Inc. Vol. 2 pp. 81-93.

Priest, F.G. (1993). *Bacillus*. In:(Biotechnology, H-J. Rehm, and G. Reed, eds., pp. 368-400).

Pugsley, A. P. (1993). The complete general secretory pathway in gram negative bacteria. Microbiol Rev. *57***:** 50–108.

Raimbault,. M. and Alzad, M. (1980). Culture method to study fungal growth in solid fermentation. Eur. J. Appl. Microbio. Biotech. *9*: 199-209.

Rao, M.B., Tanksale, A.M., Gathe, M.S., Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. Micrbiol. Mol.Biol. Rev. *62*(3): 597-635

Reddy, S., Annapoorna Nimmagadda and K. R. S. Sambasiva Rao (2003). An overview of the microbial α-amylase family. African Journal of Biotechnology *2* (12): 645-648.

Reed, G. (1975). Oxidoreductase. In: Enzyme in food processing. pp. 176 – 196. Academic Press. New York.

Robyt, J.F. and Whelan, W.J. (1968). Amylase and their action on Starch and it's derivatives. In:(Starch and it's derivatives, J.A. radley, ed., pp. 423-497), Champan and Hall, London.

Sakaki, H,. Kurosawa, K, and Takao, S. (1986). Screening of microorganisms for raw starch saccharifying enzyme production. Agric. Biol. Chem., *50*(6): 1661-1664.

Salin, M.L. and Peterson-Brown, N.J. (1993). Dealing with active oxygen intermediates. Experrentia *49*:523-529.

Salva G. and Moraes T. (1994). Effect of pH and temperature on *Bacillus subtilis* ATCC 601 □-amylase production. Some properties of the crude enzyme. *Rev. Microbiol., São Paulo, Brasil. 25*:119-25.

Sanford P.A. and Baird J. (1983). Industrial utilization of polysaccharide, 411-490. The polysaccharide .IN G.O. Aspinall (ed), Academic Press, New York.

Satoh E., Niimura Y., Uchimura T., Kozaki M., and Komagata K. (1993). Molecular Cloning and Expression of Two oc-Amylase Genes from *Streptococcus bovis* 148 in *Escherichia coli.* Appl. Environ. Microbiol. *59*(11):3669-3673.

Segal, I.H. (1975). Enzyme kinetics. Hohn Wiley and Sons, New York , London.

Shaw J., Lin F., Chen S, C., and Hsing C. C. (1995). Purification and properties of an extracellular α- amylse from *Thermus* sp. Bot. Bull Acad. Sin. *36*: 195-200.

Shaw J.F. and Ou-Lee T.M. (1984). Simultaneous purification of alpha and beta amylase from germinated rice seeds and sme factors affecting activities of purified enzyme. Bot. Bull. Acad. Sci. *25*: 197-204.

Shawn J. F. R. S. and Pan, W. H. H. (1989). influence of the pH on the inactivation of isoamylase. Bot. Bull. Acad. Sin. *30*: 91-95**.**

Shih N.J. and Labbe R. (1995). Purification and Characterization of an Extracellular a-Amylase from *Clostridium perfringens* Type A. Appl. Environ. Microbiol. *61*(5): 1776–1779.

Shinke, R., Nishira, H. and Mugibayashi, N. (1974). Isolation of β-amylase producing microorganism. Agric. Biol. Chem., *38*: 665-666.

Simpson, C. L. and Russell, R. B. (1998). Intracellular a-Amylase of *Streptococcus mutans.* J. Bacteriol. *180*(17): 4711–4717.

Sindhu G.S., Sharma P., Chakrabarti T., Gupta J.K. (1997). Strain improvement for the production of a thermostable α-amylase. Enzyme Microb. Technol. *24*: 584-589.

Sinha, S. and Chandra, A. L. (1981). Isolation of an amylase – producing *Streptomycete* from soil and its characterization . Zentralbl. Bacteriol. Parasitenkd, Infecctions Kr, Hyg., *136*(3): 209 – 214.

Sinnot M.L., (1990). Catalytic mechanism of enzymatic glycosyl transferase. Chem. Rev. *90*: 1171 – 1201.

Skov, L. K. , Osman M., Anette H., Gabrielle P. M., Magali R.S., Patricia S., Willemot R. M., Pierre M., and Michael G. (2001). Amylosucrase, a Glucan-synthesizing Enzyme from the a-Amylase Family. J. Biol. Chem. *276*(27): 25273–25278.

Star M. P. and Stephan, W. L. (1964). Pigmentation and taxanomy of the genus *Xanthomonas*. J. bacteriology *98*: 293-302.

Takasaki, Y.(1989). Nvel maltose – producing amylase from *Bacillus megateruim* G-2. Agric. Biol. Chem. *53*(2): 341-347.

Takekawa, S., Uozumi, N,. Tsukagoshi, N. and Uda, S. (1991). Proteases involved in generation of β- and α- amylase from a large amylase precursor in *Bacillus polymyxa*. J. Bacteriol *173*(21): 6820-6825.

Tang, J.-L., Gough, C. L. and Daniels, M. J. (1990). Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pathovar *campestris*. Mol Gen Genet *222*: 157-160.

Tengerdy, R.P. (1985). Solid substrate fermentation. Trends in Biotechnology 3 : 96-99.

Teodoro C. E. S. and Martins M. L. L. (2000). culture condition for the production of the thermostable α- amylase from *Bacillus* sp.. . Martins Brazilian Journal of Microbiology *31*:298-302.

Teotia S.; Gupta M.N.(2002). Alginate Beads for Purification of Some Starch Degrading Enzymes. Molecular Biotechnology *20*: 231-238

Tester R.F., Karkalas J., Qi X. (2004). Starch structure and digestibility Enzyme-Substrate relationship. World's Poultry Science Journal *60*(2): 186-195.

Tonozuka, T., Ohtsuka, M., MoGi, S-I., Sakai, H., Ohta, T. and Sakano, Y. (1993). A neeophullulanase – type α-amylase gene from *Thermoactinomyces vulgaris* R-47. Biosci. Biotech. Boichem., *57*(3): 395-401.

Tonozuka, T., Ohtsuka, M., MoGi, S-I., Sakai, H., Ohta, T. and Sakano, Y. (1993). A neeophullulanase – type α-amylase gene from *Thermoactinomyces vulgaris* R-47. Biosci. Biotech. Boichem., *57*(3): 395-401.

Tseng Y. H., Choy K., Hung C. H., Lin N. T., Liu J. Y., Lou C. H., Yang B.Y., Wen F. S., Weng S. F., and Wu J. R. (1999). Chromosome Map of *Xanthomonas campestris* pv. *campestris* 17 with Locations of Genes Involved in Xanthan Gum Synthesis and Yellow Pigmentation. J. Bacteriol. *181*(1): 117–125.

Tutino M.L., Parrilli E., Giaquinto L., Duilio A., Sannia G., Feller G., Marino G. (2002). Secretion of alpha-amylase from *Pseudoalteromonas haloplanktis* TAB23: two different pathways in different hosts, J. Bacteriol. *184*: 5814–5817.

Uguru, G. C.; Akinyanju, J. A. and Sani, A. (1997). The use of yam peel for growth of locally isolated *Aspergillus niger* and α-amylase production. Enz. Microbiol. Technol. *21*, 48-51.

Umemura, Y. and Kawano, K. (1983). Field assessment and inheritance of resistance to cassava bacterial blight. Crop Sci., *23*: 1127-1132.

Upton, M.E. and Fogarty, W.M. (1977). Production and purification of thermostable amylase and protease of *Thermomonospora viridis*. Appl. Environ. Microbiol., *33*(1): 59-64.

Vallee, B. L., Stein, E. A. Sumerwell, W.N., and Fisher, E. H. (1959). Metal content of α – amylase of various origins. J. Biol. Chem. *234*(11):2910 – 2905.

Veralag, S. (1989). Protein purification. USA, Inc, New York.

Verhasselt P., Poncelet F., Vits K., Gool `A. and Vanderleyden J. (1989). Cloning and expressin of a Clostridium acetobutylicum α- amylase gene in *Escherichia coli.* FEMS Microbioloy Letters *59*:135-140.

Verneire C.O., Cirevolo E.L., Gambin O., Jacquemont J.P., and Luesitte L. (1993). Evaluation of biological substrate utilization system to identify and assess metabolic pathways among strains of *Xanthomonas campastris* pv citri. Appl. Environm. Mocrobiol. *59*: 243-249.

Volesky, B., and Loung, I. (1985). Microbial enzymes productions purification and isolation. Reviews in Biotechnology, *2*:119 - 146.

Walker, G.J. and Whelan, W.J. (1960). The mechanism of carbohydrase action. Biochem. J., *76*: 264-268.

Wang, T.-W., and Y.-H. Tseng. (1992). Electrotransformation of *Xanthomonas campestris* by RF DNA of filamentous phage fLf. Lett. Appl. Microbiol. *14***:** 65–68.

Weiss, B. D., M. A. Capage, M. Kessel, and S. A. Benson. (1994). Isolation and characterization of a generalized transducing phage for *Xanthomonas campestris* pv. *campestris*. J. Bacteriol. *176***:** 3354–3359.

Whitaker, J.R. (1972). Principles of Enzymology for food sciences. Marcel Dekker, Inc. New York.

Whitaker, J.R. and Bernhard, R.A. (1972). Experiments for an introduction to Enzymology. The Wibber Press Davis.

Widjaja R., Suwanto A. and Tjahjono B. (1999). Genome size and macrorestriction map of *Xanthomonas campestris* pv. *glycines* YR32 chromosome. FEMS Microbiol. Lett. *175*(1): 59-68.

Windish W.W, and Mhatre N. S. (1965). Microbial amylases. In; Wayne WU,editor. Advances in Applied Microbiology *7*: 273-304.

Wojskaowicz, J. (1977). Biosynthesis of α- amylase and protease by *Streptomyces oivaceus* 142. I. Regulation of α-amylaseactivity. Acta Microbiol. Pol.,, *26*(2): 149 – 162.(cited by Fogarty and Kelly, 1980).

Summary

The ability of *Xanthomonas campestris* strain H6 for the production of α – amylase was studied and it was found that Xc H6 is capable of producing the e xtracelluar α – amylase enzyme.

Optimal conditions for the α – amylase production in PM1 medium were studied and the higher activity of this enzyme was obtained in PM1 medium supplemented with glucose 3.5 %, peptone 2 %, K_2HPO_4 2.5 % at 30 ºC and pH 7.0.

Results obtained demonstrate that α – amylase produced by Xc H6 is a constitutive enzyme that is not subjected to catabolite repression by glucose like many other amylases.

This enzyme was initially purified by ammonium sulphate precipitation with saturation ratio or 60 – 80 % which gave a specific activity of 12.97 U/mg with 1.4 purification fold, then it was purified by gel filtration using Sepharose CL – 6B which resulted in specific activity of 42.63, 4.7 purification fold and an overall yield of 21.69%.

Purity of the purified α – amylase was confirmed using poly acrylamide gel electrophoresis under non denatured condition which demonstrated only single protein band.

Characterization experiments showed that the molecular weight of the α – amylase produced by Xc H6 is about 14000 Dalton, on the other hand it was found that optimum pH for the activity of the purified enzyme was 7.5 and the enzyme retained its complete activity when it was incubated at pH 6.5 and 7.0 and it retained more that 90% of its activity at pH 7.5.

Thermostability of the purified α – amylase was tested and the enzyme showed 100% of its activity at 30, 35 and 40 ˚C and about 95% at 45 ˚C. Activity of the enzyme was 50% when it was incubated at 60 ˚C.

The effect of chelating agents on α – amylase activity was investigated and results showed that there was no significant decrease in the activity of the α – amylase when it was incubated with different concentrations of EDTA, indicating that chelating agent have no effect on α – amylase enzyme and its is a non – metalloenzyme.

Purification and Characterization of α-Amylase Produced by The Local Isolate *Xanthomonas campestris* H6

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