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Genetic and Biochemical Study of α–Amylase produced by *Bacillus stearothermophilus* M13 isolated from Iraqi soil

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جمهورية العراق
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دراسة وراثية و كيموحيوية لأنزيم إلفا- اميليز المنتج من بكتريا *Bacillus stearothermophilus* M13 المعزولة من التربة العراقية

أطروحة
مقدمة إلى مجلس كلية العلوم –جامعة النهرين
وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في التقانة الإحيائية

من قبل

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الخلاصة

عزلت ست وتسعون عزلة محلية من بكتريا *Bacillus sp.* المتحملة للحرارة و اختبرت قدرتها على انتاج الفا–املیز (EC 3.2.1.1) المفرز خارج خلوي و المحلل للنشا وبعد عدة خطوات من الغربلة الاولية في الاوساط الصلبة والمزارع المغمورة انتخبت العزلة *Bacillus stearothermophilus* M13 المشخصة كونها العزلة الاعلى انتاجا للانزيم مقارنة مع بقية العزلات واستخدمت في انتاج وتنقية وتوصيف الانزيم ومن ثم كلونة الجين المشفرللانزيم في هذه الدراسة . وهي كانت حساسةلبعض المضادات الحياتيةالتالية (Imipenem ,Cefotaxime ,Norfloxacin ,Gentamicin , Pencillin, Ampicillin) بينما اظهرت مقاومة للمضاد الحياتي(Aztreonem) .

كانت الظروف المثلى للانتاج هي بتنمية البكتريا في وسط TSM بطريقة المزارع المغمورة عند رقم هيدروجيني ابتدائي مقداره 7.0 و بدرجة حرارة 5٥ درجة مئوية لمدة 24 ساعة.

نُقي انزيم الفا–املیز المنتج من بكتريا *B. stearothermophilus* M13 وتضمنت خطوات شملت الترسيب بكبريتات الامونيوم بنسبة اشباع (٤٠–٨٠%)و كرماتوغرافيا التبادل الايوني باستخدام عمود DEAE-Sephadex و لوحظ وجود اربعة مناظرات انزيمية ، وتم انتخاب المناظر الانزيمي"b" لكونه الاعلى فعالية . تم اجراء كرماتوغرافيا الترشيح الهلامي على عمود Sephadex G-100 وكانت الفعالية النوعية النهائية له ١,٦٧٥وحدة/ملغم، بعدد مرات التنقية 30.45 وبحصيلة انزيمية50.25 %.

بينت نتائج توصيف الانزيم ان وزنه الجزيئي 56234 دالتون عند تعينه بطريقة الترشيح الهلامي، بينما كان الوزن الجزيئي عند تعينه بطريقة SDS-PAGE مساويا الى ٥٥٤٢٦ دالتون. وكانت قيم الرقم الهيدروجيني الامثل لفعالية وثبات الانزيم مساوية الى ٦,٥– ٧,٠و ٦,٥–٨,٥ على التوالي، ولوحظ ان اقصى فعالية للانزيم كانت عند درجةحرارة٦٠°م .واظهر الانزيم ثباتية ١٠٠% في درجة حرارة ٧٠°م لمدة ٣٠دقيقة ، واحتفظ الانزيم ب ٥٠% من الفعالية الاصلية عند تعريضه لدرجة حرارة٩٠°م لمدة ٣٠ دقيقة مما ساهم في تاكيد كونه انزيما متحملا للحرارة.

تم التحري عن النواتج المتكونة بفعل تاثير(تحليل) الانزيم لمادة النشا مع دالة زمن التفاعل باستحدام تقنية الكروماتوغرافي الورقي ، حيث اظهرت وجود المالتوز، الكلوكوز وبعض السكريات التي لم يتم تشخيص هويتها لعدم توفر النظائر القياسية .

اظهر الانزيم خصائص مستضدية عند تمنيع الارانب به، اذا ان الاجسام المضادة قد سببت انخفاض في الفعالية بحدود ٣٧,٤%.

تم تقييد الانزيم بطريقة الاقتناص باستخدام الجينات الكالسيوم ، واحتفظ الانزيم ب ٢٤% من الفعالية الاصلية عند اليوم ٢٥ من الخزن بدرجة حرارة ٤°م .

تم التحري عن وجود الدنا البلازميدي في العزلة *Bacillus stearothermophilus* M13 وباستخدام ثلاث طرق مختلفة هي التحلل القاعدي (Alkaline lysis, CTAB ,Melinz)حيث اظهرت النتائج عدم احتواء هذه البكتريا على أي بلازميد .ولاجل كلونة الجين المشفر لانزيم الاميليز تم استخلاص DNA الكروموسومي لهذه البكتريا وكذالك تم عزل وتنقية الناقل البلازميدي pBR*322* وباستخدام طريقة الطرد المركزي الفائق السرعة المتدرج الكثافة باستخدام السيزيوم كلورايد– الاثيديوم بروماید . تم هضم الناقل كليا، وهضم المجين جزئيا بالانزيم القاطع *Hin*d III . لحمت القطع الناتجة معا بالانزيم اللاحم T4 ligase وادخلت الجزيئات الهجينة الناتجة عن طريق التحول الى بكتريا *E.coli* MM294 وانتخبت المتحولات على اساس المقاومة للامبسلين ثم الكشف عن قدرتها على تحليل النشا .اختبرة قدرة ٣٨٤٧ مستعمرة مقاومة لامبسلين على تحليل النشا ، واظهرت خمس مستعمرات (٠,١٣%) منها فقط القدرة على افراز الانزيم في الاوساط الصلبة والسائلة ، وانتخبت العزلة الاعلى انتاجا بينها في الاوساط السائلة وسميت *E.coli* MA 1 حيث بلغت الفعالية النوعية ٠,٠٤٥ وحدة/ملغم .

Conclusions

1. *Bacillus stearothermophilus* M13 produced a valuable amount of α-Amylase among the local thermophilic isolates , that could be used in many industrial applications.
2. The optimal conditions for α-Amylase production and by *Bacillus stearothermophilus* M13 were determined ;represented by TSM medium, at 55 ºC , late log and early stationary phase of growth .
3. α-amylase showed a high activity at pH 7.0 with stability at pH range (6.5-8.0), and a high activity at 70ºC with 100% of the remaining activity .while, at 90ºC showed 55% of the remaining activity .
4. The purification protocol included DEAE –sephadex Ion exchange and gel filtration gel were recommended.
5. Four possible forms (a, b, c and d) of enzyme were eluted from ion-exchange chromatography in which "b" showed the highest specific activity among them , and the overall activity may be due to it namely.
6. Molecular weight of α-amylase was" 56234 Da." when estimated by gel filtration and "55426 Da." when estimated by SDS- PAGE technique .
7. TLC technique was applied to verify the α-Amylase time-effect –products in which monosaccharide and disaccharides were among the products.
8. α-Amylase showed antigenic properties in vivo by decreasing the activity 37.4% for the partially purified α-amylase forms.
9. The DNA fragment that coded for α-Amylase was cloned into *E .coli* MM 294 using pBR*322* as a vector, in which the length of the cloned fragment was estimated of 1790bp approximately.
10. The expression of the recombinant fragment was detected in the transformants cells on a solid medium and liquid medium, which showed a

slight decrease in the specific activity in comparison with original organism with no accompanying proteases activity.

5.2 Recommendations

1. Studying the optimization of α-Amylase production by the cloned *E .coli* MA1 using the liquid media.
2. Conducting further molecular studies for α-Amylase regulation and secretion in the original *Bacillus stearothermophilus M13* and transfrormed (cloned) *E .coli* AM1.
3. Cloning the multicopy number for α-amylase gene in pBR322 or other suitable vectors.
4. Producing and purifying Monoclonal antibodies to detect the enzyme and more accurate assay of α-amylase activity.
5. Conducting more detailed studies concerning protein engineering of α-Amylase produced by the cloned *E. coli* to undergo the industrial processes within different conditions such as alkalinity stability and more thermophilic stability .
6. Studying the kinetics of α-Amylase form(s) (if any) in the new host .
7. Founding of a specialized center to enhance the production and to improve the proteins for future important industeries in Iraq.

Summary

Sixty-nine local bacterial isolates of thermophilic *Bacillus* spp. isolated from different soil locations over Iraq land were screened for their ability to produce extracellular α-amylase (EC 3.2.1.1) in solid and submerged cultures. The isolate *Bacillus stearothermophilus* M13 was selected based on its production of enzyme among the other isolates and used in the present work to study the production,purification and chracterization of enzyme then the cloning of the α-amylase gene. It was susceptible for some antibiotics (Imipenem, Cefotaxime, Norfloxacin, Gentamicin, Pencillin, Ampicillin). while, it was resistant to Aztreonem.

The optimum conditions for α-Amylase production from *Bacillus stearothermophilus* M13 were grown in TSM medium with an initial pH 7.0 at 55^{o}C for 24 hrs of incubation, the activity of crude extract was 0.04 U/ml.

α-Amylase was purified from *Bacillus stearothermophilus* M13 isolate by several steps including saturation of crude extract with ammonium sulphate (40-80%), ion exchange chromatography by DEAE-Sephadex in which four isozymes(forms) were noticed named as (*a,b.c* and *d*). "*b* form", which showed the higher specific activity among the others, was applied to gel filtration through Sephadex-G100 that revealed overall specific activity of 1.675 U/mg ; 30.45 folds of purification and yield of 50.25 %.

The characterization of the partial purified enzyme showed that its molecular weight was "56234" dalton as determined by gel filtration technique, on the other hand the estimation of molecular weight using SDS- PAGE was "55426" dalton.

The enzyme activity was higher at pH rang 6.5-7.5 and it showed stability at pH rang 6.5-8.0. The maximum enzyme activity was reported at 60^{o}C and it

was stable at 70^{o}C for 30 min and retained 50% of its original activity at 90^{o}C, which confirmed that this enzyme is heat stable enzyme.

The α-amylase showed antigenic activity in vivo (Rabbits) ,the polyclonal antibodies could decrease the α-amylase activity with 37.4% . Then the products of α-amylase action on the starch over the reaction time had been investigated using TLC , glucose ,maltose and other unknown carbohydrates were produced that could not be identified due to unvaiability of standard analoges .

Immobilization of α-amylase via entrabment method using the calcium alginate was successful, it retained 24% of its original activity on the 25th day of storage when it kept at 4^{o}C.

α-Amylase genome was investigated for any possible plasmid DNA carrying the α-amylase gene , the plasmid DNA was isolated by three different protocols, (Alkaline lyses ;CTAP and Mielenz`s protocol) results showed no detectable plasmid.

Cloning of α-amylase gene was done ; chromosomal DNA of *B. stearothermophilus* M13was extracted and partially digested with *Hin*d III . The pBR322 was isolated and purified via CsCl-EtBr gradient ultracentrifugation, then it completely digested by *Hin*dIII. The digested chromosomal DNA and vector was ligated by T4 ligase in ratio of the inserted DNA : vector DNA as 5:1 , followed by the transformation of the host cells (*E. coli*) with hybrid vector. Transformants (3847) were selected according to the ampicillin resistance as in the primary screening then to hydrolysis of starch. Five clones (0.13 %) only out of 3847 transforments were able to produce α-amylase on solid and liquid media , the higher transformant-producer was named *E. coli* MA1 , in which the specific activity was 0.045 U/mg .

1.1 Introduction

Enzymes are complex proteins that act as catalysts for the countless biochemical reactions that keep humans, animals, plants, and microorganisms alive. Enzymes have an importance in the industrial and commercial uses as well. Since ancient times, people have observed enzymes at work when fermenting their wine and beer, turning their sour milk into cheese, and causing their bread dough to rise.

In 1833, the French chemist Anselme Payen separated a substance from an extract of malt that, he realized, seemed capable of speeding up the conversion of starch to sugar. Payen called the substance diastase (Greek expression separation, from, to stand apart; dia` through + to stand)diastase. (Webster, 1913), the first enzyme to be isolated and prepared in a concentrated form.

Starch-degrading, amylolytic enzymes are widely distributed among microbes. Several activities are required to hydrolyze starch to its glucose units. These enzymes including α-amylase, β-amylase, glucoamylase, α-glucosidase, pullulan degrading enzymes, and cyclodextrin glycosyltransferase. Properties of these enzymes vary and are somewhat linked to the environmental circumstances of the producing organisms, features of the enzymes, their action patterns, physicochemical properties, occurrence and genetics (Vihinen and Mantsala, 1989**) .**

Screening for new microbial strains producing the desired α-amylase from extreme environments has been reported (Sunna *et al.*, 1997; Niehaus *et al.*, 1999;Veille and Zeikus, 2001) .Utilization of high concentrations of starch, thermostability, and protein yield are important criteria for commercialization (Schafer *et al.*, 2000).

Genetic engineering has been used extensively for cloning of amylase-producing strains, mainly α-amylase and glucoamylase (GA), in order to

achieve desirable characteristics in the cloned host. The purpose of gene cloning can be, among others, the expression of thermostable enzymes, higher enzyme productivity, co-expression of two enzymes by the same organism or as less α-amylase contaminated with the other enzymes.

α-Amylase is found in saliva and pancreatic secretions, where they play an obvious role in polysaccharide digestion. More surprisingly, α-amylase was also found in blood, sweat, and tears, possibly for anti-bacterial activity.

α-Amylase determination has been recognized as an important diagnostic tool for many years, because the elevated levels of the enzyme are associated with liver and pancreatic disorders, as well as other diseases (Bookrags, 2006). Enzymes are the catalysts of all reactions in living systems. These reactions are catalyzed in the active sites of globular proteins. Enzymatic processes have been increasingly incorporated in textiles over the last years. Cotton, wool, flax, or starches are natural materials used in textiles that could be processed with enzymes. Enzymes have been used for desizing, scouring, polishing, washing, degumming, peroxide degradation in bleaching baths as well as for decolourisation of dye house wastewaters, bleaching of released dyestuff and inhibiting dye transfer. Furthermore, many new applications are under development such as natural and synthetic fibers modification, enzymatic dyeing and finishing. Most of the textile processes are heterogeneous where an auxiliary as a dye, enzyme, softener, or oxidant has to be taken from the solution to the fiber. These processes require the presence of surface-active agents, ionic force “balancers” buffers, stabilisers and others, and are characterized with high turbulence and mechanical agitation in the textile baths.

The present study attempts to:

1. Isolation and identification of *Bacillus stearothermophilus* from different soil samples.
2. Production, Purification and chractrization of some properties of thermoliable α-amylase.
3. producing Anti-amylae sera as polyclonal antibodies
4. Molecular cloning of α-amylase gene from *Bacillus stearothermophilus* in *E. coli* MM294 using pBR *322* vector, then investigate of the possible expression of α-amylase gene in the transformed cell(s).

2.1 *Bacillus*

Bacillus spp. as a genus, are gram positive , rodlet cells,shaped, straight or nearly so, endospores are very resistant to many adverse conditions ,formed not more than one per cell ; sporulation not repressed by exposure to environment. Aerobic or facultative anaerobic , catalase- positive ; oxidase –positive or negative ,G+C is (32-69%), singly or chains (0.5-1.2μm)–(2.5 - 10 μm) (Sneath *et al.*,1986).

The basis of classification and identification was established by Gibson and Gordon(1974), they allocate *Bacillus* spp. Into 19 species based on shape of endospores and position in the mother cells or sporangium. With the introduction of modern taxonomic techniques such as numerical phenetics ,DNA reassociation experiments have allowed DNA sequence homology between strains to estimate the range of DNA , It could be best be appreciated by numerical classification for which strains are examined for numerous physiological, biochemical and morphological characters similarities in which they were recovered in six large groups or aggregates of clusters (Nielsen *et al.*,1995).

According to the optimum temperature of growth *Bacillus* sp. could be placed in one of four classes :

1. Psychrophiles grow well at 0°C and have an optimum growth temperature of 15^{o}C or lower; the maximum is around 20^{o}C .
2. Many species can grow at 0^{o}C even though they have optimum between 20^{o}C and 30^{o}C ,and maximum at about 35^{o}C these are referred to as psychrotrophs or facultative psychrophiles .
3. Mesophiles are microorganisms with growth optima around 20-45^{o}C and a temperature maximum of 15^{o}C to 20^{o}C.

4. Thermopiles can grow at temperature of 55^{o}C or higher –their growth minimum is usually around 45^{o}C and they often have optima between 55^{o}C and 65^{o}C (Prescott *et al.*,1990)

2.2 *Bacillus stearothermophilus*

Bacillus stearothermophilus, means fat-and heat –loving, The most distinctive diagnostic characterictic is the capacity to grow at 65 ^{o}C and a limited tolerance to acid.It occurs in arctic water, ocean sediments, food and compost, mol % G+C of the mol% DNA which is reported to be(43.5-52.6) (Sneath *et al.*,1986).Thermophiles are adapted to temperature above 60 ºC in a variety of ways, often thermopiles have a high (%G + C) content in their DNA such that the melting point of the DNA (the temperature at which the strands of the double helix separate) is at least as high as the organism's maximum for growth, but this is not always the case, and the correlation is far from perfect, so thermophile DNA must be stabilized in these cells by other means. The membrane fatty of thermophilic bacteria are highly saturated allowing their membranes to remain stable and functional at high temperatures (Daron ,1970) .

Membranes of hyperthermophiles ,virtually all of which are Archaea ,are not composed of fatty acids but of repeating subunits of the C5 compound , phytane , a branched ,saturated,"isopernoid" substances ,which contributes heavily to the ability of these bacteria to live in superheated environments (Prescott *et al.*,1990).The structural proteins (e.g. ribosomal proteins, transport proteins permease) and enzymes of thermophiles and hyperthermophiles are very heat stable compared with their mesophilic counterparts .The proteins are modified in a number of ways including dehydration and through slight changes in their primary structure ,which accounts for their thermal stability (Kenneth and Todar , 2005).

2.2 Starch:

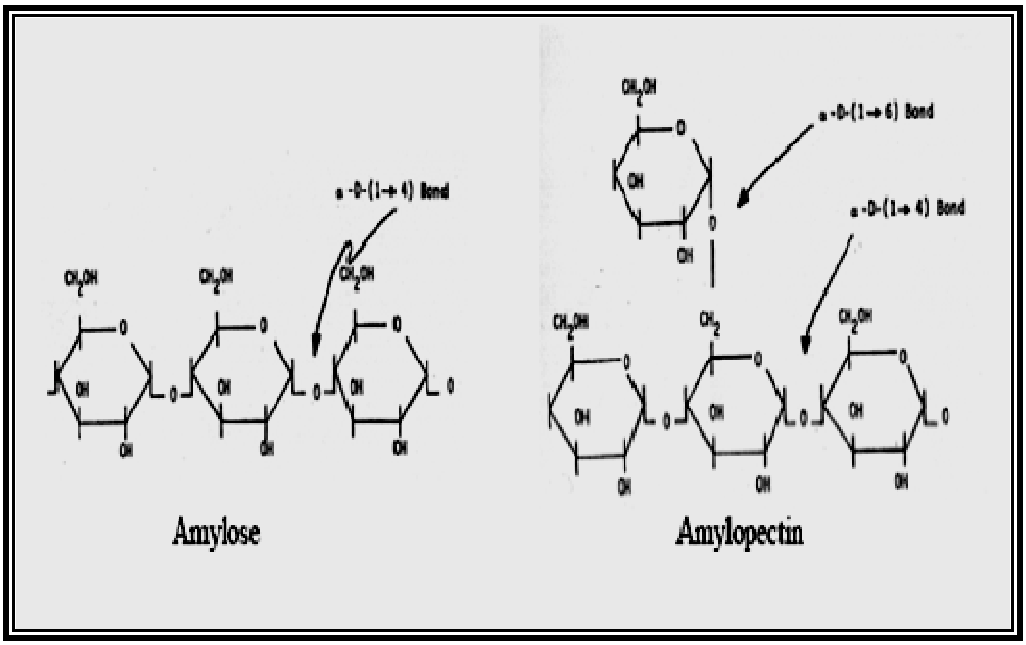
2.2.1 Molecular structure of Starch

Corn starch is found in granules within the kernel as a long polymer composed of two structural classes: amylose and amylopectin. The basic repeating unit for both types of starch is D-glucose molecules, connected by glycosidic bonds (figure 2.1). The polymer chains and the formation of the intermolecular network traps water and results in gel formation and solution thickening, after the starch is completely hydrolyzed or broken down, its basic component is D-glucose also called dextrose or corn sugar (Koivula,1996).

Amylose is a lanear polymer of short 1, 4-linked glucose chains. Typically, the amylose fraction is about 25-30% of the starch molecules found in corn and has a molecular weight of about (250 kd), The percentage of amylose in the starch is genetically determined, Genetic modifications producing high-amylose (50-70%) cornstarch are also found. amylopectin comprises about 70-75% of the starch found in the corn kernel and has a molecular weight of about 50-500 million Daltons, Amylopectin is a branched polymer of the basic repeating units of 1,4 linked glucose with branches of 1, 6 linked glucose, The branching occurs irregularly in the starch, approximately one per twenty-five glucose units (Karkallas, 1985**;** Randleman, 2000).

The corn starch separated from the kernel by the wet milling process is generally 99% pure and contains 0.25-0.35% protein, 0.5-0.6% lipid and less than 0.1% minerals. 35% of the industrially prepared cornstarch is utilized by the food industry; the remainder of the starch is further refined or modified for use in the paper and construction industries, A significant proportion of the corn starch derived from the wet-milled process used for food goes into

the fermentation of alcohol. It first has to be converted into dextrose or D-glucose(Karkallas ,1985;Bhosale *et al.*,1996).



Figure(2.1):Amylose and amylopectin of starch modified from (Koivula, 1996)

2.2.2 Starch in industry work

Enzymes of various types are used in these processes. Although starches from diverse plants may be utilised, corn is the world's most abundant source and provides most of the substrate used in the preparation of starch hydrolysates (Hazare, 2005).

There are three stages in the conversion of starch (figure 2.2) (Bhosale *et al.*, 1996)

1. Gelatinisation, involving the dissolution of the nanogram-sized starch granules to form a viscous suspension.
2. Liquefaction, involving the partial hydrolysis of the starch, with concomitant loss in viscosity.
3. Saccharification, involving the production of glucose and maltose by further hydrolysis.

Gelatinisation is achieved by heating the starch with water, and occurs necessarily and naturally when starchy foods are cooked. Gelatinised starch is readily liquefied by partial hydrolysis with enzymes or acids and saccharified by further acidic or enzymatic hydrolysis (Chaplin, 2003; Crabb and Shetty, 2003).

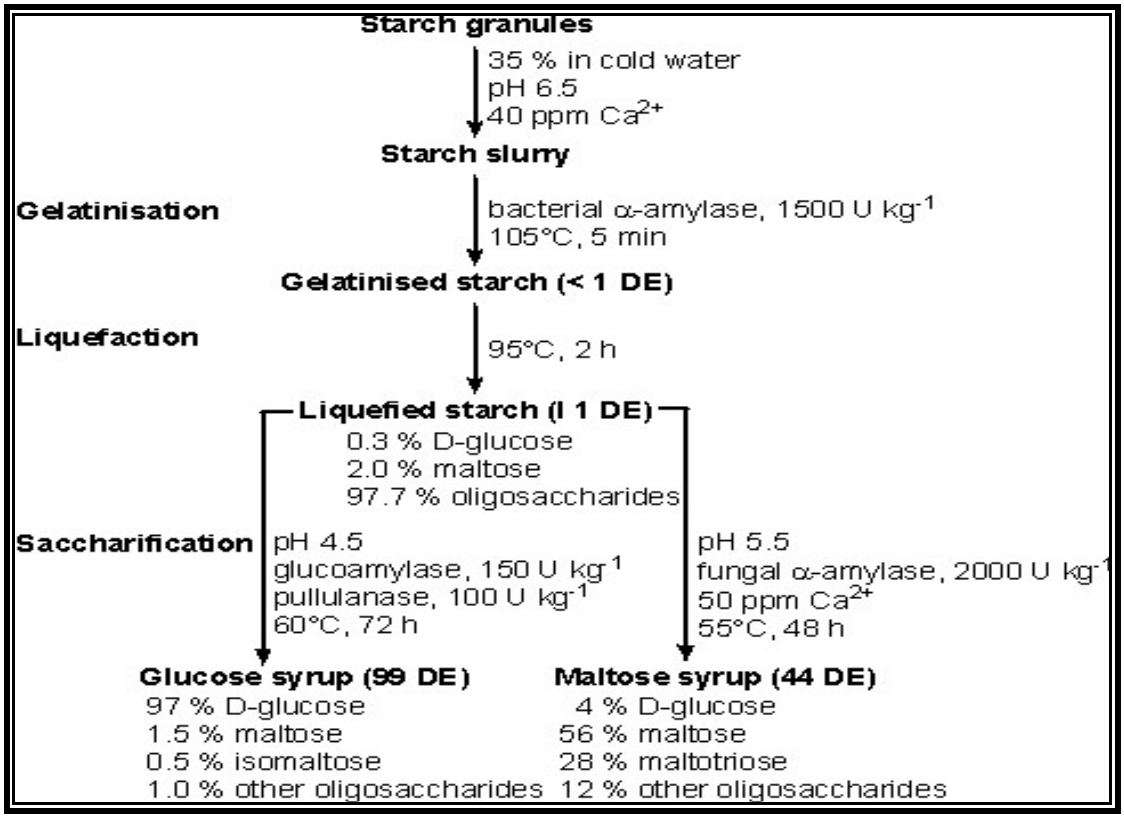


Figure (2.2) Starch hydrolysis as in the industrial applications (Chaplin , 2003).

DE, represents the percentage hydrolysis of the glycosidic linkages present

2.3 Microbial amylolytic enzymes

Starch-degrading, amylolytic enzymes are widely distributed among microbes. Several activities are required to hydrolyze starch to its glucose units. These enzymes include α-amylase, β-amylase, glucoamylase, α-glucosidase , pullulan-degrading enzymes, exo-acting enzymes yielding α-type end products, and cyclodextrin glycosyltransferase table (2.1). Properties of these enzymes vary and are somewhat linked to the

environmental circumstances of the producing organisms **(**Vihinen and Mantsala , 1989).

2.3.1 Amylases

A term that refers here to α-amylase, β-amylase and glucoamylase (Pandey *et al.,* 2000), which hydrolyze starch molecules to give diverse product including dextrin and progressively smaller polymers composed of glucose units (Reddy *et al.*, 2003).

2.3.1.1 α-amylase

With the advent of new frontiers in biotechnology, the spectrum of α-amylase application has expanded into many other fields, such as clinical, medicinal, and analytical chemistries. α- amylase has been in common use to control the viscosity of chocolate syrup since 1929 and in the brewing industry since 1936 that amylase find potential application in anywhere of industrial process such as in the food, textile and paper (Shaw *et al.*, 1995). Furthermore, α-amylases are used as targets for drug design in attempts to treat diabetes, obesity, and hyperlipidmia. The widening interest in the treatment of sugar metabolic disorders has been stimulated to search for new and efficient drugs to apply them as inhibitors of amylolytic enzymes (Kandra *et al.*, 2002).

α-amylase is a class of enzymes that is capable of digesting these glycosidic linkages found in starches, α-amylases can be derived from a variety of sources, Amylases are present in all living organisms, but the enzymes vary in activity, specificity, and requirements from species to species and even from tissue to tissue in the same organism .α-amylase (1,4α-D-Glucan-glucanohydrolase) acts upon large polymers of starch at internal bonds and cleaves them to short glucose polymers, α-amylase catalyzes the hydrolysis of internal α-1-4 glucan bonds in polysaccharides

containing 3 or more α-1-4 linkages; it results in a mixture of maltose and glucose. Amyloglucosidase works on the shorter polymers and splits off single glucose sugars. Bacterial α-amylase is particularly suited for industrial usage since it is inexpensive and is thermally stable (Karkallas, 1985).

α-amylase (EC 3.2.1.1) (IUB,1979) catalyzes the cleavage of the α-1,4-glucosidic linkages between glucose molecules in starch. This hydrolysis of starch is a common first step in conversion of starch into an utilizable substrate for fermentation or for conversion to dextrose and high fructose syrups (Pandey *et al.*,2000).In addition, α-amylases can sometimes cleave α-1,6 linkages with a low catalytic rate and they can bypass an α-D-1,6 branch linkage, (Koivula ,1996) .

α-Amylase producers of commercial importance include *Bacillus subtilis , Bacillus licheniformis, Bacillus amyloliquefaciens* and *Bacillus stearothermophilus;* a gram-positive thermophilic bacterium, produces an α-amylase during growth at temperatures of 55-70°C .This α-amylase is highly thermostable, therefore, it is more desirable for industrial applications of the enzyme(Ito and Horikoshi, 2004).

2.3.1.2 β-amylase

β-amylase (α-1.4-glucan maltohydrolase EC 3.2.1.2) is usually of plant origin ,but a few microbial strains are also known to produce it ,its an exoacting enzyme that cleaves non-reducing chains ends of amylose amylopectin and glycogen molecules. It hydrolytes alternate glycosidic linkages ,yielding maltose (β-anomeric form). β-amylase is unable to break α-1,6-glycosidic linkages in amylopectin (Hagihara *et al.*,2001;Ha *et al.*,2001).

2.3.1.3 Glucoamylase (GA)

Synonyms ;Amyloglucosidase, Glucogeninc amylase ,Starch glucogenase and γ-Amylase;exo-1,4-α-D-glucan glucanohydrolase, (EC 3.2.1.3)

hydrolase ,hydrolyze single glucose unit from the non reducing ends of amylose and amylopectin in a stepwise manner unlike α-amylase. Most glucoamylase are also able to hydrolyze the α-1,6 linkages at the branching points of amylopectin although at lower rate than 1,4- linkage; thus, maltose and limit dextrin are the end products of GA(Koviula,1996).

2.3.1.4 Pullulanase

Which is also called Neopullulanase , Amylopullulanase and Pullulanases , has important applications , particularly in the food and detergent industries. (Haseltine *et al.*, 1996).α-dextrin 6-glucanohydrolase (EC3.2.1.41) which have a primary affinity for α-1,6 linkages and catalyse the hydrolysis of α-1,6-glycosidic bonds in amylopectin and related polymers ,it is called debranching enzymes (Koivula,1996). Abe *et al*.(1994) reported that pullulnase from *Bacillus stearothernophilus* degraded the pullulan into panose, maltose, and glucose at a molar ratio of 3:1:1, with very weak activity on starchy substrates.

Table (2.1): Enzymes used in starch hydrolysis modified from (Hagihara *et al.*, 2001; Reddy *et al.*, 2003)

Enzyme	EC number	Action
α-amylase	3.2.1.1	Only α-1,4-oligosaccharide links are cleaved to give α-dextrins and predominately maltose (G2),G3,G6 and G7 oligosaccharides
β-amylase	3.2.1.2	Only α-1,4 links are cleaved ,from non-reducing ends, to give limit dextrins and β-glucose
Glucoamylase	3.2.1.3	α-1,4 and α-1,6 links cleaved ,from the non reducing ends, to give β-glucose
Pullulanase	3.2.1.41	Only α-1,6-links are cleaved to

		give panose.maltose and glucose

2.4 Structure of α-amylase

A feature common to all reported α-amylase structures is their $(\beta\alpha)_8$ catalytic core-domain, termed domain A. An excursion between barrel strand β-3 and helix α-3 forms the B domain, whilst the C domain, frequently an eight-stranded β -sheet, lies at the C-terminal extremity of the barrel. All family 13 members possess a constellation of three acidic residues, located at the C-terminal face of the $(\beta\alpha)_8$ -barrel, which are implicated in the catalysis (Brzozowski *et al.*, 2000).

α-amylases perform catalysis with net retention of anomeric configuration in a double-displacement mechanism. The mechanism involves the formation, and subsequent breakdown, of a covalent glycosyl-enzyme intermediate *via* oxocarbenium-ion like transition state. One of the catalytic acidic residues functions as the catalytic nucleophile whilst another function is the catalytic acid/base (Davies *et al.*, 1997),they possess a $(\beta/\alpha)_8$ or TIM barrel structure (which is similar to triose phosphate isomerase) containing the catalytic site residues(Reddy *et al.*,2003).While in Psychrophile *Alteromonas haloplanctis amy* gene encodes an α-amylase precursor composed of 669 amino acid residues. N- and C-terminal amino acid sequences of the native-amylase secreted by *A. haloplanctis* allow the location of three distinct functional domains of the precursor: 1- The peptide signal made of 24 residues, 2-The mature enzyme composed of 453 residues with a *M*r value of 49,340, and 3- Large C-terminal propeptide composed of 192 residues (Feller *et al* ., 1998).

The available amino acid sequences of the α-amylase family (glycosyl hydrolase family 13) have been searched to identify their domain B, A distinct domain that protrudes from the regular catalytic $(\alpha/\beta)_8$ barrel between the strand α-3 and the helix β-3.Sequence analyses and inspection of

the few available three-dimensional structures suggest that the secondary structure of domain B varies with the enzyme specificity. Domain B in these different forms. However, may still have evolved from a common ancestor. The largest number of different specificities has been found in the group with structural similarity to domain B from *Bacillus cereus* oligo-1, 6-glucosidase that contains a β-helix succeeded by a three-stranded antiparallel α-sheet (Skov *et al.*,2001).Domain B, of this type, was observed also in some mammalian proteins involved in the transport of amino acids, These proteins show remarkable similarity with $(\alpha/\beta)_8$-barrel elements throughout the entire sequence of enzymes from the oligo-1,6-glucosidase group. The transport proteins, in turn, resemble the animal 4F2 heavy-chain cell surface antigens, for which the sequences either lack domain B or contain only parts (Janecek *et al.*, 1997).

2.5 Structural and functional characteristics

α-Amylases are often divided into two categories according to the degree of hydrolysis of the substrate (Fukumoto and Okada, 1963), Saccharifying α-amylases hydrolysis 50 to 60 % , and liquefying α-amylases about 30 to 40 % of the glycosidic linkages of starch. However, this division is not an absolute one. Some bacteria produce an extracellular enzyme cyclodextrin glycosyltransferase (CGTase) (1,4-α-D-glucan4-α-D-(1,4-α-D-glucano) transferase , EC (2.4.1.19), which hydrolysis α-1,4-glycosidic bonds of starch to produce cyclodextrins (cyclic compounds of six to eight a-1,4-linked glucose units) via an intramolecular transglycosylation reaction. In addition, CGTases can display coupling, disproportionation and hydrolysing activities, and are functionally related to α-amylases(Macgregor and Svensson , 1989).

The primary structures of CGTases and α-amylases from different organisms share about 30 % amino acid sequence identity and all belong to the same

glycosyl hydrolase family 13 (Henrissat and Bairoch, 1993). Other related amylolytic enzyme in family 13 is pullulanases (Cheong *et al.*, 2002).

The 3D structures of α-amylases have revealed monomeric, calcium-containing enzymes, with a single polypeptide chain folded into three domains (A-C) figure(2.2). While, the polypeptide chains of CGTases fold into five domains (A-E), three of these correspond to α-amylase domains and domain E has been recognized as having a similar fold as the starch-binding domain of glucoAmylases). Domain D of CGTases has an immunoglobulin-type fold, the function of which is not yet known (Koivula ,1996).

Dauter *et al.(*1999)described maltogenic α-amylase of *B. stearothermophilus* which is composed of five domains that is similar to the CGTases structure using X-ray technique, figure (2.3).The catalytic domain A of α-amylases and all family 13 hydrolases folds into an $(a/b)_8$ barrel protein. The active site is created by residues located at the C-terminus of the b strands and in the helix-connecting loops extending from these ends. This is typical to all enzymes belonging to the a/b -barrel protein family .The domain B protrudes from the middle of the barrel at the C-terminal side and forms a small separate, Ca^{2+} -stabilized structural domain, which has also been recognized in a number of other amylolytic enzymes (Jespersen *et al.*, 1991; Aghajari *et al.*,2002).The sequence of this domain varies most; in *Bacillus* α- amylases, the sequence is relatively long and folds into a more complex structure of b-strands (Machius *et al.*, 1995), whereas in barley α-amylase it is an irregularly structured domain of 64 residues (Kadziola *et al.*, 1994).

The open active site cleft is formed between domains A and B, so that residues from domain B also participate in substrate binding. Based on the solved 3D structures it could be deduced that the conserved regions of α-amylases and CGTases involve the calcium-binding site between domains A

and B, and regions belonging to the active site (Macgregor and Svensson, 1989; Klein and Schultz, 1991; Lawson *et al.*, 1994).

α-Amylases have a domain C which is relatively conserved and folds into an antiparallel β-barrel, The orientation of domain C relative to domain A varies depending on the type and source of Amylase (Brayer *et al.*, 1995). The function of this domain is not known (koivula, 1996).

A study about *B. circulans* CGTase (Lawson *et al.*, 1994) and another from pig pancreatic α-amylase (Qian *et al.*, 1994), both clearly showed based on electron densities, that maltose units stack against the aromatic side-chain of a tryptophan situated on the surface of domain C (Fig. 2.2,2.3). The truncation of domain C more than 30 to 40 amino acids from the C-terminus was shown to be deleterious to the proper folding of the *B. stearothermophilus* α-amylases (Vihinen *et al.*, 1994).

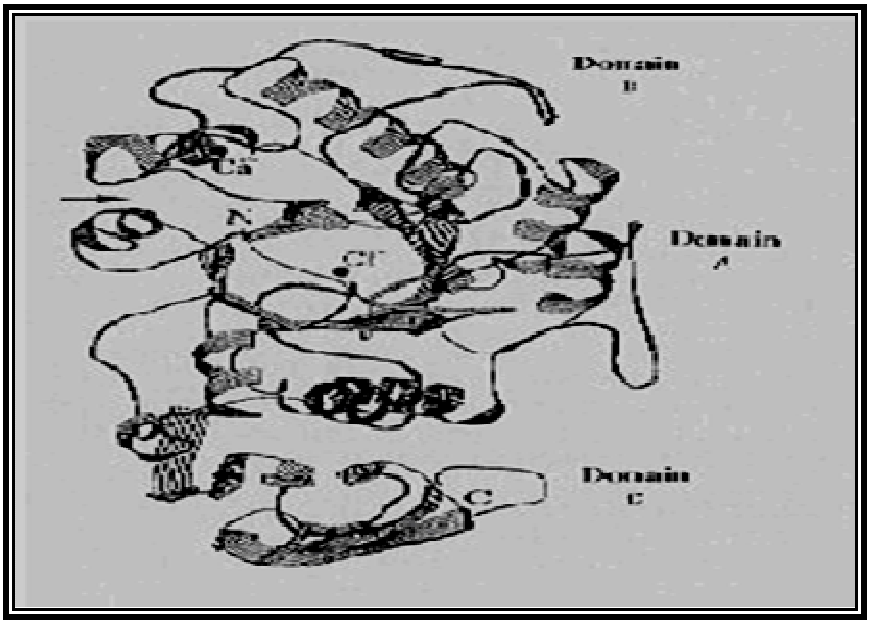


Figure (2.2): Ribbon presentation of human pancreatic α-amylase is composed of three domains (A-C) (Koivula, 1996)

While Aghajari *et al.(*2002) described that α-amylase produced by animals and *Pseudoalteromonas haloplanktis* activated by chloride .

The thermostability and structural integrity of the α-amylase due to the presence of three Ca^{+2} (Srivastava and Baruah,1986) and one Na^{+} ions has been found in *Bacillus licheniformis* , a lanear triad CaI- Na-CaII arrangement figure (2.4) ,is located at the interface between domain A and B. another Ca^{2+} (CaIII) bridges between domains A and C. (Ito and Horikoshi, 2004).In addition, the prolane residues are to be the backbone rotation proposed a prolane of β- turn make α-amylase more stability (Matthews *et al.*, 1987).

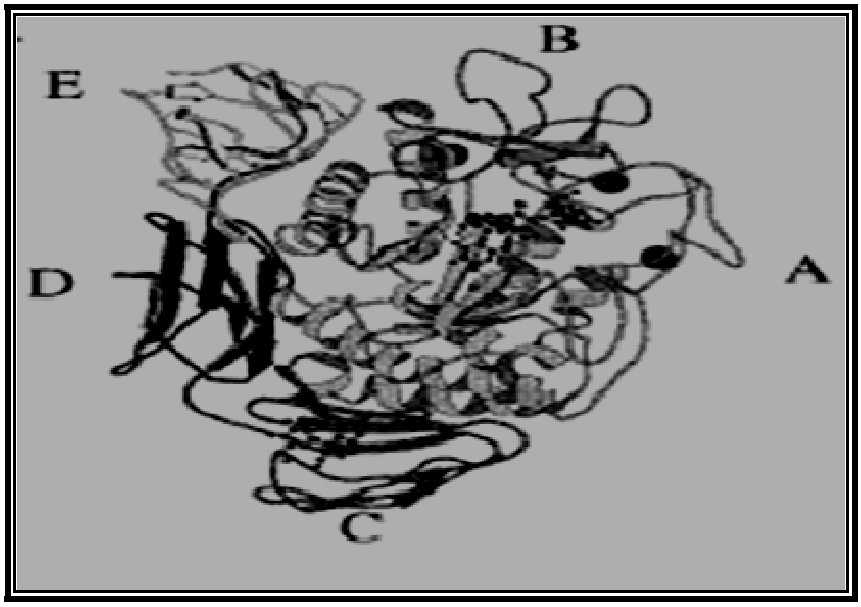


Figure (2.3):Divergent stereo schematic diagram of the maltogenic α-amylase , novamyl, from *B. stearothermophilus* (Dauter *et al.*,1999)

All α-amylases bind at least one strongly conserved Ca^{2+} ion that is required for structural integrity and for enzymatic activity (Aghajari *et al.*, 2002).Feller *et al.(*1996) showed that the chloride free α-amylase display a low level of basal activity , typically < 0.5 % , and chloride follows a simple binding isotherm . Activation is also provided, but to a lower level, by other small monovalent anions and notably by NO_3^- or ClO_3^- indicated that the negative charge is essential for the amylolytic reaction (Aghajari *et al.*, 2002).

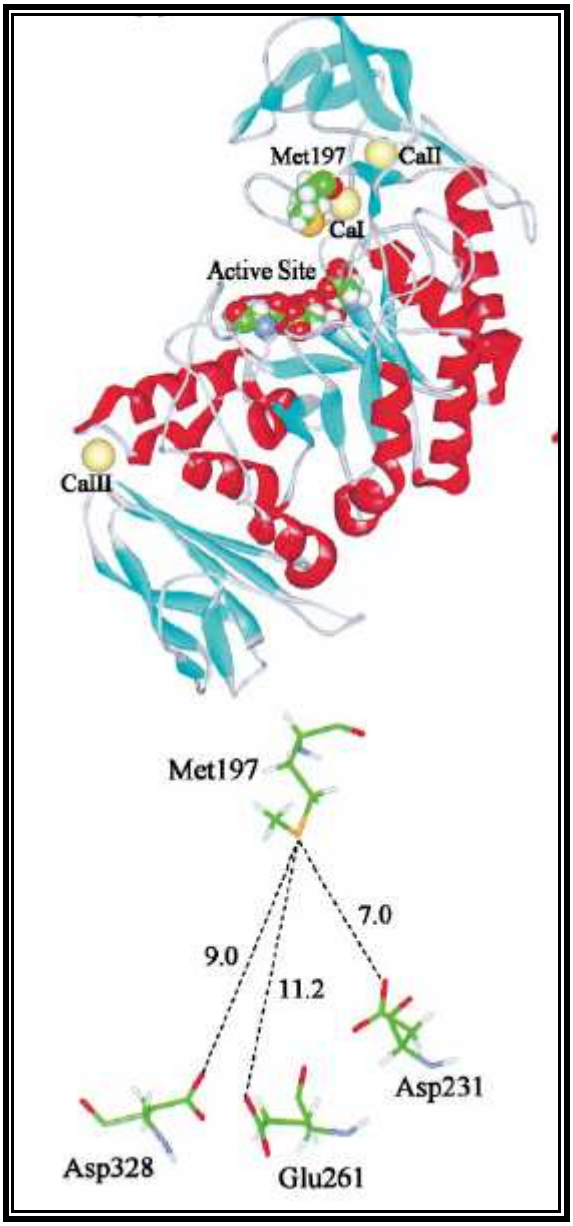


Figure (2.4): Ribbon representation of the crystal structure of *Bacillus licheniformis* template active site . α helices and β sheets are shown in red and cyan , respectively. The catalytic residues Asp231, Glu261, and Asp328 along with the subsite Met197. Yellow spheres represent Ca ions (I, II, III). oxygen atoms in red, nitrogen atoms in purple, carbon atoms in green, and hydrogen atoms in white (Hagihara *et al.*,2001)

2.5 Breaking down of *O*-glycosidic bond

Amylases, like all glycosidases, catalyze the transfer of a glycosyl group to water utilizing general acid catalysis. The enzymatic breakdown of a glycosidic bond is also a stereo- selective process, in which configuration about the anomeric centre (C1 carbon) can either be inverted or retained. Different mechanisms are required for such different stereochemical outcomes, as suggested over 50 years ago by Koshland (1953); both mechanisms contain a pair of carboxylic acid residues suitably disposed on either side of the bond to be cleaved. The roles of these two residues are different. Inverting enzymes use a single-displacement mechanism involving a general acid and a base. In this mechanism, a general acid (AH) donates a proton to the leaving glycosidic oxygen and a general base (B-) assists the nucleophilic attack of water.

O-Glycosylhydrolases (EC 3.2.1.) are a widespread group of enzymes that hydrolyze the glycosidic bond α-amylase family in that of the α- retaining double displacement (van der Maarel *et al.*, 2002)**.** α-retaining mechanism is the characteristic feature of the enzymes from the α-amylase family. They vary widely in their reaction specificities. The attachments of different domains to the catalytic site or to extra sugar binding subsites around the catalytic site are the prime reason for these differences (Van der Maarel *et al.*, 2002).

The catalytic domain A is the most conserved domain in the α-amylase family, It consists of an amino terminal $(\beta/\alpha)_8$ barrel structure. (Matsuura *et al.*, 1984; Boel *et al.*, 1990).The mechanism for retaining glycosidases involves a double displacement reaction in which a covalent glycosyl-enzyme intermediate has been shown to be formed with many but not all retaining enzymes (Skory and Freer, 1995), As a first step the deprotonated carboxylate acting as a nucleophile attacks at the anomeric centre and displaces the glycosidic oxygen. This process is assisted by a general acid

catalytic residue (AH) which is deprotonated (A-), In the second step water attacks at the anomeric centre with general base-catalytic assistance (A-) displacing the nucleophile and releasing the product sugar (Koshland, 1953; Sinnott, 1990; Withers and Aebersold, 1995).

The stereochemical course of the hydrolysis is usually determined by proton NMR, in which the A- and B-anomeric protons give different chemical shifts (Withers *et al.,* 1986).In most cases that studied the same enzymatic mechanism, (inverting or retaining) has been conserved within a glycosyl hydrolase family (Gebler *et al.,* 1992). Thus, both the fold of the protein and the stereochemistry of the hydrolysis reaction appear to be conserved within a given family.

Retaining enzymes are also able to bring about transglycosylation which, in the second step of the reaction another oligosaccharide instead of a water molecule, attacks at the anomeric centre, leading to elongation of the saccharide chain , figure (2-5) (Sinnott, 1990).

Transglycosylation was described to play a role in the induction of cellulase promoters (Biely *et al.,* 1991; Fowler *et al.*, 1993; Nevalainen and Penttila, 1995) and it could be applied in the enzymatic synthesis of oligosaccharides (Ogawa *et al.,* 1990; Gusakov *et al.,* 1991).

Inverting amylases such as glucoamylases have been shown to be able to reverse the hydrolysis by condensation reaction (Nikolov *et al.,* 1989;Sinnott, 1990). This ability has also been used in the synthesis of some gluco-oligosaccharides (Nakano *et al.,* 1995) .With some retaining enzymes, most studied with lysozyme, attempts to prove the existence of a covalent glycosyl-enzyme intermediate have failed (Sinnott, 1990). Another type of mechanism has been suggested in which the positively charged oxocarbonium is stabilized by the catalytic nucleophile through electrostatic interactions (Sinnott, 1990; Koivula, 1999).

In addition, ring distortion at the subsite preceding the scissile bond was suggested as an element of the catalytic machinery for both inverting and retaining glycosyl hydrolases (Harris *et al.,* 1993; Strynadka and James, 1991; Kuroki *et al.,* 1993; Varghese *et al.,* 1992). The identification of active site carboxylic acid residues usually occurs through X-ray crystallographic determination of the 3D structure of protein (Dauter *et al.,* 1999). In the case of retaining enzymes, the active site carboxylic acids have been identified by the use of affinity labels or mechanism-based inactivators to specifically derivatives the key amino acids. 2- Deoxy-2-fluoro-derivatives of glucose and cellobiose have successfully been used as mechanism-based inhibitors, which covalently bind the nucleophile in the retaining enzymes (McCarter and Withers, 1994; Withers and Aebersold, 1995).

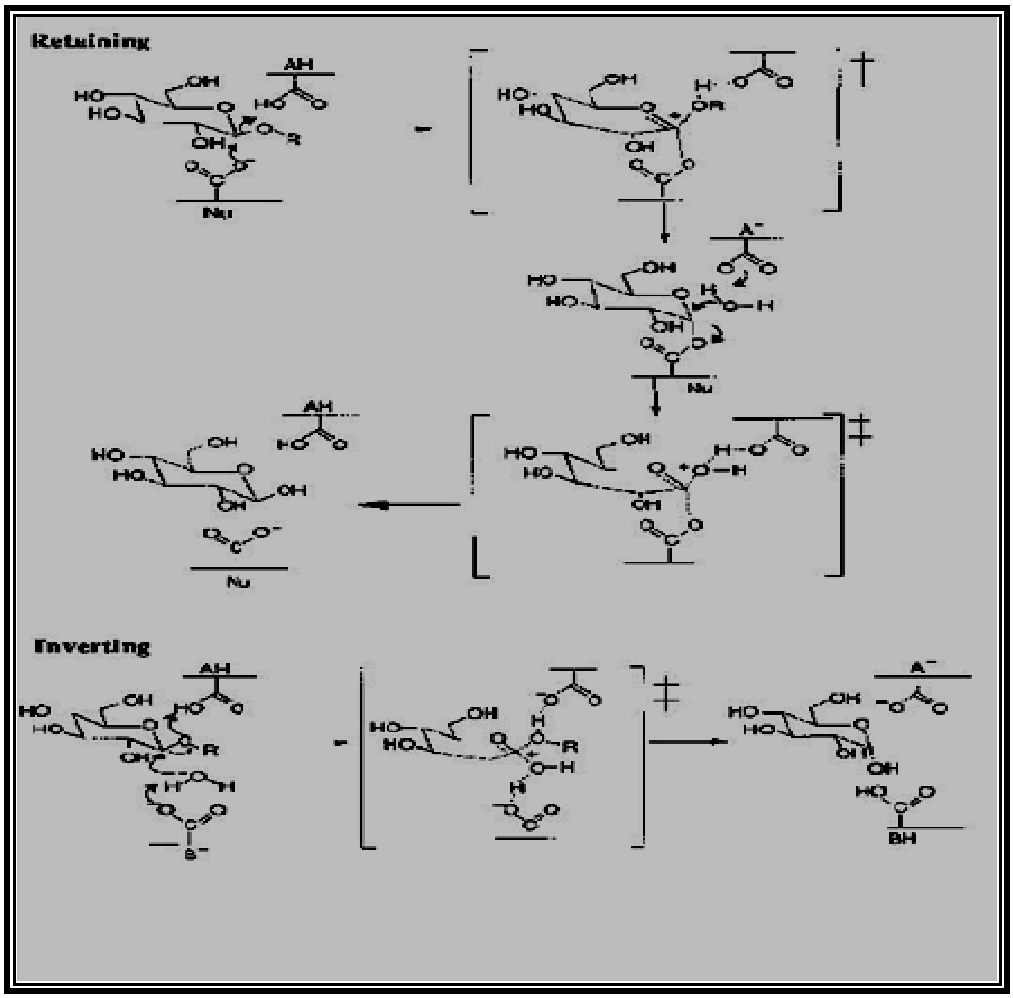


Figure (2.5) Two different reaction mechanisms of glycosyl hydrolases (Koivula ,1996).

2.6 α-Amylase isoenzymes (Forms)

Electrophoretic analyses of barley endosperm at different stages after germination allowed the detection of 16 principal amylase bands plus one secondary band(Maris,1992).Amylases showed less polymorphism than other enzymes such as esterases, acid phosphatases, malate dehydrogenases, leucine aminopeptidases (George and Cavali,2003).

Amylases are hydrolases involved in peripheral metabolism, and for this reason are considered potentially polymorphic enzymes (Gillespie and Langley, 1974). *Bacillus* sp. showed two and three isoenzymes of α-amylase (Lin *et al.,* 1998).Brazilian barley cultivars showe different isoenzymes ,the major differences in Amylase patterns found when barley varieties were analyzed at the same developmental stage were at the regulatory level. These differences are probably due to differential gene activation in the different genotypes (George, 1996).

The reasons behind the existence of more than one form could be the purification procedures, protease hydrolysis and deamination (Shih and Labbe,1995). The regulatory difference is of a greater adaptive and evolutionary significance than structural gene changes (Soule ,1973 ;Wilson, 1976) emphasized that the process involved in species differentiation requires changes mainly at the gene regulatory level. Differently, at the intraspecific level, gene regulation follows a standard development program with precisely coordinated sequences, although some regulatory mutants have been detected in different species (Holmes *et al.,* 1983; Doane *et al.,* 1983; Scandalios, 1983).

2.7 Methods of α-amylase quantitative estimations

Several methods were developed to detect of the amylolytic activity and to estimate the amylase activity precisely:

2.7.1 Viscometric method (Maslow and Davison)

It is based on the change in the viscosity of starch due to the amylases activity.

2.7.2 Polariscopic method (Maslow and Davison)

1. It is based on determining the reduction in light rotation to the right due to maltose and the maltosaccharides by the activity of amylases on starch.

2.7.3 Caldwell – Hildebrand method

This method is based on the direct determination of the residual starch or amylose at any stage in its hydrolysis, and depends upon its quantitative precipitation, free from dextrin and maltose, by ethyl alcohol. The difference between the weight of original amylose and the weight of residual amylose gives a measure of the amyloclastic activity of the enzyme analogous to the measurements of its saccharogenic activity which are based upon the determination of reducing the sugar formed.

2.7.4 Photometric method

This method depends on the difference in the intensity of the color produced with iodine by a measured amount of soluble starch before and after hydrolysis by the α-amylase.

2.7.6 Somogy-Nelson Colorimetric method

A photometric method, in which one unit of α-amylase activity is defined as the amount of enzyme that librates one µmol of reducing sugar equivalent per minute, using the glucose as a standard under the specified conditions (Abe *et al*., 1994)

2.7.5 Fuwa`s colorimetric method (Fuwa, 1954)

It depends on iodine –starch color reaction. One unit of α-amylase activity is defined as the amount of enzyme, which decreases the absorbance at 660 nm, by 1.0 in 10 min.

2.7.7 Bernfield`s method (Bernfield, 1955)

A colorimetric method depends on using 3,5 dinitrosalicylic acid. One unit of amylase activity was defined as the amount of enzyme that librates one µmol of reducing sugar, with maltose as the standard, per minute under the specified conditions (Aiyer, 2004).

2.8 Engineering of α-amylase to improve the stability.

Since a thermostable α-amylase originating from *Bacillus licheniformis* was first employed in the starch liquefaction process in the early 70's ,α-amylases have been introduced into various industries.

The starch liquefaction process is still the main application of α- amylases, while the application to detergent (additive to detergent formulations) has been growing very much both for laundering and dish washing (Ito and Horikoshi, 2004). Protein engineering techniques have been employed for α-amylase development, which are to improve the characteristics of a targeted enzyme by introducing mutations in α-amylase encoding DNA sequence. The methods for protein engineering could be roughly categorized into two: site-directed mutagenesis and random mutagenesis (Miyoko and Henrik, 2000).

Site-directed mutagenesis is to introduce additions, deletions and substitutions of specific amino acids, and structural information of the targeted enzyme is essential to make those alternations (Bhosale *et al.*, 1996; Miyoko and Henrik,2000).

Computer technology is done to make molecular dynamics calculations and predictions for the structure when certain amino acids are substituted. (Laderman *et al.*, 1993; Hagihara *et al.*,2001).

Random mutagenesis is to introduce mutations at random along the entire length of a gene using error-prone PCR, DNA shuffling , chemical mutagenesis and UV irradiation. This method could develop new generation of α-amylases as a detergent (Yoneda and Maruo, 1975).Miyoko and Henrik (2000), α-amylase could obtain improved a detergent alkalane stable and calcium independent by site-directed mutagenesis after microbial screening. Engineering of the available commercial α-amylases have been advocated hybrids of two homologous strains of the *B. licheniformis* and *B. amyloliquefaciens* (Suzuki *et al.*, 1989) and two regions that are important for thermostability has been identified. To improve the stability, disulphide bonds are introduced in the enzyme and alteration of the amino acids prone to the oxidation by an amino acid that is unaffected by oxidative agents (Barnett *et al.,* 1998;Agihara *et al.*2001).

Engineering α-amylase for changed pH of activity profiles, also would be in favor of the stability of the enzymes (Nielsen and Borchert, 2000;van der Maarel *et al*. ,2000 ; Gupta *et al*. ,2003) . Declereck *et al.*(2002) engineered a thermostable α-amylase from *B. licheniformis* using a structure based on mutagenesis, which could be used for an operation occurring over 50°C without perturbing significantly .

2.9 Regulation of α-amylase synthesis

It has been reported that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subject to catabolic repression by readily metabolically substrates such as glucose (Lin *et al.*,1998).

The addition of glucose to the culture has diminished greatly the synthesis of α-amylase similar to the findings reported by Haseltine *et al.(* 1996), who observed that glucose represses the production of α-amylase in the hyperthermophilic archaeon *Sulfolobus solfataricus*. According to them, glucose prevents α-amylase gene expression and not merely secretion of preformed enzyme, Levels of the α-amylase in crude culture supernatants vary greatly in response to the sole carbon source used for growth of the organism(Srivastava and Baruah,1986). Substrate induction of this enzyme by starch emanated relatively early under all growth conditions tested, glucose has also been observed, which indicated that such repression of further enzyme synthesis might be due to the accumulation of starch hydrolytic products, including glucose (Saito and Yamamoto,1975).

The maltase is likely to play an important metabolic role in the accumulation of repressing levels of glucose, as the maltase exhibits considerable activity against dextrins which are the immediate products of α-amylase action repression of α-amylase synthesis by glucose represents one type of catabolic control over this enzyme and the second type which is the graded production of catabolic enzyme levels resulting from growth on other control is independent of the presence of starch,these two forms of regulation of α-amylase production may represent a generalized response to carbon source quality or availability (Rolfsmeier and Blum 1995; Haseltine *et al.*, 1996) .

α-amylase of *Streptomyces lividans* is regulated by *reg1*, a Pleiotropic regulatory gene *reg1* acts as a repressor of α-amylase genes in *S. lividans*. no other regulatory gene (repressor or activator) seems to control the expression of the α-amylase genes(Nguyen *et al.*, 1997;Van wezel *et al.*,1997) , since the α-amylases is at a maximum level in a minimal medium and is not further inducible by maltose when *reg1* is disrupted.*reg1* seems to be solely responsible for glucose catabolite repression of the α-amylase genes, which

is completely lost upon *reg1* disruption. The dual role of *reg1* resembles that of *gylR*, the repressor of the *gyl* operon in *S. coelicolor* which is also involved in catabolite repression of this operon (Hindle and Smith, 1994). However, the relative locations of the two regulatory genes are different, as *gylR* is adjacent to the *gyl* operon under its control in *S. coelicolor* whereas *reg1* is not adjacent to α-amylase genes in *S. lividans* . By analogy with *malR* (Van Wezel *et al.*, 1997) it is likely that *reg1* is adjacent to the *malE* homolog of *S. lividans* and therefore also ensures a dual control of the expression of this gene in *S. lividans*. (Nguyen *et al.*, 1997).

2.10 Secretion and Transport of α-amylase

Amylase secretion is mostly started at the logarithmic growth phase, in which extracellular secretion starts in the stationary growth phase parallel to degradation of cell wall components in the course of increased autolytic activity (Saito and Yamamoto,1975).

Permeability studies applying the (SUMs), S-layer ultrafiltarion membranes technique, and using culture supernatants for ultra filtration experiments showed that the S-layer lattice completely rejected the enzymes once they had assumed the final three dimensional structure, for *B. stearothermophilus* ,the passage of three Amylase through the pores in the S-layer lattice is only possible for enzyme molecules perfolded into smaller domains. After passing through the cell wall, complete folding of enzymes into their final three-dimensional structure will occur either on the cell surface or after the release into the culture fluid, it is unknown how the amylase is associated with S-layer protein. Generally, three possibilities exist 1- adhesion to S-layer surface 2- incorprtion of amylase into the S-layer lattice 3- binding of the enzyme to S-layer protein domain (Egelseer *et al.*,1995) .

Transport of protein by the major secretary pathways cross the bacterial wall in wall is a two-step mechanism via the periplasm ,Exporting, taking this two-step route, possesses an N-terminal signal peptide and use the general sec machinery for inner membrane translocation. Transport across the outer membrane in the second step requires a secretory apparatus encoded by several exproteins, when the *B. licheniformis* α-amylase, AmyL, is secreted from *B. subtilis*, it is subjected to considerable cell-associated proteolytic degradation (Stephenson, 1996).This proteolysis results in only a proportion of the newly synthesized α-amylase being released into the culture medium. AmyL, an extracellular α-amylase from *Bacillus licheniformis*, is resistant to extracellular proteases secreted by *Bacillus subtilis* during growth. Nevertheless, when AmyL is produced and secreted by *B. subtilis*, it is subject to considerable cell-associated proteolysis. (Stephenson and Harwood, 1998).

In the gram-positive bacterium as *B. subtilis*, the only one protein outside the cytoplasmic membrane, PrsA, is known to be involved in protein secretion. PrsA is a lipoprotein that consists of a 33-kDa lysine-rich protein part and the N-terminal cysteine with a thiol-linked diacylglycerol anchoring the protein to the outer leaflet of the cytoplasmic membrane (Kontinen, *et al.,* 1991; Kontinen and Sarvas**,** 1993).

The PrsA protein is crucial for the efficient secretion of a number of exoproteins. In *prsA* mutants, the secretion and stability of some model proteins is decreased, while overproduction of PrsA enhances the secretion of exoproteins engineered to be expressed at a high level (Kontinen *et al.,* 1991).

Although the nature of the PrsA protein hints at an activity outside the cytoplasmic membrane, its mode of action and interaction with other components of the secretion apparatus and the specific steps of secretion in which it is involved remains to be elucidated.(Vitikainen *et al.,* 2001)

2.11 Amylase gene cloning

Genetic engineering has been used extensively for the cloning of amylase gene of producing strains, mainly *a*-amylase and GA, in order to achieve desirable characteristics in the cloned host. The purpose of gene cloning can be, amongst others, the expression of thermostable enzymes, higher enzyme productivity, and co-expression of two enzymes by the same organism.

1.8 KB fragment containing the amylase gene is sequenced(figure 2.6) .The fragment of the open reading frame (ORF) which begins with an ATG codon at nucleotide 1 and ends with TAA codon at nucleotide 1545 in the 1786 bp nucleotide sequence is determined in *Bacillus subtilis* (figure2.7) (Igarashi *et al.*,1998) .Upstream from this ORF ,the putative ribosome-binding sequence AGGAGA is found(Igarashi *et al.,* 1998,Hagihara *et al.,* 2001).The α-amylase gene in *Pyrococcus furiosus* encompasses 1950 nucleotides, with the initiation codon GTG at position 715 (Laderman *et al.*, 1993). Many genes involved in the synthesis of extracellular α-amylase have been identified in *B. subtilis*. The structural gene for the α-amylase enzyme, amyE, and its regulatory gene, (amyR), are closely linked on the genetic map of the *B. subtilis* chromosome. Furthermore, the gene order surrounding this α-amylase cistron is similar for three distinct strains of *B. subtilis*. The location of the gene for α-amylase from *B. stearothermophilus* has been examined by using recombinant DNA methods. In contrast to the gene for α-amylase from *B. subtilis*, the gene for α-amylase in *B. stearothermophilus* has been reported in one study that is located on a naturally occurring plasmid (Mielenz , 1983).

Igarashi *et al.*(1998) cloned 1.8 Kb fragment carrying the entire gene of α-amylase into *E. coli* using pUC19 as vector , which was actually expressed . The gene encoding β-amylase 1.8 kb was cloned from *Bacillus polymyxa* 72

into *E. coli* HB101 by inserting *Hin*d Ill generated DNA fragments into the *Hin*d III site of pBR*322* (Kawazu *et al.*,1987).

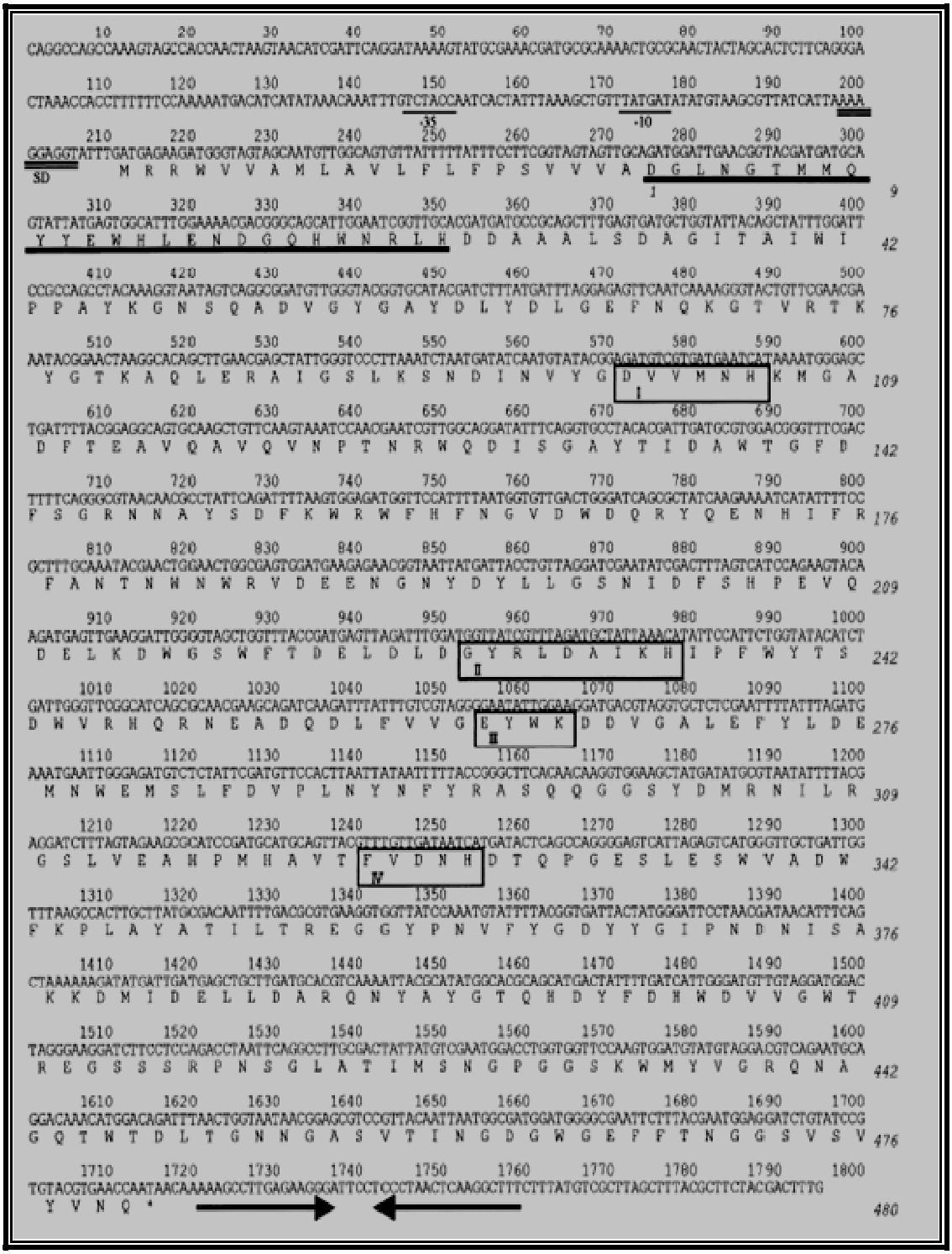


Figure (2.6): The sequence of α-amylase gene in *Bacillus subtilis*(Igarashi *et al.*,1998)

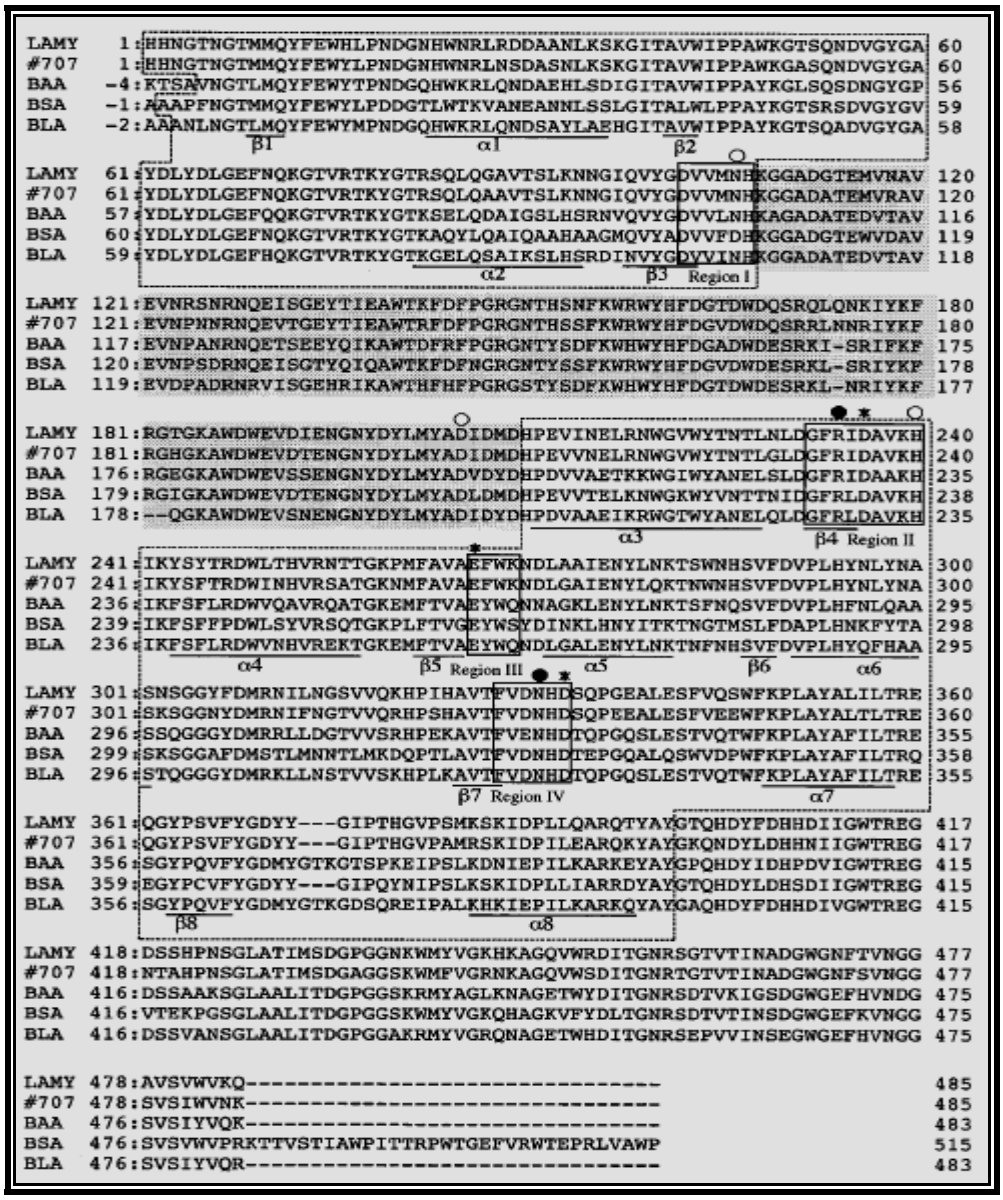


Figure (2.7) :Amino acid sequence alignment of α-amylase in *Bacillus amyloliquefaciens* (BAA), *Bacillus stearothermophilus* (BSA), and *Bacillus licheniformis* (BLA), Novel liquifiying α-amylase (AMYL) (Igarashi *et al.*,,1998)

The α-amylase genes of *Streptococcus bovis* 148 were cloned in *E. coli* MC1061, using pBR*322* in which it was cleaved by *Bam*H1 and Among about 50000 ampicillin resistant transformants, 11 colonies showed clear halos detected with I_2 (Satoh *et al.*,1993).(Sidhu *et al.*, 1997) could clone in *E. coli* pBR322 systems and the 4.8 kb cloned fragment was mapped with restriction enzymes. Mielenz,(1983) reported cloning of α-amylase gene

fragment carried on plasmid of *B. stearothermophilus* using pBR*322* vector cleaved with *Hin*d III, expressed *E. coli* , the fragment was 5.4 Kb that result in chimeric plasmid of 9.7kb length .

α-amylase gene from *B. stearothermophilus* was cloned in *B. stearothermophilus* and *B. subtilis* using pATB90 and pATB53, they expressed the trait (Aiba *et al.*,1983). A gene encoding the periplasmic α-amylase of *Xanthomonas campestris* K-11151 was cloned into *E. coli* using pUC19 as a vector (Abe *et al.*,1996).Thermostable α-amylase of *Bacillus* sp. was cloned in *E. coli*, the fragment length was 3 kb (Ali *et al.*, 1999).The genomic DNA of *B. licheniformis* was double-digested with *Eco*RI and *Bam*HI and ligated the pBR*322* in which the gene fragment was 3.5 kb (Iefuji *et al.*, 1996) . The detection methods for cloned fragments were different ,in which they depended on the formation of halo zones around the transformed cells , antibiotic resistance and sensitivity or both(Satoh *et al.*, 1993; Aiba *et al.*,1983).

A great deal of work has been done on the cloning of α-amylase genes in different microbes, mostly in *E. coli* or *Saccharomyces cerevisiae*.

Liebl *et al.*, (1997) described the gene structure of the *a*-amylase from *Thermotogamaritima* MSB8in which it was located on chromosome , *a*-amylase gene, designated *amyA*, and was predicted to code for a 553-amino acid preprotein with significant amino acid sequence. The *T. maritima a*-amylase appeared to be the first known example of a lipoprotein *a*-amylase. Following the signal peptide, a 25- residue putative linker sequence rich in serine and threonine residues was found.

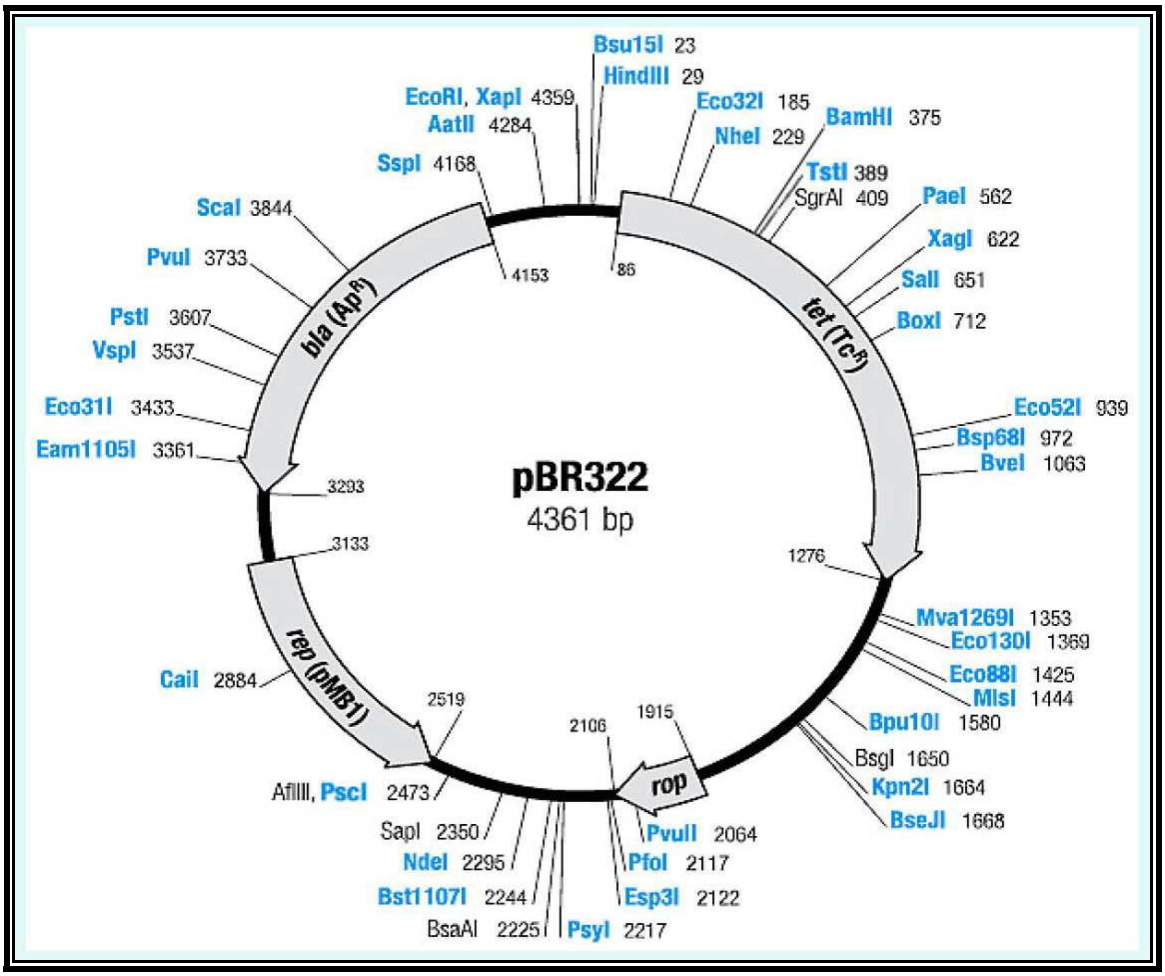
Tsao *et al.*(1993) could clone the α-amylase gene from *Streptomyces lividans* into *E .coli*. The amylase gene was expressed in *E. coli*. Suganuma *et al.*, (1996) studied the N-terminal sequence of the amino acids of the *a*-amylase from *Aspergillus usanii*. The sequence of the first 20 amino acids was identical to the *a*-Amylase from *A. niger*. Marco *et al.*, (1996) inserted *B.*

subtilis α-amylase gene into a plasmid, which was transferred to *E. coli*. And he could replace 171 C-terminal with new 33 amino acid in order to achieve extensive activity .In animals, α-amylase genes have a complex structure with an interrupted coding sequence, in the mollusk *Crassostrea gigas,* the two α-amylase genes are characterized by the same organization, with the existence of eight exons separated by seven introns (Sellos and Wormhoudt , 2002).

2.12 pBR *322* cloning vector

The plasmid pBR322 is one of the most commonly used *E.coli* cloning vectors,pBR*322* is 4361 bp in length and contains: 1- the replicon *rep* responsible for the replication of plasmid (source - plasmid pMB1); 2- *rop* gene coding for the Rop protein, which promotes conversion of the unstable RNA I - RNA II complex to a stable complex and serves to decrease the copy number (source - plasmid pMB1); 3- *bla* gene, coding for beta-lactamase that confers resistance to ampicillin (source - transposon Tn3); 4- *tet* gene, encoding tetracyclane resistance protein (source – plasmid pSC101) figure(2.8) (Rodriguez and Tait,1983).

The circular sequence is numbered such that 1 is the first T of the unique *Eco*RI site GAATTC and the count increases first through the *tet* gene, the pMB1 material, and finally through the Tn3 region. The map shows enzymes that cut pBR322 DNA once,The exact position of genetic elements is shown on the map (termination codons included),the *bla* gene nucleotides 4153-4085 complementary strand) code for a signal peptide. The indicated *rep* region is sufficient to promote replication. DNA replication initiates at position 2533 (+/- 1) and proceeds in the direction indicated. (Hardy,1988;Fermentas, 2006)



Figure(2.8):Description and restriction map of pBR 322 (Fermentas , 2006)

2.13 Optimal conditions for α-amylase production

2.13.1 Culture conditions

α-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 (forgaty and Kelly,1980; Hewitt and Solomons, 1996; Hillier *et al*., 1996).

In production of α-amylase from *Bacillus* , the addition of yeast extract (0.5%) and peptone (1%) 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 favored for the

growth and synthesis of amylase by the organism studied (Saito and Yamatomo,1975; Krishnan and Chandra ,1983).

The starch is very important to stimulate the production of α-amylase in *B. stearothermophilus* (Aiba *et al.*,1983). While, Chakraborty *et al.(*1997) reported that the best source for α-amylase production by *B. stearothermophilus* was starch 1.25%. Teodero and Martins (2000) used 1% soluble starch in production media for α-amylase by *Bacillus* sp.

Srivastava and Baruah (1986) described the culture conditions for the production of the enzyme by *B. stearothermophilus,* in which they mentioned peptone, starch had enhanced the production of the enzyme , while the synthesis was suppressed greatly by glucose , maltose and sucrose .

Saito and Yamamoto (1975) reported that the glucose repressed the production of α-amylase ,while the starch , glycogen and oligosaccharides induced the formation of it at the end of logarithmic phase .

Concerning the inorganic nitrogenous compound, diamonium hydrogen phosphate and ammonium dihyrogen phosphate were found to be better than Ammonium sulphate in synthesis of enzyme (Srivastava and Baruah, 1986; Aiyer,2004). While, Chakraborty *et al.(*1997) reported that the best source for α-amylase production by *B. stearothermophilus* was ammonium sulphate .The addition of 10 -20 mM calcium to the liquid medium improved the growth and amylase production,since the enzyme is known to be a calcium metalloenzyme, it is possible that the results found were because of the more availability of calcium ion (Srivastava and Baruah, 1986).These results are similar to the findings of (Hewitt and Solomons, 1996) with cultures of *Bacillus amyloliquefaciens*.

2.13.2 Optimal growth temperature

The temperature is one of the important factors that control the production of α-amylase. Srivastava and Baruah (1986) noticed that the

optimal growth temperature for α-amylase production by *B. stearothermophilus* was 50°C, while the optimal growth temperature for that one used in the study of Welker and Campbell(1963) was 55-70°C.while Chakraborty *et al.(*1997) reported that the optimal temperature for production α-amylase by *B. stearothermophilus* was 50 °C.

Saito(1973) noticed the optimal temperature for production by *B. licheniformis* was 50°C,in contrast (Aiyar,2004) reported the optimal temperture for *B. licheniformis* SPT 27 was 37°C.

More recently**,** The optimum temperature for amylase production by thermophilic and alkaliphilic *Bacillus* sp. was 55 °C (Ito and Horikoshi , 2004).

2.13.3 Initial pH of the media

It is clear that the optimal pH of α-amylase production depends on the strain or the nature of the producer microorganism . The research of Mazza and Ertola (1976) on the production of α-amylase by *B. subtilis* showed the optimal pH was 7.2 ,using fermentor ,that could be attributed to the influence of pH on enzyme formation, or on the release of enzyme from cells, and to consider strain differences as well as the influence of the components of the medium. While, Chakraborty *et al.(*1997)reported that the optimal initial pH for the production α-amylase *B. stearothermophilus* was 7.2 .

Foosi *et al.*(2005) mentioned that the initial pH of media for amylase production by yeast was 4.5 , while Lin *et al.*(1998) reported that optimal initial pH for production by thermophilic and alkaliphilic *Bacillus* sp. was 8.5 .The pH for the production by *Rhizomucor pusillus* was 7.0. Saito(1973); Saito and yamamoto (1975); Kochhar and Dua(1983) ;Turchi and Becker (1987) reported that the initial pH for the production by *B. licheniformis* was 7.0, while Aiyer(2004) reported that the optimal pH for *B. licheniformis* SPT 27 was 9.0 .

2.14 Purification and properties of α-amylase

Enzyme application in pharmaceutical and clinical sectors requires high-purity amylases. Thus, it is significant to develop economic processes for their purification to obtain chemically pure enzymes with maximum specific activity. Traditionally, the purification of α-amylases from fermentation media has been done in several steps, which include centrifugation of the culture (a step of extraction may be required for solid media), selective concentration of the supernatant, usually by ultrafiltration, and selective precipitation of the enzyme by ammonium sulphate and chromatography methods such as affinity chromatography or ion-exchange chromatography and gel filtration.

Extracellular α-amylase enzymes extracellular α-amylase from *Clostridium perfringens* Concentrated by polyethylene glycol were separated by DEAE-Sephacel chromatography (Shih and. Labbe, 1995).The precipitated of crude enzyme (cell –fraction broth)contained six amylolytic isoenzymes that were detected by isoelectric focusing and polyacrylamide gel electrophoresis,one of these amylases was purified by diethylaminoethyl-Sephadex A-50 ion-exchange chromatography and gel filtration by Sephadex G-200(McWethy and Hartman, 1977).

The extracellular α-amylase from *Clostridium acetobutylicum* ATCC 824 was purified to homogeneity by anion-exchange chromatography (Mono Q) and gel filtration (Paquet *et al.*,1991).As bacterial α-amylases have generally been produced from the strains belonging to genus *Bacillus*, several attempts have been made at their purification and characterization, from both mesophilic and thermophilic strains.

Furthermore, α-amylase was purified from Alkaliphilic *Bacillus* sp. using DEAE –Toyopearl and CM-Toyopearl obtaining a specific activity of 5009.3 U/mg protein and 35 % yield (Igarashi *et al.,* 1998). While, Lin *et al.(* 1998) could use Sephacryl S-100 and HiTrap Q in a procedure to purify the α-

amylase from thermophilc alkaliphilic *Bacillus* sp. , they could achieve specific activity of 921.0 U/mg protein , purification folds of 708 .5 and yield (13.2%) .It was purified from *B. stearothermophilus* using DEAE-Sephadex A 25 to get specific activity 100 U/mg protein, and 57 % yield (Aiba *et al*. ,1983).

Krishnan and Chandra(1983)and Bolton *et al.(* 1997) purified an α-amylase to homogeneity using a combination of ammonium sulphate precipitation, ion-exchange chromatography and gel filtration.

An extracellular thermostable *a*-amylase produced by *B. licheniformis* was purified by two a phase separation in a polyethylene glycol / dextran system followed by gel filtration and ion-exchange chromatography (Ivanova ,1993),while Kochhar and Dua(1983) applied heat treatment , Ion- exchange chromatography and gel filtration procedures in the purification the α-amylase and the purified enzyme showed activity of 0.8×10^9 units .

Saito (1973) used DEAE-cellulose, CM- cellulose and Sephadex G-100 in the purification of the enzyme from *B. licheniformis* . Hanzawa *et al.(*1986) purified the α-amylase from *Aspergillus oryzae* using DEAE- cellulose and gel filtration using Sephadex G-75 .Affinity chromatography and HPLC technique were also applied in the purification of enzyme from *Rhizomucor pusillus.* Katoh *et al.*, (1997) purified an α-amylase, produced by recombinant cells, by a specific elution out of anti-peptide antibodies.

2.15 Characterization of α-amylase

2.15.1 Optimal pH for activity and stability

The optimal pH of the activity and stability were different according to the nature of the source organism.

The optimal pH for α-amylase produced by *Bacillus amyloliquefaciens* was 6.0 (Kochhar and Dua ,1983).while, Bakhmatova *et al*.(1984) indicted that the optimal pH for α- amylases produced by variants of *Bacillus subtilis*

was within the acidic range (4.5-5.0),which is alike to optimal pH for the activity of that produced by *Clostridium perfringens* (Shih and Labbe ,1995) .

The optimal pH for the maximal activity of α-amylase produced by *Pyrococcus furiosus* was 5.5 (Dong *et al.*, 1997). Saito (1973) mentioned that the optimal pH for α-amylase produced by *B. licheniformis* was at 9.0 , which is similar to the findings of Krishnan and Chandra(1983) in the activity of α-amylase from *B. licheniformis* was at pH of 9.0 , also the activity of enzyme was reduced when Tris- hydrochloride and carbonate-bicarbonate buffers were used .

Concerning the stability of α-amylase, the α-amylase produced by *Bacillus stearothermophilus* was stable at pH range (6.0-11) while the α-amylase produced by *B. amyloliquefaciens* was stable at pH (6-9.5) for 24 h. Kochhar and Dua (1983) in the presence of calcium ion , while α-amylase produced by *Bacillus licheniformis* was stable at pH rang (6- 11) after the incubation for 30min and 24 hr. and in another study it was stable at pH range (7.0-9.0) (Krishnan and Chandra, 1983) .

2.15.2 Optimal temperature for activity and stability

The optimal temperature for the activity and stability of enzyme is different from each organism to the other, the optimal temperature for thermostable α-amylase activity produced by *Bacillus stearothermophilus* was at 40°C (Aiba *et al.*, 1983). While in another study, it was at 82°C (Srivastava and Baruah, 1986).

The maximal activity for α-amylase produced by *Bacillus licheniformis* was at 90°C (Krishnan and Chandra, 1983). While Saito (1973) reported that the optimal temperature for the enzyme was at 76°C .The α-amylase produced by *B. amyloliquefaciens* showed the maximal activity at the temperature of 65°C. (Kochhar and Dua, 1983).

Concerning the temperature of stability, the results revealed that the α-amylase produced by *Clostridium perfringens* was stable in the presence of Ca^{2+} with 100% of remaining activity up to 45°C, and then the stability was lowered (Shih and Labbe, 1995),while that produced by *C . acetobutylicum* retained about 30% of the remaining activity after treatment at 45°C for 60 min.(Paquet *et al.*,1991), in which the Ca^{2+} plays an important role in the thermostability of enzyme (Lin *et al.*, 1998).

2-16 Immobilization of α-amylase

Immobilized enzymes are used in food technology, biotechnology, biomedicine, and analytical chemistry,they have various advantages over free enzymes including easy separation of the reactants, products, and reaction media, easy recovery of the enzyme, and repeated or continuous reuse (varavinit *et al.,* 2002).

Immobilization is the technique of binding the biocatalysts to a carrier as means of increasing their activity, stability and improving the technological application of the reaction (Hazare, 2005). The immobilization of enzyme on insoluble supports provides a stabilization effect at elevated temperature and pH.(Tzanov *et al.,* 2003).

Many techniques for immobilization of enzymes have been used in which Chalation of Glucose oxidase (GOD) on hydrous transition metals oxides $CrCl_3$,$CuCl_2$, $CoCl_2$, $Zncl_2$ and $FeCl_2$ (Aziz ,1997).

The immobilization of glucoamylase has been studied extensively in which The enzyme is immobilized on a multitude of different carriers by entrapment, adsorption, ion exchange, and covalent bonding (Reilly, 1979; Schafhauser and Storey, 1992). Glycosylated enzymes immobilized on (Concanavalin A) supports showed impressive gains in resistance to inactivation induced by heat, chemical denaturizing, proteolysis, storage and long-term continuous operation for several weeks (Mislovicova *et al.* ,2000).

Biomimetic silica support was used recently in immobilization of enzymes (Lauckarift *et al.*, 2004).Immobilized enzymes were coupled *in situ* with polyurethane polymer. Immobilized cholanesterase was stable for long periods (LeJeune *et al.*, 1997).

The greatest number of attempts to immobilize glucoamylase have been applied using a covalent attachment to organic and inorganic carriers, including low-cost magnetic support (Pieters & Bardeletti, 1992). Among these attempts, the most common method is to employ nitrous acid or glutaraldehyde to link the enzyme to amine-activated porous glass or silica.
The immobilized glucoamylases treated with glutaraldehyde approximately 45% of the original activity is retained upon immobilization (Reilly, 1976).
Cellulose fibers of bagasse were oxidized by periodic acid at positions 2 and 3 of the an hydro-glucose unit to obtain dialdehyde cellulose the aldehyde groups of the dialdehyde cellulose were able to react with amino groups of a thermostable alpha-amylase to form covalent bonds and resulted in a dialdehyde cellulose immobilized enzyme. The activity yield of the immobilized enzyme was 44% (varavinit *et al.*, 2002). Alginate was commonly used in immobilization of enzyme; it was derived from algae and stabilized by a divalent cation. It consists of 1-4 bonded D-mannuronic and L-gluronic acid groups (Ivanova *et al.*,1995; Rodziewicz and Rymowicz ; 1999) used the calcium alginate in the immobilized *Bacillus polymyxa* cells for α-amylase production in continuous biosynthesis .
Gels are formed due to binding of divalent cations to the guluronic acid groups ,The strength of the gel depends strongly on the origin of the alginate, the strength of the matrix improves with an increasing amount of guluronic acid groups in the alginate, The type of divalent cation used also regulates the gel strength ,The strength of an alginate gel increases with the affinity of the used cation according to the following order:

$Pb^{2+} > Cu^{2+} = Ba^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$ (Leenen, 2001).

3.1 Materials and Methods

3.1.1 Equipments and Apparatus

The following equipments have been used throughout the study.

Equipment	Company
Autoclave	**Gallenkamp (England)**
Compound microscope	**Olympus (Japan)**
Ultracentrifuge	**Beckman (USA)**
Cooled centrifuge	**M.SE (U.K.)**
Micro centrifuge	**Sigma (Germany)**
Cooled shaker incubator	**Gallenkamp (U.K.)**
Distillatory unit	**Kent (England)**
Lyophilizer	**LKB (Switzerland)**
Hot plate magnetic stirrer	**Stuart scientific (U.K.)**
Incubator	**Gallenkamp(U.K.)**
Millipore filters	**Millipore and Whatman(England)**
Oven	**Memmert (Germany)**
pH-meter	**Metter-Toledo(U.K.)**
Sensitive balance	**Sartorius (Germany)**
Shaker incubator	**GFL (Germany)**
Shaker water bath	**Kotterman (France)**
Spectrophotometer	**Aurora instrument Ltd. UV 201 (U.K.)**
Vacuum Pump	**Robin Air (USA)**
Vortex mixer	**Buchi (Switzerland)**
DNA –Gel Electrophoresis	**Bio-Rad (USA)**
Protein –Gel Electrophoresis	**Lkb(Sweden)**
Micropipettes	**Witeg (USA)**
Ultracentrifuge	**Beckman(USA)**

and Biological Items 3.1.2 Chemical

The following chemical and biological items have been used throughout the study.

Materials	Manufacturer
Absolute ethanol, Glacial acetic acid, Potassium chloride, Casein hydrolysates , Dihydrogen potassium phosphate, Dipotassium hydrogen phosphate, Glycine , Sucrose, Potassium iodide , Paraffin ,Glycerol, hydrochloric acid, Isoamyle alcohol , Methylene blue ,Iodine , Safranine, Dimethyl-α-naphthalane , α-Naphthylamine ,Sulphanilic acid	**BDH (England)**
Yeast extract, Agar, Nutrient broth, Gelatin.	**Biolife (Italy)**
Soluble corn-Starch, Urea, Maltose	**Difco(USA)**
Sodium lauryl sulphate,Tris(hydroxyl methyl) amino methane base ,Sodium hydroxide, Hydrogen peroxide ,Na_2EDTA, Sodium azide	**Fluka (Switzerland)**
Calcium chloride hydrate, Magnesium sulphate hydrate , Bromophenol blue ,Ammonium chloride ,Glucose, Sodium chloride, Ethidium bromide	**Reidel-DeHaeny Germany**
2-Merceptoethanol	**Calbiochem-USA**
Ampicillin ,Tetracyclane ,Chloramphenicol	**Sinochem jiangsu (China)**
Cesium chloride, 2,3-dinitrosalicylic acid ,TEMED, Ammonium persulfate , Complete freund`s adjuvant	**Sigma**

3.1.3 Culture media:

3.1.3.1 Ready to use media

Media	Manufactured company
Brain heart infusion agar	Biolife (Italy)
MacConky agar	Oxoid (England)
Nutrient agar	Oxoid (England)
Nutrient broth	Oxoid (USA)
Simmon`s citrate medium	Difco (USA)
Triple sugar iron (TSI)	Difco(USA)
Tryptic soy agar	Biolife(Italy)
Urea agar Base	Biolife
Muller-hinton medium	Difco

3.1.3.2 Laboratory prepared media

The autoclaving was done for the all autoclvable media at 121ºC for 15min.

3.1.3.2.1 Blood Agar (Atlas *et .al.*,1995)

It was prepared by dissolving 37 g of blood base agar in 950 ml of distilled water then the pH was adjusted to 7.0 and autoclaved , after cooling to 50ºC, the blood was added to final concentration of 5 %, mixed well and poured into Petri dishes .

3.1.3.2.2 Nitrate broth medium (Cruickshank *et al.*, 1975)

It is composed of:-

Beef-extract	3 g.
Peptone	5 g.
Potassium nitrate	0.2 g.
Distilled water	Up to 1 L.

The pH was adjusted to 7, distributed into tubes and autoclaved at 121C^{o} for 15 minutes

3.1.3.2.3 Carbohydrate fermentation medium (Parry *et al.*,1983)

Ammonium salt sugars medium (A.S.S) was used; it is composed of :-

Ammonium diphosphate	1g.
Potassium chloride	0.2 g.
Hydrous magnesium sulphate	0.2 g.
Yeast extract	0.2 g.
Agar-agar	14 g.
Bromophenol blue solution 1%	3.2 ml
D.W.	Up to 1 L

pH was adjusted to 7.0 and sterilized by autoclave. Then desired sugar solution was filtrated and added to be 1% as a final solution and kept as slants.

3.1.3.2.4 Gelatin medium (Cruickshank *et al.*, 1975)

Twelve gram of gelatin were dissolved and completed to 100 ml of nutrient broth medium. Distributed in tubes and autoclaved .

3.1.3.2.5 Sporulation medium (Cruickshank *et al.*, 1975)

Nutrient broth	6 g.
Hydrous magnesium sulphate	0.03 g.
Potassium phosphate monobasic	0.25 g.
Agar-agar	20 g.
D.W	Up to 1 L

The pH was adjusted to be 7.0, distributed into tubes and autoclaved , and then kept as slants.

3.1.3.2.6 Methyl red and Voges- proskauer (MR-VP) medium (Cruickshank *et al.*, 1975)

It is composed of :-

Glucose	5 g.
Peptone	7 g.
K_2HPO_4	5 g.
D.W	Up to 1L

The pH was adjusted to be 7.0, distributed into tubes and autoclaved, then the filtrated glucose was added.

3.1.3.2.7 Peptone water medium (Atlas *et al.*, 1995)

It is composed of:-

Peptone	2 g.
Sodium Chloride	0.5 g.
D.W	Up to 100 ml.

pH adjusted to 7.0,the media were distributed into tubes and autoclaved.

3.1.3.2.8 Urea agar medium (Collee *et al.*,1996)

It was prepared by adjusting the pH of the urea agar base (Christensen's media) (3.1.3.1) to 7.0, autoclaved, allowed to cool to 50ºC. Then 50 ml of 40% urea (sterilized by filtration) mixed together then distributed into sterilized tubes, kept as slant.

3.1.3.2.9 Casein medium (Cruickshank *et al.*, 1975)

It is composed of :-

Skim milk	5 g.
Agar-agar	2 g.
D.W	U to 100 ml.

The pH was adjusted to 7.0 and autoclaved for 10 min.

3.1.3.2.10 Starch- agar I medium

Nutrient agar supplied with 1.5 % soluble starch, the pH was adjusted to 7.0 then autoclaved.

3.1.3.2.11 Luria –Bertani (LB) broth (Maniatis *et al.*, 1982)

It is composed of :-

Tryptone	10g
Yeast extract	5g
NaCl	5g
Glucose	1g
D.W.	Up to 1L

The pH was adjusted to 7.5 and autoclaved.

3.1.3.2.12 Semi -solid agar medium (Collee *et al.*, 1996)

It was prepared by dissolving 0.7 % of agar, then distributed into tubes, autoclaved and allowed to stand in a vertical position.

3.1.3.2.13 starch- agar media II (Mielenz, 1983)

It is composed of :-

Na_2HPO_4	0.6 g.
KH_2PO_4	0.3g.
NaCl	0.05g.
NH_4Cl	0.1g.
Yeast extract	1g.
Peptone	1.0g.
Soluble starch	1.0g.
Agar-agar	2 g.
D.W.	Up to 100ml

The pH was adjusted to be 7.0 , and autoclaved

3.1.3.2.14 SH media (Saito and Yamamoto, 1975)

It is composed of :-

Soluble starch	40 g.
Ammonium hydrogen phosphate	5 g.
Yeast extract	5 g.
Magnesium sulfate heptahydrate	0. 5 g.
sodium citrate	2 g.
Calcium chloride	0.08g.
D.W.	Up to 1 L.

The pH was adjusted to 7.2, then autoclaved at 121°C for 15 min.

3.1.3.2.15 TSM media (Campbell, 1954)

It is composed of :-

A) Stock elements solution :

Magnesium chloride hexahydrate	0.5 g.
Calcium chloride	0.5 g.
Iron(II) chloride tetrahydrate	0.3 g.
D.W	Up to 100ml

The pH was adjusted to 7.2, then autoclaved at 121°C for 10 min.

B) Media Solution :

Soluble starch	10 g.
Trypton	20 g.
Sodium phosphate dibasic	2.5 g.
potassium phosphate monobasic	1 g.
Sodium chloride	1 g.
D.W	Up to 999 ml

The pH was adjusted to 7.2 and autoclaved at 121°C for 15 min, then the stock (1 ml) elements solution was added and mixed

3.1.3.2.16 Nutrient broth with 1% soluble starch (Aiyar, 2004)

Nutrient broth supplemented with 1 % soluble starch, was prepared and pH was adjusted to 7.0, then autoclaved.

3.1.4 Reagents and Dyes

3.1.4.1 Catalase reagent (Atlas *et al.*,1995)

It is composed of 3% hydrogen peroxide

3.1.4.2 Oxidase reagent (Collins and Lyne ,1987)

One gram of Tetramethyl-*P*-phenylene –diamine dihydrochloride was dissolved in 100 ml distilled water, kept in a dark bottle at 4°C.

3.1.4.3(Lugol`s solution) Starch hydrolysis reagent(Collins and Lyne, 1987)

Potassium iodide	10 g.
Iodine	2 g.
D.W	100 ml

Kept in dark bottle at 4°C.

3.1.4.4 Nitrate reduction reagent (Cruickshank *et al.*,1975)

Reagent A: 0.8 % sulphanilic acid was dissolved in 5N acetic acid over heated.

Reagent B: 0.5 % α-naphthylamine was dissolved in 5N acetic acid over heated .Equal volumes of A and B were mixed together .

C: Zink dust

3.1.4.5 (Barritt`s indicator) Voges –proskauer (Cruickshank *et al.*, 1975)

- **Solution A** : Potassium hydroxide 40 %
- **Solution B**: α-naphthol 5% in absolute Ethanol.

3.1.4.6 Methyl red indicator (Collee *et al.*, 1996)

It is composed of :-

Methyl Red	0.025 g.
Ethanol (95 % V/V)	75 ml
D.W	50 ml

3.1.4.7 Kovac`s reagent

It is composed of :-

p-dimethylaminobenzaldehyde	5 g.
Isoamyl alcohol	75 ml.
Con. HCl	25 ml.

P-dimethylaminobenzaldehyde was slowly dissolved in isoamylacohol with heating in a water bath at 50ºC, until it became cold; HCl was added, kept in a dark bottle at 4ºC .

3.1.4.8 3, 5-Dinitrosalicylic acid (DNSA) (Aiba *et al.*, 1983)

It was prepared by dissolving 1g. of DNSA in 50ml of D.W then 20 ml of NaOH 2M was added ,until it completely dissolved ,30g.of Potassium –sodium tartrate teterahydrat- (Rochelle Salt)gradually was added to be completely dissolved , then the total volume brought to 100 ml, and kept in a dark bottle .

3.1.4.9 3, 5-Dinitrosalicylic acid for TLC products detection

It was prepared by dissolving 0.5g of DNSA in 100 ml of NaOH solution (3.1.5.16) (Caraway, 1976).

3.1.4.10 Coomassie brilliant blue G 250(Bradford, 1976)

Coomassie brilliant blue G-250 (0.1 g) was dissolved in 50 ml of 95% ethanol, then 100 ml of 85 %*O*-phosphoric acid was added ,the volume completed to one liter with distilled water .

3.1.4.11 Bromophenol blue (0.25%) (Shi and Jackowsk, 1998)

It is composed of :-

Bromophenol blue	0.25 g.
D.W	Up to 100 ml

3.1.4.12 Coomassie brilliant blue R-250 (Shi and Jackowsk, 1998)

Coomassie brilliant blue R-250 (2g.) was dissolved in 500 ml of methanol, 100 ml of glacial acetic acid then 400 ml of D.W . The dye was filtrated through Whatman No.1 filter paper and kept in a dark bottle at room temperature.

3.1.4.13 Methyl Red Indicator (Collee *et al.*, 1996)

3.1.4.14 Gram stain (Atlas *et al.*,, 1995)

3.1.4.15 Ethidium bromide solution (10 mg/ml) (Maniatis *et al.*,,1982)

Ethidium bromide (0.1 g) was dissolved in 10 ml of D.W and stirred with a magnetic stirrer for six hours to ensure the complete dissolving, then it filtrated and stored in a dark bottle, wrapped with aluminum foil at 4°C.

3.1.4.16 Staining solution (for SDS-PAGE and Non-SDS) (Shi and Jackowsk, 1998)

Methanol	250 ml
Glacial acetic acid	50 ml
Coomassie brilliant Blue R-250	1 gm
D. W	up to 500ml

3.1.4.17 Destaining solution (Shi and Jackowsk, 1998)

Methanol	200 ml
Glacial acetic acid	50 ml
D.W	up to 250 ml

3.1.4.18 McFarland standard turbidity suspension (Tube 0.5)

It is composed of :-

Barium chloride	1.175 %	0.5 ml
Sulfuric acid	0.36 N	99.5 ml

Which equals to 1.5×10^8 ,in general

3.1.5 Buffers and Solutions

3.1.5.1 TE buffer pH 8.0 (Maniatis *et al.*, 1982)

It is composed of :-

Tris-HCl	10 mM
EDTA	1 mM

3.1.5.2 Potassium phosphate buffer (100 mM) pH 7.0

It is composed of :-

K_2HPO_4	100 mM
KH_2PO_4	100 mM

3.1.5.3 EDTA – SDS buffer pH 8.0

It is composed of :-

EDTA	50mM
SDS	1%

3.1.5.4 TEG buffer pH 8.0(Kieser, 1995)

It is composed of :-

Tris-HCl	25 mM
EDTA	10 mM
Glucose	50 mM
RNase A	5µg/ml

3.1.5.5 TE25S(Kieser, 1995)

It is composed of :-

Tris-Base	**25**	**mM**
Na-EDTA	**25**	**mM**
Sucrose	**300**	**mM**

3.1.5.6 CTAB/NaCl (Kieser, 1995)

It is composed of :-

CTAB	10 g.
NaCl	4g.
D.W.	100 ml

3.1.5.7 5M NaCl (Kieser, 1995)

It was prepared by dissolving 29.22 g. in 100 ml D.W

3.1.5.8 Solution I for DNA isolation (Maniatis *et al.*, 1982)

It is composed of :-

	Glucose	50mM
Tris.HCl	(pH 8.0)	25mM
	EDTA	10mM

Autoclaved, stored at 4°C and Lysozyme dissolved in solution just before use in concentration of 5mg/ml.

3.1.5.9 Solution II for DNA isolation (Maniatis *et al.*, 1982)

It was prepared freshly, as stock solution of 10 N NaOH and 20 % SDS , pH was adjusted to 10.5 . The concentration of the working solution should be:

NaOH	0.2 N
SDS	1%

3.1.5.10 Lyses buffer

It is composed of:-

Sucrose	12.5 %	
Tris-Base	25 mM	pH 8.0
Lysozyme	2 mg/ml	

The pH was adjusted to 8.0

3.1.5.11 TBE buffer 5x (Maniatis *et al.*, 1982)

It is composed of :-

Tris-Base	54 g	
Boric acid	27.5 gm	
EDTA 0.5M (pH8)	20 ml	

The volume was brought to up 1 L and autoclaved

3.1.5.12 Potassium acetate pH 4.8(Maniatis *et al.*, 1982)

To 60 ml of 5M potassium acetate, 11.5 ml of glacial acetic acid was added and then 28.5 ml of D.W was added, mixed together, and allowed to stand on ice for 10 min. before use.

3.1.5.13 SET buffer (Kieser, 1995)

It is composed of :-

NaCl	٧٥ mM
EDTA pH 8.0	25 mM
Tris-Base	20 mM

3.1.5.14 TEGRLR buffer pH 8.0 (Kieser, 1995)

It is composed of :-

Tris-base pH 8.0	25 mM
EDTA	10 mM
Glucose	50mM
Lysozyme	1mg/ml
RNase	10 µg/ml

3.1.5.15 Gel loading buffer 6X (Maniatis *et al.*, 1982)

It is composed of :-

Bromophenol Blue	0.25 g.
Sucrose	40 g.
D.W	100 ml

Kept at 4°C

3.1.5.16 1 M NaOH

3.1.5.17 0.005 M Potassium phosphate buffer -0.025M $CaCl_2$ pH 7.0

3.1.5.18 0.25 M NaCl –0.25 M NaOH

3.1.5.19 0.25 N HCL

3.1.5.20 0.005 M Potassium phosphate buffer pH 7.0

3.1.5.21 0.01 M Potassium phosphate buffer pH 7.0

3.1.5.22 Starch solution 0.5 %

It was prepared by dissolving 0.25 g. of corn starch in 25 ml of potassium phosphate buffer-$CaCl_2$ (3.1.5.18) dissolving solution and then 25 ml of boiled potassium phosphate buffer-$CaCl_2$ (3.1.5.18) dissolving solution was added, boiled for two minutes, until it completely dissolved ,then allowed to cool .

3.1.5.23 Maltose stock solution 1 mg/ml

It was prepared by dissolving 0.1 g. of Maltose in 100ml of D.W.

3.1.5.24 Sodium alginate 4 % solution

It was prepared by dissolving 4 g. of sodium alginate in 100 ml of D.W and autoclaved for 10 min. at 121°C.

3.1.5.25 $CaCl_2$ 1 % solution

It was prepared by dissolving 1 g. of $CaCl_2$ in 100 ml of D.W and autoclaved for 10 min. at 121°C.

3.1.5.26 Tris-acetate-phosphate buffer (Eliss and Morrison, 1982)

It was prepared by mixing Tris, acetate and phosphoric acid with final concentration of 0.2 M (using prepared computer program). The final pH was adjusted according to the needed values.

3.1.5.27 Tris.Cl 10mM / EDTA 1mM pH 7.5

3.1.5.28 Chloroform/ Isoamyl alcohol solution (Kieser, 1995)

Potassium phosphate buffer-$CaCl_2$

Chloroform	24 ml
Isoamyl alcohol	1 ml

3.1.5.29 Transformation buffer

Potassium phosphate buffer-$CaCl_2$

Tris.base pH 8.0	10 mM
$CaCl_2$	50mM

3.1.5.30 Discontinues SDS – PAGE and (Non-SDS)-PAGE buffers and solutions based on (Shi and Jackowski, 1998)

Acrylamide /Bisacrylamide 30% stock solution

It is composed of :-

Acrylamide	29.22 g.
Bisacrylamide	0.78 g.
D.W	Up to 100 ml

Stock solution filtrated through 0.45 µm filter and stored in dark bottle.

3.1.5.31 Stacking gel buffer solution 4 X (0.5 M Tris.base pH 6.8)

It is composed of :-

Tris –Base	121.1 g
D.W	Up to 200 ml

The pH was adjusted with 6M HCl

3.1.5.32 Resolving gel buffer 4 X (1.5 M Tris-HCl pH 8.8)

It is composed of :-

Tris.base	36.3 g.
D.W	Up to 200 ml

The pH was adjusted to 8.8 with 6 N HCl.

3.1.5.33 Running buffer 5 X (pH 8.3) for SDS-PAGE

It is composed of :-

Tris- Base	15 g.
Glycine	72 g.
SDS	5 g.
D.W	Up to 1L.

The pH should be 8.3 without adjustment, the buffer kept in dark bottle at room temperature.

3.1.5.34 Running buffer 5 X (pH 8.3) for PAGE

It is composed of :-

Tris- Base	15 g.
Glycine	72 g.
D.W	Up to 1L.

The pH should be 8.3 without adjustment, the buffer kept in dark bottle at room temperature.

3.1.5.35 Sample buffer 2X (SDS- PAGE)

It is composed of :-

Stacking Gel Buffer	2.0 ml
Glycerol	1.6 ml
10 % SDS	3.2 ml
2-mercaptoethanol	0.8 ml
Bromophenol Blue in D.W(0.1% w/v)	0.4 ml

kept at 4°C in dark bottle.

3.1.5.36 Sample buffer 2X (PAGE)

It is composed of :-

Stacking Gel Buffer	2.0 ml
Glycerol	1.6 ml
D.W	4 ml
Bromophenol Blue in D.W(0.1% w/v)	0.4 ml

Kept at 4°C in dark bottle.

3.1.5.37 Ammonium persulfate solution 1.5%

It is composed of :-

Ammonium Persulfate	0.15 g.
D.W	10 ml

It was freshly prepared

3.1.5.38 Fixing solution

It is composed of:-

Methanol	40 %
TCA(Tri-Chloroacetic acid)	10 %

3.1.5.39 TLC separation system (Aiba *et al.*,1983)

It was prepared by mixing n-butanol /glacial acetic acid/ distilled water as ratio of 5:4:1.

3.1.5.40 Glucose 1% solution

3.1.5.41 0.005M potassium buffer –1M NaCl pH 7.0

3.1.5.42 Enzymes and Nucleic acids

Lysozyme	Sigma
λ DNA digested with *Pst* I	sigma
*Hin*dIII	Fermentas
Pronase	Sigma
RNase- free DNase	Sigma
Alkalane phosphatase	Sigma
T4 Ligase	Fermentas

3.1.5.43 Bacterial standard strains

E.coli HB101	*hsdS20,recA-13,ara-14,proA2, lacY1, galK2, rpsI* (Sm^-),*ryl-5,mtl-1,supE44*	**Genetic Engineering and Biotechnology Institute**

		University of Baghdad, Iraq
E.coli MM294	*hsd R⁻,dsd M⁺,thi⁻,end A*	**Genetic Engineering and Biotechnology Institute University of Baghdad, Iraq**

3.1.6 Antibiotic

3.1.6.1 Antibiotic disks

The source of these disks was (Bioanalyse, Turkey):

Antibiotic	Abbreviation	Content (µg or U)
Imipenem	IPM	10
Cefotaxime	CTX	30
Norfloxacin	NOR	10
Gentamicin	CN	١٠
Penicillin	P	10 U
Amipcillin	AM	١٠
Aztreonam	ATM	30

3.1.6.2 Ampcillin stock solution (25mg/ml) (Maniatis *et al.*, 1982)

Ampcillin (25 mg) was dissolved in 1 ml of D.W then filtrated through Millipore and stored at -20°C in dark as aliquots.

3.1.6.3 Tetracyclane stock solution (12.5 mg/ml) (Maniatis *et al.*, 1982)

Tetracyclane (12.5 mg/ml) was prepared in Ethanol /water (50% V/V) then filtrated through Millipore and stored at -20°C in dark as aliquots.

3.1.6.4 Chloramphenicol stock solution (34 mg/ml) (Maniatis *et al.*, 1982)

Chloramphenicol (34 mg /ml) was prepared in Ethanol then filtrated through Millipore and stored at -20°C in dark as aliquots..

3.1.7 Enzyme solutions (Maniatis *et al.*, 1982)

3.1.7.1 Lysozyme solution (50mg/ml) in lysis buffer

3.1.7.2 Pronase solution (20 mg/ml)

Pronase 20mg/ml was prepared and incubated at 37°C for 2 hours in water bath to inactivate possible DNase and RNase contaminants , distrubted into aliquots and stored at-20ºC.

3.1.7.3 RNase Solution (10 mg/ml)

RNase 10mg/ml was prepared in 10 mM Tris.HCl and 15 mM NaCl, Heated to 100°C for 10 min. allowed to cool slowly at room temperature. Then distributed into aliquots and stored at – 20°C.

3.2 Methods

3.2.1 Isolation of thermophilic bacteria producing α-amylase (Teodoro and Martins, 2000)

Sixty nine soil samples were collected from different sites in Baghdad, Babylon, Dyhala and Diwaniyha districts .They transported to the lab., using sterilized nylon sacs, 10 grams of each sample had been added to 90 ml. D.W, mixed vigorously and heated to 80 °C for 30 min. with shaking in water bath, the pH of the soil sample was measured for next studies.Serial dilutions of each sample using sterilized phosphate buffer were set up from 10^{-1} through 10^{-6}. (0.1) ml of appropriate dilutions were spreaded onto Petri dishes of Starch –Agar medium (3.1.3.2.10) , incubated at 65 C^{o} for two days . Replica plating was made for the growing colonies then starch hydrolysis test was done for one of the replica plates by flooding plates with

lugol`s reagent(3.1.4.3). The colonies that showed a halo diameter zones (Z/G) ratio ; halo diameter: colony growth diameter ,were picked to repeat the same test to confirm the result.

3.2.2 Microscopic and morphological identification of the isolated bacteria (Harley and Prescott, 1996).

The morphology ,size and the margin of isolated colonies grown on nutrient agar (3.1.3.1) and blood agar (3.1.3.2.1) media were studied. 18 hour old colonies were subjected to a microscopic examination of their shape ,size and gram's stain. The sporulation ability was studied by cultivating the isolate on sporulation medium (3.1.3.2.5) for 72 hrs. Spores were been examined of their shape and position within the cell using Phase contrast Microscope.

3.2.3 Biochemical tests.

The colonies that produced amylase and showed characteristics similar to *Bacillus* as a genus have been chosen for further examinations to determine the genus and species accurately.

3.2.3.1 Catalase test (Cruickshank *et al.*, 1975)

This test was performed by adding drops of hydrogen peroxide (H_2O_2) 3% (3.1.4.1) on a single colony grown overnight, placed on glass slide. The production of gaseous bubbles indicated the presence of catalase.

3.2.3.2 Oxidase test (Atlas *et al.*, 1995)

Filter paper was moistening with the substrate solution (3.1.4.2), colony of bacteria grown overnight was rubbed on the filter paper by a sterile wooden applicator stick. An immediate color change to a deep blue indicated a positive test result.

3.2.3.3 Starch hydrolysis test (Harley and Prescott, 1996)

The ability of bacteria to hydrolyze the starch was performed by inoculating the bacteria on the plate of starch –agar medium (3.1.3.2.10) by making a single streak of the bacteria and by incubating at 55ºC for 48 hour.

After incubation, the plate was flooded with lugol`s solution(3.1.4.3).The presence of halo zone around the colonies indicated hydrolysis of starch.

3.2.3.4 Urease test (Atlas *et al.*, 1995)

This test was used to examine the presence of urease, which hydrolyzes urea to ammonia, and Co_2.Christensen urea agar slants were inoculated with single colony of bacteria, incubated at 45ºC for 4-5 days. The appearance of pink color slant indicated a positive test.

3.2.3.5 Triple sugar iron (TSI) test (Atlas *et al.*, 1995)

Isolates were cultured on TSI agar slants by stapping into the agar and streaking on the surface, and then incubated for 48 hrs. at 55°C .Bacteria that only ferment glucose produce an alkalane (red slant) and an acid (yellow) butt. Bacteria that ferment lactose or (and) sucrose as well as glucose produce an acid (yellow) slant and an acid (yellow) butt. Bacteria that do not ferment glucose, lactose, or sucrose produce an alkalane (red) slant and an alkalane (red) butt. Bacteria that produce gas during fermentation form bubbles or cracks in the medium. Bacteria that produce H_2S gas turn the medium black.

3.2.3.6 Citrate utilization (Atlas *et al.*, 1995)

This test was used to examine the ability of bacteria to utilize citrate as a sole source of carbon. In this test, a colony was inoculated on the surface of simmon`s citrate medium (3.1.3.1) slant and the medium was incubated overnight at 55ºC. The change of the color to blue indicated the positive result.

3.2.3.7 Methyl red and voges- proskauer (Collee *et al.*, 1996)

The methyl red test was used to determine the pH of the end products of glucose fermentation.

Tubes of MR-VP liquid media were inoculated and incubated at 55ºC for 48hrs. Both tests were performed from the same inoculums suspension, which was divided for testing. The methyl red test was performed after adding about five drops of methyl-red reagent (3.1.4.6) the positive test was bright red and negative test was yellow.To detect the formation of Acetoin , Barritt`s solution (3.1.4.5) (600µl of 5 % α-naphthol and 200 µl of 40% NaOH) were added to the 1 ml bacterial culture (3.1.3.2.6), the appearance of red color within minutes indicated the formation of intermediate -compound Acetyl-methyl carbionl(Acetoin).

3.2.3.8 Carbohydrates fermentation (Parry *et al.*, 1983)

Bacteria were grown on ASS (3.1.3.2.3) medium containing the sugar to be tested and incubated at 45C^{o} for 3-5 days, the turn of reagent's color into yellow indicated utilization of the sugar and the forming of an acid.

3.2.3.9 Nitrate reduction test (Cruickshank *et al.*, 1975)

One hundred µL of each test reagent solution A and B (3.1.4.4) was added to the culture in medium (3.1.3.2.2). A red color developed within few minutes indicated the ability of the organism to reduce nitrate to nitrite.

3.2.3.10 Gelatin liquefaction test (Cruickshank *et al.*, 1975)

Tubes of gelatin media (3.1.3.2.4) were inoculated with bacteria and incubated at 45C^{o} for 5 days then transferred to the refrigerator for an hour, the liquefaction of tubes indicated positive results to be compared with the control one .

3.2.3.11 Indol formation test (Collee *et al.*, 1996)

The tubes of peptone water media (3.1.3.2.7)were inoculated with bacteria ,and incubated at 45ºC for 48 hrs , then 100µl of kovac`s reagent (3.1.4.6) was added to the culture . the Formation of red ring near the surface indicated the positive result due to the formation of tryptophanase.

3.2.3.12 Motility test (Collee *et al.*, 1996)

Semi solid agar medium (3.1.3.2.12) was inoculated with bacteria by stapping in the center of agar , to half of it , incubating at 55 °C for 48 hrs., a diffuse and hazy growth that spread slightly throughout the medium indicated positive results .

3.2.4 Maintenance of bacterial isolates

3.2.4.1 Short term storage

Bacteria were maintained in slant culture for period of a month. Such cultures were grown on slant surface in tubes containing 5-8ml of nutrient agar medium and stored at 4ºC .

3.2.4.2 Storage in soil

clean soil was sterilized successive times using autoclave , then its aseptic condition was tested by inoculating a sample of it on nutrient agar for three days , no visible growth indicated its sterilization .Then 1 ml of spores suspension was mixed with it, allowed to dry and kept at 4ºC for undetermined period.

3.2.4.3 Storage in 15 % glycerol.

Bacteria could be stored for a relatively long time in media containing 15% glycerol at a low temperature without a significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria grown in nutrient broth in a screw-capped bottle in final volume 10 ml and stored at -20ºC.

3.2.5 Preparation of Spores

B. stearothermophilus M13 was cultured on the surface of sporulation media(3.1.1.2.5) in slant tubes at 55ºC for three days ,then sterilized D.W was added to the culture and centrifuged at 3000xg for 5 min., discarding the

supernatant, the same procedure was repeated. The spore suspension was obtained and kept onto the soil at 4ºC.

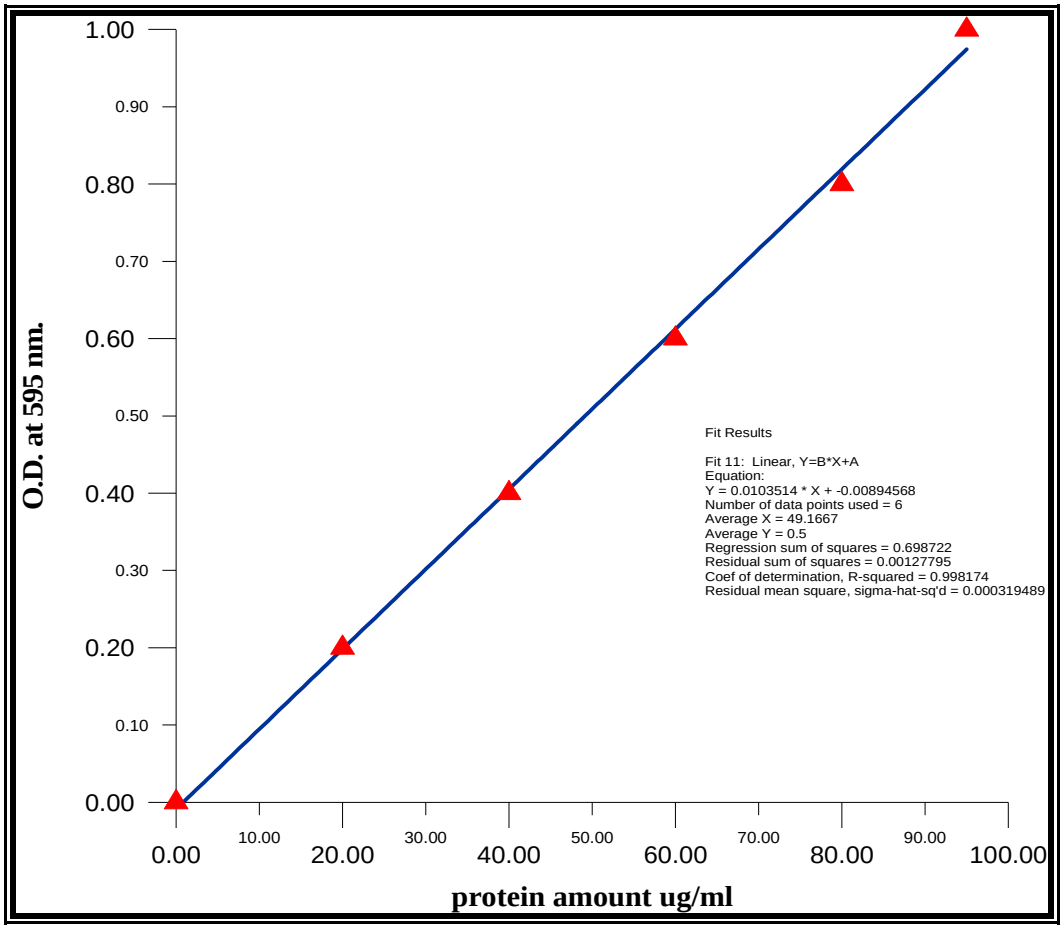
3.2.6 Estimation of protein (Bradford,1976)

Protein was estimated by dye-binding (Coomassie G-250) method in which the bovine serum albumin used as a standard protein .

1. The standard protein bovine serum albumin (BSA) was prepared by dissolving 10 mg (BSA) in 10 ml of 0.05 M phosphate buffer pH 7.0
2. Several dilutions of standard protein (BSA)(1µg/µl) were performed in the same buffer and according to the following volumes :

BSA µL	Buffer µL	Protein amount µg	Final volume ml
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1
80	20	80	0.1
100	0	100	0.1

3. Twenty µl of each concentration were mixed with 50µl of NaOH 1M (3.1.5.16)
4. Two hundred and fifty µl of the dye reagent (3.1.4.10) were added, mixed, and allowed to stand for 10 minutes at room temperature.
5. The absorbance at 595 nm was measured; the blank was prepared from 0.1 ml of the buffer and 2.5 ml of the dye reagent.
6. A standard curve was plotted between the amounts of protein in the given sample against the corresponding absorbance. The protein concentrations of unknown sample were calculated from the standard curve (Fig. 3.1).



Figure(3.1):Standard curve for protein estimation by Bradford`s method.

3.2.7 α-amylase activity assay

Method of Bernfield (1955) was used for α-amylase assay; one unit enzyme activity is defined as the amount of enzyme that liberates one µmole of reducing sugar and measured as maltose per minute under the conditions of assay.

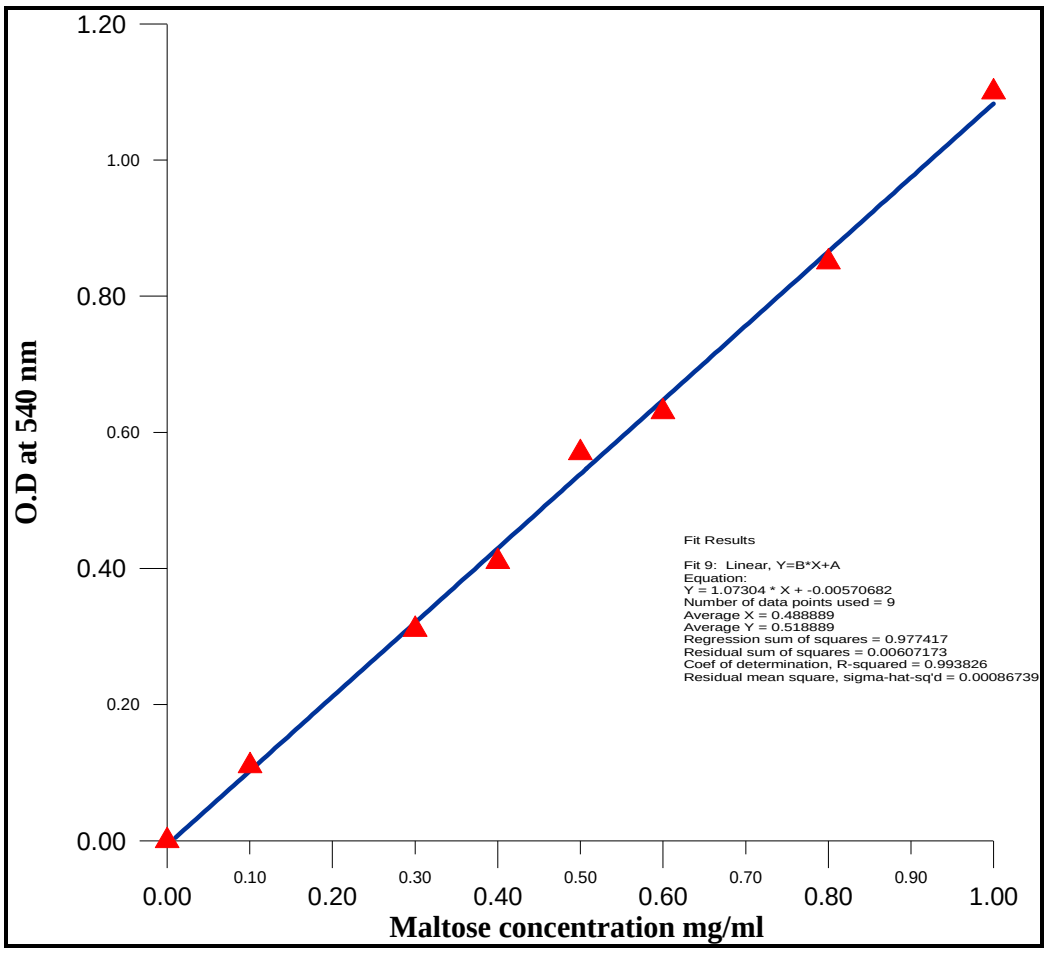
3.2.7.1 Standard curve of maltose solution

1. the following volumes of standard maltose stock solution (1mg/ml) (3.1.5.23) had been distributed into test tubes as duplicates for each single volume , then the appropriate volumes of D.W, had been added for each, as follows;

Tube No.	Stock standard maltose(ml)	D.W (ml)	Final conc. maltose(mg/ml)
Blank	0	1	0

1	0.1	0.9	0.1
2	0.3	0.7	0.3
3	0.4	0.6	0.4
4	0.5	0.5	0.5
5	0.6	0.4	0.6
6	0.8	0.2	0.8
7	1.0	0	1

2. One ml of DNSA Reagent (3.1.4.8) was added to each tube.
3. Tubes were incubated in boiling water bath for 5 min.
4. Tubes were removed from the boiling water bath and immediately placed into ice bath.
5. Ten ml of D.W. were added to each tube and well mixed.
6. Reading the absorbency for each sample at 540nm. Versus blank.
7. The Absorbency values at 540nm were plotted against maltose concentration (mg/ml), then the activity was calculated .



Figure(3.2):Standard curve for maltose, using DNSA as blocking reagent

3.2.7.2 Assay method

1. Enzyme solution (0.1 ml) was added to 0.9 ml of starch solution (3.1.5.24) in tubes and incubated at 60°C for 10 min.
2. The reaction was stopped by adding 1ml of DNSA, and then the tubes were incubated in boiling water bath for 5 min.
3. Tubes removed from the boiling water bath and immediately placed into an ice bath.
4. Ten ml of D.W was added to each tube.
5. The absorbency for duplicate tubes was measured at 540 nm , the blank was created by adding the enzyme solution after DNSA addition (blocking solution).

3.2.8 Optimum conditions for α-amylase production in liquid media

The main optimal conditions for amylase production have been studied in order to produce it with higher amount using the batch cultures.

3.2.8.1 Determination of optimal α-amylase production media

The colonies that revealed halo zone of hydrolysis on media (3.1.3.2.10) and classified as *B. stearothermophilus* were picked for the next experiments in order to choose the most efficient isolate by inoculating on SH medium.*B. stearothermophilus* was grown in nutrient broth for overnight , then three duplicated flasks containing SH medium (3.1.3.2.14) , TSM medium (3.1.3.2.15) and nutrient broth supplemented with 1% soluble potato starch (3.1.3.2.16) were inoculated with 1 ml of the previous grown culture at log phase of growth . Then flasks were incubated in a shaker incubator (120 rpm, 55˚C, 24 hrs.). The cultures were centrifuged at 3000xg for 5 min and supernatant was used for assaying the α-amylase activity (3.2.7).

3.2.8.2 Optimal temperature for α-amylase production

B. stearothermophilus M13 was grown in TSM medium broth (in which it was the best among the media) and incubated in different temperatures (35, 40, 45, 50, 55, 60, and 65˚C) in a shaker incubator (120 rpm, 48hrs). The cultures were centrifuged at 3000rpm for 5 min and supernatant was used for assaying of α-amylase specific activity as described in (3.2.7).

3.2.8.3 Optimal Initial pH for α-amylase production

The optimal pH for production of α-amylase was determined by preparing the TSM medium with different pH, ranging from (6, 6.5, 7, 7.5 and 8) .Media were inoculated and incubated in shaker incubator (120 rpm, 55˚C, 24 hr.). The culture was centrifuged at 3000rpm for 5 min and supernatant was used for assaying of α-amylase specific activity as described in (3.2.7).

3.2.8.4 Measurement of bacterial growth (Growth curve) and optimal growth phase for α-amylase production.

Growth of bacteria was monitored by measuring the optical density of a liquid culture using spectrophotometer at 600nm, at interval time of 2 hours .The corresponding α-amylase activity was determined as described in (3.2.7) after centrifugation of the samples at 3000xg .

3.2.9 Antibiotic susceptibility (Aslim *et al.*, 2002)

B. stearothermophilus M13 was inoculated into nutrient broth and incubated at 55°C for several hours until the turbidity was easily visible. The turbidity was adjusted by nutrient broth against McFarland (3.1.4.18) standard tube. Then 100 µl of bacteria suspension was transferred to the surface of Muller-Hinton agar plates , spreaded and incubated for 15 min. at 55°C .Antibiotic discs were placed on muller-hinton agar medium seeded with bacteria and the diameters of inhibition zones that formed following 24 h. incubation were measured and test results of antibiotic susceptibility were determined according to the inhibition zone diameter (NCCLs, 1991).

3.2.10 α-amylase purification

3.2.10.1 Precipitation by the Ammonium sulphate (Segel, 1976)

One hundred ml of the crude extract were made up to 40 % saturation with ammonium sulphate stirred on the ice for 1 hour,then centrifuged at 9000 xg for 20 min. at 4^{o}C . Ammonium sulphate was added again to supernatant to raise saturation ratio to 80% using the same procedure. The mixture was centrifuged, then the supernatant was discarded. The precipitated was dissolved in 5 ml of the potassium phosphate buffer 0.005M – $CaCl_2$ 0.025M pH 7.0 (3.1.5.17). Then enzymatic activity and the protein concentration were measured after dialysis.

3.2.10.1.1 Preparation of dialysis tubing with10000 d. cutoff (Maniatis *et al.*, 1982)

1. Cut the tubing into pieces of 15 cm length.
2. Boiled for 10 min., with 100 ml of 2% sodium bicarbonate and 1mM EDTA.
3. The tubing was rinsed thoroughly in distilled water.
4. Boiled for 10 min. in 0.001 M EDTA.
5. Allowed to cool and stored at 4°C in D.W
6. Washed with D.W before application.

3.2.10.1.2 Dialysis

The supernatant of the last step was dialyzed against the following buffers, taking into consideration changing the solution many times during the process

1. Distilled water.
2. potassium phosphate buffer –$CaCl_2$ (3.1.5.17)

3.2.10.2 Preparation of the ion exchange chromatography column (LKB)

The DEAE-sepahdex was prepared according to the manufacturer (LKB), in which the exchanger was washed with NaCl-NaOH solution (3.1.5.18). Using the buchner funnel as a ratio 440ml : 20 g. of DEAE-Sephadex, filtrated under vacuum through Wattman paper No.1. then washed for many times over running distilled water, suspended by HCl solution (3.1.5.19) then washed for many times by potassium phosphate buffer (3.1.5.20), and resuspend onto it, until the pH became 7.0 ,allowed to stand for 1 hour ,then the fines were removed , washing with distilled water was repeated to ensure a better removal of fines or impurities and degassed . Glass wool was inserted at the bottom of column, and then the slurry was poured into column,allowed to be packed in glass column to be (1.3 x 35) cm. The equilibration was done overnight using potassium phosphate buffer solution (3.1.5.20) at speed of 40 ml/hour.

3.2.10.2.1 Sample application

The concentrated enzyme (4ml) was added to the side wall using a glass rod gently , then it was washed by potassium phosphate buffer solution (3.1.5.20) ,the washing fractions were collected at speed of 5 ml/frac, the optical density was measured for each fraction at 280 nm. The declane of the optical density readings to the base lane indicated that the washing process of column was completed.

3.2.10.2.2 Elution of the enzyme

A laner salt gradient of (potassium buffer –NaCl) (3.1.5.41) in order to recover the enzyme from Ion- exchanger, the protein for each fraction was estimated at 280 nm. The enzymatic activity for each fraction was assayed as described in (3.2.7); the curve of enzymatic activity (unit/ml) was plotted against the O.D.280nm.

The fractions that revealed significant peak of activity were mixed together.

3.2.10.2.3 Dialysis

The enzymatic solution that recovered from the last step was dialyzed against potassium phosphate buffer 0.005 M – $Cacl_2$ 0.025 M pH 7.0(3.1.5.20) , and for several times until the next day in order to remove all the salt used in the recovery , then it was lyophilized .

3.2.10.3 Gel filtration chromatography (Laue and Rhodes, 1990)

3.2.10.3.1 Preparation of Sephadex G-100 Column

The gel was prepared according to the instructions of the manufacturer (LKB) in which the gel was washed with potassium phosphate buffer solution (3.1.5.21), then it was suspended in the same solution overnight at 4°C to allow swelling .The fines were removed . Degassing was done using vacuum , the slurry was poured into the column and allowed to be packed in the glass column(2.5x 35) cm , the washing, overnight, was done with solution (3.1.5.21) at a flow rate of 30 ml /h. . The void volume was measured by blue dxtran- 2000 (V_o)at concentration of 5mg/ ml , the blue dextran -2000

was recovered from the column by potassium phosphate buffer solution (3.1.5.21) in which it was collected at speed of the 5 ml /frac. , at a flow rate of 30 ml /h., the absorbency of each fraction was measured at 600 nm. The lyophilized enzyme was dissolved in 2 ml of (0.005 M phosphate buffer – 0.002 $CaCl_2$) then was gently added to the side wall of column using the glass rod of the gel, the elution was done by the same buffer , 5ml / fraction at flow rate of 30 5ml /h. using same conditions of that for the blue dextran - 2000 recovery .

The fractions that revealed the protein and enzymatic activity in the same peak was mixed and dialyzed against solution (3.1.5.21), then protein and enzymatic activity were assayed once again for the partially purified enzyme.

3.2.11 Determination of α-amylase purity and estimation of its molecular weight .

3.2.11.1 SDS- Polyacrylamide gel electrophoresis of partial purified α-amylase (Shi and Jackowski, 1998)

Tubes used were soaked overnight in 95% ethanol, then allowed to dry. Each tube was sealed from the bottom side by the clean parafilm and aligned in casting stand board.

The separating (resolving) gel was prepared by adding 7.5 ml of (acrylamide and bisacrylamide) solution (3.1.5.30) , 7.5 ml of resolving gel buffer 1X (3.1.5.32) pH 8.8, 0.3 ml of 10% SDS solution and 14.49 ml of distilled water . The solution was degassed for 10 min. using a vacuum pump , then 150 µl of 1.5% ammonium persulphate (3.1.5.37) and 15µl of TEMED were added to the degassed solution and mixed gently. using Pasteur pipette ,the separating gel was transferred to PAGE tubes using another pipette ; the top of the gel was covered slowly with "Isobutyl alcohol " and allowed the gel to be polymerized for two hours at room temperature . Then the layer of isobutyl alcohol poured off.

Stacking gel was prepared by adding 3.9 ml of (acrylamide and bisacrylamide) solution (3.1.5.30),7.5 ml of stacking gel buffer 1X(3.1.5.31) ,0.3 ml of 10 % SDS solution and 18.3 ml of distilled water .The solution was degassed for 10 min., using a vacuum pump, 50 µl of 4.5 % ammonium persulphate (3.1.5.37) and 5 µl of TEMED were added to the degassed solution and mixed gently. Using Pasteur pipette, the stacking gel was transferred slowly to cover the upper surface of the separating gel to polymerization about 2 hr at room temperature. Samples for SDS –PAGE were prepared by adding equal volumes of a sample buffer solution 1X (3.1.5.35) to the sample and mixed well. The samples were incubated in water bath at 90 C^o for 5 min. and cooled to 25°C .

The PAGE tubes were submerged in running buffer 1X (3.1.5.33) and 100 µl of the prepared sample was loaded on the gel .The power supply was connected to the samples and run at 2.5mA tube for 30 min. then it was raised to 6mA. The run was kept at 4°C until the tracking dye reached a narrow distance just before the end of gel .

3.2.11.2 Standard Proteins Solution

It was prepared by dissolving the following proteins` molecular weights (150 , 80, 67, 50, 20, 14 kDa) as a concentration of 3 mg/ml in sample buffer (3.1.5.35). Then the same procedures for electrophoresis were applied.

3.2.11.3 Detection of protein bands on the polyacrylamid gel.

Polyacrylamid gels were removed from the SDS-PAGE tubes and placed separately in test tubes .Gels were covered with fixing solution (3.1.5.38) for 1 hour; fixing solution was poured off and the gels were covered with staining solution (3.1.4.10) for 3 hrs . Then a staining solution was poured off and the gels were immerged with destaining solution (3.1.4.16) The destining process

continued with changing the solution several times until blue bands were obtained with white background, gels were stored in 10 % acetic acid.

The molecular weight was determined using specialized software PhotoCapMw.Also, based on the application of the following equation to measure the (Relative mobility R_f) (Elyassaki,1991)

$$R_f = M \times L/M' \times L'$$

Where

M = Migration of protein band

M' = Migration of tracking dye

L= Length of gel before staining

L'= Length of gel after staining

In order to confirm the value of R_f of the markers were plotted against their known molecular weight on a semi logarithmic scale and the samples were calculated in accordance.

3.2.11.4 Determination of α-amylase molecular weight by sephadex G-100

3.2.11.4.1 Standard Protein solution

(lysozyme 14400, ovalbumin 43000, bovine serum albumin 67000 and Alkalane phosphatase 160000 Dalton), were prepared at concentration 10 mg/ml in phosphate buffer 0.005 M pH 7.0 (3.1.5.20).

3.2.11.4.2 Gel Filtration

Gel filtration was done using Sephadex G-100 for the standard protein , well-known molecular weights for each one alone, following the same procedures and under the same conditions applied for the α-amylase and blue dextran (3.2.11.3) (Whitaker, 1963) .

The protein was estimated in each fraction by calculating the elution volume V_e for each one, then the ratio between recovery volume for each protein to

void volume (V_e/V_o) for column as measured by the blue dextran-2000 (3.2.11.3).

A standard curve was drawn by plotting the value of (V_e/V_o) against log molecular weight for each single standard protein, then the α-amylase molecular weight was estimated by plotting its (V_e/V_o) value against the corresponding log value of it .

3.2.12 α-amylase characterization

For all reactions the mixture of enzyme and substrate, were supplemented with 0.005 M $CaCl_2$ and assayed as described in (3.2.7).

3.2.12.1 Determination of pH effect on α-amylase activity

Using buffer solution described in (3.1.5.26) which had been distributed equally into aliquots, the desired pH was adjusted according to the what was described by Ellis and Morrison(1982). 100µl of purified α-amylase was added to 900µl of each one of the different pH solutions ranged (5.5-8.5)containing soluble starch 0.5 % as a substrate. The activity of α-amylase was assayed and plotted against the corresponding pH values to determine the optimal pH for α-amylase activity.

3.2.12.2 Determination of pH effects on α-amylase stability.

Equal volumes of purified α-amylase and buffer solution of (3.1.5.26) with pH range 5.0 through 9.0 were incubated in a water bath at 55°C for 45 min. then transferred immediately into an ice bath .The enzymatic activity for each one was determined.

The remaining activity (%) for enzyme was measured and plotted against the corresponding pH values of solution to determine the optimal pH for α-amylase stability in industry.

3.2.12.3 Determination the optimal temperature for α-amylase activity

The enzyme was incubated with the substrate, in water bath at different temperatures (30, 40, 50, 60,70,80,90 and 100°C) for ten min. Enzymatic activity was measured and the enzyme activity was plotted against the temperature.

3.2.12.4 Determination of thermal stability for α-amylase activity

The purified enzyme was incubated at different temperatures (30, 40, 50, 60,70,80,90 and 100°C) for 30 min., then immediately transferred into an ice bath. Enzymatic activity was assayed and the remaining activity (%) was plotted against corresponding temperature.

3.2.13 products separation by ascending paper chromatography analysis

Hydrolysis products from soluble starch were analyzed by paper chromatography, as described by (Hatada *et al.*, 1996; Safar,1998) with modifications .

1. One hundred µl of purified enzymatic solution was mixed with 900µl of the 0.5%-starch solution (3.1.5.22).
2. The reaction solution was incubated at 60 ^{o}C for (0, 15, 30, and 75 min) , then transferred immediately to an ice bath .
3. Thirty µl of the product were transferred to the TLC paper wattman No.1 with 0.16 mm thickness, allowed to dry , then it was twice to hold a high concentration of possible products , after that the same procedures were applied to standard maltose (3.2.7.1) and glucose (3.1.5.40) solutions
4. The paper was placed in the glass jar containing separation system (3.1.5.39) and the jar was covered, the process was stopped when the solvent reached the end lane

5. After determining the distances of the solvent, the paper was removed from the jar and allowed to dry at room temperature, then it was treated with DNSA (3.1.4.8).

6. The paper was baked in an oven at 100 °C for 20 min. to complete the reaction , then the R_f was calculated for each sample according to the formula (Ghosh and Chandra ,1980).

$$R_f = \frac{\text{Distance immigrant by solute}}{\text{Distance immigrant by solvent}}$$

3.2.14 Immobilization of α-amylase.

The α-amylase was immobilized by an entrapment method using sodium alginate according to (Guo *et al.,* 1990)

1. The enzymatic solution was added as 0.2U/ml to sodium alginate solution (3.1.5.24) and mixed well for 10 min .
2. Then the mixture was pulled in by a syringe and dropped into a beaker containing cooled calcium chloride solution (3.1.5.25)to form beads ,allowed to solidify, then washed off many times with a calcium chloride solution to remove the remaining of unimmobilized enzyme .

3.2.14.1 Determination of the efficiency of immobilized α-amylase

Twenty five grams of granules in which the enzyme was immobilized to them was placed into 25 ml of 0.5% starch. Incubated at 60°C degree in a shaker incubator 50 rpm for 10 min. The efficiency was measured according to the formula below (Varavinit *et al.,* 2002) . The immobilized α-amylase was kept in the phosphate buffer at 4°C for monitoring the activity over 30 days.

Activity yield (%) = 100 x activity of immobilized enzyme / A-B

Where:

A: The activity of free enzyme added .

B: The activity of the unimmobilized α-amylase (remaining enzyme and unimmobilized α-amylase in washed water).

3.2.15 Preparation of the anti- amylase sera (Yamaguchi *et al.*, 1974 a,b;1999)

A mixture of the α-amylase (2mg in 0.5 ml of 0.85 NaCl) and 0.5 ml of complete freund`s adjuvant was injected subcutaneously into a mature white rabbits (two rabbits-New Zeeland with two rabbits as a control).Two weeks later, the rabbits received an additional subcutaneous injection of the α-amylase –adjuvant mixture (2 mg of the α-amylase). The mixture was injected into rabbits. Booster injections of the samples were given in the same manner after 4 and 6 weeks, and the blood was collected a week after the last booster injection, sera were separated and incubated at 56 ºC for 30 min to inactive complement, and stored at -20^{o}C .

3.2.15.1 Neutralization of α-amylase with anti-amylase Sera(Yamaguchi *et al.*, 1974a)

Five hundred µl volume of the α-amylase solution was mixed with an equal volume of the antiserum .After incubation at 40^{o}C for 30 minutes; the remaining enzyme activity was assayed .The neutralization was almost completed within 60 minutes. After the reaction completed, the mixture was centrifuged at 6000xg for 20 minutes, then α-amylase activity was assayed.

3.2.16 Genetic analysis of *B. stearothermophilus* M13

3.2.16.1 Isolation of plasmid DNA from *B. stearothermophilus* M13 (Mielenz ,1983)

In order to analyze the genetic potential of *B. stearothermophilus* M13 concerning the possibility of harboring a plasmid , the following procedure was applied.

1. *B. stearothermophilus* was cultured in 25 ml nutrient broth overnight with shaking (120 rpm)at 55 °C ,then 250 ml of fresh nutrient broth was cultured with 2.5 ml of 18 hrs. old growth , incubated at 55°C with shaking(120) rpm overnight.

2. *B. stearothermophilus* M13 cells were harvested at 6000g , 4°C using cooling centrifuge.
3. Cells were resuspended in 5 ml of lysis buffer (3.1.5.10) and incubated for 1 hr. on an ice .
4. Lysate was allowed to stand at room temperature, then 5 ml of SDS solution (3.1.5.3) was added , after 15 min. 10 mg of pronase was added and incubated at 37°C for 30 min.
5. The mixture was diluted with 2.5 ml buffer (3.1.5.27) pH 7.5.
6. An equal volume of (chloroform /Isoamyl alcohol) solution (3.1.5.28) was added, and centrifuged at 12000xg for 15 min.
7. The aqueous phase was transferred to a clean tube , 0.6 volume of chilled isopropanol was added , and centrifuged at 12000xg for 10 min.

The DNA pellet was washed with 70% ethanol , allowed to dry , and dissolved in 750 µl of TE buffer

3.2.16.2 Isolation of genomic DNA of *B. stearothermophilus* M13

1- By Salting out procedure (Pospiech and Neumann, 1995; Kieser, 1995)

The genomic DNA of *B. stearothermophilus* M13 was isolated by salting out procedure with some modifications applied through the study.

1. *B. stearothermophilus* was cultured in 25 ml nutrient broth overnight with shaking (120 rpm) at 55 °C , then 250 ml of fresh nutrient broth was cultured with 2.5 ml of 18 hrs. old growth , incubated at 55°C with shaking(120 rpm) overnight.
2. Cells harvested from 250 ml of bacterial culture using cooling centrifugation 6000g,at 4ºC.
3. Resuspended in the 5 ml SET buffer, then 200 µl Lysozyme solution (3. 1.7.1) and 60 µl of RNase solution (3.1.7.3) were added, incubated for 1 hour at 37 ^{o}C.

4. Three hundred µl pronase solution (3.1.7.2) was added and incubated at 37°C for 45 min., then 600 µl 10% SDS was added , mixed by inversion, incubated 2hrs. at 55 ^{o}C, inverted occasionally.
5. Two ml of NaCl (5M) solution (3.1.5.7) was added, mixed thoroughly by inversion, allowed to stand at 37 ^{o}C.
6. Equal volume of (chloroform /Isoamyl alcohol) solution (3.1.5.28) was added, mixed by inversion for 30 min at 25 ^{o}C.
7. Centrifuge 15 min, 12000 xg at 20°C.
8. Supernatant transferred to a fresh propylene tube add 0.6 volume chilled Isopropanol ,mixed by inversion then left for 2 hours in refrigerator then centrifuged , DNA was rinsed with 70% ethanol ,allowed to dry ,and dissolved in 750 µl TE at 50 ^{o}C .
9. Kept at -20°C.

2- By CTAP Procedure (Rogers and Bendich, 1988; Kieser, 1995)

The genomic DNA of *B. stearothermophilus* M13 was isolated by CTAP procedure with some modifications applied through the study .

1. Cells harvested from 250 ml of bacterial culture by cold centrifugation
2. Resuspended in the 5 ml TE25S buffer (3.1.5.5), then 200 µl lysozyme solution (3. 1.7.1) and 60 µl of RNase solution (3.1.7.3) were added, incubated for 1 hour at 37 ^{o}C.
3. Three hundred µl of pronase solution (3.1.7.2) were added ,incubated at 37°C for 45 min. , then 600 µl of 10% SDS was added , mixed by inversion, incubated 2hrs. at 55 ^{o}C, inverted occasionally.
4. Two ml of NaCl (5M)solution (3.1.5.7) was added, mixed thoroughly by inversion, allowed to be at 37 ^{o}C.
5. 700 µl of CTAB/NaCl (3.1.5.6) mixed thoroughly and incubated for 15 min at 55 °C, then cooled to 37°C .
6. An equal volume of (chloroform /Isoamyl alcohol) solution (3.1.5.28) was added, mixed by inversion for 30 min at 25 ^{o}C.

7. Centrifuge 20 min, 12000xg at 20 °C .

8. Supernatant transferred to a fresh propylene tube; 0.6 volume chilled Isopropanol was added ,mixed by inversion then left for 1 hours in refrigerator then centrifuged at 12000xg for 15 min. , DNA was rinsed with 70% ethanol ,air dry ,and dissolved in 750 µl TE at 50 ^{o}C .

9. Kept at -20°C.

3.2.17 Isolation and purification of pBR *322* vector from *E. coli* HB 101 by Cesium chloride–Ethidium bromide density gradients ultracentrifugation method (Maniatis *et al.*, 1982).

1- Isolation

1. Ten ml of LB medium containing ampicillin (50µg/ml) was inoculated with single colony of *E. coli* HB101 at 37 °C overnight with shaking.

2. Two hundred and fifty ml of LB medium containing ampicillin (50µg/ml) was inoculated with 0.25 ml of *E.coli* HB101(18 hrs old). incubated at 37 °C with shaking. When the OD_{600} of culture was 0.5 , 1.25 ml of Chloramphenicol solution(34mg/ml in 95% ethanol) was added in which the final concentration was 170µg/ml .

3. The culture was incubated at 37°C for further 12-16 hrs.

4. The bacteria were harvested by centrifugation at 5000xg for 10 min. at 4°C.

5. Wash with 50 ml of ice-cold STE Buffer (3.1.5.10) twice.

6. The cells were resuspended in 10 ml of solution I –lyses solution(3.1.5.8) containing 10 mg/ml Lysozyme, incubated at 37°C for 30 min. in polypropylene tube.

7. Twenty ml of freshly prepared solution II (3.1.5.9) was added, gently mixed for 5 min., allowed to stand on ice bath for 10 min.

8. Fifteen ml of an ice-cold solution of potassium acetate solution (3.1.5.12)(pH 4.8),then an equal volume of chloroform was added, mixed immediately by inversion and allowed to stand for 5 min.on an ice bath.

9. Centrifuged for 20 min, 10000xg at 4°C

10. Aqueous phase was transferred to clean polypropylene tube and 0.6 volume of isopropanol was added , mixed well and allowed to stand at room temperature for 15 min.

11. Centrifuge 20min, 10000 rpm.

12. The supernatant was discarded, the pellet was washed with ethanol 70%, and then the pellet was dried at room temperature.

13. The pellets were dissolved in 5 ml of TE buffer, kept at – 20 °C.

14. Ten µl of the sample was added to 990 µl of TE buffer in a quartz cuvette and the absorbency at 260nm and 280nm was measured after calibration based on blank of TE buffer at 260nm and 280nm., the calculations were done according to the following formula:-

Concentration of double strand DNA (µg/ml) in the sample

= (OD_{260} x 50 x 100)

O.D 260/ O.D280≥ 1.8 provides an estimate the purity of DNA

OD_{260} 1= 50 µg/ml of double strand DNA and 20 µg/ml forsingle stranded oligonucleotides.

2-Purification

1. The concentration of DNA in TE buffer was estimated, 1.10 g. CsCl per 1 ml of DNA solution (4.29 g to 3.9 ml) was added, mixed and dissolved.

2. Ethidium bromide solution 234 µl of (10 mg/ml) concentration was added and mixed immediately by inversion in which the final concentration of the ethidium bromide was 600 µg/ml, it was centrifuged 10 min. ,8000rpm at 20°C.

3. The empty part of the tube was filled with light paraffin oil and sealed using electric-thermo fuser.

4. The tubes were centrifuged 45 000xg for 36 hours at 20°C.

5. The tubes were removed from the rotor, and fixed to aboard , a small hole at the top tube was made , then # 21 hypodermic needle inserted into the side of the tubes just below the plasmid band , and withdrawal completely.

3.2.18 Removal of Ethidium bromide (Maniatis *et al.*, 1982).

1. An equal volume of 1-butanol saturated with water was added to the cesium chloride –ethidium bromide solution containing the plasmids.
2. The two phases were mixed by pipetting vigorously and Centrifuged at 3000xg for 3 min. at room temperature.
3. The lower aqueous phase was transferred by a clean pipette; the same steps mentioned above were repeted until the pink color disappeared completely from the aqueous solution.
4. The aqueous solution was dialyzed against the TE buffer overnight (pH 8.0), three times, and dissolved in 500 µl , kept at -20ºC.

3.2.19 Digestion of DNA by *Hin*d III (Maniatis *et al.*, 1982)

All items used in the restriction experiment were kept on ice during the experiments, and then transferred to the water bath of 37°C in which the reaction was occurred.

One unit of *Hin*d III is defined as the amount required digesting 1µg of lambda DNA in 1 hour at 37°C in 50µl of assay buffer.

3.2.19.1 Partial digestion of *B. stearothermophilus* genomic DNA (Rodriguez and Tait, 1983)

For partial digestion, four duplicate pilot experiments were made,

The DNA concentration was set up to be 1 µg / µl, and the stock restriction enzyme 10U/µl was diluted using the dilution buffer according to the manufacture to be 1U/µl.

	Experiment One	Experiment Two	Experiment Three	Control
ddH_2O	12 µl	12 µl	12 µl	14 µl
Buffer 10 X	2 µl	2 µl	2 µl	2 µl
DNA volume µl	4 µl	4 µl	4 µl	4 µll
***Hin*d III 1U/µl**	2 µl	2 µl	2 µl	-
Total Reaction volume µl	20 µl	20 µl	20 µl	20 µl
Reaction Time	20 min.	30 min.	40 min.	1 hour

The reaction was stopped by the addition of 4 µl of 0.5 M EDTA pH 7.5 and by being mixed together.

3.2.19.2 Digestion of pBR322 plasmid with *Hin*d III (Rodriguez and Tait, 1983)

ddH_2O	14 µl
Buffer 10X	2 µl
*Hin*d III 1U/µl	2 µl
pBR 322(1µg/µl)	2µl
Total reaction volume	20 µl
Time	2 hours

The reaction was stopped by the addition of 4 µl of 0.5 M EDTA pH 7.5 and mixing gently.

3.2.20 Dephosphorylation of p*BR* 322 (Elyassaki, 1991)

ddH_2O	22 µl
CIP buffer 10X	5 µl
pBR 322(2µg/20µl)	20 µl 10 mM Tris-HCl (pH 7.8)
Intestinal calf alkalane phosphatase	3 µl
Total volume	50 µl

The mixture was incubated at 37°C for 15 min., then switched to 50°C for 20 min., additional 3µl of enzyme was edded, and the same procedure was repeated.

TE buffer was added to the reaction mixture to bring the total volume to 250µl, chloroform extraction was applied, followed by chilled ethanol precipitation, and the pellet was then resuspended in 50 µl of sterilized D.W

3.2.21. Ligation (Elyassaki ,1991, Sambrook and Russell,2001)

Three duplicates of pilot experiments were carried out in order to achieve successful ligation reaction, optimum ligation was performed according to the formula (with some modifications concerning the concentrations of the nucleic acids)

Insert concentration / vector concentration = 5:1 as a ratio

	Experiment 1	Experiment 2	Experiment 3	Control
ddH_2O	10 µl	9 µl	8 µl	12 µl
Ligation buffer 10X	2 µl	2 µl	2µl	2µl
Insert DNA	5 µl	5 µl	5 µl	5 µl
pBR322(10ng/ml)	1 µl	1 µl	1 µl	1 µl
T4 ligase(30U/µl)	2 µl	3 µl	4 µl	-
Total volume µl	20 µl	20 µl	20 µl	20 µl

The mixture was incubated in a water bath at 16°C for 18 hrs. then it was diluted to a final concentration of 2 ng/µl

3.2.22 Agarose gel (For DNA analysis):

Agarose 0.9 % concentration was used, dissolved in TBE 1X (3.1.5.11)

3.2.23 Transformation by Calcium chloride and detection of transformants (Maniatis *et al.*,1982; Hanahan ,1983; Mielenz ,1983;Tang *et al.*,1994).

1. A hundred ml of LB was inoculated with 1 ml of an overnight culture of *E.coli* M M294 , in flask and incubated at 37ºC with shaking until the O.D 600 nm was 0.2 (~ 5 x 10 cells/ ml) .
2. The culture was chilled on an ice bath for 5 min., and then centrifuged at 4000xg for 5 min. at 4ºC.
3. The supernatant was discarded, and the cells resuspended in 50 ml of sterile solution of 50 mM $CaCl_2$,10 mM Tris.Cl (3.1.5.29)pH 8.0.
4. The cell suspension was placed into ice bath for 15 min., and then it was centrifuged at 4000xg for 5 min. at 4ºC.
5. The supernatant was discarded, and the cells resuspended in 7 ml of ice, sterile buffer solution (3.1.5.29) (pH 8.0), the cells were dispensed into 0.2 ml prechilled tubes, and kept at overnight at 4ºC .
6. Ligated DNA dissolved in TE buffer in concentration of 30 ng / 100 µL was added to the 200 µl of cell suspension, mixed and stored into an ice bath for 30 min, then transferred to a water bath, preheated to 42 ºC for 2 min., then transferred to an ice bath for 1 min.
7. One hundred µl of LB was added to each tube ,and incubated at 37 ºC for an hour without shaking
8. Serial dilutions for the cells after the incubation period were made, then the cells were spreaded over nutrient agar plates containing ampicillin (50 µg /ml) and incubated at 37ºC overnight .

9. Replica plating technique was applied for the single Amp^r colonies , assuming the master plate is nutrient agar plate , while the detection plate for the expression of possible α- amylase gene was plates containing detection media(3.1.3.2.13) , incubated at 37ºC for 24 hours . Positive colonies were surrounded by a clear zone on the plating medium, while negative control was *E. coli* MM294 before transformation.
10. Positive transormant(s) –α-amylase producing colonies were cultured in liquid-starch media (3.1.3.2.16) to assay the specific activity of the α-amylase encoded by the possible recombinant α-amylase gene as mentioned in (3.2.7).

1.1 Introduction

Enzymes are complex proteins that act as catalysts for the countless biochemical reactions that keep humans, animals, plants, and microorganisms alive. Enzymes have an importance in the industrial and commercial uses as well. Since ancient times, people have observed enzymes at work when fermenting their wine and beer, turning their sour milk into cheese, and causing their bread dough to rise.

In 1833, the French chemist Anselme Payen separated a substance from an extract of malt that, he realized, seemed capable of speeding up the conversion of starch to sugar. Payen called the substance diastase (Greek expression separation, from, to stand apart; dia` through + to stand)diastase. (Webster, 1913), the first enzyme to be isolated and prepared in a concentrated form.

Starch-degrading, amylolytic enzymes are widely distributed among microbes. Several activities are required to hydrolyze starch to its glucose units. These enzymes including α-amylase, β-amylase, glucoamylase, α-glucosidase, pullulan degrading enzymes, and cyclodextrin glycosyltransferase. Properties of these enzymes vary and are somewhat linked to the environmental circumstances of the producing organisms, features of the enzymes, their action patterns, physicochemical properties, occurrence and genetics (Vihinen and Mantsala, 1989**) .**

Screening for new microbial strains producing the desired α-amylase from extreme environments has been reported (Sunna *et al.*, 1997; Niehaus *et al.*, 1999;Veille and Zeikus, 2001) .Utilization of high concentrations of starch, thermostability, and protein yield are important criteria for commercialization (Schafer *et al.*, 2000).

Genetic engineering has been used extensively for cloning of amylase-producing strains, mainly α-amylase and glucoamylase (GA), in order to

achieve desirable characteristics in the cloned host. The purpose of gene cloning can be, among others, the expression of thermostable enzymes, higher enzyme productivity, co-expression of two enzymes by the same organism or as less α-amylase contaminated with the other enzymes.

α-Amylase is found in saliva and pancreatic secretions, where they play an obvious role in polysaccharide digestion. More surprisingly, α-amylase was also found in blood, sweat, and tears, possibly for anti-bacterial activity.

α-Amylase determination has been recognized as an important diagnostic tool for many years, because the elevated levels of the enzyme are associated with liver and pancreatic disorders, as well as other diseases (Bookrags, 2006). Enzymes are the catalysts of all reactions in living systems. These reactions are catalyzed in the active sites of globular proteins. Enzymatic processes have been increasingly incorporated in textiles over the last years. Cotton, wool, flax, or starches are natural materials used in textiles that could be processed with enzymes. Enzymes have been used for desizing, scouring, polishing, washing, degumming, peroxide degradation in bleaching baths as well as for decolourisation of dye house wastewaters, bleaching of released dyestuff and inhibiting dye transfer. Furthermore, many new applications are under development such as natural and synthetic fibers modification, enzymatic dyeing and finishing. Most of the textile processes are heterogeneous where an auxiliary as a dye, enzyme, softener, or oxidant has to be taken from the solution to the fiber. These processes require the presence of surface-active agents, ionic force “balancers” buffers, stabilisers and others, and are characterized with high turbulence and mechanical agitation in the textile baths.

The present study attempts to:

1. Isolation and identification of *Bacillus stearothermophilus* from different soil samples.
2. Production, Purification and chractrization of some properties of thermoliable α-amylase.
3. producing Anti-amylae sera as polyclonal antibodies
4. Molecular cloning of α-amylase gene from *Bacillus stearothermophilus* in *E. coli* MM294 using pBR *322* vector, then investigate of the possible expression of α-amylase gene in the transformed cell(s).

2.1 *Bacillus*

Bacillus spp. as a genus, are gram positive , rodlet cells,shaped, straight or nearly so, endospores are very resistant to many adverse conditions ,formed not more than one per cell ; sporulation not repressed by exposure to environment. Aerobic or facultative anaerobic , catalase- positive ; oxidase –positive or negative ,G+C is (32-69%), singly or chains (0.5-1.2μm)–(2.5 - 10 μm) (Sneath *et al.*,1986).

The basis of classification and identification was established by Gibson and Gordon(1974), they allocate *Bacillus* spp. Into 19 species based on shape of endospores and position in the mother cells or sporangium. With the introduction of modern taxonomic techniques such as numerical phenetics ,DNA reassociation experiments have allowed DNA sequence homology between strains to estimate the range of DNA , It could be best be appreciated by numerical classification for which strains are examined for numerous physiological, biochemical and morphological characters similarities in which they were recovered in six large groups or aggregates of clusters (Nielsen *et al.*,1995).

According to the optimum temperature of growth *Bacillus* sp. could be placed in one of four classes :

1. Psychrophiles grow well at 0°C and have an optimum growth temperature of 15^{o}C or lower; the maximum is around 20^{o}C .
2. Many species can grow at 0^{o}C even though they have optimum between 20^{o}C and 30^{o}C ,and maximum at about 35^{o}C these are referred to as psychrotrophs or facultative psychrophiles .
3. Mesophiles are microorganisms with growth optima around 20-45^{o}C and a temperature maximum of 15^{o}C to 20^{o}C.

4. Thermopiles can grow at temperature of 55^{o}C or higher –their growth minimum is usually around 45^{o}C and they often have optima between 55^{o}C and 65^{o}C (Prescott *et al.*,1990)

2.2 *Bacillus stearothermophilus*

Bacillus stearothermophilus, means fat-and heat –loving, The most distinctive diagnostic characterictic is the capacity to grow at 65 ^{o}C and a limited tolerance to acid.It occurs in arctic water, ocean sediments, food and compost, mol % G+C of the mol% DNA which is reported to be(43.5-52.6) (Sneath *et al.*,1986).Thermophiles are adapted to temperature above 60 ºC in a variety of ways, often thermopiles have a high (%G + C) content in their DNA such that the melting point of the DNA (the temperature at which the strands of the double helix separate) is at least as high as the organism's maximum for growth, but this is not always the case, and the correlation is far from perfect, so thermophile DNA must be stabilized in these cells by other means. The membrane fatty of thermophilic bacteria are highly saturated allowing their membranes to remain stable and functional at high temperatures (Daron ,1970) .

Membranes of hyperthermophiles ,virtually all of which are Archaea ,are not composed of fatty acids but of repeating subunits of the C5 compound , phytane , a branched ,saturated,"isopernoid" substances ,which contributes heavily to the ability of these bacteria to live in superheated environments (Prescott *et al.*,1990).The structural proteins (e.g. ribosomal proteins, transport proteins permease) and enzymes of thermophiles and hyperthermophiles are very heat stable compared with their mesophilic counterparts .The proteins are modified in a number of ways including dehydration and through slight changes in their primary structure ,which accounts for their thermal stability (Kenneth and Todar , 2005).

2.2 Starch:

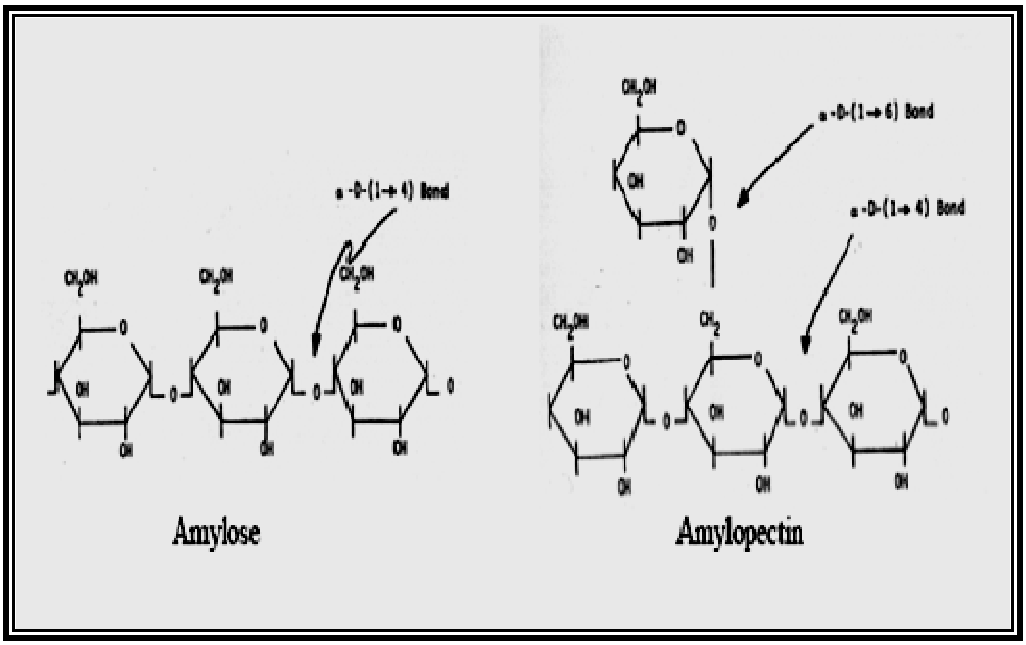
2.2.1 Molecular structure of Starch

Corn starch is found in granules within the kernel as a long polymer composed of two structural classes: amylose and amylopectin. The basic repeating unit for both types of starch is D-glucose molecules, connected by glycosidic bonds (figure 2.1). The polymer chains and the formation of the intermolecular network traps water and results in gel formation and solution thickening, after the starch is completely hydrolyzed or broken down, its basic component is D-glucose also called dextrose or corn sugar (Koivula,1996).

Amylose is a lanear polymer of short 1, 4-linked glucose chains. Typically, the amylose fraction is about 25-30% of the starch molecules found in corn and has a molecular weight of about (250 kd), The percentage of amylose in the starch is genetically determined, Genetic modifications producing high-amylose (50-70%) cornstarch are also found. amylopectin comprises about 70-75% of the starch found in the corn kernel and has a molecular weight of about 50-500 million Daltons, Amylopectin is a branched polymer of the basic repeating units of 1,4 linked glucose with branches of 1, 6 linked glucose, The branching occurs irregularly in the starch, approximately one per twenty-five glucose units (Karkallas, 1985**;** Randleman, 2000).

The corn starch separated from the kernel by the wet milling process is generally 99% pure and contains 0.25-0.35% protein, 0.5-0.6% lipid and less than 0.1% minerals. 35% of the industrially prepared cornstarch is utilized by the food industry; the remainder of the starch is further refined or modified for use in the paper and construction industries, A significant proportion of the corn starch derived from the wet-milled process used for food goes into

the fermentation of alcohol. It first has to be converted into dextrose or D-glucose(Karkallas ,1985;Bhosale *et al.*,1996).



Figure(2.1):Amylose and amylopectin of starch modified from (Koivula, 1996)

2.2.2 Starch in industry work

Enzymes of various types are used in these processes. Although starches from diverse plants may be utilised, corn is the world's most abundant source and provides most of the substrate used in the preparation of starch hydrolysates (Hazare, 2005).

There are three stages in the conversion of starch (figure 2.2) (Bhosale *et al.*, 1996)

1. Gelatinisation, involving the dissolution of the nanogram-sized starch granules to form a viscous suspension.
2. Liquefaction, involving the partial hydrolysis of the starch, with concomitant loss in viscosity.
3. Saccharification, involving the production of glucose and maltose by further hydrolysis.

Gelatinisation is achieved by heating the starch with water, and occurs necessarily and naturally when starchy foods are cooked. Gelatinised starch is readily liquefied by partial hydrolysis with enzymes or acids and saccharified by further acidic or enzymatic hydrolysis (Chaplin, 2003; Crabb and Shetty, 2003).

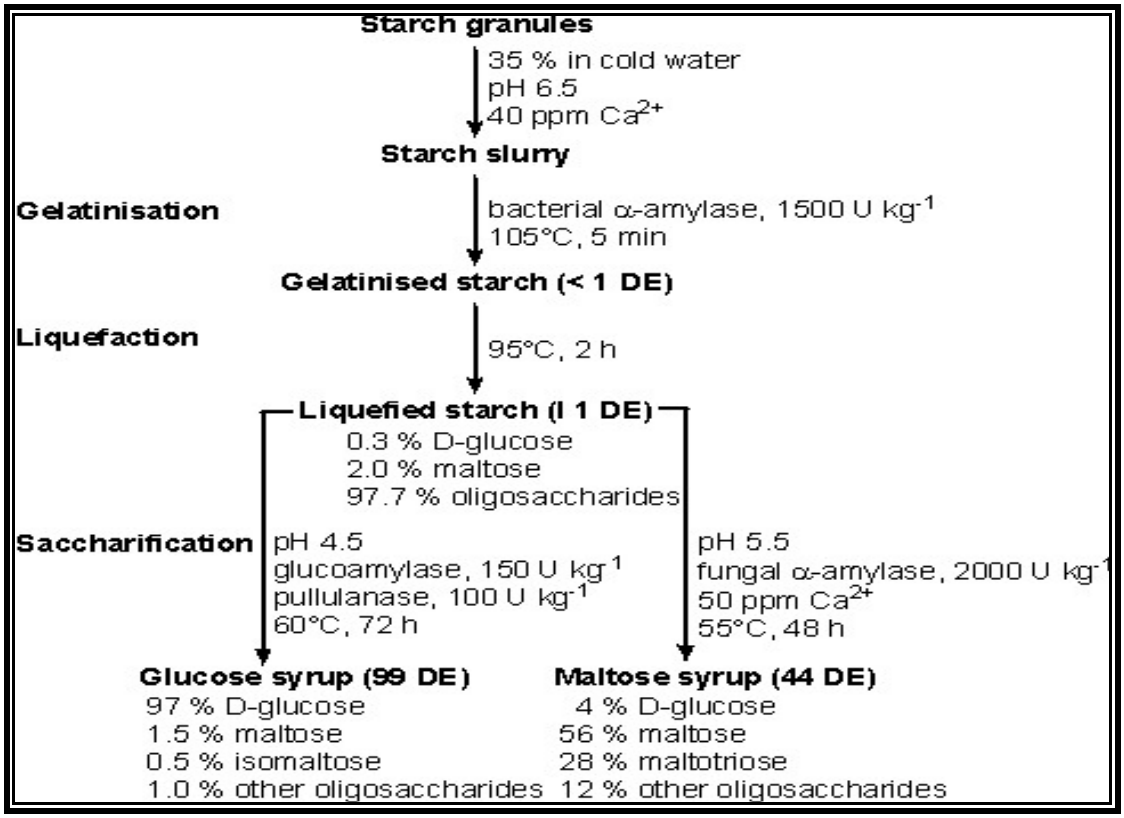


Figure (2.2) Starch hydrolysis as in the industrial applications (Chaplin , 2003).

DE, represents the percentage hydrolysis of the glycosidic linkages present

2.3 Microbial amylolytic enzymes

Starch-degrading, amylolytic enzymes are widely distributed among microbes. Several activities are required to hydrolyze starch to its glucose units. These enzymes include α-amylase, β-amylase, glucoamylase, α-glucosidase , pullulan-degrading enzymes, exo-acting enzymes yielding α-type end products, and cyclodextrin glycosyltransferase table (2.1). Properties of these enzymes vary and are somewhat linked to the

environmental circumstances of the producing organisms **(**Vihinen and Mantsala , 1989).

2.3.1 Amylases

A term that refers here to α-amylase, β-amylase and glucoamylase (Pandey *et al.,* 2000), which hydrolyze starch molecules to give diverse product including dextrin and progressively smaller polymers composed of glucose units (Reddy *et al.*, 2003).

2.3.1.1 α-amylase

With the advent of new frontiers in biotechnology, the spectrum of α-amylase application has expanded into many other fields, such as clinical, medicinal, and analytical chemistries. α- amylase has been in common use to control the viscosity of chocolate syrup since 1929 and in the brewing industry since 1936 that amylase find potential application in anywhere of industrial process such as in the food, textile and paper (Shaw *et al.*, 1995). Furthermore, α-amylases are used as targets for drug design in attempts to treat diabetes, obesity, and hyperlipidmia. The widening interest in the treatment of sugar metabolic disorders has been stimulated to search for new and efficient drugs to apply them as inhibitors of amylolytic enzymes (Kandra *et al.*, 2002).

α-amylase is a class of enzymes that is capable of digesting these glycosidic linkages found in starches, α-amylases can be derived from a variety of sources, Amylases are present in all living organisms, but the enzymes vary in activity, specificity, and requirements from species to species and even from tissue to tissue in the same organism .α-amylase (1,4α-D-Glucan-glucanohydrolase) acts upon large polymers of starch at internal bonds and cleaves them to short glucose polymers, α-amylase catalyzes the hydrolysis of internal α-1-4 glucan bonds in polysaccharides

containing 3 or more α-1-4 linkages; it results in a mixture of maltose and glucose. Amyloglucosidase works on the shorter polymers and splits off single glucose sugars. Bacterial α-amylase is particularly suited for industrial usage since it is inexpensive and is thermally stable (Karkallas, 1985).

α-amylase (EC 3.2.1.1) (IUB,1979) catalyzes the cleavage of the α-1,4-glucosidic linkages between glucose molecules in starch. This hydrolysis of starch is a common first step in conversion of starch into an utilizable substrate for fermentation or for conversion to dextrose and high fructose syrups (Pandey *et al.*,2000).In addition, α-amylases can sometimes cleave α-1,6 linkages with a low catalytic rate and they can bypass an α-D-1,6 branch linkage, (Koivula ,1996) .

α-Amylase producers of commercial importance include *Bacillus subtilis , Bacillus licheniformis, Bacillus amyloliquefaciens* and *Bacillus stearothermophilus;* a gram-positive thermophilic bacterium, produces an α-amylase during growth at temperatures of 55-70°C .This α-amylase is highly thermostable, therefore, it is more desirable for industrial applications of the enzyme(Ito and Horikoshi, 2004).

2.3.1.2 β-amylase

β-amylase (α-1.4-glucan maltohydrolase EC 3.2.1.2) is usually of plant origin ,but a few microbial strains are also known to produce it ,its an exoacting enzyme that cleaves non-reducing chains ends of amylose amylopectin and glycogen molecules. It hydrolytes alternate glycosidic linkages ,yielding maltose (β-anomeric form). β-amylase is unable to break α-1,6-glycosidic linkages in amylopectin (Hagihara *et al.*,2001;Ha *et al.*,2001).

2.3.1.3 Glucoamylase (GA)

Synonyms ;Amyloglucosidase, Glucogeninc amylase ,Starch glucogenase and γ-Amylase;exo-1,4-α-D-glucan glucanohydrolase, (EC 3.2.1.3)

hydrolase ,hydrolyze single glucose unit from the non reducing ends of amylose and amylopectin in a stepwise manner unlike α-amylase. Most glucoamylase are also able to hydrolyze the α-1,6 linkages at the branching points of amylopectin although at lower rate than 1,4- linkage; thus, maltose and limit dextrin are the end products of GA(Koviula,1996).

2.3.1.4 Pullulanase

Which is also called Neopullulanase , Amylopullulanase and Pullulanases , has important applications , particularly in the food and detergent industries. (Haseltine *et al.*, 1996).α-dextrin 6-glucanohydrolase (EC3.2.1.41) which have a primary affinity for α-1,6 linkages and catalyse the hydrolysis of α-1,6-glycosidic bonds in amylopectin and related polymers ,it is called debranching enzymes (Koivula,1996). Abe *et al*.(1994) reported that pullulnase from *Bacillus stearothernophilus* degraded the pullulan into panose, maltose, and glucose at a molar ratio of 3:1:1, with very weak activity on starchy substrates.

Table (2.1): Enzymes used in starch hydrolysis modified from (Hagihara *et al.*, 2001; Reddy *et al.*, 2003)

Enzyme	EC number	Action
α-amylase	**3.2.1.1**	**Only α-1,4-oligosaccharide links are cleaved to give α-dextrins and predominately maltose (G2),G3,G6 and G7 oligosaccharides**
β-amylase	**3.2.1.2**	**Only α-1,4 links are cleaved ,from non-reducing ends, to give limit dextrins and β-glucose**
Glucoamylase	**3.2.1.3**	**α-1,4 and α-1,6 links cleaved ,from the non reducing ends, to give β-glucose**
Pullulanase	**3.2.1.41**	**Only α-1,6-links are cleaved to give panose.maltose and glucose**

2.4 Structure of α-amylase

A feature common to all reported α-amylase structures is their $(\beta\alpha)_8$ catalytic core-domain, termed domain A. An excursion between barrel strand β-3 and helix α-3 forms the B domain, whilst the C domain, frequently an eight-stranded β -sheet, lies at the C-terminal extremity of the barrel. All family 13 members possess a constellation of three acidic residues, located at the C-terminal face of the $(\beta\alpha)_8$ -barrel, which are implicated in the catalysis (Brzozowski *et al.*, 2000).

α-amylases perform catalysis with net retention of anomeric configuration in a double-displacement mechanism. The mechanism involves the formation, and subsequent breakdown, of a covalent glycosyl-enzyme intermediate *via* oxocarbenium-ion like transition state. One of the catalytic acidic residues functions as the catalytic nucleophile whilst another function is the catalytic acid/base (Davies *et al.*, 1997),they possess a $(\beta/\alpha)_8$ or TIM barrel structure (which is similar to triose phosphate isomerase) containing the catalytic site residues(Reddy *et al.*,2003).While in Psychrophile *Alteromonas haloplanctis amy* gene encodes an α-amylase precursor composed of 669 amino acid residues. N- and C-terminal amino acid sequences of the native-amylase secreted by *A. haloplanctis* allow the location of three distinct functional domains of the precursor: 1- The peptide signal made of 24 residues, 2-The mature enzyme composed of 453 residues with a *M*r value of 49,340, and 3- Large C-terminal propeptide composed of 192 residues (Feller *et al* ., 1998).

The available amino acid sequences of the α-amylase family (glycosyl hydrolase family 13) have been searched to identify their domain B, A distinct domain that protrudes from the regular catalytic $(\alpha/\beta)_8$ barrel between the strand α-3 and the helix β-3.Sequence analyses and inspection of the few available three-dimensional structures suggest that the secondary

structure of domain B varies with the enzyme specificity. Domain B in these different forms. However, may still have evolved from a common ancestor. The largest number of different specificities has been found in the group with structural similarity to domain B from *Bacillus cereus* oligo-1, 6-glucosidase that contains a β-helix succeeded by a three-stranded antiparallel α-sheet (Skov *et al.*,2001).Domain B, of this type, was observed also in some mammalian proteins involved in the transport of amino acids, These proteins show remarkable similarity with $(\alpha/\beta)_8$-barrel elements throughout the entire sequence of enzymes from the oligo-1,6-glucosidase group. The transport proteins, in turn, resemble the animal 4F2 heavy-chain cell surface antigens, for which the sequences either lack domain B or contain only parts (Janecek *et al.,* 1997).

2.5 Structural and functional characteristics

α-Amylases are often divided into two categories according to the degree of hydrolysis of the substrate (Fukumoto and Okada, 1963), Saccharifying α-amylases hydrolysis 50 to 60 % , and liquefying α-amylases about 30 to 40 % of the glycosidic linkages of starch. However, this division is not an absolute one. Some bacteria produce an extracellular enzyme cyclodextrin glycosyltransferase (CGTase) (1,4-α-D-glucan4-α-D-(1,4-α-D-glucano) transferase , EC (2.4.1.19), which hydrolysis α-1,4-glycosidic bonds of starch to produce cyclodextrins (cyclic compounds of six to eight a-1,4-linked glucose units) via an intramolecular transglycosylation reaction. In addition, CGTases can display coupling, disproportionation and hydrolysing activities, and are functionally related to α-amylases(Macgregor and Svensson , 1989).

The primary structures of CGTases and α-amylases from different organisms share about 30 % amino acid sequence identity and all belong to the same

glycosyl hydrolase family 13 (Henrissat and Bairoch, 1993). Other related amylolytic enzyme in family 13 is pullulanases (Cheong *et al.*, 2002).

The 3D structures of α-amylases have revealed monomeric, calcium-containing enzymes, with a single polypeptide chain folded into three domains (A-C) figure(2.2). While, the polypeptide chains of CGTases fold into five domains (A-E), three of these correspond to α-amylase domains and domain E has been recognized as having a similar fold as the starch-binding domain of glucoAmylases). Domain D of CGTases has an immunoglobulin-type fold, the function of which is not yet known (Koivula ,1996).

Dauter *et al.(*1999)described maltogenic α-amylase of *B. stearothermophilus* which is composed of five domains that is similar to the CGTases structure using X-ray technique, figure (2.3).The catalytic domain A of α-amylases and all family 13 hydrolases folds into an $(a/b)_8$ barrel protein. The active site is created by residues located at the C-terminus of the b strands and in the helix-connecting loops extending from these ends. This is typical to all enzymes belonging to the a/b -barrel protein family .The domain B protrudes from the middle of the barrel at the C-terminal side and forms a small separate, Ca^{2+} -stabilized structural domain, which has also been recognized in a number of other amylolytic enzymes (Jespersen *et al.*, 1991; Aghajari *et al.*,2002).The sequence of this domain varies most; in *Bacillus* α- amylases, the sequence is relatively long and folds into a more complex structure of b-strands (Machius *et al.*, 1995), whereas in barley α-amylase it is an irregularly structured domain of 64 residues (Kadziola *et al.*, 1994).

The open active site cleft is formed between domains A and B, so that residues from domain B also participate in substrate binding. Based on the solved 3D structures it could be deduced that the conserved regions of α-amylases and CGTases involve the calcium-binding site between domains A

and B, and regions belonging to the active site (Macgregor and Svensson, 1989; Klein and Schultz, 1991; Lawson *et al.*, 1994).

α-Amylases have a domain C which is relatively conserved and folds into an antiparallel β-barrel, The orientation of domain C relative to domain A varies depending on the type and source of Amylase (Brayer *et al.*, 1995). The function of this domain is not known (koivula, 1996).

A study about *B. circulans* CGTase (Lawson *et al.*, 1994) and another from pig pancreatic α-amylase (Qian *et al.*, 1994), both clearly showed based on electron densities, that maltose units stack against the aromatic side-chain of a tryptophan situated on the surface of domain C (Fig. 2.2,2.3). The truncation of domain C more than 30 to 40 amino acids from the C-terminus was shown to be deleterious to the proper folding of the *B. stearothermophilus* α-amylases (Vihinen *et al.*, 1994).

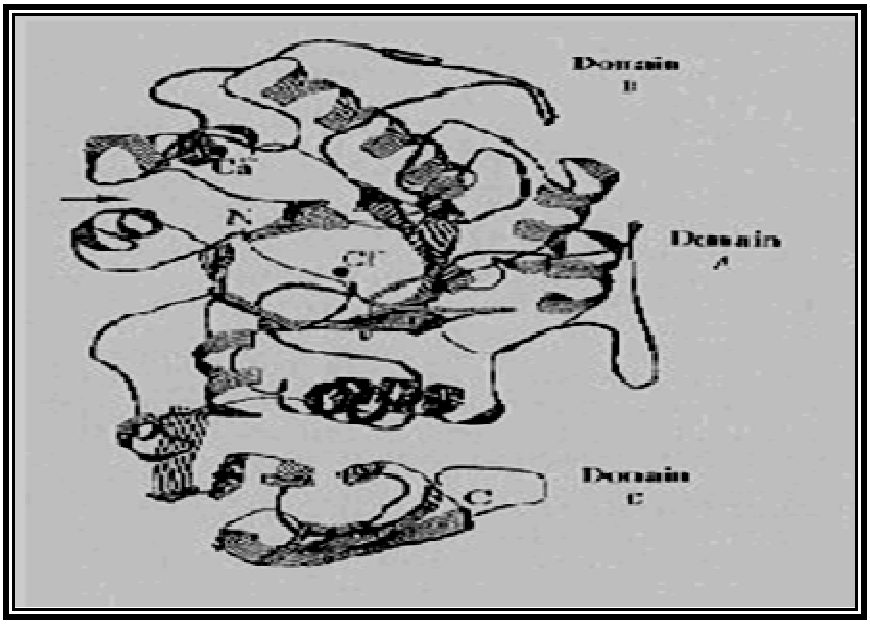


Figure (2.2): Ribbon presentation of human pancreatic α-amylase is composed of three domains (A-C) (Koivula, 1996)

While Aghajari *et al.(*2002) described that α-amylase produced by animals and *Pseudoalteromonas haloplanktis* activated by chloride .

The thermostability and structural integrity of the α-amylase due to the presence of three Ca^{+2} (Srivastava and Baruah,1986) and one Na^{+} ions has been found in *Bacillus licheniformis* , a lanear triad CaI- Na-CaII arrangement figure (2.4) ,is located at the interface between domain A and B. another Ca^{2+} (CaIII) bridges between domains A and C. (Ito and Horikoshi, 2004).In addition, the prolane residues are to be the backbone rotation proposed a prolane of β- turn make α-amylase more stability (Matthews *et al.*, 1987).

Figure (2.3):Divergent stereo schematic diagram of the maltogenic α-amylase , novamyl, from *B. stearothermophilus* (Dauter *et al.*,1999)

All α-amylases bind at least one strongly conserved Ca^{2+} ion that is required for structural integrity and for enzymatic activity (Aghajari *et al.*, 2002).Feller *et al.(*1996) showed that the chloride free α-amylase display a low level of basal activity , typically < 0.5 % , and chloride follows a simple binding isotherm . Activation is also provided, but to a lower level, by other small monovalent anions and notably by NO_3^- or ClO_3^- indicated that the negative charge is essential for the amylolytic reaction (Aghajari *et al.*, 2002).

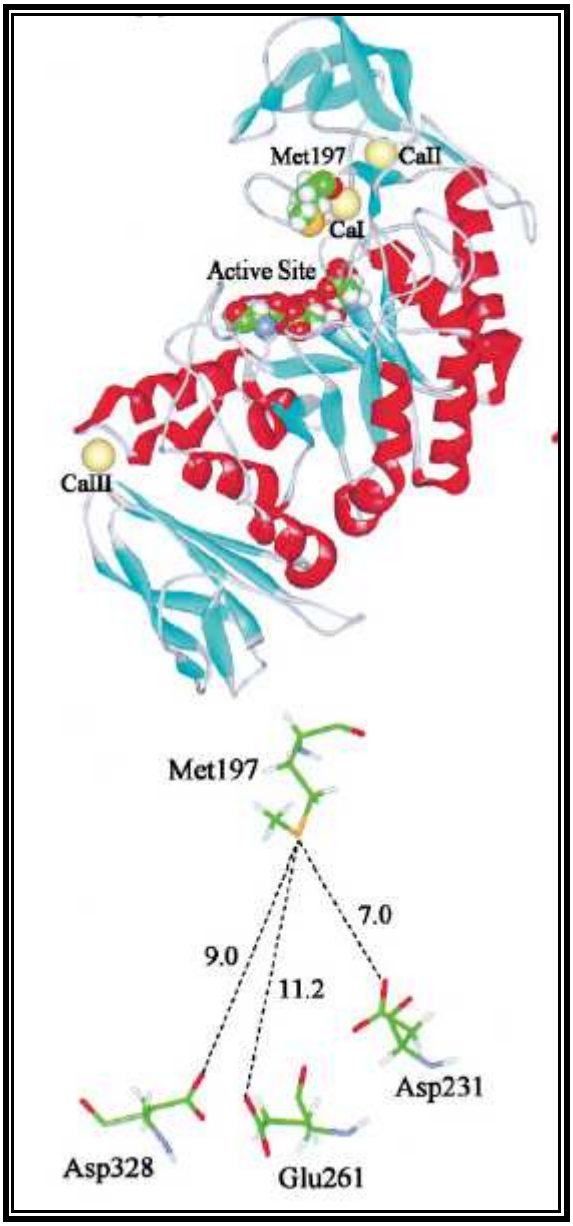


Figure (2.4): Ribbon representation of the crystal structure of *Bacillus licheniformis* template active site . α helices and β sheets are shown in red and cyan , respectively. The catalytic residues Asp231, Glu261, and Asp328 along with the subsite Met197. Yellow spheres represent Ca ions (I, II, III). oxygen atoms in red, nitrogen atoms in purple, carbon atoms in green, and hydrogen atoms in white (Hagihara *et al.*,2001)

2.5 Breaking down of *O*-glycosidic bond

Amylases, like all glycosidases, catalyze the transfer of a glycosyl group to water utilizing general acid catalysis. The enzymatic breakdown of a glycosidic bond is also a stereo- selective process, in which configuration about the anomeric centre (C1 carbon) can either be inverted or retained. Different mechanisms are required for such different stereochemical outcomes, as suggested over 50 years ago by Koshland (1953); both mechanisms contain a pair of carboxylic acid residues suitably disposed on either side of the bond to be cleaved. The roles of these two residues are different. Inverting enzymes use a single-displacement mechanism involving a general acid and a base. In this mechanism, a general acid (AH) donates a proton to the leaving glycosidic oxygen and a general base (B-) assists the nucleophilic attack of water.

O-Glycosylhydrolases (EC 3.2.1.) are a widespread group of enzymes that hydrolyze the glycosidic bond α-amylase family in that of the α- retaining double displacement (van der Maarel *et al.*, 2002)**.** α-retaining mechanism is the characteristic feature of the enzymes from the α-amylase family. They vary widely in their reaction specificities. The attachments of different domains to the catalytic site or to extra sugar binding subsites around the catalytic site are the prime reason for these differences (Van der Maarel *et al.*, 2002).

The catalytic domain A is the most conserved domain in the α-amylase family, It consists of an amino terminal $(\beta/\alpha)_8$ barrel structure. (Matsuura *et al.*, 1984; Boel *et al.*, 1990).The mechanism for retaining glycosidases involves a double displacement reaction in which a covalent glycosyl-enzyme intermediate has been shown to be formed with many but not all retaining enzymes (Skory and Freer, 1995), As a first step the deprotonated carboxylate acting as a nucleophile attacks at the anomeric centre and displaces the glycosidic oxygen. This process is assisted by a general acid

catalytic residue (AH) which is deprotonated (A-), In the second step water attacks at the anomeric centre with general base-catalytic assistance (A-) displacing the nucleophile and releasing the product sugar (Koshland, 1953; Sinnott, 1990; Withers and Aebersold, 1995).

The stereochemical course of the hydrolysis is usually determined by proton NMR, in which the A- and B-anomeric protons give different chemical shifts (Withers *et al.,* 1986).In most cases that studied the same enzymatic mechanism, (inverting or retaining) has been conserved within a glycosyl hydrolase family (Gebler *et al.,* 1992). Thus, both the fold of the protein and the stereochemistry of the hydrolysis reaction appear to be conserved within a given family.

Retaining enzymes are also able to bring about transglycosylation which, in the second step of the reaction another oligosaccharide instead of a water molecule, attacks at the anomeric centre, leading to elongation of the saccharide chain , figure (2-5) (Sinnott, 1990).

Transglycosylation was described to play a role in the induction of cellulase promoters (Biely *et al.,* 1991; Fowler *et al.,* 1993; Nevalainen and Penttila, 1995) and it could be applied in the enzymatic synthesis of oligosaccharides (Ogawa *et al.,* 1990; Gusakov *et al.,* 1991).

Inverting amylases such as glucoamylases have been shown to be able to reverse the hydrolysis by condensation reaction (Nikolov *et al.,* 1989;Sinnott, 1990). This ability has also been used in the synthesis of some gluco-oligosaccharides (Nakano *et al.,* 1995) .With some retaining enzymes, most studied with lysozyme, attempts to prove the existence of a covalent glycosyl-enzyme intermediate have failed (Sinnott, 1990). Another type of mechanism has been suggested in which the positively charged oxocarbonium is stabilized by the catalytic nucleophile through electrostatic interactions (Sinnott, 1990; Koivula, 1999).

In addition, ring distortion at the subsite preceding the scissile bond was suggested as an element of the catalytic machinery for both inverting and retaining glycosyl hydrolases (Harris *et al.,* 1993; Strynadka and James, 1991; Kuroki *et al.,* 1993; Varghese *et al.,* 1992). The identification of active site carboxylic acid residues usually occurs through X-ray crystallographic determination of the 3D structure of protein (Dauter *et al.,* 1999). In the case of retaining enzymes, the active site carboxylic acids have been identified by the use of affinity labels or mechanism-based inactivators to specifically derivatives the key amino acids. 2- Deoxy-2-fluoro-derivatives of glucose and cellobiose have successfully been used as mechanism-based inhibitors, which covalently bind the nucleophile in the retaining enzymes (McCarter and Withers, 1994; Withers and Aebersold, 1995).

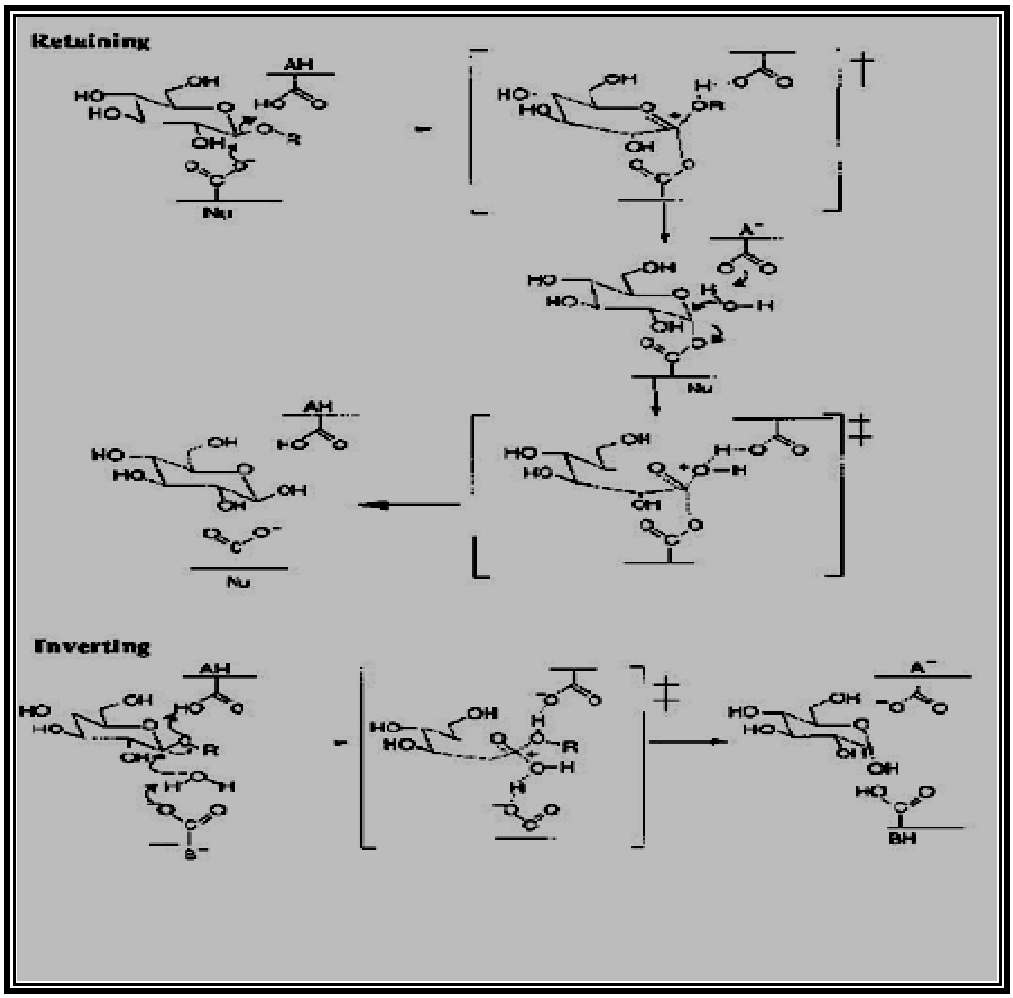


Figure (2.5) Two different reaction mechanisms of glycosyl hydrolases (Koivula ,1996).

2.6 α-Amylase isoenzymes (Forms)

Electrophoretic analyses of barley endosperm at different stages after germination allowed the detection of 16 principal amylase bands plus one secondary band(Maris,1992).Amylases showed less polymorphism than other enzymes such as esterases, acid phosphatases, malate dehydrogenases, leucine aminopeptidases (George and Cavali,2003).

Amylases are hydrolases involved in peripheral metabolism, and for this reason are considered potentially polymorphic enzymes (Gillespie and Langley, 1974). *Bacillus* sp. showed two and three isoenzymes of α-amylase (Lin *et al.,* 1998).Brazilian barley cultivars showe different isoenzymes ,the major differences in Amylase patterns found when barley varieties were analyzed at the same developmental stage were at the regulatory level. These differences are probably due to differential gene activation in the different genotypes (George, 1996).

The reasons behind the existence of more than one form could be the purification procedures, protease hydrolysis and deamination (Shih and Labbe,1995). The regulatory difference is of a greater adaptive and evolutionary significance than structural gene changes (Soule ,1973 ;Wilson, 1976) emphasized that the process involved in species differentiation requires changes mainly at the gene regulatory level. Differently, at the intraspecific level, gene regulation follows a standard development program with precisely coordinated sequences, although some regulatory mutants have been detected in different species (Holmes *et al.,* 1983; Doane *et al.,* 1983; Scandalios, 1983).

2.7 Methods of α-amylase quantitative estimations

Several methods were developed to detect of the amylolytic activity and to estimate the amylase activity precisely:

2.7.1 Viscometric method (Maslow and Davison)

It is based on the change in the viscosity of starch due to the amylases activity.

2.7.2 Polariscopic method (Maslow and Davison)

It is based on determining the reduction in light rotation to the right due to maltose and the maltosaccharides by the activity of amylases on starch.

2.7.3 Caldwell – Hildebrand method

This method is based on the direct determination of the residual starch or amylose at any stage in its hydrolysis, and depends upon its quantitative precipitation, free from dextrin and maltose, by ethyl alcohol. The difference between the weight of original amylose and the weight of residual amylose gives a measure of the amyloclastic activity of the enzyme analogous to the measurements of its saccharogenic activity which are based upon the determination of reducing the sugar formed.

2.7.4 Photometric method

This method depends on the difference in the intensity of the color produced with iodine by a measured amount of soluble starch before and after hydrolysis by the α-amylase.

2.7.6 Somogy-Nelson Colorimetric method

A photometric method, in which one unit of α-amylase activity is defined as the amount of enzyme that librates one µmol of reducing sugar equivalent per minute, using the glucose as a standard under the specified conditions (Abe *et al.*, 1994)

2.7.5 Fuwa`s colorimetric method (Fuwa, 1954)

It depends on iodine –starch color reaction. One unit of α-amylase activity is defined as the amount of enzyme, which decreases the absorbance at 660 nm, by 1.0 in 10 min.

2.7.7 Bernfield`s method (Bernfield, 1955)

A colorimetric method depends on using 3,5 dinitrosalicylic acid. One unit of amylase activity was defined as the amount of enzyme that librates one µmol of reducing sugar, with maltose as the standard, per minute under the specified conditions (Aiyer, 2004).

2.8 Engineering of α-amylase to improve the stability.

Since a thermostable α-amylase originating from *Bacillus licheniformis* was first employed in the starch liquefaction process in the early 70's ,α-amylases have been introduced into various industries.

The starch liquefaction process is still the main application of α- amylases, while the application to detergent (additive to detergent formulations) has been growing very much both for laundering and dish washing (Ito and Horikoshi, 2004). Protein engineering techniques have been employed for α-amylase development, which are to improve the characteristics of a targeted enzyme by introducing mutations in α-amylase encoding DNA sequence. The methods for protein engineering could be roughly categorized into two: site-directed mutagenesis and random mutagenesis (Miyoko and Henrik, 2000).

Site-directed mutagenesis is to introduce additions, deletions and substitutions of specific amino acids, and structural information of the targeted enzyme is essential to make those alternations (Bhosale *et al.*, 1996; Miyoko and Henrik,2000).

Computer technology is done to make molecular dynamics calculations and predictions for the structure when certain amino acids are substituted. (Laderman *et al.*, 1993; Hagihara *et al.*,2001).

Random mutagenesis is to introduce mutations at random along the entire length of a gene using error-prone PCR, DNA shuffling , chemical mutagenesis and UV irradiation. This method could develop new generation

of α-amylases as a detergent (Yoneda and Maruo, 1975).Miyoko and Henrik (2000), α-amylase could obtain improved a detergent alkalane stable and calcium independent by site-directed mutagenesis after microbial screening. Engineering of the available commercial α-amylases have been advocated hybrids of two homologous strains of the *B. licheniformis* and *B. amyloliquefaciens* (Suzuki *et al.*, 1989) and two regions that are important for thermostability has been identified. To improve the stability, disulphide bonds are introduced in the enzyme and alteration of the amino acids prone to the oxidation by an amino acid that is unaffected by oxidative agents (Barnett *et al.*, 1998;Agihara *et al.*2001).

Engineering α-amylase for changed pH of activity profiles, also would be in favor of the stability of the enzymes (Nielsen and Borchert, 2000;van der Maarel *et al*. ,2000 ; Gupta *et al*. ,2003) . Declereck *et al.*(2002) engineered a thermostable α-amylase from *B. licheniformis* using a structure based on mutagenesis, which could be used for an operation occurring over 50°C without perturbing significantly .

2.9 Regulation of α-amylase synthesis

It has been reported that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subject to catabolic repression by readily metabolically substrates such as glucose (Lin *et al.*,1998).

The addition of glucose to the culture has diminished greatly the synthesis of α-amylase similar to the findings reported by Haseltine *et al.(* 1996), who observed that glucose represses the production of α-amylase in the hyperthermophilic archaeon *Sulfolobus solfataricus*. According to them, glucose prevents α-amylase gene expression and not merely secretion of preformed enzyme, Levels of the α-amylase in crude culture supernatants vary greatly in response to the sole carbon source used for growth of the

organism(Srivastava and Baruah,1986). Substrate induction of this enzyme by starch emanated relatively early under all growth conditions tested, glucose has also been observed, which indicated that such repression of further enzyme synthesis might be due to the accumulation of starch hydrolytic products, including glucose (Saito and Yamamoto,1975).

The maltase is likely to play an important metabolic role in the accumulation of repressing levels of glucose, as the maltase exhibits considerable activity against dextrins which are the immediate products of α-amylase action repression of α-amylase synthesis by glucose represents one type of catabolic control over this enzyme and the second type which is the graded production of catabolic enzyme levels resulting from growth on other control is independent of the presence of starch,these two forms of regulation of α-amylase production may represent a generalized response to carbon source quality or availability (Rolfsmeier and Blum 1995; Haseltine *et al.*, 1996) .

α-amylase of *Streptomyces lividans* is regulated by *reg1*, a Pleiotropic regulatory gene *reg1* acts as a repressor of α-amylase genes in *S. lividans*. no other regulatory gene (repressor or activator) seems to control the expression of the α-amylase genes(Nguyen *et al.*, 1997;Van wezel *et al.*,1997) , since the α-amylases is at a maximum level in a minimal medium and is not further inducible by maltose when *reg1* is disrupted.*reg1* seems to be solely responsible for glucose catabolite repression of the α-amylase genes, which is completely lost upon *reg1* disruption. The dual role of *reg1* resembles that of *gylR*, the repressor of the *gyl* operon in *S. coelicolor* which is also involved in catabolite repression of this operon (Hindle and Smith, 1994). However, the relative locations of the two regulatory genes are different, as *gylR* is adjacent to the *gyl* operon under its control in *S. coelicolor* whereas *reg1* is not adjacent to α-amylase genes in *S. lividans* . By analogy with *malR* (Van Wezel *et al.*, 1997) it is likely that *reg1* is adjacent to the *malE*

homolog of *S. lividans* and therefore also ensures a dual control of the expression of this gene in *S. lividans*. (Nguyen *et al.*, 1997).

2.10 Secretion and Transport of α-amylase

Amylase secretion is mostly started at the logarithmic growth phase, in which extracellular secretion starts in the stationary growth phase parallel to degradation of cell wall components in the course of increased autolytic activity (Saito and Yamamoto,1975).

Permeability studies applying the (SUMs), S-layer ultrafiltarion membranes technique, and using culture supernatants for ultra filtration experiments showed that the S-layer lattice completely rejected the enzymes once they had assumed the final three dimensional structure, for *B. stearothermophilus* ,the passage of three Amylase through the pores in the S-layer lattice is only possible for enzyme molecules perfolded into smaller domains. After passing through the cell wall, complete folding of enzymes into their final three-dimensional structure will occur either on the cell surface or after the release into the culture fluid, it is unknown how the amylase is associated with S-layer protein. Generally, three possibilities exist 1- adhesion to S-layer surface 2- incorprtion of amylase into the S-layer lattice 3- binding of the enzyme to S-layer protein domain (Egelseer *et al.*,1995) .

Transport of protein by the major secretary pathways cross the bacterial wall in wall is a two-step mechanism via the periplasm ,Exporting, taking this two-step route, possesses an N-terminal signal peptide and use the general sec machinery for inner membrane translocation. Transport across the outer membrane in the second step requires a secretory apparatus encoded by several exproteins, when the *B. licheniformis* α-amylase, AmyL, is secreted from *B. subtilis*, it is subjected to considerable cell-associated proteolytic degradation (Stephenson, 1996).This proteolysis results in only a

proportion of the newly synthesized α-amylase being released into the culture medium. AmyL, an extracellular α-amylase from *Bacillus licheniformis,* is resistant to extracellular proteases secreted by *Bacillus subtilis* during growth. Nevertheless, when AmyL is produced and secreted by *B. subtilis*, it is subject to considerable cell-associated proteolysis. (Stephenson and Harwood, 1998).

In the gram-positive bacterium as *B. subtilis*, the only one protein outside the cytoplasmic membrane, PrsA, is known to be involved in protein secretion. PrsA is a lipoprotein that consists of a 33-kDa lysine-rich protein part and the N-terminal cysteine with a thiol-linked diacylglycerol anchoring the protein to the outer leaflet of the cytoplasmic membrane (Kontinen, *et al.*, 1991; Kontinen and Sarvas**,** 1993).

The PrsA protein is crucial for the efficient secretion of a number of exoproteins. In *prsA* mutants, the secretion and stability of some model proteins is decreased, while overproduction of PrsA enhances the secretion of exoproteins engineered to be expressed at a high level (Kontinen *et al.*, 1991).

Although the nature of the PrsA protein hints at an activity outside the cytoplasmic membrane, its mode of action and interaction with other components of the secretion apparatus and the specific steps of secretion in which it is involved remains to be elucidated.(Vitikainen *et al.*, 2001)

2.11 Amylase gene cloning

Genetic engineering has been used extensively for the cloning of amylase gene of producing strains, mainly *a*-amylase and GA, in order to achieve desirable characteristics in the cloned host. The purpose of gene cloning can be, amongst others, the expression of thermostable enzymes, higher enzyme productivity, and co-expression of two enzymes by the same organism.

1.8 KB fragment containing the amylase gene is sequenced(figure 2.6) .The fragment of the open reading frame (ORF) which begins with an ATG codon at nucleotide 1 and ends with TAA codon at nucleotide 1545 in the 1786 bp nucleotide sequence is determined in *Bacillus subtilis* (figure2.7) (Igarashi *et al.*,1998) .Upstream from this ORF ,the putative ribosome-binding sequence AGGAGA is found(Igarashi *et al.,* 1998,Hagihara *et al.,* 2001).The α-amylase gene in *Pyrococcus furiosus* encompasses 1950 nucleotides, with the initiation codon GTG at position 715 (Laderman *et al.,* 1993). Many genes involved in the synthesis of extracellular α-amylase have been identified in *B. subtilis.* The structural gene for the α-amylase enzyme, amyE, and its regulatory gene, (amyR), are closely linked on the genetic map of the *B. subtilis* chromosome. Furthermore, the gene order surrounding this α-amylase cistron is similar for three distinct strains of *B. subtilis*. The location of the gene for α-amylase from *B. stearothermophilus* has been examined by using recombinant DNA methods. In contrast to the gene for α-amylase from *B. subtilis*, the gene for α-amylase in *B. stearothermophilus* has been reported in one study that is located on a naturally occurring plasmid (Mielenz , 1983).

Igarashi *et al.*(1998) cloned 1.8 Kb fragment carrying the entire gene of α-amylase into *E. coli* using pUC19 as vector , which was actually expressed . The gene encoding β-amylase 1.8 kb was cloned from *Bacillus polymyxa* 72 into *E. coli* HB101 by inserting *Hin*d Ill generated DNA fragments into the *Hin*d III site of pBR*322* (Kawazu *et al.*,1987).

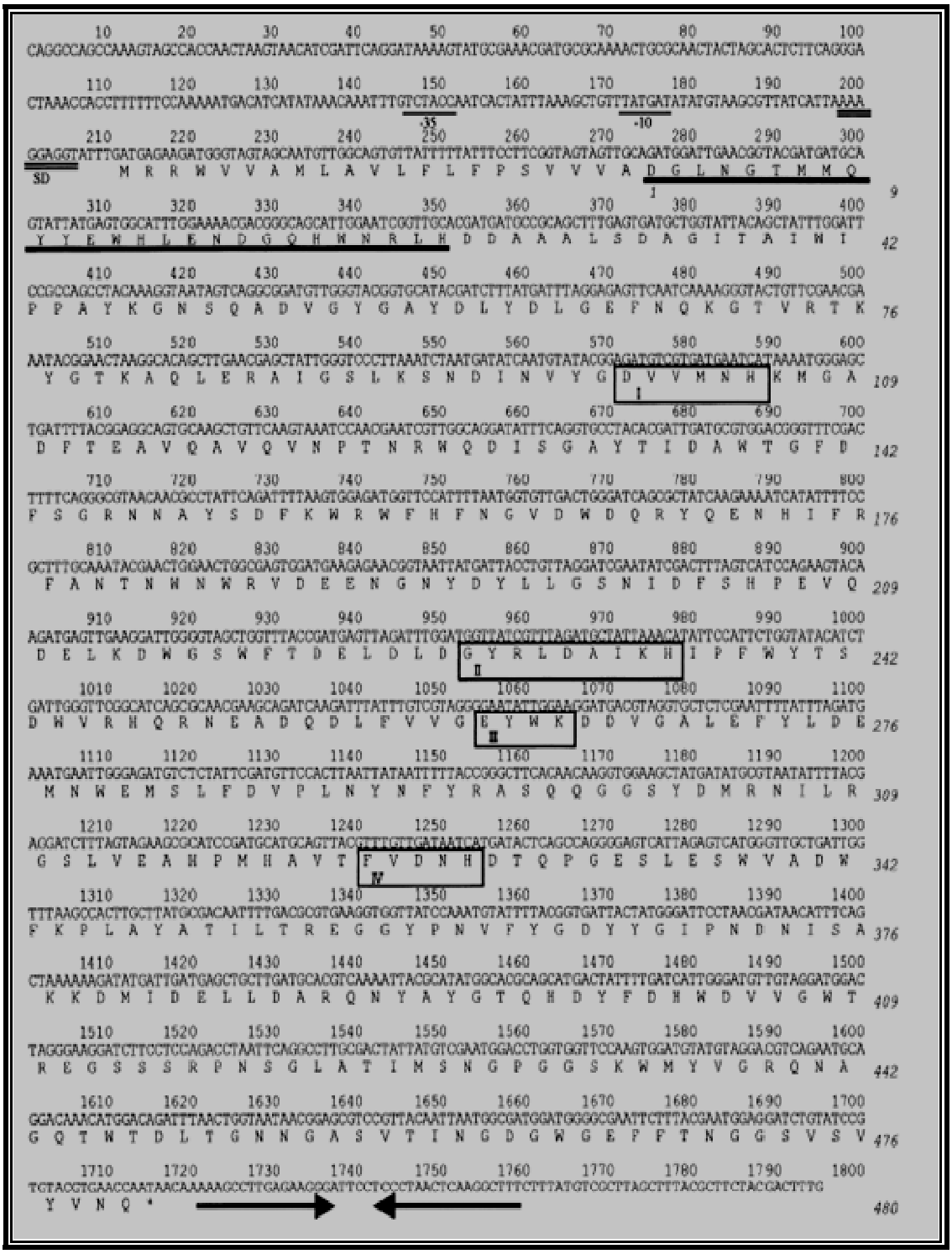


Figure (2.6): The sequence of α-amylase gene in *Bacillus subtilis*(Igarashi *et al.*,1998)

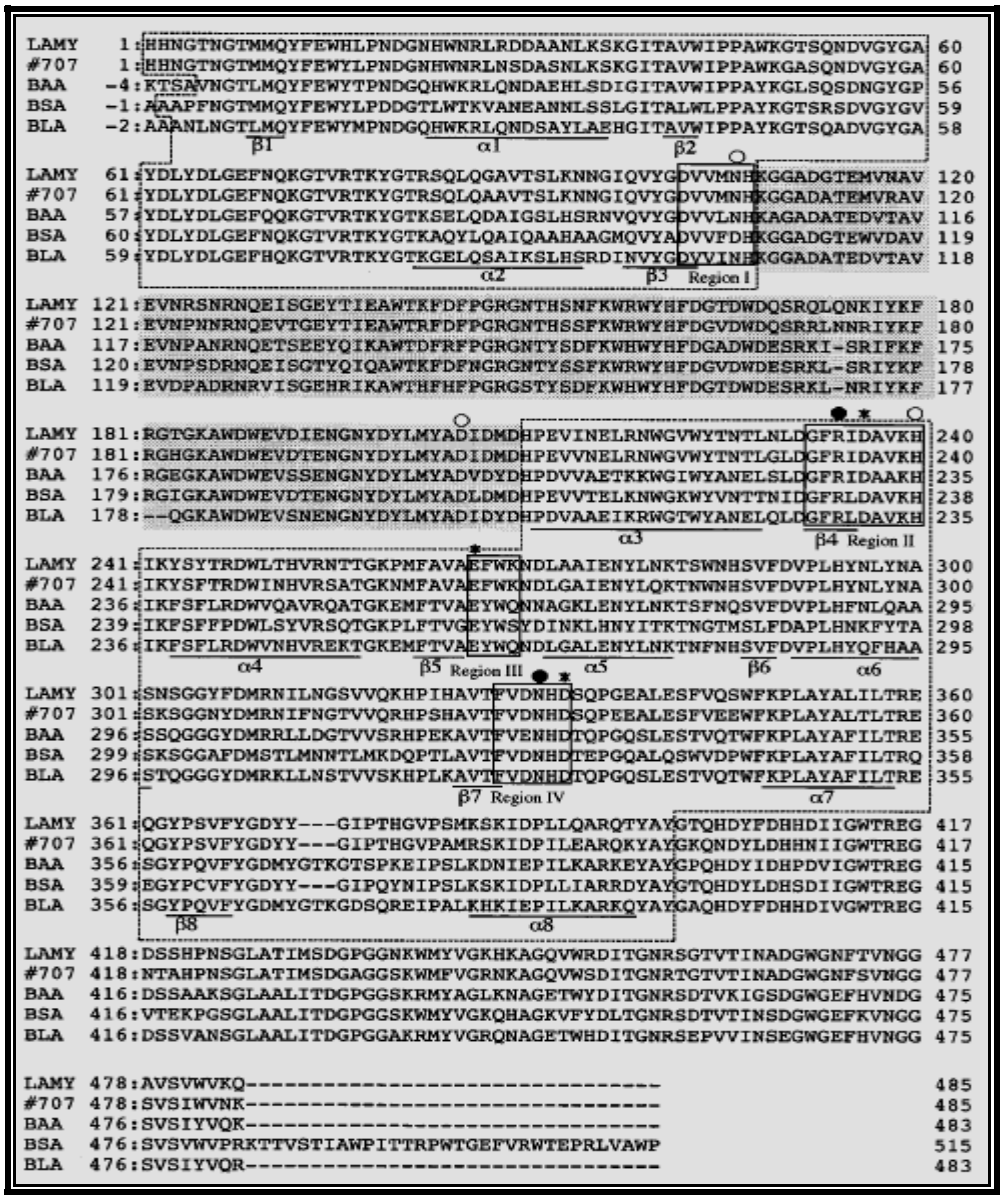


Figure (2.7) :Amino acid sequence alignment of α-amylase in *Bacillus amyloliquefaciens* (BAA), *Bacillus stearothermophilus* (BSA), and *Bacillus licheniformis* (BLA), Novel liquifiying α-amylase (AMYL) (Igarashi *et al.*,,1998)

The α-amylase genes of *Streptococcus bovis* 148 were cloned in *E. coli* MC1061, using pBR*322* in which it was cleaved by *Bam*H1 and Among about 50000 ampicillin resistant transformants, 11 colonies showed clear halos detected with I_2 (Satoh *et al.*,1993).(Sidhu *et al.*, 1997) could clone in

E. coli pBR322 systems and the 4.8 kb cloned fragment was mapped with restriction enzymes. Mielenz,(1983) reported cloning of α-amylase gene fragment carried on plasmid of *B. stearothermophilus* using pBR*322* vector cleaved with *Hin*d III, expressed *E. coli* , the fragment was 5.4 Kb that result in chimeric plasmid of 9.7kb length .

α-amylase gene from *B. stearothermophilus* was cloned in *B. stearothermophilus* and *B. subtilis* using pATB90 and pATB53, they expressed the trait (Aiba *et al.*,1983). A gene encoding the periplasmic α-amylase of *Xanthomonas campestris* K-11151 was cloned into *E. coli* using pUC19 as a vector (Abe *et al.*,1996).Thermostable α-amylase of *Bacillus* sp. was cloned in *E. coli*, the fragment length was 3 kb (Ali *et al.*, 1999).The genomic DNA of *B. licheniformis* was double-digested with *Eco*RI and *Bam*HI and ligated the pBR*322* in which the gene fragment was 3.5 kb (Iefuji *et al.*, 1996) . The detection methods for cloned fragments were different ,in which they depended on the formation of halo zones around the transformed cells , antibiotic resistance and sensitivity or both(Satoh *et al.*, 1993; Aiba *et al.*,1983).

A great deal of work has been done on the cloning of α-amylase genes in different microbes, mostly in *E. coli* or *Saccharomyces cerevisiae*.

Liebl *et al.*, (1997) described the gene structure of the *a*-amylase from *Thermotogamaritima* MSB8in which it was located on chromosome , *a*-amylase gene, designated *amyA*, and was predicted to code for a 553-amino acid preprotein with significant amino acid sequence. The *T. maritima a*-amylase appeared to be the first known example of a lipoprotein *a*-amylase. Following the signal peptide, a 25- residue putative linker sequence rich in serine and threonine residues was found.

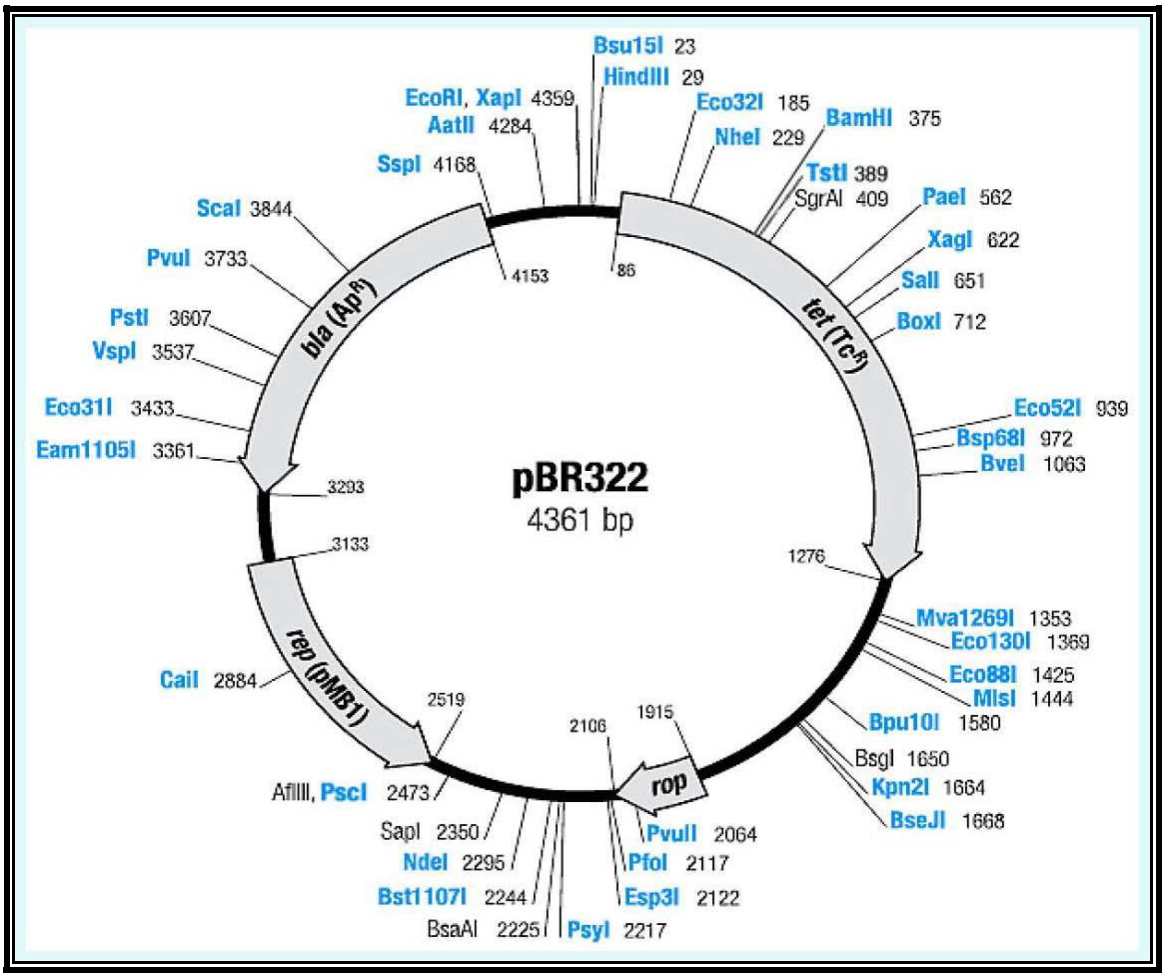
Tsao *et al.*(1993) could clone the α-amylase gene from *Streptomyces lividans* into *E .coli*. The amylase gene was expressed in *E. coli*. Suganuma *et al.*, (1996) studied the N-terminal sequence of the amino acids of the *a*-amylase

from *Aspergillus usanii*. The sequence of the first 20 amino acids was identical to the *a*-Amylase from *A. niger*. Marco *et al.*, (1996) inserted *B. subtilis* α-amylase gene into a plasmid, which was transferred to *E. coli*. And he could replace 171 C-terminal with new 33 amino acid in order to achieve extensive activity .In animals, α-amylase genes have a complex structure with an interrupted coding sequence, in the mollusk *Crassostrea gigas*, the two α-amylase genes are characterized by the same organization, with the existence of eight exons separated by seven introns (Sellos and Wormhoudt , 2002).

2.12 pBR *322* cloning vector

The plasmid pBR322 is one of the most commonly used *E.coli* cloning vectors,pBR*322* is 4361 bp in length and contains: 1- the replicon *rep* responsible for the replication of plasmid (source - plasmid pMB1); 2- *rop* gene coding for the Rop protein, which promotes conversion of the unstable RNA I - RNA II complex to a stable complex and serves to decrease the copy number (source - plasmid pMB1); 3- *bla* gene, coding for beta-lactamase that confers resistance to ampicillin (source - transposon Tn3); 4- *tet* gene, encoding tetracyclane resistance protein (source – plasmid pSC101) figure(2.8) (Rodriguez and Tait,1983).

The circular sequence is numbered such that 1 is the first T of the unique *Eco*RI site GAATTC and the count increases first through the *tet* gene, the pMB1 material, and finally through the Tn3 region. The map shows enzymes that cut pBR322 DNA once,The exact position of genetic elements is shown on the map (termination codons included),the *bla* gene nucleotides 4153-4085 complementary strand) code for a signal peptide. The indicated *rep* region is sufficient to promote replication. DNA replication initiates at position 2533 (+/- 1) and proceeds in the direction indicated. (Hardy,1988;Fermentas, 2006)



Figure(2.8):Description and restriction map of pBR 322 (Fermentas , 2006)

2.13 Optimal conditions for α-amylase production

2.13.1 Culture conditions

α-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 (forgaty and Kelly,1980; Hewitt and Solomons, 1996; Hillier *et al.*, 1996).

In production of α-amylase from *Bacillus* , the addition of yeast extract (0.5%) and peptone (1%) 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 favored for the growth and synthesis of amylase by the organism studied (Saito and Yamatomo,1975; Krishnan and Chandra ,1983).

The starch is very important to stimulate the production of α-amylase in *B. stearothermophilus* (Aiba *et al.*,1983). While, Chakraborty *et al.(*1997) reported that the best source for α-amylase production by *B. stearothermophilus* was starch 1.25%. Teodero and Martins (2000) used 1% soluble starch in production media for α-amylase by *Bacillus* sp.

Srivastava and Baruah (1986) described the culture conditions for the production of the enzyme by *B. stearothermophilus*, in which they mentioned peptone, starch had enhanced the production of the enzyme , while the synthesis was suppressed greatly by glucose , maltose and sucrose .

Saito and Yamamoto (1975) reported that the glucose repressed the production of α-amylase ,while the starch , glycogen and oligosaccharides induced the formation of it at the end of logarithmic phase .

Concerning the inorganic nitrogenous compound, diamonium hydrogen phosphate and ammonium dihyrogen phosphate were found to be better than Ammonium sulphate in synthesis of enzyme (Srivastava and Baruah, 1986; Aiyer,2004). While, Chakraborty *et al.(*1997) reported that the best source for α-amylase production by *B. stearothermophilus* was ammonium sulphate .The addition of 10 -20 mM calcium to the liquid medium improved the growth and amylase production,since the enzyme is known to be a calcium metalloenzyme, it is possible that the results found were because of the more availability of calcium ion (Srivastava and Baruah, 1986).These results are similar to the findings of (Hewitt and Solomons, 1996) with cultures of *Bacillus amyloliquefaciens*.

2.13.2 Optimal growth temperature

The temperature is one of the important factors that control the production of α-amylase. Srivastava and Baruah (1986) noticed that the optimal growth temperature for α-amylase production by *B. stearothermophilus* was 50°C, while the optimal growth temperature for that one used in the study of Welker and Campbell(1963) was 55-70°C.while Chakraborty *et al.(*1997) reported that the optimal temperature for production α-amylase by *B. stearothermophilus* was 50 °C.

Saito(1973) noticed the optimal temperature for production by *B. licheniformis* was 50°C,in contrast (Aiyar,2004) reported the optimal temperture for *B. licheniformis* SPT 27 was 37°C.

More recently**,** The optimum temperature for amylase production by thermophilic and alkaliphilic *Bacillus* sp. was 55 °C (Ito and Horikoshi , 2004).

2.13.3 Initial pH of the media

It is clear that the optimal pH of α-amylase production depends on the strain or the nature of the producer microorganism . The research of Mazza and Ertola (1976) on the production of α-amylase by *B. subtilis* showed the optimal pH was 7.2 ,using fermentor ,that could be attributed to the influence of pH on enzyme formation, or on the release of enzyme from cells, and to consider strain differences as well as the influence of the components of the medium. While, Chakraborty *et al.(*1997)reported that the optimal initial pH for the production α-amylase *B. stearothermophilus* was 7.2 .

Foosi *et al.*(2005) mentioned that the initial pH of media for amylase production by yeast was 4.5 , while Lin *et al.*(1998) reported that optimal initial pH for production by thermophilic and alkaliphilic *Bacillus* sp. was 8.5 .The pH for the production by *Rhizomucor pusillus* was 7.0. Saito(1973); Saito and yamamoto (1975); Kochhar and Dua(1983) ;Turchi and Becker

(1987) reported that the initial pH for the production by *B. licheniformis* was 7.0, while Aiyer(2004) reported that the optimal pH for *B. licheniformis* SPT 27 was 9.0 .

2.14 Purification and properties of α-amylase

Enzyme application in pharmaceutical and clinical sectors requires high-purity amylases. Thus, it is significant to develop economic processes for their purification to obtain chemically pure enzymes with maximum specific activity. Traditionally, the purification of α-amylases from fermentation media has been done in several steps, which include centrifugation of the culture (a step of extraction may be required for solid media), selective concentration of the supernatant, usually by ultrafiltration, and selective precipitation of the enzyme by ammonium sulphate and chromatography methods such as affinity chromatography or ion-exchange chromatography and gel filtration.

Extracellular α-amylase enzymes extracellular α-amylase from *Clostridium perfringens* Concentrated by polyethylene glycol were separated by DEAE-Sephacel chromatography (Shih and. Labbe, 1995).The precipitated of crude enzyme (cell –fraction broth)contained six amylolytic isoenzymes that were detected by isoelectric focusing and polyacrylamide gel electrophoresis,one of these amylases was purified by diethylaminoethyl-Sephadex A-50 ion-exchange chromatography and gel filtration by Sephadex G-200(McWethy and Hartman, 1977).

The extracellular α-amylase from *Clostridium acetobutylicum* ATCC 824 was purified to homogeneity by anion-exchange chromatography (Mono Q) and gel filtration (Paquet *et al.*,1991).As bacterial α-amylases have generally been produced from the strains belonging to genus *Bacillus*, several attempts have been made at their purification and characterization, from both mesophilic and thermophilic strains.

Furthermore, α-amylase was purified from Alkaliphilic *Bacillus* sp. using DEAE –Toyopearl and CM-Toyopearl obtaining a specific activity of 5009.3 U/mg protein and 35 % yield (Igarashi *et al.,* 1998). While, Lin *et al.(* 1998) could use Sephacryl S-100 and HiTrap Q in a procedure to purify the α-amylase from thermophilc alkaliphilic *Bacillus* sp. , they could achieve specific activity of 921.0 U/mg protein , purification folds of 708 .5 and yield (13.2%) .It was purified from *B. stearothermophilus* using DEAE-Sephadex A 25 to get specific activity 100 U/mg protein, and 57 % yield (Aiba *et al*. ,1983).

Krishnan and Chandra(1983)and Bolton *et al.(* 1997) purified an α-amylase to homogeneity using a combination of ammonium sulphate precipitation, ion-exchange chromatography and gel filtration.

An extracellular thermostable *a*-amylase produced by *B. licheniformis* was purified by two a phase separation in a polyethylene glycol / dextran system followed by gel filtration and ion-exchange chromatography (Ivanova ,1993),while Kochhar and Dua(1983) applied heat treatment , Ion- exchange chromatography and gel filtration procedures in the purification the α-amylase and the purified enzyme showed activity of 0.8 x 10^9 units .

Saito (1973) used DEAE-cellulose, CM- cellulose and Sephadex G-100 in the purification of the enzyme from *B. licheniformis* . Hanzawa *et al.(*1986) purified the α-amylase from *Aspergillus oryzae* using DEAE- cellulose and gel filtration using Sephadex G-75 .Affinity chromatography and HPLC technique were also applied in the purification of enzyme from *Rhizomucor pusillus.* Katoh *et al.*, (1997) purified an α-amylase, produced by recombinant cells, by a specific elution out of anti-peptide antibodies.

2.15 Characterization of α-amylase

2.15.1 Optimal pH for activity and stability

The optimal pH of the activity and stability were different according to the nature of the source organism.

The optimal pH for α-amylase produced by *Bacillus amyloliquefaciens* was 6.0 (Kochhar and Dua ,1983).while, Bakhmatova *et al.*(1984) indicted that the optimal pH for α- amylases produced by variants of *Bacillus subtilis* was within the acidic range (4.5-5.0),which is alike to optimal pH for the activity of that produced by *Clostridium perfringens* (Shih and Labbe ,1995) .

The optimal pH for the maximal activity of α-amylase produced by *Pyrococcus furiosus* was 5.5 (Dong *et al.,* 1997). Saito (1973) mentioned that the optimal pH for α-amylase produced by *B. licheniformis* was at 9.0 , which is similar to the findings of Krishnan and Chandra(1983) in the activity of α-amylase from *B. licheniformis* was at pH of 9.0 , also the activity of enzyme was reduced when Tris- hydrochloride and carbonate-bicarbonate buffers were used .

Concerning the stability of α-amylase, the α-amylase produced by *Bacillus stearothermophilus* was stable at pH range (6.0-11) while the α-amylase produced by *B. amyloliquefaciens* was stable at pH (6-9.5) for 24 h. Kochhar and Dua (1983) in the presence of calcium ion , while α-amylase produced by *Bacillus licheniformis* was stable at pH rang (6- 11) after the incubation for 30min and 24 hr. and in another study it was stable at pH range (7.0-9.0) (Krishnan and Chandra, 1983) .

2.15.2 Optimal temperature for activity and stability

The optimal temperature for the activity and stability of enzyme is different from each organism to the other, the optimal temperature for

thermostable α-amylase activity produced by *Bacillus stearothermophilus* was at 40°C (Aiba *et al.*, 1983). While in another study, it was at 82°C (Srivastava and Baruah, 1986).

The maximal activity for α-amylase produced by *Bacillus licheniformis* was at 90°C (Krishnan and Chandra, 1983). While Saito (1973) reported that the optimal temperature for the enzyme was at 76°C .The α-amylase produced by *B. amyloliquefaciens* showed the maximal activity at the temperature of 65°C. (Kochhar and Dua, 1983).

Concerning the temperature of stability, the results revealed that the α-amylase produced by *Clostridium perfringens* was stable in the presence of Ca^{2+} with 100% of remaining activity up to 45°C, and then the stability was lowered (Shih and Labbe, 1995),while that produced by *C . acetobutylicum* retained about 30% of the remaining activity after treatment at 45°C for 60 min.(Paquet *et al.*,1991), in which the Ca^{2+} plays an important role in the thermostability of enzyme (Lin *et al.*, 1998).

2-16 Immobilization of α-amylase

Immobilized enzymes are used in food technology, biotechnology, biomedicine, and analytical chemistry,they have various advantages over free enzymes including easy separation of the reactants, products, and reaction media, easy recovery of the enzyme, and repeated or continuous reuse (varavinit *et al.*, 2002).

Immobilization is the technique of binding the biocatalysts to a carrier as means of increasing their activity, stability and improving the technological application of the reaction (Hazare, 2005). The immobilization of enzyme on insoluble supports provides a stabilization effect at elevated temperature and pH.(Tzanov *et al.*, 2003).

Many techniques for immobilization of enzymes have been used in which Chalation of Glucose oxidase (GOD) on hydrous transition metals oxides $CrCl_3$,$CuCl_2$, $CoCl_2$, $Zncl_2$ and $FeCl_2$ (Aziz ,1997).

The immobilization of glucoamylase has been studied extensively in which The enzyme is immobilized on a multitude of different carriers by entrapment, adsorption, ion exchange, and covalent bonding (Reilly, 1979; Schafhauser and Storey, 1992). Glycosylated enzymes immobilized on (Concanavalin A) supports showed impressive gains in resistance to inactivation induced by heat, chemical denaturizing, proteolysis, storage and long-term continuous operation for several weeks (Mislovicova *et al.* ,2000).

Biomimetic silica support was used recently in immobilization of enzymes (Lauckarift *et al.,* 2004).Immobilized enzymes were coupled *in situ* with polyurethane polymer. Immobilized cholanesterase was stable for long periods (LeJeune *et al.,* 1997).

The greatest number of attempts to immobilize glucoamylase have been applied using a covalent attachment to organic and inorganic carriers, including low-cost magnetic support (Pieters & Bardeletti, 1992). Among these attempts, the most common method is to employ nitrous acid or glutaraldehyde to link the enzyme to amine-activated porous glass or silica.
The immobilized glucoamylases treated with glutaraldehyde approximately 45% of the original activity is retained upon immobilization (Reilly, 1976).
Cellulose fibers of bagasse were oxidized by periodic acid at positions 2 and 3 of the an hydro-glucose unit to obtain dialdehyde cellulose the aldehyde groups of the dialdehyde cellulose were able to react with amino groups of a thermostable alpha-amylase to form covalent bonds and resulted in a dialdehyde cellulose immobilized enzyme. The activity yield of the immobilized enzyme was 44% (varavinit *et al.,* 2002). Alginate was commonly used in immobilization of enzyme; it was derived from algae and

stabilized by a divalent cation. It consists of 1-4 bonded D-mannuronic and L-gluronic acid groups (Ivanova *et al.*,1995; Rodziewicz and Rymowicz ; 1999) used the calcium alginate in the immobilized *Bacillus polymyxa* cells for α-amylase production in continuous biosynthesis .

Gels are formed due to binding of divalent cations to the guluronic acid groups ,The strength of the gel depends strongly on the origin of the alginate, the strength of the matrix improves with an increasing amount of guluronic acid groups in the alginate, The type of divalent cation used also regulates the gel strength ,The strength of an alginate gel increases with the affinity of the used cation according to the following order:

$Pb^{2+} > Cu^{2+} = Ba^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$ (Leenen, 2001).

3.1 Materials and Methods

3.1.1 Equipments and Apparatus

The following equipments have been used throughout the study.

Equipment	Company
Autoclave	**Gallenkamp (England)**
Compound microscope	**Olympus (Japan)**
Ultracentrifuge	**Beckman (USA)**
Cooled centrifuge	**M.SE (U.K.)**
Micro centrifuge	**Sigma (Germany)**
Cooled shaker incubator	**Gallenkamp (U.K.)**
Distillatory unit	**Kent (England)**
Lyophilizer	**LKB (Switzerland)**
Hot plate magnetic stirrer	**Stuart scientific (U.K.)**
Incubator	**Gallenkamp(U.K.)**
Millipore filters	**Millipore and Whatman(England)**
Oven	**Memmert (Germany)**
pH-meter	**Metter-Toledo(U.K.)**
Sensitive balance	**Sartorius (Germany)**
Shaker incubator	**GFL (Germany)**
Shaker water bath	**Kotterman (France)**
Spectrophotometer	**Aurora instrument Ltd. UV 201 (U.K.)**
Vacuum Pump	**Robin Air (USA)**
Vortex mixer	**Buchi (Switzerland)**
DNA –Gel Electrophoresis	**Bio-Rad (USA)**
Protein –Gel Electrophoresis	**Lkb(Sweden)**
Micropipettes	**Witeg (USA)**
Ultracentrifuge	**Beckman(USA)**

3.1.2 Chemical and Biological Items

The following chemical and biological items have been used throughout the study.

Materials	Manufacturer
Absolute ethanol, Glacial acetic acid, Potassium chloride, Casein hydrolysates , Dihydrogen potassium phosphate, Dipotassium hydrogen phosphate, Glycine , Sucrose, Potassium iodide , Paraffin ,Glycerol, hydrochloric acid, Isoamyle alcohol , Methylene blue ,Iodine , Safranine, Dimethyl-α-naphthalane , α-Naphthylamine ,Sulphanilic acid	**BDH (England)**
Yeast extract, Agar, Nutrient broth, Gelatin.	**Biolife (Italy)**
Soluble corn-Starch, Urea, Maltose	**Difco(USA)**
Sodium lauryl sulphate,Tris(hydroxyl methyl) amino methane base ,Sodium hydroxide, Hydrogen peroxide ,Na_2EDTA, Sodium azide	**Fluka (Switzerland)**
Calcium chloride hydrate, Magnesium sulphate hydrate , Bromophenol blue ,Ammonium chloride ,Glucose, Sodium chloride, Ethidium bromide	**Reidel- DeHaeny Germany**
2-Merceptoethanol	**Calbiochem-USA**
Ampicillin ,Tetracyclane ,Chloramphenicol	**Sinochem jiangsu (China)**
Cesium chloride, 2,3-dinitrosalicylic acid ,TEMED, Ammonium persulfate , Complete freund`s adjuvant	**Sigma**

3.1.3 Culture media:

3.1.3.1 Ready to use media

Media	**Manufactured company**
Brain heart infusion agar	Biolife (Italy)
MacConky agar	Oxoid (England)
Nutrient agar	Oxoid (England)
Nutrient broth	Oxoid (USA)
Simmon`s citrate medium	Difco (USA)
Triple sugar iron (TSI)	Difco(USA)
Tryptic soy agar	Biolife(Italy)
Urea agar Base	Biolife
Muller-hinton medium	Difco

3.1.3.2 Laboratory prepared media

The autoclaving was done for the all autoclvable media at 121ºC for 15min.

3.1.3.2.1 Blood Agar (Atlas *et .al.*,1995)

It was prepared by dissolving 37 g of blood base agar in 950 ml of distilled water then the pH was adjusted to 7.0 and autoclaved , after cooling to 50ºC, the blood was added to final concentration of 5 %, mixed well and poured into Petri dishes .

3.1.3.2.2 Nitrate broth medium (Cruickshank *et al.*, 1975)

It is composed of:-

Beef-extract	3 g.
Peptone	5 g.
Potassium nitrate	0.2 g.
Distilled water	Up to 1 L.

The pH was adjusted to 7, distributed into tubes and autoclaved at 121C^{o} for 15 minutes

3.1.3.2.3 Carbohydrate fermentation medium (Parry *et al.*,1983)

Ammonium salt sugars medium (A.S.S) was used; it is composed of :-

Ammonium diphosphate	1g.
Potassium chloride	0.2 g.
Hydrous magnesium sulphate	0.2 g.
Yeast extract	0.2 g.
Agar-agar	14 g.
Bromophenol blue solution 1%	3.2 ml
D.W.	Up to 1 L

pH was adjusted to 7.0 and sterilized by autoclave. Then desired sugar solution was filtrated and added to be 1% as a final solution and kept as slants.

3.1.3.2.4 Gelatin medium (Cruickshank *et al.*, 1975)

Twelve gram of gelatin were dissolved and completed to 100 ml of nutrient broth medium. Distributed in tubes and autoclaved .

3.1.3.2.5 Sporulation medium (Cruickshank *et al.*, 1975)

Nutrient broth	6 g.
Hydrous magnesium sulphate	0.03 g.
Potassium phosphate monobasic	0.25 g.
Agar-agar	20 g.
D.W	Up to 1 L

The pH was adjusted to be 7.0, distributed into tubes and autoclaved , and then kept as slants.

3.1.3.2.6 Methyl red and Voges- proskauer (MR-VP) medium (Cruickshank *et al.*, 1975)

It is composed of :-

Glucose	5 g.
Peptone	7 g.
K_2HPO_4	5 g.
D.W	Up to 1L

The pH was adjusted to be 7.0, distributed into tubes and autoclaved, then the filtrated glucose was added.

3.1.3.2.7 Peptone water medium (Atlas *et al.*, 1995)

It is composed of:-

Peptone	2 g.
Sodium Chloride	0.5 g.
D.W	Up to 100 ml.

pH adjusted to 7.0,the media were distributed into tubes and autoclaved.

3.1.3.2.8 Urea agar medium (Collee *et al.*,1996)

It was prepared by adjusting the pH of the urea agar base (Christensen's media) (3.1.3.1) to 7.0, autoclaved, allowed to cool to 50ºC. Then 50 ml of 40% urea (sterilized by filtration) mixed together then distributed into sterilized tubes, kept as slant.

3.1.3.2.9 Casein medium (Cruickshank *et al.*, 1975)

It is composed of :-

Skim milk	5 g.
Agar-agar	2 g.
D.W	U to 100 ml.

The pH was adjusted to 7.0 and autoclaved for 10 min.

3.1.3.2.10 Starch- agar I medium

Nutrient agar supplied with 1.5 % soluble starch, the pH was adjusted to 7.0 then autoclaved.

3.1.3.2.11 Luria –Bertani (LB) broth (Maniatis *et al.*, 1982)

It is composed of :-

Tryptone	10g
Yeast extract	5g
NaCl	5g
Glucose	1g
D.W.	Up to 1L

The pH was adjusted to 7.5 and autoclaved.

3.1.3.2.12 Semi -solid agar medium (Collee *et al.*, 1996)

It was prepared by dissolving 0.7 % of agar, then distributed into tubes, autoclaved and allowed to stand in a vertical position.

3.1.3.2.13 starch- agar media II (Mielenz, 1983)

It is composed of :-

Na_2HPO_4	0.6 g.
KH_2PO_4	0.3g.
NaCl	0.05g.
NH_4Cl	0.1g.
Yeast extract	1g.
Peptone	1.0g.
Soluble starch	1.0g.
Agar-agar	2 g.
D.W.	Up to 100ml

The pH was adjusted to be 7.0 , and autoclaved

3.1.3.2.14 SH media (Saito and Yamamoto, 1975)

It is composed of :-

Soluble starch	40 g.
Ammonium hydrogen phosphate	5 g.
Yeast extract	5 g.
Magnesium sulfate heptahydrate	0. 5 g.
sodium citrate	2 g.
Calcium chloride	0.08g.
D.W.	Up to 1 L.

The pH was adjusted to 7.2, then autoclaved at 121°C for 15 min.

3.1.3.2.15 TSM media (Campbell, 1954)

It is composed of :-

A) Stock elements solution :

Magnesium chloride hexahydrate	0.5 g.
Calcium chloride	0.5 g.
Iron(II) chloride tetrahydrate	0.3 g.
D.W	Up to 100ml

The pH was adjusted to 7.2, then autoclaved at 121°C for 10 min.

B) Media Solution :

Soluble starch	10 g.
Trypton	20 g.
Sodium phosphate dibasic	2.5 g.
potassium phosphate monobasic	1 g.
Sodium chloride	1 g.
D.W	Up to 999 ml

The pH was adjusted to 7.2 and autoclaved at 121°C for 15 min, then the stock (1 ml) elements solution was added and mixed

3.1.3.2.16 Nutrient broth with 1% soluble starch (Aiyar, 2004)

Nutrient broth supplemented with 1 % soluble starch, was prepared and pH was adjusted to 7.0, then autoclaved.

3.1.4 Reagents and Dyes

3.1.4.1 Catalase reagent (Atlas *et al.*,1995)

It is composed of 3% hydrogen peroxide

3.1.4.2 Oxidase reagent (Collins and Lyne ,1987)

One gram of Tetramethyl-*P*-phenylene –diamine dihydrochloride was dissolved in 100 ml distilled water, kept in a dark bottle at 4°C.

3.1.4.3(Lugol`s solution) Starch hydrolysis reagent(Collins and Lyne, 1987)

Potassium iodide	10 g.
Iodine	2 g.
D.W	100 ml

Kept in dark bottle at 4°C.

3.1.4.4 Nitrate reduction reagent (Cruickshank *et al.*,1975)

Reagent A: 0.8 % sulphanilic acid was dissolved in 5N acetic acid over heated.

Reagent B: 0.5 % α-naphthylamine was dissolved in 5N acetic acid over heated .Equal volumes of A and B were mixed together .

C: Zink dust

3.1.4.5 (Barritt`s indicator) Voges –proskauer (Cruickshank *et al.*, 1975)

- **Solution A** : Potassium hydroxide 40 %

- **Solution B**: α-naphthol 5% in absolute Ethanol.

3.1.4.6 Methyl red indicator (Collee *et al.*, 1996)

It is composed of :-

Methyl Red	0.025 g.
Ethanol (95 % V/V)	75 ml
D.W	50 ml

3.1.4.7 Kovac`s reagent

It is composed of :-

p-dimethylaminobenzaldehyde	5 g.
Isoamyl alcohol	75 ml.
Con. HCl	25 ml.

P-dimethylaminobenzaldehyde was slowly dissolved in isoamylacohol with heating in a water bath at 50ºC, until it became cold; HCl was added, kept in a dark bottle at 4ºC .

3.1.4.8 3, 5-Dinitrosalicylic acid (DNSA) (Aiba *et al.*, 1983)

It was prepared by dissolving 1g. of DNSA in 50ml of D.W then 20 ml of NaOH 2M was added ,until it completely dissolved ,30g.of Potassium –sodium tartrate teterahydrat- (Rochelle Salt)gradually was added to be completely dissolved , then the total volume brought to 100 ml, and kept in a dark bottle .

3.1.4.9 3, 5-Dinitrosalicylic acid for TLC products detection

It was prepared by dissolving 0.5g of DNSA in 100 ml of NaOH solution (3.1.5.16) (Caraway, 1976).

3.1.4.10 Coomassie brilliant blue G 250(Bradford, 1976)

Coomassie brilliant blue G-250 (0.1 g) was dissolved in 50 ml of 95% ethanol, then 100 ml of 85 %*O*-phosphoric acid was added ,the volume completed to one liter with distilled water .

3.1.4.11 Bromophenol blue (0.25%) (Shi and Jackowsk, 1998)

It is composed of :-

Bromophenol blue	0.25 g.
D.W	Up to 100 ml

3.1.4.12 Coomassie brilliant blue R-250 (Shi and Jackowsk, 1998)

Coomassie brilliant blue R-250 (2g.) was dissolved in 500 ml of methanol, 100 ml of glacial acetic acid then 400 ml of D.W . The dye was filtrated through Whatman No.1 filter paper and kept in a dark bottle at room temperature.

3.1.4.13 Methyl Red Indicator (Collee *et al.*, 1996)

3.1.4.14 Gram stain (Atlas *et al.*,, 1995)

3.1.4.15 Ethidium bromide solution (10 mg/ml) (Maniatis *et al.*,,1982)

Ethidium bromide (0.1 g) was dissolved in 10 ml of D.W and stirred with a magnetic stirrer for six hours to ensure the complete dissolving, then it filtrated and stored in a dark bottle, wrapped with aluminum foil at 4°C.

3.1.4.16 Staining solution (for SDS-PAGE and Non-SDS) (Shi and Jackowsk, 1998)

Methanol	250 ml
Glacial acetic acid	50 ml
Coomassie brilliant Blue R-250	1 gm
D. W	up to 500ml

3.1.4.17 Destaining solution (Shi and Jackowsk, 1998)

Methanol	200 ml
Glacial acetic acid	50 ml
D.W	up to 250 ml

3.1.4.18 McFarland standard turbidity suspension (Tube 0.5)

It is composed of :-

Barium chloride 1.175 %	0.5 ml
Sulfuric acid 0.36 N	99.5 ml

Which equals to 1.5×10^8 ,in general

3.1.5 Buffers and Solutions

3.1.5.1 TE buffer pH 8.0 (Maniatis *et al.*, 1982)

It is composed of :-

Tris-HCl	10 mM
EDTA	1 mM

3.1.5.2 Potassium phosphate buffer (100 mM) pH 7.0

It is composed of :-

K_2HPO_4	100 mM
KH_2PO_4	100 mM

3.1.5.3 EDTA – SDS buffer pH 8.0

It is composed of :-

EDTA	50mM
SDS	1%

3.1.5.4 TEG buffer pH 8.0(Kieser, 1995)

It is composed of :-

Tris-HCl	25 mM
EDTA	10 mM
Glucose	50 mM
RNase A	5µg/ml

3.1.5.5 TE25S(Kieser, 1995)

It is composed of :-

Tris-Base	**25 mM**
Na-EDTA	**25 mM**
Sucrose	**300 mM**

3.1.5.6 CTAB/NaCl (Kieser, 1995)

It is composed of :-

CTAB	10 g.
NaCl	4g.
D.W.	100 ml

3.1.5.7 5M NaCl (Kieser, 1995)

It was prepared by dissolving 29.22 g. in 100 ml D.W

3.1.5.8 Solution I for DNA isolation (Maniatis *et al.*, 1982)

It is composed of :-

Glucose	50mM
Tris.HCl (pH 8.0)	25mM
EDTA	10mM

Autoclaved, stored at 4°C and Lysozyme dissolved in solution just before use in concentration of 5mg/ml.

3.1.5.9 Solution II for DNA isolation (Maniatis *et al.*, 1982)

It was prepared freshly, as stock solution of 10 N NaOH and 20 % SDS , pH was adjusted to 10.5 . The concentration of the working solution should be:

NaOH	0.2 N
SDS	1%

3.1.5.10 Lyses buffer

It is composed of:-

Sucrose	12.5 %
Tris-Base	25 mM pH 8.0
Lysozyme	2 mg/ml

The pH was adjusted to 8.0

3.1.5.11 TBE buffer 5x (Maniatis *et al.*, 1982)

It is composed of :-

Tris-Base	54 g
Boric acid	27.5 gm
EDTA 0.5M (pH8)	20 ml

The volume was brought to up 1 L and autoclaved

3.1.5.12 Potassium acetate pH 4.8(Maniatis *et al.*, 1982)

To 60 ml of 5M potassium acetate, 11.5 ml of glacial acetic acid was added and then 28.5 ml of D.W was added, mixed together, and allowed to stand on ice for 10 min. before use.

3.1.5.13 SET buffer (Kieser, 1995)

It is composed of :-

NaCl	٧٥ mM
EDTA pH 8.0	25 mM
Tris-Base	20 mM

3.1.5.14 TEGRLR buffer pH 8.0 (Kieser, 1995)

It is composed of :-

Tris-base pH 8.0	25 mM
EDTA	10 mM
Glucose	50mM
Lysozyme	1mg/ml
RNase	10 µg/ml

3.1.5.15 Gel loading buffer 6X (Maniatis *et al.*, 1982)

It is composed of :-

Bromophenol Blue	0.25 g.
Sucrose	40 g.
D.W	100 ml

Kept at 4°C

3.1.5.16 1 M NaOH

3.1.5.17 0.005 M Potassium phosphate buffer -0.025M $CaCl_2$ pH 7.0

3.1.5.18 0.25 M NaCl –0.25 M NaOH

3.1.5.19 0.25 N HCL

3.1.5.20 0.005 M Potassium phosphate buffer pH 7.0

3.1.5.21 0.01 M Potassium phosphate buffer pH 7.0

3.1.5.22 Starch solution 0.5 %

It was prepared by dissolving 0.25 g. of corn starch in 25 ml of potassium phosphate buffer-$CaCl_2$ (3.1.5.18) dissolving solution and then 25 ml of boiled potassium phosphate buffer-$CaCl_2$ (3.1.5.18) dissolving solution was added, boiled for two minutes, until it completely dissolved ,then allowed to cool .

3.1.5.23 Maltose stock solution 1 mg/ml

It was prepared by dissolving 0.1 g. of Maltose in 100ml of D.W.

3.1.5.24 Sodium alginate 4 % solution

It was prepared by dissolving 4 g. of sodium alginate in 100 ml of D.W and autoclaved for 10 min. at 121°C.

3.1.5.25 $CaCl_2$ 1 % solution

It was prepared by dissolving 1 g. of $CaCl_2$ in 100 ml of D.W and autoclaved for 10 min. at 121°C.

3.1.5.26 Tris-acetate-phosphate buffer (Eliss and Morrison, 1982)

It was prepared by mixing Tris, acetate and phosphoric acid with final concentration of 0.2 M (using prepared computer program). The final pH was adjusted according to the needed values.

3.1.5.27 Tris.Cl 10mM / EDTA 1mM pH 7.5

3.1.5.28 Chloroform/ Isoamyl alcohol solution (Kieser, 1995)

Potassium phosphate buffer-$CaCl_2$

Chloroform	24 ml
Isoamyl alcohol	1 ml

3.1.5.29 Transformation buffer

Potassium phosphate buffer-$CaCl_2$

Tris.base pH 8.0	10 mM
$CaCl_2$	50mM

3.1.5.30 Discontinues SDS – PAGE and (Non-SDS)-PAGE buffers and solutions based on (Shi and Jackowski, 1998)

Acrylamide /Bisacrylamide 30% stock solution

It is composed of :-

Acrylamide	29.22 g.
Bisacrylamide	0.78 g.
D.W	Up to 100 ml

Stock solution filtrated through 0.45 µm filter and stored in dark bottle.

3.1.5.31 Stacking gel buffer solution 4 X (0.5 M Tris.base pH 6.8)

It is composed of :-

Tris –Base	121.1 g
D.W	Up to 200 ml

The pH was adjusted with 6M HCl

3.1.5.32 Resolving gel buffer 4 X (1.5 M Tris-HCl pH 8.8)

It is composed of :-

Tris.base	36.3 g.
D.W	Up to 200 ml

The pH was adjusted to 8.8 with 6 N HCl.

3.1.5.33 Running buffer 5 X (pH 8.3) for SDS-PAGE

It is composed of :-

Tris- Base	15 g.
Glycine	72 g.
SDS	5 g.
D.W	Up to 1L.

The pH should be 8.3 without adjustment, the buffer kept in dark bottle at room temperature.

3.1.5.34 Running buffer 5 X (pH 8.3) for PAGE

It is composed of :-

Tris- Base	15 g.
Glycine	72 g.
D.W	Up to 1L.

The pH should be 8.3 without adjustment, the buffer kept in dark bottle at room temperature.

3.1.5.35 Sample buffer 2X (SDS- PAGE)

It is composed of :-

Stacking Gel Buffer	2.0 ml
Glycerol	1.6 ml
10 % SDS	3.2 ml
2-mercaptoethanol	0.8 ml
Bromophenol Blue in D.W(0.1% w/v)	0.4 ml

kept at 4°C in dark bottle.

3.1.5.36 Sample buffer 2X (PAGE)

It is composed of :-

Stacking Gel Buffer	2.0 ml
Glycerol	1.6 ml
D.W	4 ml
Bromophenol Blue in D.W(0.1% w/v)	0.4 ml

Kept at 4°C in dark bottle.

3.1.5.37 Ammonium persulfate solution 1.5%

It is composed of :-

Ammonium Persulfate	0.15 g.
D.W	10 ml

It was freshly prepared

3.1.5.38 Fixing solution

It is composed of:-

Methanol	40 %
TCA(Tri-Chloroacetic acid)	10 %

3.1.5.39 TLC separation system (Aiba *et al.*,1983)

It was prepared by mixing n-butanol /glacial acetic acid/ distilled water as ratio of 5:4:1.

3.1.5.40 Glucose 1% solution

3.1.5.41 0.005M potassium buffer –1M NaCl pH 7.0

3.1.5.42 Enzymes and Nucleic acids

Lysozyme	Sigma
λ DNA digested with *Pst* I	sigma
*Hin*dIII	Fermentas
Pronase	Sigma
RNase- free DNase	Sigma
Alkalane phosphatase	Sigma
T4 Ligase	Fermentas

3.1.5.43 Bacterial standard strains

E.coli HB101	*hsdS20,recA-13,ara-14,proA2, lacY1, galK2, rpsI* (Sm^-),*ryl-5,mtl-1,supE44*	**Genetic Engineering and Biotechnology Institute University of Baghdad, Iraq**
E.coli MM294	*hsd* R^-,*dsd* M^+,*thi*$^-$,*end A*	**Genetic Engineering and Biotechnology Institute University of Baghdad, Iraq**

3.1.6 Antibiotic

3.1.6.1 Antibiotic disks

The source of these disks was (Bioanalyse, Turkey):

Antibiotic	Abbreviation	Content (µg or U)
Imipenem	IPM	10
Cefotaxime	CTX	30
Norfloxacin	NOR	10
Gentamicin	CN	١٠
Penicillin	P	10 U
Amipcillin	AM	١٠
Aztreonam	ATM	30

3.1.6.2 Ampcillin stock solution (25mg/ml) (Maniatis *et al.*, 1982)

Ampcillin (25 mg) was dissolved in 1 ml of D.W then filtrated through Millipore and stored at -20°C in dark as aliquots.

3.1.6.3 Tetracyclane stock solution (12.5 mg/ml) (Maniatis *et al.*, 1982)

Tetracyclane (12.5 mg/ml) was prepared in Ethanol /water (50% V/V) then filtrated through Millipore and stored at -20°C in dark as aliquots.

3.1.6.4 Chloramphenicol stock solution (34 mg/ml) (Maniatis *et al.*, 1982)

Chloramphenicol (34 mg /ml) was prepared in Ethanol then filtrated through Millipore and stored at -20°C in dark as aliquots..

3.1.7 Enzyme solutions (Maniatis *et al.*, 1982)

3.1.7.1 Lysozyme solution (50mg/ml) in lysis buffer

3.1.7.2 Pronase solution (20 mg/ml)

Pronase 20mg/ml was prepared and incubated at 37°C for 2 hours in water bath to inactivate possible DNase and RNase contaminants , distrubted into aliquots and stored at-20ºC.

3.1.7.3 RNase Solution (10 mg/ml)

RNase 10mg/ml was prepared in 10 mM Tris.HCl and 15 mM NaCl, Heated to 100°C for 10 min. allowed to cool slowly at room temperature. Then distributed into aliquots and stored at – 20°C.

3.2 Methods

3.2.1 Isolation of thermophilic bacteria producing α-amylase (Teodoro and Martins, 2000)

Sixty nine soil samples were collected from different sites in Baghdad, Babylon, Dyhala and Diwaniyha districts .They transported to the lab., using sterilized nylon sacs, 10 grams of each sample had been added to 90 ml. D.W, mixed vigorously and heated to 80 °C for 30 min. with shaking in water bath, the pH of the soil sample was measured for next studies.Serial dilutions of each sample using sterilized phosphate buffer were set up from 10^{-1} through 10^{-6}. (0.1) ml of appropriate dilutions were spreaded onto Petri

dishes of Starch –Agar medium (3.1.3.2.10) , incubated at 65 C^o for two days . Replica plating was made for the growing colonies then starch hydrolysis test was done for one of the replica plates by flooding plates with lugol`s reagent(3.1.4.3). The colonies that showed a halo diameter zones (Z/G) ratio ; halo diameter: colony growth diameter ,were picked to repeat the same test to confirm the result.

3.2.2 Microscopic and morphological identification of the isolated bacteria (Harley and Prescott, 1996).

The morphology ,size and the margin of isolated colonies grown on nutrient agar (3.1.3.1) and blood agar (3.1.3.2.1) media were studied. 18 hour old colonies were subjected to a microscopic examination of their shape ,size and gram's stain. The sporulation ability was studied by cultivating the isolate on sporulation medium (3.1.3.2.5) for 72 hrs. Spores were been examined of their shape and position within the cell using Phase contrast Microscope.

3.2.3 Biochemical tests.

The colonies that produced amylase and showed characteristics similar to *Bacillus* as a genus have been chosen for further examinations to determine the genus and species accurately.

3.2.3.1 Catalase test (Cruickshank *et al.*, 1975)

This test was performed by adding drops of hydrogen peroxide (H_2O_2) 3% (3.1.4.1) on a single colony grown overnight, placed on glass slide. The production of gaseous bubbles indicated the presence of catalase.

3.2.3.2 Oxidase test (Atlas *et al.*, 1995)

Filter paper was moistening with the substrate solution (3.1.4.2), colony of bacteria grown overnight was rubbed on the filter paper by a sterile wooden applicator stick. An immediate color change to a deep blue indicated a positive test result.

3.2.3.3 Starch hydrolysis test (Harley and Prescott, 1996)

The ability of bacteria to hydrolyze the starch was performed by inoculating the bacteria on the plate of starch –agar medium (3.1.3.2.10) by making a single streak of the bacteria and by incubating at 55ºC for 48 hour. After incubation, the plate was flooded with lugol`s solution(3.1.4.3).The presence of halo zone around the colonies indicated hydrolysis of starch.

3.2.3.4 Urease test (Atlas *et al.*, 1995)

This test was used to examine the presence of urease, which hydrolyzes urea to ammonia, and Co_2.Christensen urea agar slants were inoculated with single colony of bacteria, incubated at 45ºC for 4-5 days. The appearance of pink color slant indicated a positive test.

3.2.3.5 Triple sugar iron (TSI) test (Atlas *et al.*, 1995)

Isolates were cultured on TSI agar slants by stapping into the agar and streaking on the surface, and then incubated for 48 hrs. at 55°C .Bacteria that only ferment glucose produce an alkalane (red slant) and an acid (yellow) butt. Bacteria that ferment lactose or (and) sucrose as well as glucose produce an acid (yellow) slant and an acid (yellow) butt. Bacteria that do not ferment glucose, lactose, or sucrose produce an alkalane (red) slant and an alkalane (red) butt. Bacteria that produce gas during fermentation form bubbles or cracks in the medium. Bacteria that produce H_2S gas turn the medium black.

3.2.3.6 Citrate utilization (Atlas *et al.*, 1995)

This test was used to examine the ability of bacteria to utilize citrate as a sole source of carbon. In this test, a colony was inoculated on the surface of simmon`s citrate medium (3.1.3.1) slant and the medium was incubated overnight at 55ºC. The change of the color to blue indicated the positive result.

3.2.3.7 Methyl red and voges- proskauer (Collee *et al.*, 1996)

The methyl red test was used to determine the pH of the end products of glucose fermentation.

Tubes of MR-VP liquid media were inoculated and incubated at 55ºC for 48hrs. Both tests were performed from the same inoculums suspension, which was divided for testing. The methyl red test was performed after adding about five drops of methyl-red reagent (3.1.4.6) the positive test was bright red and negative test was yellow.To detect the formation of Acetoin , Barritt`s solution (3.1.4.5) (600µl of 5 % α-naphthol and 200 µl of 40% NaOH) were added to the 1 ml bacterial culture (3.1.3.2.6), the appearance of red color within minutes indicated the formation of intermediate -compound Acetyl-methyl carbionl(Acetoin).

3.2.3.8 Carbohydrates fermentation (Parry *et al.*, 1983)

Bacteria were grown on ASS (3.1.3.2.3) medium containing the sugar to be tested and incubated at 45C^{o} for 3-5 days, the turn of reagent's color into yellow indicated utilization of the sugar and the forming of an acid.

3.2.3.9 Nitrate reduction test (Cruickshank *et al.*, 1975)

One hundred µL of each test reagent solution A and B (3.1.4.4) was added to the culture in medium (3.1.3.2.2). A red color developed within few minutes indicated the ability of the organism to reduce nitrate to nitrite.

3.2.3.10 Gelatin liquefaction test (Cruickshank *et al.*, 1975)

Tubes of gelatin media (3.1.3.2.4) were inoculated with bacteria and incubated at 45C^{o} for 5 days then transferred to the refrigerator for an hour, the liquefaction of tubes indicated positive results to be compared with the control one .

3.2.3.11 Indol formation test (Collee *et al.*, 1996)

The tubes of peptone water media (3.1.3.2.7)were inoculated with bacteria ,and incubated at 45ºC for 48 hrs , then 100µl of kovac`s reagent (3.1.4.6) was added to the culture . the Formation of red ring near the surface indicated the positive result due to the formation of tryptophanase.

3.2.3.12 Motility test (Collee *et al.*, 1996)

Semi solid agar medium (3.1.3.2.12) was inoculated with bacteria by stapping in the center of agar , to half of it , incubating at 55 °C for 48 hrs., a diffuse and hazy growth that spread slightly throughout the medium indicated positive results .

3.2.4 Maintenance of bacterial isolates

3.2.4.1 Short term storage

Bacteria were maintained in slant culture for period of a month. Such cultures were grown on slant surface in tubes containing 5-8ml of nutrient agar medium and stored at 4ºC .

3.2.4.2 Storage in soil

clean soil was sterilized successive times using autoclave , then its aseptic condition was tested by inoculating a sample of it on nutrient agar for three days , no visible growth indicated its sterilization .Then 1 ml of spores suspension was mixed with it, allowed to dry and kept at 4ºC for undetermined period.

3.2.4.3 Storage in 15 % glycerol.

Bacteria could be stored for a relatively long time in media containing 15% glycerol at a low temperature without a significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria grown in nutrient broth in a screw-capped bottle in final volume 10 ml and stored at -20ºC.

3.2.5 Preparation of Spores

B. stearothermophilus M13 was cultured on the surface of sporulation media(3.1.1.2.5) in slant tubes at 55ºC for three days ,then sterilized D.W was added to the culture and centrifuged at 3000xg for 5 min., discarding the supernatant, the same procedure was repeated. The spore suspension was obtained and kept onto the soil at 4ºC.

3.2.6 Estimation of protein (Bradford,1976)

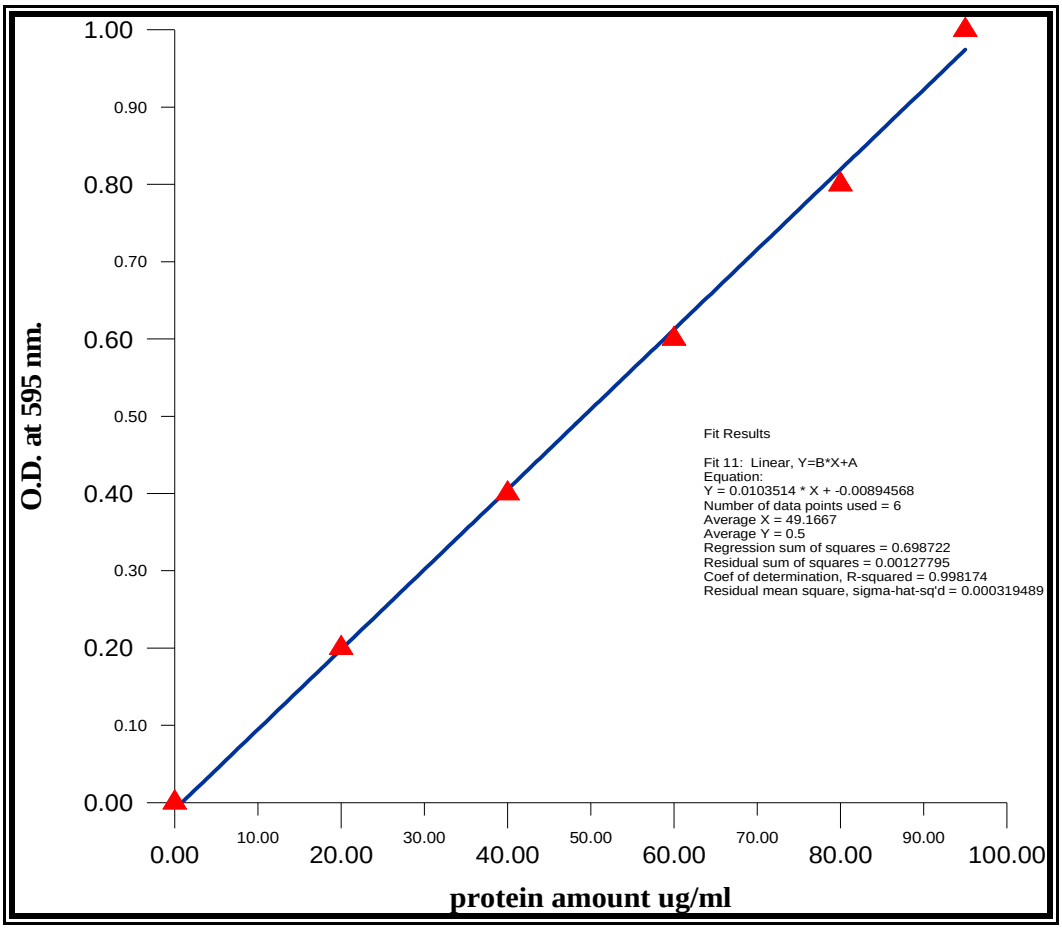
Protein was estimated by dye-binding (Coomassie G-250) method in which the bovine serum albumin used as a standard protein .

1. The standard protein bovine serum albumin (BSA) was prepared by dissolving 10 mg (BSA) in 10 ml of 0.05 M phosphate buffer pH 7.0
2. Several dilutions of standard protein (BSA)(1µg/µl) were performed in the same buffer and according to the following volumes :

BSA µL	Buffer µL	Protein amount µg	Final volume ml
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1
80	20	80	0.1
100	0	100	0.1

3. Twenty µl of each concentration were mixed with 50µl of NaOH 1M (3.1.5.16)
4. Two hundred and fifty µl of the dye reagent (3.1.4.10) were added, mixed, and allowed to stand for 10 minutes at room temperature.
5. The absorbance at 595 nm was measured; the blank was prepared from 0.1 ml of the buffer and 2.5 ml of the dye reagent.

6. A standard curve was plotted between the amounts of protein in the given sample against the corresponding absorbance. The protein concentrations of unknown sample were calculated from the standard curve (Fig. 3.1).



Figure(3.1):Standard curve for protein estimation by Bradford`s method.

3.2.7 α-amylase activity assay

Method of Bernfield (1955) was used for α-amylase assay; one unit enzyme activity is defined as the amount of enzyme that liberates one µmole of reducing sugar and measured as maltose per minute under the conditions of assay.

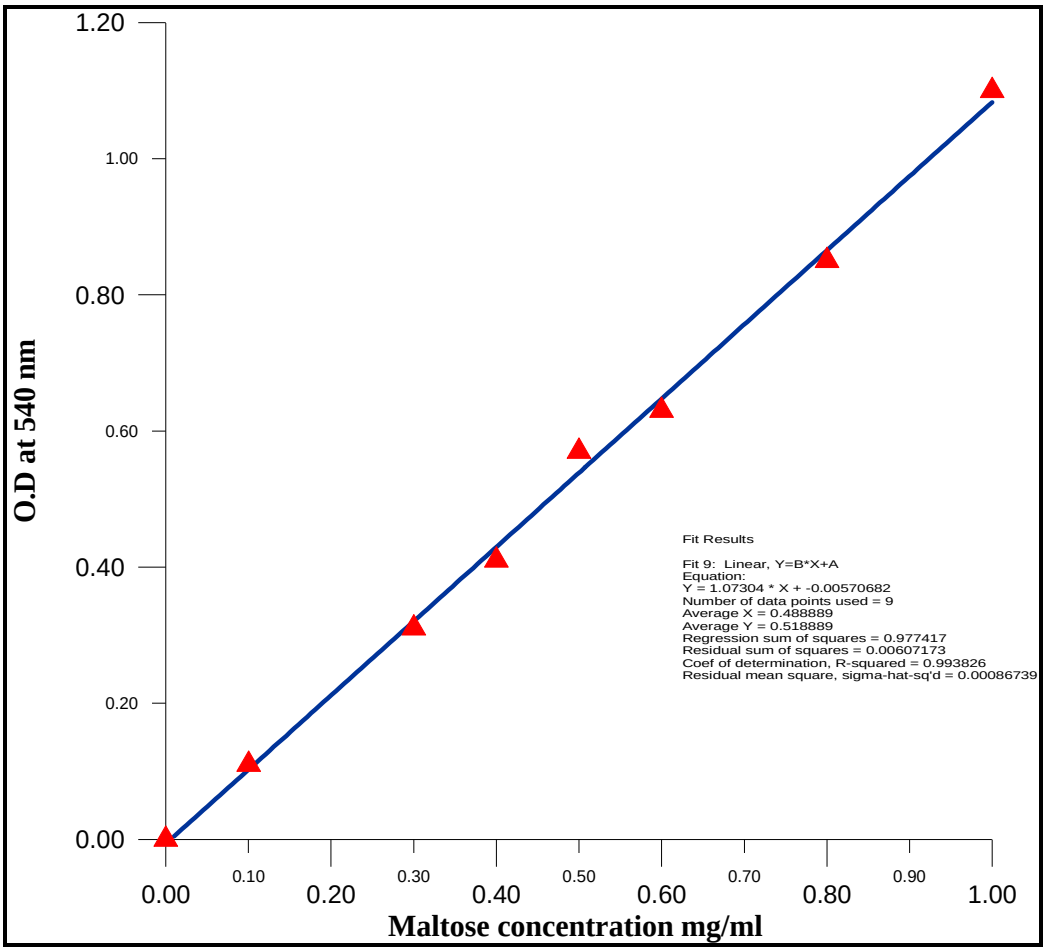
3.2.7.1 Standard curve of maltose solution

1. the following volumes of standard maltose stock solution (1mg/ml) (3.1.5.23) had been distributed into test tubes as duplicates for each

single volume , then the appropriate volumes of D.W, had been added for each, as follows;

Tube No.	**Stock standard maltose(ml)**	**D.W (ml)**	**Final conc. maltose(mg/ml)**
Blank	0	1	0
1	0.1	0.9	0.1
2	0.3	0.7	0.3
3	0.4	0.6	0.4
4	0.5	0.5	0.5
5	0.6	0.4	0.6
6	0.8	0.2	0.8
7	1.0	0	1

2. One ml of DNSA Reagent (3.1.4.8) was added to each tube.
3. Tubes were incubated in boiling water bath for 5 min.
4. Tubes were removed from the boiling water bath and immediately placed into ice bath.
5. Ten ml of D.W. were added to each tube and well mixed.
6. Reading the absorbency for each sample at 540nm. Versus blank.
7. The Absorbency values at 540nm were plotted against maltose concentration (mg/ml), then the activity was calculated .



Figure(3.2):Standard curve for maltose, using DNSA as blocking reagent

3.2.7.2 Assay method

1. Enzyme solution (0.1 ml) was added to 0.9 ml of starch solution (3.1.5.24) in tubes and incubated at 60°C for 10 min.
2. The reaction was stopped by adding 1ml of DNSA, and then the tubes were incubated in boiling water bath for 5 min.
3. Tubes removed from the boiling water bath and immediately placed into an ice bath.
4. Ten ml of D.W was added to each tube.
5. The absorbency for duplicate tubes was measured at 540 nm , the blank was created by adding the enzyme solution after DNSA addition (blocking solution).

3.2.8 Optimum conditions for α-amylase production in liquid media

The main optimal conditions for amylase production have been studied in order to produce it with higher amount using the batch cultures.

3.2.8.1 Determination of optimal α-amylase production media

The colonies that revealed halo zone of hydrolysis on media (3.1.3.2.10) and classified as *B. stearothermophilus* were picked for the next experiments in order to choose the most efficient isolate by inoculating on SH medium.*B. stearothermophilus* was grown in nutrient broth for overnight , then three duplicated flasks containing SH medium (3.1.3.2.14) , TSM medium (3.1.3.2.15) and nutrient broth supplemented with 1% soluble potato starch (3.1.3.2.16) were inoculated with 1 ml of the previous grown culture at log phase of growth . Then flasks were incubated in a shaker incubator (120 rpm, 55˚C, 24 hrs.). The cultures were centrifuged at 3000xg for 5 min and supernatant was used for assaying the α-amylase activity (3.2.7).

3.2.8.2 Optimal temperature for α-amylase production

B. stearothermophilus M13 was grown in TSM medium broth (in which it was the best among the media) and incubated in different temperatures (35, 40, 45, 50, 55, 60, and 65˚C) in a shaker incubator (120 rpm, 48hrs). The cultures were centrifuged at 3000rpm for 5 min and supernatant was used for assaying of α-amylase specific activity as described in (3.2.7).

3.2.8.3 Optimal Initial pH for α-amylase production

The optimal pH for production of α-amylase was determined by preparing the TSM medium with different pH, ranging from (6, 6.5, 7, 7.5 and 8) .Media were inoculated and incubated in shaker incubator (120 rpm, 55˚C, 24 hr.). The culture was centrifuged at 3000rpm for 5 min and supernatant was used for assaying of α-amylase specific activity as described in (3.2.7).

3.2.8.4 Measurement of bacterial growth (Growth curve) and optimal growth phase for α-amylase production.

Growth of bacteria was monitored by measuring the optical density of a liquid culture using spectrophotometer at 600nm, at interval time of 2 hours .The corresponding α-amylase activity was determined as described in (3.2.7) after centrifugation of the samples at 3000xg .

3.2.9 Antibiotic susceptibility (Aslim *et al.*, 2002)

B. stearothermophilus M13 was inoculated into nutrient broth and incubated at 55°C for several hours until the turbidity was easily visible. The turbidity was adjusted by nutrient broth against McFarland (3.1.4.18) standard tube. Then 100 µl of bacteria suspension was transferred to the surface of Muller-Hinton agar plates , spreaded and incubated for 15 min. at 55°C .Antibiotic discs were placed on muller-hinton agar medium seeded with bacteria and the diameters of inhibition zones that formed following 24 h. incubation were measured and test results of antibiotic susceptibility were determined according to the inhibition zone diameter (NCCLs, 1991).

3.2.10 α-amylase purification

3.2.10.1 Precipitation by the Ammonium sulphate (Segel, 1976)

One hundred ml of the crude extract were made up to 40 % saturation with ammonium sulphate stirred on the ice for 1 hour,then centrifuged at 9000 xg for 20 min. at 4^{o}C . Ammonium sulphate was added again to supernatant to raise saturation ratio to 80% using the same procedure. The mixture was centrifuged, then the supernatant was discarded. The precipitated was dissolved in 5 ml of the potassium phosphate buffer 0.005M – $CaCl_2$ 0.025M pH 7.0 (3.1.5.17). Then enzymatic activity and the protein concentration were measured after dialysis.

3.2.10.1.1 Preparation of dialysis tubing with10000 d. cutoff (Maniatis *et al.*, 1982)

1. Cut the tubing into pieces of 15 cm length.
2. Boiled for 10 min., with 100 ml of 2% sodium bicarbonate and 1mM EDTA.
3. The tubing was rinsed thoroughly in distilled water.
4. Boiled for 10 min. in 0.001 M EDTA.
5. Allowed to cool and stored at 4°C in D.W
6. Washed with D.W before application.

3.2.10.1.2 Dialysis

The supernatant of the last step was dialyzed against the following buffers, taking into consideration changing the solution many times during the process

1. Distilled water.
2. potassium phosphate buffer –$CaCl_2$ (3.1.5.17)

3.2.10.2 Preparation of the ion exchange chromatography column (LKB)

The DEAE-sepahdex was prepared according to the manufacturer (LKB), in which the exchanger was washed with NaCl-NaOH solution (3.1.5.18). Using the buchner funnel as a ratio 440ml : 20 g. of DEAE-Sephadex, filtrated under vacuum through Wattman paper No.1. then washed for many times over running distilled water, suspended by HCl solution (3.1.5.19) then washed for many times by potassium phosphate buffer (3.1.5.20), and resuspend onto it, until the pH became 7.0 ,allowed to stand for 1 hour ,then the fines were removed , washing with distilled water was repeated to ensure a better removal of fines or impurities and degassed . Glass wool was inserted at the bottom of column, and then the slurry was poured into column,allowed to be packed in glass column to be (1.3 x 35) cm. The equilibration was done overnight using potassium phosphate buffer solution (3.1.5.20) at speed of 40 ml/hour.

3.2.10.2.1 Sample application

The concentrated enzyme (4ml) was added to the side wall using a glass rod gently , then it was washed by potassium phosphate buffer solution (3.1.5.20) ,the washing fractions were collected at speed of 5 ml/frac, the optical density was measured for each fraction at 280 nm. The declane of the optical density readings to the base lane indicated that the washing process of column was completed.

3.2.10.2.2 Elution of the enzyme

A laner salt gradient of (potassium buffer –NaCl) (3.1.5.41) in order to recover the enzyme from Ion- exchanger, the protein for each fraction was estimated at 280 nm. The enzymatic activity for each fraction was assayed as described in (3.2.7); the curve of enzymatic activity (unit/ml) was plotted against the O.D.280nm.

The fractions that revealed significant peak of activity were mixed together.

3.2.10.2.3 Dialysis

The enzymatic solution that recovered from the last step was dialyzed against potassium phosphate buffer 0.005 M – $Cacl_2$ 0.025 M pH 7.0(3.1.5.20) , and for several times until the next day in order to remove all the salt used in the recovery , then it was lyophilized .

3.2.10.3 Gel filtration chromatography (Laue and Rhodes, 1990)

3.2.10.3.1 Preparation of Sephadex G-100 Column

The gel was prepared according to the instructions of the manufacturer (LKB) in which the gel was washed with potassium phosphate buffer solution (3.1.5.21), then it was suspended in the same solution overnight at 4°C to allow swelling .The fines were removed . Degassing was done using vacuum , the slurry was poured into the column and allowed to be packed in the glass column(2.5x 35) cm , the washing, overnight, was done with solution (3.1.5.21) at a flow rate of 30 ml /h. . The void volume was measured by

blue dxtran- 2000 (V_o)at concentration of 5mg/ ml , the blue dextran -2000 was recovered from the column by potassium phosphate buffer solution (3.1.5.21) in which it was collected at speed of the 5 ml /frac. , at a flow rate of 30 ml /h., the absorbency of each fraction was measured at 600 nm. The lyophilized enzyme was dissolved in 2 ml of (0.005 M phosphate buffer – 0.002 $CaCl_2$) then was gently added to the side wall of column using the glass rod of the gel, the elution was done by the same buffer , 5ml / fraction at flow rate of 30 5ml /h. using same conditions of that for the blue dextran -2000 recovery .

The fractions that revealed the protein and enzymatic activity in the same peak was mixed and dialyzed against solution (3.1.5.21), then protein and enzymatic activity were assayed once again for the partially purified enzyme.

3.2.11 Determination of α-amylase purity and estimation of its molecular weight .

3.2.11.1 SDS- Polyacrylamide gel electrophoresis of partial purified α-amylase (Shi and Jackowski, 1998)

Tubes used were soaked overnight in 95% ethanol, then allowed to dry. Each tube was sealed from the bottom side by the clean parafilm and aligned in casting stand board.

The separating (resolving) gel was prepared by adding 7.5 ml of (acrylamide and bisacrylamide) solution (3.1.5.30) , 7.5 ml of resolving gel buffer 1X (3.1.5.32) pH 8.8, 0.3 ml of 10% SDS solution and 14.49 ml of distilled water . The solution was degassed for 10 min. using a vacuum pump , then 150 µl of 1.5% ammonium persulphate (3.1.5.37) and 15µl of TEMED were added to the degassed solution and mixed gently. using Pasteur pipette ,the separating gel was transferred to PAGE tubes using another pipette ; the top of the gel was covered slowly with "Isobutyl alcohol " and allowed the gel to

be polymerized for two hours at room temperature . Then the layer of isobutyl alcohol poured off.

Stacking gel was prepared by adding 3.9 ml of (acrylamide and bisacrylamide) solution (3.1.5.30),7.5 ml of stacking gel buffer 1X(3.1.5.31) ,0.3 ml of 10 % SDS solution and 18.3 ml of distilled water .The solution was degassed for 10 min., using a vacuum pump, 50 µl of 4.5 % ammonium persulphate (3.1.5.37) and 5 µl of TEMED were added to the degassed solution and mixed gently. Using Pasteur pipette, the stacking gel was transferred slowly to cover the upper surface of the separating gel to polymerization about 2 hr at room temperature. Samples for SDS –PAGE were prepared by adding equal volumes of a sample buffer solution 1X (3.1.5.35) to the sample and mixed well. The samples were incubated in water bath at 90 C^o for 5 min. and cooled to 25°C .

The PAGE tubes were submerged in running buffer 1X (3.1.5.33) and 100 µl of the prepared sample was loaded on the gel .The power supply was connected to the samples and run at 2.5mA tube for 30 min. then it was raised to 6mA. The run was kept at 4°C until the tracking dye reached a narrow distance just before the end of gel .

3.2.11.2 Standard Proteins Solution

It was prepared by dissolving the following proteins` molecular weights (150 , 80, 67, 50, 20, 14 kDa) as a concentration of 3 mg/ml in sample buffer (3.1.5.35). Then the same procedures for electrophoresis were applied.

3.2.11.3 Detection of protein bands on the polyacrylamid gel.

Polyacrylamid gels were removed from the SDS-PAGE tubes and placed separately in test tubes .Gels were covered with fixing solution (3.1.5.38) for 1 hour; fixing solution was poured off and the gels were covered with staining solution (3.1.4.10) for 3 hrs . Then a staining solution was poured off and the gels were immerged with destaining solution

(3.1.4.16) The destining process continued with changing the solution several times until blue bands were obtained with white background, gels were stored in 10 % acetic acid.

The molecular weight was determined using specialized software PhotoCapMw.Also, based on the application of the following equation to measure the (Relative mobility R_f) (Elyassaki,1991)

R_f = M x L/M' x L'

Where

M = Migration of protein band

M' = Migration of tracking dye

L= Length of gel before staining

L'= Length of gel after staining

In order to confirm the value of R_f of the markers were plotted against their known molecular weight on a semi logarithmic scale and the samples were calculated in accordance.

3.2.11.4 Determination of α-amylase molecular weight by sephadex G-100

3.2.11.4.1 Standard Protein solution

(lysozyme 14400, ovalbumin 43000, bovine serum albumin 67000 and Alkalane phosphatase 160000 Dalton), were prepared at concentration 10 mg/ml in phosphate buffer 0.005 M pH 7.0 (3.1.5.20).

3.2.11.4.2 Gel Filtration

Gel filtration was done using Sephadex G-100 for the standard protein , well-known molecular weights for each one alone, following the same procedures and under the same conditions applied for the α-amylase and blue dextran (3.2.11.3) (Whitaker, 1963) .

The protein was estimated in each fraction by calculating the elution volume V_e for each one, then the ratio between recovery volume for each protein to

void volume (V_e/V_o) for column as measured by the blue dextran-2000 (3.2.11.3).

A standard curve was drawn by plotting the value of (V_e/V_o) against log molecular weight for each single standard protein, then the α-amylase molecular weight was estimated by plotting its (V_e/V_o) value against the corresponding log value of it .

3.2.12 α-amylase characterization

For all reactions the mixture of enzyme and substrate, were supplemented with 0.005 M $CaCl_2$ and assayed as described in (3.2.7).

3.2.12.1 Determination of pH effect on α-amylase activity

Using buffer solution described in (3.1.5.26) which had been distributed equally into aliquots, the desired pH was adjusted according to the what was described by Ellis and Morrison(1982). 100µl of purified α-amylase was added to 900µl of each one of the different pH solutions ranged (5.5-8.5)containing soluble starch 0.5 % as a substrate. The activity of α-amylase was assayed and plotted against the corresponding pH values to determine the optimal pH for α-amylase activity.

3.2.12.2 Determination of pH effects on α-amylase stability.

Equal volumes of purified α-amylase and buffer solution of (3.1.5.26) with pH range 5.0 through 9.0 were incubated in a water bath at 55°C for 45 min. then transferred immediately into an ice bath .The enzymatic activity for each one was determined.

The remaining activity (%) for enzyme was measured and plotted against the corresponding pH values of solution to determine the optimal pH for α-amylase stability in industry.

3.2.12.3 Determination the optimal temperature for α-amylase activity

The enzyme was incubated with the substrate, in water bath at different temperatures (30, 40, 50, 60,70,80,90 and 100°C) for ten min. Enzymatic activity was measured and the enzyme activity was plotted against the temperature.

3.2.12.4 Determination of thermal stability for α-amylase activity

The purified enzyme was incubated at different temperatures (30, 40, 50, 60,70,80,90 and 100°C) for 30 min., then immediately transferred into an ice bath. Enzymatic activity was assayed and the remaining activity (%) was plotted against corresponding temperature.

3.2.13 products separation by ascending paper chromatography analysis

Hydrolysis products from soluble starch were analyzed by paper chromatography, as described by (Hatada *et al.*, 1996; Safar,1998) with modifications .

1. One hundred µl of purified enzymatic solution was mixed with 900µl of the 0.5%-starch solution (3.1.5.22).
2. The reaction solution was incubated at 60 ^{o}C for (0, 15, 30, and 75 min) , then transferred immediately to an ice bath .
3. Thirty µl of the product were transferred to the TLC paper wattman No.1 with 0.16 mm thickness, allowed to dry , then it was twice to hold a high concentration of possible products , after that the same procedures were applied to standard maltose (3.2.7.1) and glucose (3.1.5.40) solutions
4. The paper was placed in the glass jar containing separation system (3.1.5.39) and the jar was covered, the process was stopped when the solvent reached the end lane

5. After determining the distances of the solvent, the paper was removed from the jar and allowed to dry at room temperature, then it was treated with DNSA (3.1.4.8).

6. The paper was baked in an oven at 100 ^{o}C for 20 min. to complete the reaction , then the R_f was calculated for each sample according to the formula (Ghosh and Chandra ,1980).

$$\mathbf{R_f = \frac{\text{Distance immigrant by solute}}{\text{Distance immigrant by solvent}}}$$

3.2.14 Immobilization of α-amylase.

The α-amylase was immobilized by an entrapment method using sodium alginate according to (Guo *et al.,* 1990)

1. The enzymatic solution was added as 0.2U/ml to sodium alginate solution (3.1.5.24) and mixed well for 10 min .

2. Then the mixture was pulled in by a syringe and dropped into a beaker containing cooled calcium chloride solution (3.1.5.25)to form beads ,allowed to solidify, then washed off many times with a calcium chloride solution to remove the remaining of unimmobilized enzyme .

3.2.14.1 Determination of the efficiency of immobilized α-amylase

Twenty five grams of granules in which the enzyme was immobilized to them was placed into 25 ml of 0.5% starch. Incubated at 60^{o}C degree in a shaker incubator 50 rpm for 10 min. The efficiency was measured according to the formula below (Varavinit *et al.,* 2002) . The immobilized α-amylase was kept in the phosphate buffer at 4°C for monitoring the activity over 30 days.

Activity yield (%) = 100 x activity of immobilized enzyme / A-B

Where:

A: The activity of free enzyme added .

B: The activity of the unimmobilized α-amylase (remaining enzyme and unimmobilized α-amylase in washed water).

3.2.15 Preparation of the anti- amylase sera (Yamaguchi *et al.*, 1974 a,b;1999)

A mixture of the α-amylase (2mg in 0.5 ml of 0.85 NaCl) and 0.5 ml of complete freund`s adjuvant was injected subcutaneously into a mature white rabbits (two rabbits-New Zeeland with two rabbits as a control).Two weeks later, the rabbits received an additional subcutaneous injection of the α-amylase –adjuvant mixture (2 mg of the α-amylase). The mixture was injected into rabbits. Booster injections of the samples were given in the same manner after 4 and 6 weeks, and the blood was collected a week after the last booster injection, sera were separated and incubated at 56 ºC for 30 min to inactive complement, and stored at -20^{o}C .

3.2.15.1 Neutralization of α-amylase with anti-amylase Sera(Yamaguchi *et al.*, 1974a)

Five hundred µl volume of the α-amylase solution was mixed with an equal volume of the antiserum .After incubation at 40^{o}C for 30 minutes; the remaining enzyme activity was assayed .The neutralization was almost completed within 60 minutes. After the reaction completed, the mixture was centrifuged at 6000xg for 20 minutes, then α-amylase activity was assayed.

3.2.16 Genetic analysis of *B. stearothermophilus* M13

3.2.16.1 Isolation of plasmid DNA from *B. stearothermophilus* M13 (Mielenz ,1983)

In order to analyze the genetic potential of *B. stearothermophilus* M13 concerning the possibility of harboring a plasmid , the following procedure was applied.

1. *B. stearothermophilus* was cultured in 25 ml nutrient broth overnight with shaking (120 rpm)at 55 °C ,then 250 ml of fresh nutrient broth was cultured with 2.5 ml of 18 hrs. old growth , incubated at 55°C with shaking(120) rpm overnight.

2. *B. stearothermophilus* M13 cells were harvested at 6000g , 4°C using cooling centrifuge.
3. Cells were resuspended in 5 ml of lysis buffer (3.1.5.10) and incubated for 1 hr. on an ice .
4. Lysate was allowed to stand at room temperature, then 5 ml of SDS solution (3.1.5.3) was added , after 15 min. 10 mg of pronase was added and incubated at 37°C for 30 min.
5. The mixture was diluted with 2.5 ml buffer (3.1.5.27) pH 7.5.
6. An equal volume of (chloroform /Isoamyl alcohol) solution (3.1.5.28) was added, and centrifuged at 12000xg for 15 min.
7. The aqueous phase was transferred to a clean tube , 0.6 volume of chilled isopropanol was added , and centrifuged at 12000xg for 10 min.

The DNA pellet was washed with 70% ethanol , allowed to dry , and dissolved in 750 µl of TE buffer

3.2.16.2 Isolation of genomic DNA of *B. stearothermophilus* M13

1- By Salting out procedure (Pospiech and Neumann, 1995; Kieser, 1995)

The genomic DNA of *B. stearothermophilus* M13 was isolated by salting out procedure with some modifications applied through the study.

1. *B. stearothermophilus* was cultured in 25 ml nutrient broth overnight with shaking (120 rpm) at 55 °C , then 250 ml of fresh nutrient broth was cultured with 2.5 ml of 18 hrs. old growth , incubated at 55°C with shaking(120 rpm) overnight.
2. Cells harvested from 250 ml of bacterial culture using cooling centrifugation 6000g,at 4ºC.
3. Resuspended in the 5 ml SET buffer, then 200 µl Lysozyme solution (3. 1.7.1) and 60 µl of RNase solution (3.1.7.3) were added, incubated for 1 hour at 37 ^{o}C.

4. Three hundred µl pronase solution (3.1.7.2) was added and incubated at 37°C for 45 min., then 600 µl 10% SDS was added , mixed by inversion, incubated 2hrs. at 55 °C, inverted occasionally.
5. Two ml of NaCl (5M) solution (3.1.5.7) was added, mixed thoroughly by inversion, allowed to stand at 37 °C.
6. Equal volume of (chloroform /Isoamyl alcohol) solution (3.1.5.28) was added, mixed by inversion for 30 min at 25 °C.
7. Centrifuge 15 min, 12000 xg at 20°C.
8. Supernatant transferred to a fresh propylene tube add 0.6 volume chilled Isopropanol ,mixed by inversion then left for 2 hours in refrigerator then centrifuged , DNA was rinsed with 70% ethanol ,allowed to dry ,and dissolved in 750 µl TE at 50 °C .
9. Kept at -20°C.

2- By CTAP Procedure (Rogers and Bendich, 1988; Kieser, 1995)

The genomic DNA of *B. stearothermophilus* M13 was isolated by CTAP procedure with some modifications applied through the study .

1. Cells harvested from 250 ml of bacterial culture by cold centrifugation
2. Resuspended in the 5 ml TE25S buffer (3.1.5.5), then 200 µl lysozyme solution (3. 1.7.1) and 60 µl of RNase solution (3.1.7.3) were added, incubated for 1 hour at 37 °C.
3. Three hundred µl of pronase solution (3.1.7.2) were added ,incubated at 37°C for 45 min. , then 600 µl of 10% SDS was added , mixed by inversion, incubated 2hrs. at 55 °C, inverted occasionally.
4. Two ml of NaCl (5M)solution (3.1.5.7) was added, mixed thoroughly by inversion, allowed to be at 37 °C.
5. 700 µl of CTAB/NaCl (3.1.5.6) mixed thoroughly and incubated for 15 min at 55 °C, then cooled to 37°C .
6. An equal volume of (chloroform /Isoamyl alcohol) solution (3.1.5.28) was added, mixed by inversion for 30 min at 25 °C.

7. Centrifuge 20 min, 12000xg at 20 °C .
8. Supernatant transferred to a fresh propylene tube; 0.6 volume chilled Isopropanol was added ,mixed by inversion then left for 1 hours in refrigerator then centrifuged at 12000xg for 15 min. , DNA was rinsed with 70% ethanol ,air dry ,and dissolved in 750 µl TE at 50 ^{o}C .
9. Kept at -20°C.

3.2.17 Isolation and purification of pBR *322* vector from *E. coli* HB 101 by Cesium chloride–Ethidium bromide density gradients ultracentrifugation method (Maniatis *et al.*, 1982).

1- Isolation

1. Ten ml of LB medium containing ampicillin (50µg/ml) was inoculated with single colony of *E. coli* HB101 at 37 °C overnight with shaking.
2. Two hundred and fifty ml of LB medium containing ampicillin (50µg/ml) was inoculated with 0.25 ml of *E.coli* HB101(18 hrs old). incubated at 37 °C with shaking. When the OD_{600} of culture was 0.5 , 1.25 ml of Chloramphenicol solution(34mg/ml in 95% ethanol) was added in which the final concentration was 170µg/ml .
3. The culture was incubated at 37°C for further 12-16 hrs.
4. The bacteria were harvested by centrifugation at 5000xg for 10 min. at 4°C.
5. Wash with 50 ml of ice-cold STE Buffer (3.1.5.10) twice.
6. The cells were resuspended in 10 ml of solution I –lyses solution(3.1.5.8) containing 10 mg/ml Lysozyme, incubated at 37°C for 30 min. in polypropylene tube.
7. Twenty ml of freshly prepared solution II (3.1.5.9) was added, gently mixed for 5 min., allowed to stand on ice bath for 10 min.
8. Fifteen ml of an ice-cold solution of potassium acetate solution (3.1.5.12)(pH 4.8),then an equal volume of chloroform was added, mixed immediately by inversion and allowed to stand for 5 min.on an ice bath.

9. Centrifuged for 20 min, 10000xg at 4°C
10. Aqueous phase was transferred to clean polypropylene tube and 0.6 volume of isopropanol was added , mixed well and allowed to stand at room temperature for 15 min.
11. Centrifuge 20min, 10000 rpm.
12. The supernatant was discarded, the pellet was washed with ethanol 70%, and then the pellet was dried at room temperature.
13. The pellets were dissolved in 5 ml of TE buffer, kept at – 20 °C.
14. Ten µl of the sample was added to 990 µl of TE buffer in a quartz cuvette and the absorbency at 260nm and 280nm was measured after calibration based on blank of TE buffer at 260nm and 280nm., the calculations were done according to the following formula:-

Concentration of double strand DNA (µg/ml) in the sample
= (OD_{260} x 50 x 100)

O.D 260/ O.D280≥ 1.8 provides an estimate the purity of DNA
OD_{260} 1= 50 µg/ml of double strand DNA and 20 µg/ml for single stranded oligonucleotides.

2-Purification

1. The concentration of DNA in TE buffer was estimated, 1.10 g. CsCl per 1 ml of DNA solution (4.29 g to 3.9 ml) was added, mixed and dissolved.
2. Ethidium bromide solution 234 µl of (10 mg/ml) concentration was added and mixed immediately by inversion in which the final concentration of the ethidium bromide was 600 µg/ml, it was centrifuged 10 min. ,8000rpm at 20°C.
3. The empty part of the tube was filled with light paraffin oil and sealed using electric-thermo fuser.
4. The tubes were centrifuged 45 000xg for 36 hours at 20°C.

5. The tubes were removed from the rotor, and fixed to aboard , a small hole at the top tube was made , then # 21 hypodermic needle inserted into the side of the tubes just below the plasmid band , and withdrawal completely.

3.2.18 Removal of Ethidium bromide (Maniatis *et al.*, 1982).

1. An equal volume of 1-butanol saturated with water was added to the cesium chloride –ethidium bromide solution containing the plasmids.
2. The two phases were mixed by pipetting vigorously and Centrifuged at 3000xg for 3 min. at room temperature.
3. The lower aqueous phase was transferred by a clean pipette; the same steps mentioned above were repeted until the pink color disappeared completely from the aqueous solution.
4. The aqueous solution was dialyzed against the TE buffer overnight (pH 8.0), three times, and dissolved in 500 µl , kept at -20ºC.

3.2.19 Digestion of DNA by *Hin*d III (Maniatis *et al.*, 1982)

All items used in the restriction experiment were kept on ice during the experiments, and then transferred to the water bath of 37°C in which the reaction was occurred.

One unit of *Hin*d III is defined as the amount required digesting 1µg of lambda DNA in 1 hour at 37°C in 50µl of assay buffer.

3.2.19.1 Partial digestion of *B. stearothermophilus* genomic DNA (Rodriguez and Tait, 1983)

For partial digestion, four duplicate pilot experiments were made,

The DNA concentration was set up to be 1 µg / µl, and the stock restriction enzyme 10U/µl was diluted using the dilution buffer according to the manufacture to be 1U/µl.

	Experiment One	**Experiment Two**	**Experiment Three**	**Control**
ddH_2O	12 µl	12 µl	12 µl	14 µl
Buffer 10 X	2 µl	2 µl	2 µl	2 µl
DNA volume µl	4 µl	4 µl	4 µl	4 µll
***Hin*d III 1U/µl**	2 µl	2 µl	2 µl	-
Total Reaction volume µl	20 µl	20 µl	20 µl	20 µl
Reaction Time	20 min.	30 min.	40 min.	1 hour

The reaction was stopped by the addition of 4 µl of 0.5 M EDTA pH 7.5 and by being mixed together.

3.2.19.2 Digestion of pBR322 plasmid with *Hin*d III (Rodriguez and Tait, 1983)

ddH_2O	14 µl
Buffer 10X	2 µl
*Hin*d III 1U/µl	2 µl
pBR 322(1µg/µl)	2µl
Total reaction volume	20 µl
Time	2 hours

The reaction was stopped by the addition of 4 µl of 0.5 M EDTA pH 7.5 and mixing gently.

3.2.20 Dephosphorylation of p*BR* 322 (Elyassaki, 1991)

ddH_2O	22 µl
CIP buffer 10X	5 µl
pBR 322(2µg/20µl)	20 µl 10 mM Tris-HCl (pH 7.8)
Intestinal calf alkalane phosphatase	3 µl
Total volume	50 µl

The mixture was incubated at 37°C for 15 min., then switched to 50°C for 20 min., additional 3µl of enzyme was edded, and the same procedure was repeated.

TE buffer was added to the reaction mixture to bring the total volume to 250µl, chloroform extraction was applied, followed by chilled ethanol precipitation, and the pellet was then resuspended in 50 µl of sterilized D.W

3.2.21. Ligation (Elyassaki ,1991, Sambrook and Russell,2001)

Three duplicates of pilot experiments were carried out in order to achieve successful ligation reaction, optimum ligation was performed according to the formula (with some modifications concerning the concentrations of the nucleic acids)

Insert concentration / vector concentration = 5:1 as a ratio

	Experiment 1	**Experiment 2**	**Experiment 3**	**Control**
ddH_2O	10 µl	9 µl	8 µl	12 µl
Ligation buffer 10X	2 µl	2 µl	2µl	2µl
Insert DNA	5 µl	5 µl	5 µl	5 µl
pBR322(10ng/ml)	1 µl	1 µl	1 µl	1 µl
T4 ligase(30U/µl)	2 µl	3 µl	4 µl	-
Total volume µl	20 µl	20 µl	20 µl	20 µl

The mixture was incubated in a water bath at 16°C for 18 hrs. then it was diluted to a final concentration of 2 ng/µl

3.2.22 Agarose gel (For DNA analysis):

Agarose 0.9 % concentration was used, dissolved in TBE 1X (3.1.5.11)

3.2.23 Transformation by Calcium chloride and detection of transformants (Maniatis *et al.*,1982; Hanahan ,1983; Mielenz ,1983;Tang *et al.*,1994).

1. A hundred ml of LB was inoculated with 1 ml of an overnight culture of *E.coli* M M294 , in flask and incubated at 37ºC with shaking until the O.D 600 nm was 0.2 (~ 5 x 10 cells/ ml) .
2. The culture was chilled on an ice bath for 5 min., and then centrifuged at 4000xg for 5 min. at 4ºC.
3. The supernatant was discarded, and the cells resuspended in 50 ml of sterile solution of 50 mM $CaCl_2$,10 mM Tris.Cl (3.1.5.29)pH 8.0.
4. The cell suspension was placed into ice bath for 15 min., and then it was centrifuged at 4000xg for 5 min. at 4ºC.
5. The supernatant was discarded, and the cells resuspended in 7 ml of ice, sterile buffer solution (3.1.5.29) (pH 8.0), the cells were dispensed into 0.2 ml prechilled tubes, and kept at overnight at 4ºC .
6. Ligated DNA dissolved in TE buffer in concentration of 30 ng / 100 µL was added to the 200 µl of cell suspension, mixed and stored into an ice bath for 30 min, then transferred to a water bath, preheated to 42 ºC for 2 min., then transferred to an ice bath for 1 min.
7. One hundred µl of LB was added to each tube ,and incubated at 37 ºC for an hour without shaking
8. Serial dilutions for the cells after the incubation period were made, then the cells were spreaded over nutrient agar plates containing ampicillin (50 µg /ml) and incubated at 37ºC overnight .
9. Replica plating technique was applied for the single Amp^r colonies , assuming the master plate is nutrient agar plate , while the detection plate for the expression of possible α- amylase gene was plates containing detection media(3.1.3.2.13) , incubated at 37ºC for 24 hours . Positive colonies were surrounded by a clear zone on the

plating medium, while negative control was *E. coli* MM294 before transformation.

10. Positive transormant(s) –α-amylase producing colonies were cultured in liquid-starch media (3.1.3.2.16) to assay the specific activity of the α-amylase encoded by the possible recombinant α-amylase gene as mentioned in (3.2.7).

4. Results and Discussion

4.1 Isolation of *Bacillus* spp. producing α-amylase

Ninety six soil samples were collected from different places over Iraq (table 4.1).This showed that sixty nine isolates were thermophilic bacilli; sixty three (60.4%) isolates were α-amylase producing according to the clear zone around the growing colony on the 1.5% starch for 18 hours at 65^{o}C (March and Larson, 1953). Twelve isolates were recognized by the higher production of the α-amylase on the solid media supplemented with 1.5% soluble starch as shown zin the table (4.1), in which the (Z/G) ratio was in the range (1.5 – 2.0) due to enzymatic hydrolysis of starch.

These isolates (12) that showed the highest productivity on the solid medium were chosen and named as (**M1, M9, M13, M23, M24, M26, M29 , M31 ,M36 ,M40 , M60** and **M69**) for further biochemical tests to select *B. stearothermophilus.* The primary screening for the organism that is able to produce an enzyme is considered one of the important steps for enzyme production(Maza *et al.*,1997;Teodoro and martins ,2000).

Table (4.1): Screening of thermophilic *Bacillus.* spp. isolates for their ability to produce α-amylase.

Isolate No.	Growth diameter (mm)	Halo (clear zone) diameter (mm)	Z / G ratio	Isolate No.	Growth diameter (mm)	Halo (clear zone) diameter (mm)	Z / G ratio
M1**	2	4	2	**M50**	3	4	1.33
M2	3	4	1.33	**M51**	4	5	1.25
M3	2	-	-	**M52**	4	-	-
M4	5	6	1.2	**M53**	5	-	-
M5	4	5	1.25	**M54**	5	6	1.2
M6	3	4	1.33	**M55**	3	4	1.33
M7	4	5	1.25	**M56**	4	5	1.25

M8	2	-	-	**M57**	3	4	1.33
M9*	2	3	1.5	**M58**			
M10	4	-	-	**M59**	3	4	1.33
M11	5	6	1.2	**M60***	2	3	1.5
M12	3	4	1.33	**M61**	4	-	-
M13**	2	4	2	**M62**	4	5	1.25
M14	2	3	1.33	**M63**	3	-	-
M15	5	7	1.4	**M64**	3	-	-
M16	3	4	1.33	**M65**	3	4	1.33
M17	2	-	-	**M66**	3	-	-
M18	2	-	-	**M67**			
M19	3	-	-	**M68**	3	4	1.33
M20	3	4	1.33	**M69***	2	3	1.5
M21	3	-	-	**M70**	2	-	-
M22	3	-	-	**M71**	3	4	1.33
M23**	3	6	2	**M72**	4	5	1.25
M24	3	5	1.66	**M73**	2	3	1.5
M25**	3	-	-	**M74**	2	-	-
M26	3	5	1.66	**M75**	3	5	1.25
M27	3	4	1.33	**M76**	3	4	1.33
M28	2	-	-	**M77**	5	6	1.2
M29**	2	4	2	**M78**	3	4	1.33
M30	2	-	-	**M79**	5	6	1.2
M31**	3	5	1.66	**M80**	3	-	-
M32	3	4	1.33	**M81**	2	-	-
M33	3	4	1.33	**M82**	2	-	-
M34	5	-	-	**M83**	3	4	1.33
M35	5	-	-	**M84**	4	5	1.25
M36*	2	3	1.5	**M85**	3	4	1.33
M37	3	-	-	**M86**	2	-	-
M38	5	6	1.2	**M87**	4	5	1.25
M39	4	-	-	**M88**	4	5	1.25
M40	2	3	1.5	**M89**	5	6	1.2
M41	3	-	-	**M90**	4	5	1.25

M42	3	-	-	**M91**	3	4	1.33
M43	3	4	1.3	**M92**	4	5	1.25
M44	3	4	1.3	**M93**	5	6	1.2
M45	4	5	1.25	**M94**	3	-	-
M46	3	-	-	**M95**	2	-	-
M47	2	-	-	**M96**	3	-	-
M48	2	-	-	**-**			
M49	3	4	1.3	**-**			

* **Thermophilic *Bacillus* sp., amylase producing**
**** *Bacillus stearothermophilus*, amylase producing**
H: halo, clear zone
G: Growth diameter
pH range of soil samples was (7.1-7.4);(slightly alkalane).

4.2 Identification of the isolates

Twelve isolates were subjected to morphological, microscopical , biochemical and physiological tests to identify *B. stearothermophilus* producing α-amylase, isolates were gram positive bacilli, negative on the Voges-Proskauer test at (pH 7.2), motile, central spores and predominantly unswollen cylindrical sporangia.The morphological and physiological characteristics for seven isolates of them showed that they were to be *Bacillus stearothermophilus* according to the Claus and Berkeley (1986) in which the growing colonies on the tryptic soya agar at 55 ^{o}C for 18 hours had the circular edges, irregular and dark rough surface have a diameter of (1.5-3.0 mm.) recognized by an unacceptable odor (Welker and Campbell ,1963) ,while on the nutrient starch agar and nutrient agar, colonies had a regular edge and a rough surface with (2-5mm.). The cells were examined by dying with gram stains; the results showed they are gram positive, spore forming in which they had terminal spore causing the inflammation of the cells (figure4.1). Their growing in the nutrient broth appeared as fine granules causing the turbidity of the medium. The biochemical tests were done on

these isolates to determine the species as pointed out in the table (4.2). The results were closely matching the properties of *B. stearothermophilus.* From these results , the bacteria were identified as *B. stearothermophilus* by the criteria of Bergey's Manual of Systematic Bacteriology, in which they were named as (*B. stearothermophilus* **M1, M13, M23, M24, M26, M29,** and **M31**).

Table (4.2):Biochemical and physiological tests for identification of *Bacillus stearothermophilus* producing α-amylase

Biochemical Tests		M1	M9	M13	M23	M24	M26	M29	M31	M36	M40	M60	M69
Starch hydrolysis		+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis		+	+	+	+	-	+	+	+	+	-	-	-
Growth in 7% NaCl		-	-	-	-	-	-	-	-	-	-	-	-
Aerobic growth		+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth		-	-	-	-	-	-	-	-	-	-	-	-
Growth at	40 °C	+	+	+	+	+	+	+	+	+	+	+	+
	50 °C	+	+	+	+	+	+	+	+	+	+	+	+
	60 °C	+	+	+	+	+	+	+	+	+	+	+	+
	65 °C	+	+	+	+	+	+	+	+	-	+	-	+
Motility		+	+	+	+	+	+	+	+	-	-	-	+
V-P reaction		-	-	-	-	-	-	-	-	-	-	+	+
Citrate utilization		+	+	+	+	+	+	-	+	-	-	-	-
Catalase		+	+	+	+	+	+	+	+	-	+	-	-
Nitrate to nitrite		+	+	+	-	+	+	-	-	+	-	-	+
Gas production		-	-	-	-	-	-	-	+	+	-	+	+
indol production		-	-	-	-	-	-	-	-	+	+	+	+

(+) positive result (-) negative results

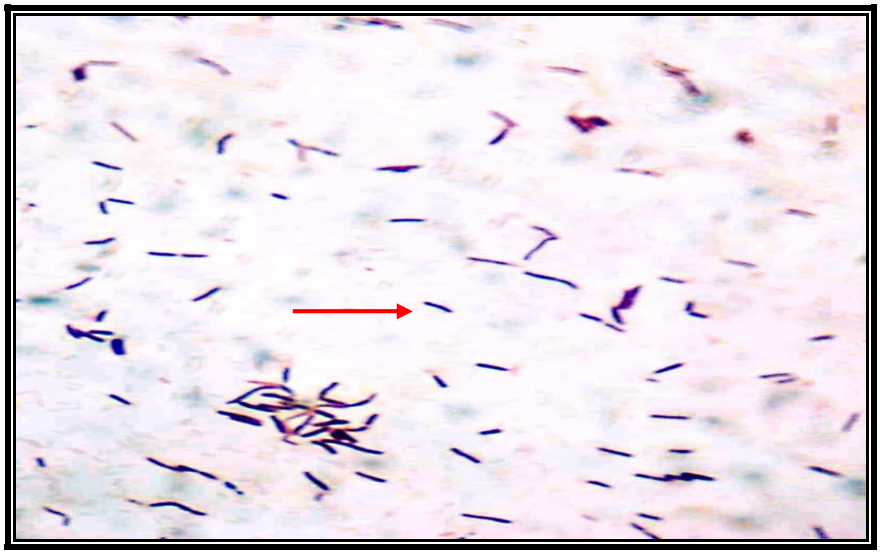


Figure (4.1): Microscopic field of *Bacillus stearothermophilus* M13 (red arrow)(growing on surface of nutrient Agar at 55°C for 24 hrs)stained with Gr.stain 1000X

4. 3 α-amylase Production in the submerged cultures

The seven isolates that have been chosen for the α-amylase production in the submerged cultures based on their production in the solid medium to determine the highest one in the production of α-amylase using N.B. medium supplemented with 1.5 % starch and to determine the optimal temperature for the α-amylase activity assay. The results, shown table (4.3) for specific activity of α-amylase, produced by the seven selected isolates M1, M13, M23, M24, M25, M26, M29 and M31.That isolate M13 was the highest one among them ,which was 0.054 U/mg in the crude extract . While, the specific activity range for the others were (0.025-0.036) U/mg. when the assay done at 60°C . While the assay at 40°C was lower with about (20.3 %) as losing activity .According to those results, M13 was considered as the most efficient isolate for α-amylase production for this study and for accurate selection for the most efficient producer α-amylase. These isolates (7) were tested again to confirm the results. Then three subculture cycles were carried out for *B. stearothermophilus* M13 and revealed approximately constant specific activity values.

Bacillus stearothermophilus can produce large amounts of extracellular enzymes such as amylases and proteases, the α-amylase is highly thermostable and is desirable for the industrial applications (Mielenz , 1983 , Egelseer *et al.*, 1995).

Table (4.3) Quantitative screening of α-amylase production from *B. stearothermophilus* for in N.B medium supplied with 1.5 % starch ,incubated at 55ºC for 24 hrs.

Isolate No.	Specific Activity U/mg protein assayed at 40 °C	Specific Activity U/mg protein assayed at 60 °C
M1	0.012	0.03
M13	0.011	0.054
M23	0.01	0.028
M24	0.015	0.032
M26	0.012	0.036
M29	0.013	0.025
M31	0.012	0.032

4.4 Optimization of the α-amylase production in liquid culture.

4.4.1 The effect of media on α-amylase production

The seven isolates that have been chosen for being *Bacillus stearothermophilus* producing α-amylase were inoculated in three different liquid media SH, TSM and(N.B supplemented with 1.5 % potato starch), in which the pH was 7.0 for the all . According to the results shown table (4.4), the isolate M13 was the most efficient in the production of α-amylase using TSM medium in which the specific activity was 0.055 U/mg , and in order to confirm those results, a second screening was done for those isolates to

confirm the specific activity using the same medium , the results were similar.

Table (4.4): α-amylase production from *B. stearothermophilus* isolates in different media, incubated at 55ºC for 24 hrs , pH 7.0

Isolate No.	TSM Medium specific activity u/mg	N. B with (starch 1.5%) specific activity u/mg	SH Medium specific activity u/mg
M 1	0.039	0.03	0.025
M 13	0.055	0.054	0.037
M23	0.034	0.028	0.033
M24	0.037	0.032	0.028
M26	0.032	0.036	0.034
M29	0.031	0.025	0.021
M31	0.031	0.032	0.024

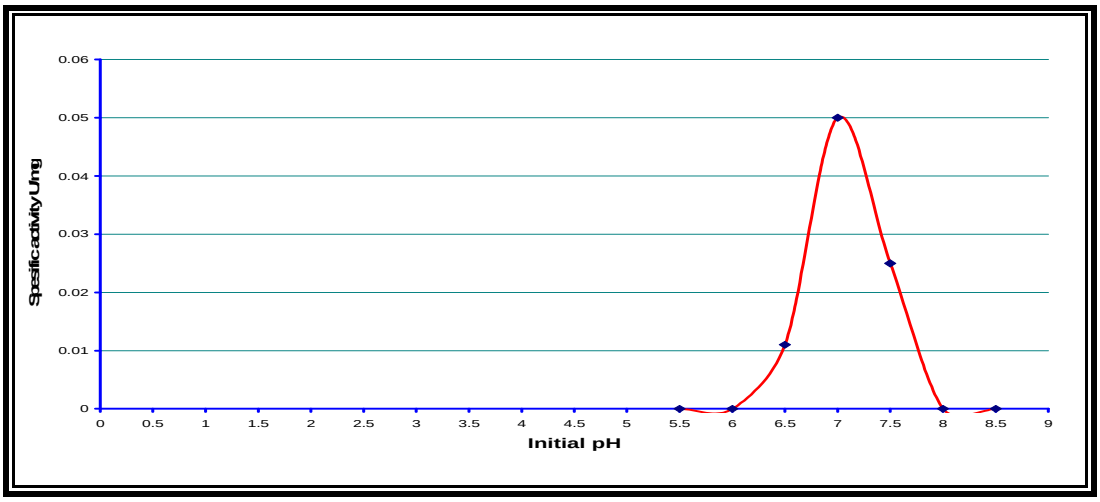
As table (4.4) illustrated that *B. stearothermophilus* M13 isolate was the most efficient one in the production of α-amylase in which the specific activity was 0.055 U/mg using the TSM medium. TSM medium was the best for the production of α-amylase among the tested media, which enhanced the production of α-amylase by the isolate *B. stearothermophilus* M13 .It was recommended by some previous studies (Saito and Yamamoto, 1975; Krishnan and Chandara,1983). The ability to support the production may be attributed to its composition, The composition and concentration of nutrients and factors in the media greatly affect the growth and production of extracellular amylases in bacteria (Srivastava and Baruah, 1986 ; Shih and Labbe,1995),yeast (Fossi *et al.*,2005)and fungus(Zangirolami *et al.*,2002) .The production of α-amylase could be affected by the amount and the nature of nitrogen source as trypton ; besides the variability of divalent cations Ca^{2+} ,Mg^{2+} that were tested for the effect production in which they

slightly induced the production ,while the trivalent cations such as Fe^{+3},As^{+3} and Au^{+3} were inhibitors at certain morality concentrations (Srivastava and Baruah , 1986) .The form and quantity of carbon source in culture media is very important for both growth and production of α-amylase in *Bacillus* . Ajayi and Fagade (2006)revealed the presence of soluble potato starch as the sole carbon source as an inducer of the production of enzyme of *B. licheniformis* because it had the α 1-4 glycosidic bond .While Aiyer (2004) reported that *B. licheniformis* could produce the enzyme in the absence of starch . On other hand, the ratio of C:N in the media could affect α-amylase production , Aiyer (2004) mentioned that the best ratio for production was "1" to be applied for *B. licheniformis* . While the ratio noticed in TSM was" 2 "this could be explained by the difference of the physiological and special requirements of α-amylase production between the *B. stearothermophilus* M13 and *B. licheniformis*.So, the TSM medium was the main medium applied during the present study, otherwise it indicated somewhere in the study.

4.4.2 The optimal initial pH for α-amylase production

To investigate the effect of initial pH medium on α-amylase production, *B. stearothermophilus* M13 was grown on TSM medium with different pH values .The results (figure 4.2) showed that initial pH for a higher production of α-amylase was close to optimal pH of bacterial growth which was (7.0), while there was no growth at pH values of < 6.0 and > 8.0 , in which we could suggest the higher production linked to the higher growth of the bacteria , that result was much similar to what Teodoro and Martins (2000) reported about the production of thermostable amylase by *Bacillus* sp. , Srivastava and Baruah,(1986) reported that the initial pH for the production of thermostable amylase by *B. stearothermophilus* was (6.9). The effect of the pH value on the enzyme production resulted from its role in the

solubility of nutrients, ionization of the substrates and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme (Bull and Bushnell, 1972). Also, dissolved CO_2 in the medium affected the buffering capacity (Segel, 1976). The low pH reduced the solubility of CO_2 in the growth medium and limited its availability for assimilation by the anaplerotic enzyme pyruvate carboxylase (Maheshwari *et al*., 2000). The research of Mazza and Ertola (1976) on the production of α-amylase by *B. subtilis* showed that the optimal pH was 7.2 using fermentor made him to attribute the influence of initial pH on enzyme formation, or on the release of enzyme from cells, and to consider strain differences as well as the influence of the components of the medium. It is clear that the optimal pH of α-amylase reproduction depends on the strain or the nature of the producer microorganism . Foosi *et al*.(2005) mentioned that the initial pH of media for amylase production by yeast was 4.5 . While, Lin *et al*,(1998) reported that optimal initial pH for the production by thermophilic and alkaliphilic *Bacillus* sp. was 8.5 . Furthermore, the pH affects the active sides on enzymes which are frequently composed of ionizable groups that must be in the proper ionic form in order to maintain the conformation of the active site (Segel, 1976) .



Figure(4.2):Optimal pH for amylase production by *B .stearothermophilus* M13 in TSM medium incubated at 55ºC for 18 hrs with shaking 120 rpm

4.4.3 Optimal temperature for α-amylase production

The results in (figure 4.3) showed that extracellular α-amylase activity increased with the temperature elevation from 20°C with specific activity of 0.01 U/mg to 60°C with 0.05 U/mg, in which the optimal temperature for the production of α-amylase was 60 °C with activity 0.055 U/mg , then the activity will decrease .

Srivastava and Baruah (1986) reported the optimal temperature for production of thermostable amylase by *B. stearothermophilus.* was 50 ± 5°C. while Lin *et al.*, (1998) reported that the optimal temperature for the production of thermostable from thermophilic and alkaliphilic *Bacillus* sp. was 55°C which is also the optimum for growth. In another study on production of α-amylase by *B. licheniformis* SPT 27 was 37°C (Aiyer, 2004) .

Furthermore, Teodoro and Martins (2000) reported that the optimal temperature for growth and production α-amylase by *Bacillus* sp. were 50ºC . While these results are in contrast with the findings reported by Chandra *et al.(*1980)which reveled that the optimal temperature of growth for *B.licheniformis*CUM 305 was 30ºC , but it never produced the α-amylase at that temperature . In addition, Saito and Yamamoto (1975) studied a *B. licheniformis* which produced α-amylase at temperatures around 50°C and never produced the enzyme at temperatures lower than 45°C.

In general, the perish of enzyme activity with elevation or decreasing the temperature may be attributed to its effects on the growth of the microorganism and velocity of the enzymatic reactions inside the cells reflecting the properties (Cornish, 1979) .

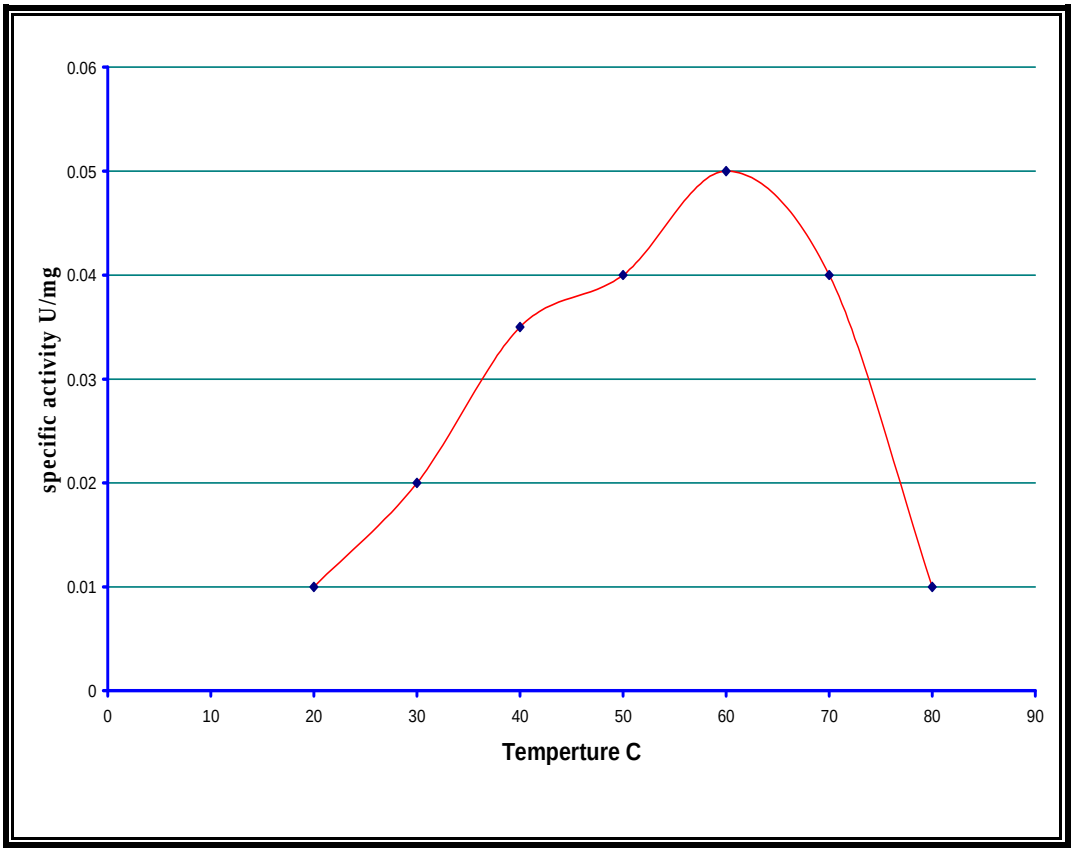


Figure (4.3): Optimal temperature for α-amylase production by *B. stearothermophilus* M13 , incubated at 55ºC ,pH =7,18 hrs with shaking 120rpm

As a fact large number of weak non-covalent bonds maintain the tertiary of the structure of an enzyme, when it absorbs too much energy the tertiary structure will disrupt, and the enzyme will be denaturized. temperature affects the production of enzyme through some aspects like O_2 concentration, medium moisture and enzymatic reactions inside the cell (Segel, 1976).

4.4.4 Optimal growth phase for α-amylase production

Measurements of the enzyme activity and cell growth of *B. stearothermophilus* M13 at a number of time intervals are shown in Figure (4.4). The addition of 10 mM calcium chloride to the liquid medium improved the growth and amylase production since the enzyme is known to be a calcium metalloenzyme as reported by Dauter *et al.*(1999).

The activity of α-amylase produced by *B. stearothermophilus* M13 during growth was investigated in the cell extracts at different time intervals which they covered the lag, log, stationary and the declane phases.

The results in (figure 4.4) showed that the growth entered the stationary phase at the age of about 18 h. in which the $O.D_{600nm}$ was 1.6 , while the growth at the late log had the higher α-amylase specific activity which was 0.05 U/mg than the early log and stationary phases .So it was concluded that the production of α-amylase depends on which phase the growth is passing through, making the highest production at the late log phase.

Teodoro and martins (2000) mentioned that the α-amylase produced by *Bacillus* sp. was at late log phase ,and in similar results Fossi *et al.*(2005) registered that the Amylase production by yeast was correlated to its biomass which was the highest at the late log phase .Concerning the *Bacillus stearothermophilus* , Srivastava and Baruah (1986) noticed that the amylase get the maximal production during post-exponential and stationary growth phases .

The termination of α-amylase production observed during the growth on starch might reflect the accumulation of glucose or maltose as repressing metabolite for α-amylase production. The other type of control over this enzyme is the graded production of the catabolic enzyme levels resulting from growth on other specific carbon sources which are independent of the presence of starch (Nagata *et al.*, 1974; Nicholson and Chambliss, 1985; Srivastava and Baruah ,1986; Haseltine *et al.*, 1996). On other hand, Zangirolami *et al.* (2002) set up a model for growth and α-amylase formation by *Aspergillus oryzae* , the specific rate of α-amylase production increased with the specific growth rate, α-amylase strongly repressed by residual glucose concentration .

Those results could be interpreted that the log phase is characterized by an increase in the biomass with highly active cells in most physiological activities which greatly affected enzyme production (Mischack *et al.*, 1985), while the elongation of fermentation time caused undesired effects on the production of α-amylase and its activity because environmental changes in media ,autolysis resulting in releasing a lot of material and intracellular lytic enzymes that could destroy the α-amylase (Lonsane and Ramesh ,1990) .

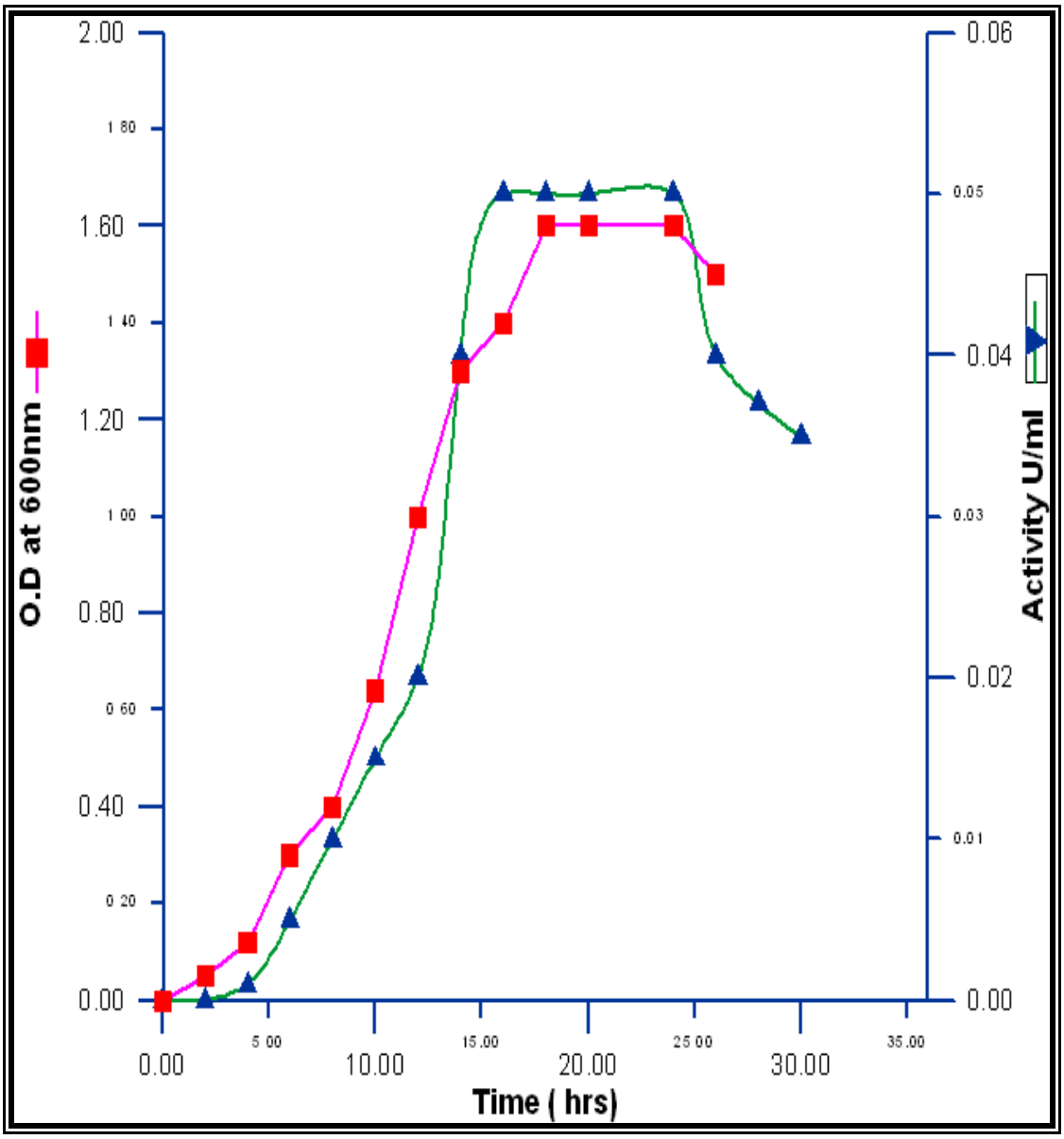


Figure: (4.4) growth curve and α-amylase production phase for the *B. stearothermophilus* M13 in TSM medium, incubated at 55°C , pH (7.0) with shaking 120 rpm

4.5 Purification of α-amylase of *B .stearothermophilus* M13

α-amylase applications in pharmaceutical ,industrial and clinical sectors requires high- purified enzyme or partial purified enzymes depending on the process taking into consideration the costs of each one related to that(Deutscher, 1990) .

4.5.1 Crude enzyme concentration by Ammonium sulfate

In order to concentrate on the crude α-amylase in culture supernatant and remove as much water and some protein molecules as possible, saturation ratio (40-80 %)was used to achieve that through two addition steps, the first step is increasing the saturation ratio from (0 - 40 %) ,which showed low specific activity of precipitated fraction of 0.002 U/mg .when (40-80)% saturation ratio was applied to the supernatant fraction , it achieved a higher specific activity to 0.051 U/mg , 0.92 purification folds with 57 % yield . From the results demonstrated above, approximately 96% of activity is within the (40-80) %. But, when we compare between the specific activity of crude to that precipitated by Ammonium sulfate , it was found the specific activity of crude is higher than resulted by precipitation ,so it is concluded that the Ammonium sulfate step is not properly recommended to this particularly stage of purification . Concerning the other studies, those results are in contrast with Sarivstava and Baruah (1987) who obtained specific activity of 210.6 U/mg for α-amylase produced by *B. stearothermophilus M13.* Through Ammonium sulfate precipitation ratio of (45-60)% ,while Aiba *et al.*(1983) obtained specific activity of 8 U/mg with 22% recovery from *B. stearothermophilus* and *B. subtilis* in successive steps using(30-45)% saturation ratio. While, Fossi *et al.* (2005) used 65% (w/v) sodium sulphate to achieve partial purification of α-amylase produced by yeast.

Furthermore, α-amylase produced by *Xanthomonas campestris* was precipitated by (45-70) % to achieve specific activity of 4.51 U/mg with 91% Recovery(Abe *et al.*, 1994) . In general, Ammonium sulfate is favored in precipitation step due to its high solubility, availability, being cheap and that it does not damage most enzymes (Volesky and Luong ,1985) .

The theory that explain the mechanism of salting out is combined with the old theory; salting-out occurs as a result of a decrease in the hydration of proteins in favor of the that of the ions of the neutral salt , the hydration layer surrounding the surface of the protein is transferred to the ions of the salt at a rate depending on the charge of the protein ,pH and temperature (Schutte, 2003) . And the recent theory is that a salt ion has a repulsive interaction with an image charge inside a low dielectric cavity (Zhou, 2005).

4.5.2 Ion –Exchange and Gel-filtration chromatography

The results in (table 4.5)showed that α-amylase purification could be interpreted that the first step after the centrifugation of culture extract contained the growth biomass , was to be concentrated, and dissolved in 20 ml of the distilled water containing 0.025 M of $CaCl_2$, then it was dialyzed (4.5 by 10-cm bags;10,000 kDa cutoff) against distilled water, phosphate buffer 0.005 M (pH7.0) supplied with 0.025 M $CaCl_2$ over four times . It achieved less specific activity to be compared with the crude with 57% yield ,0.92 folds of purification , but higher activity of 0.114 U/ml , which is considered as a step for the partial purification of some commercial important enzymes , in which it could be used at this step for further usages . Then anion ion-exchange chromatography using DEAE-Sephadex step was set up, initially it was equilibrated with the same buffer of dialysis 0.005 M phosphate buffer pH 7.0 . After applying the sample to the column, washing with the same buffer was run. the protein profile (figure 4.5)showed three peaks , but no activity was indicated for α-amylase , that could because of

those proteins have a similar charge to the anion (positively charged) or zero charge because when the pH ≤ pI for the enzyme , it will make the enzyme be marked with a positive charge . DEAE-sephadex is a weak anion exchanger with excellent flow properties and high capacity for proteins of most pI values with high resolution. The ion exchange functional group is diethylaminoethyl that remains charged and maintains consistently high capacities over the entire working range (LKB). Therefore, it could be concluded that α-amylase has been bounded onto the matrix of the exchanger, those protein fractions were washed out. then the elution was run using lanear salt gradient 0.005 M potassium phosphate – 1 M NaCl (pH 7.0) which could detect five peaks for protein and four main peaks for α-amylase activity figure (4.6), which marked as peak {***a,b,c*** and ***d*** } .Then the fractions for each single peak were collected with each other and concentrated using sucrose through dialysis bag , and dialyzed using the same procedure after the concentration by ammonium sulphate . The peak "***b***" showed the higher specific activity of 1.475 U/mg with 26.8 folds of purification and 73.75 % yield that made it as a good source for further purification among the others ,in which the peak "***a*"** showed a specific activity of 0.416 U/mg with 7.56 folds of purification , peak "***c***" showed a specific activity of 0.20 U/mg with 3.63 folds of purification and peak "***d*"** showed a specific activity of 0.043 U/mg with 0.78 folds of purification , that made an assumption of being participating to a large extent in the overall specific activity of the produced present α-amylase . The presence of four activity peaks for the α-amylase produced by *B. stearothermophilusM13* means that those could be forms (Isoenzymes) or " Isozymes" for the α-amylase which is in the first time a ,study showing such a number for *Bacillus stearothermophilus;* concerning the other studies of isoenzymes , Zhang *et al.*(1994) reported that three forms for α-amylase produced by *Bacillus* sp. with molecular weight

(Mr)110-140 kDa, two forms produced by *Bacillus* sp. with Mr of 150-42 kDa were purified by Lin *et al.*, (1998).

Furthermore, most of the studies showed a single form for α-amylase produced by *B. flavothermus* (Bolton *et al.*,, 1997) , *B. licheniformi* (Kim *et al.*, 1992; Ivanova *et al.*, 1993) . There are several possible explanations for being different Isozymes of α-amylase, they are either purification procedures due to protease(s) hydrolysis (Koch *et al.*, 1990; Pompeyo *et al.*, 1993), carbohydrate contents or protein aggregations (Spreinat *et al.*, 1990; Giraud *et al.*, 1993 ;) or deamination of α-amylase (Park *et al.*, 1991).

The fraction termed as (peak *b*) was collected together and concentrated by osmosis against sucrose crystals, then applied to Sepahdex G-100 gel filtration column in order to obtain more purified α-amylase . (G-100) is characterized by being easily prepared, long term stability and high speed flow rate and the ability to separate a broad range of the molecular weights for proteins . Sepahdex G-100 had a unique characteristic of being a combine between affinity chromatography and gel filtration properties on which it had a tendency to bind with α-amylase causing delaying in elution time of it (Scopes, 1987).

α-amylase was partially purified , Figure (4.7) showed two peaks for protein from fraction 25th through fraction 30th and matching approximately single peak for the α-amylase activity from fraction 27th through 30th which is an indicator for a partial purity of α-amylase after the gel filtration step, of specific activity 1.675 U/mg , folds of purification 30.45 and 50.25 % yield , which are considered as important results when compared to other studies . Shaw *et al.*(1995) obtained α-amylase purified from *Thermus* sp. of activity 2.22 U and 2.6 % yield using hydrophobic interaction chromatography step and followed by affinity chromatography onto corn starch .Paquet *et al.* (1991) purified the α-amylase from *Clostridium acetobutylicum* ATCC 824 using ion-exchange and gel filtration to get amylase with specific activity

57.7 U/mg , purification folds 22.2 and yield 23.2 % .while, shih and Labbe (1995) could purify the α-amyalse produced by *Clostridium perfringens* Type A using double filtration through DEAE-sephacryel achieving specific activity of 34.6 U/mg ,purification folds 20.4 and yield 11.3 %.α-amylase was purified from *B. stearothermophilus* using DEAE-Sephadex A 25 to get specific activity 100 U/mg, and 57 % yield(Aiba *et al.* ,1983).

Furthermore, α-amylase was purified from Alkaliphilic *Bacillus* sp. using DEAE –Toyopearl and CM-Toyopearl obtaining specific activity 5,009.3 U/mg and 35 % yield (Igarashi *et al.*, 1998). While, Lin *et al.*, (1998) used Sephacryel S-100 and HiTrap Q in a procedure to purify the α-amylase from Thermophilic alkaliphilic *Bacillus* sp. , they obtained a specific activity of 921.0 U/mg , purification folds of 708 .5 and yield 13.2 % ,Abe *et.al*,(1994) purified α-amylase from *Xanthomonas campestris* K-1151 by means of Cm- Toyopearl and phenyl-Toyopearl achieving a specific activity of 256 U/mg and 36% yield .

Table (4.5): purification steps of α-amylase produced by *B. stearothermophilus* M13 in TSM medium

Steps of purification	Activity U/ml	Volume ml	Protein conc. mg/ml	Specific activity U/mg	Total activity	Folds of purification	Yield %
Crude extract	0.04	100	.725	0.055	4	1	100
Ammonium sulphate precipitation 40-80%	0.114	20	2.2	0.051	2.28	0.92	57
Ion-exchange *a*	0.125	10	0.3	0.416	1.25	7.56	31.25
Ion-exchange *b*	0.295	10	0.2	1.475	2.95	26.8	73.75
Ion-exchange *c*	0.11	5	0.525	0.209	0.55	3.8	13.75
Ion-exchange *d*	0.08	10	1.825	0.043	0.8	0.78	20
Gel-filtration	0.201	10	0.12	1.675	2.01	30.45	50.25

a **stands for peak 1**
b **stands for peak 2**
c **stands for peak 3**
d **stands for peak 4**

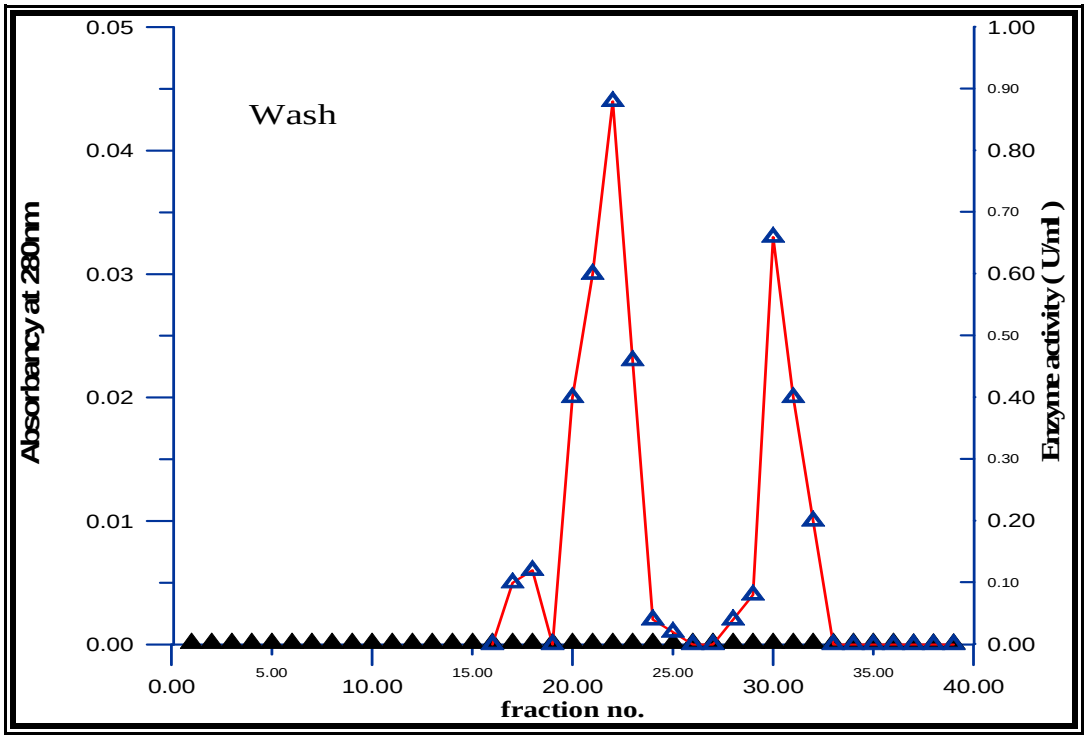
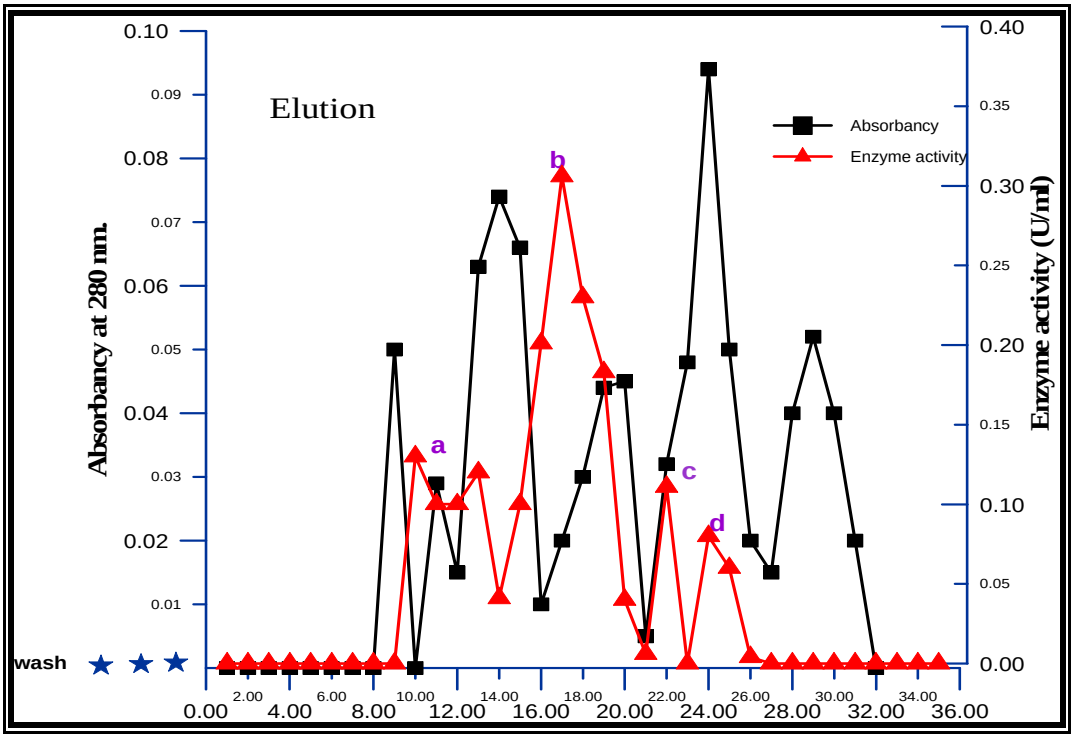


Figure (4. 5) : DEAE-Sephadex Ion- Exchange Chromatography column (2.5 X 35)cm .washing step with 0.005 M Phosphate Buffer pH 7.0 at flow rate of 30 ml/h. and 5ml /frac.



Figure(4.6):DAEA-Sephadex ion Exchange chromatography column (2.5 X35) cm.(Elution step) ,enzyme recovered with lanear salt gradient (0.005 M Phosphate Buffer – 1 M NaCl)pH 7.0 at flow rate 30 ml/h. 5ml/frac.

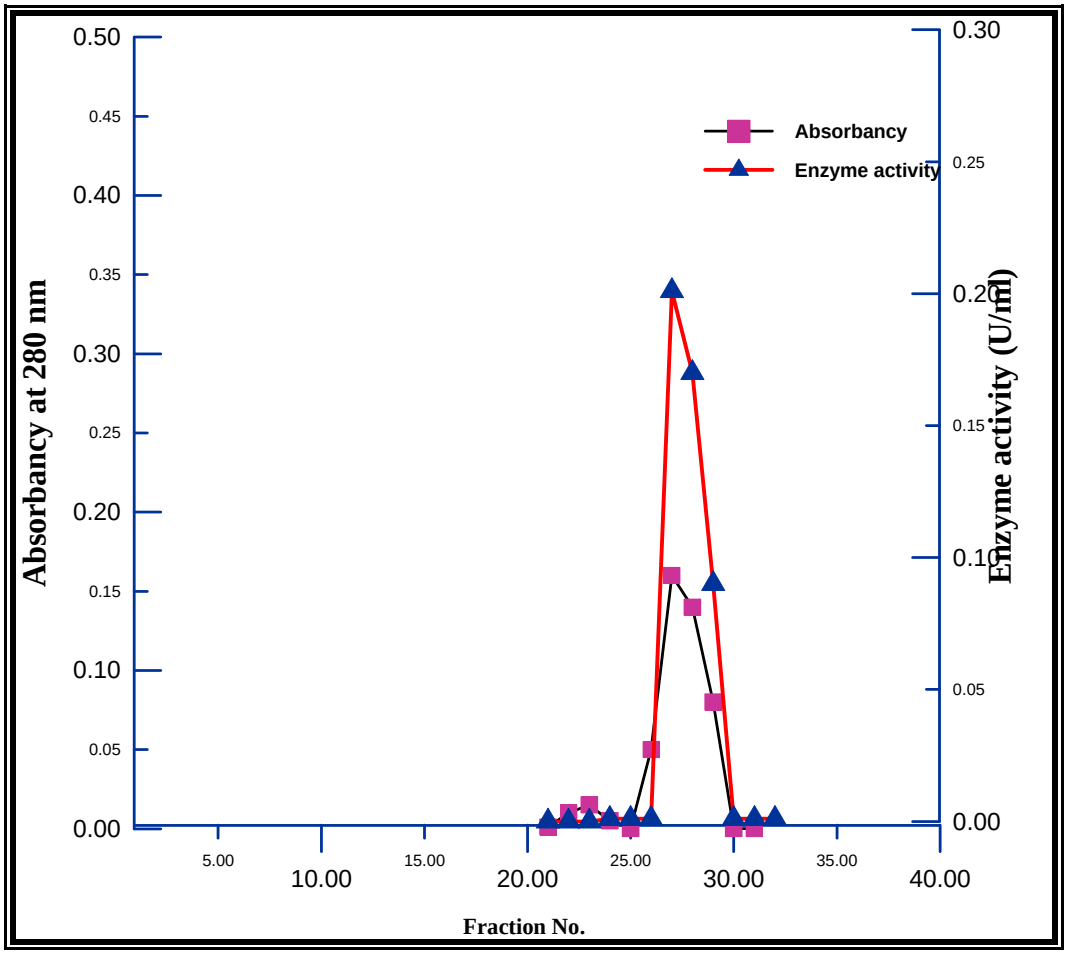


Figure :(4.7) Sephadex G-100 gel filtration, chromatography for active fraction from DEAE- Sephadex "peak *b* ", column was run onto 1.3 X 35-cm Sephadex G-100. The column was equilibrated with 0.005M phosphate buffer (pH 7.0), the elution at rate of 30 ml/hr, 5ml/ frac.

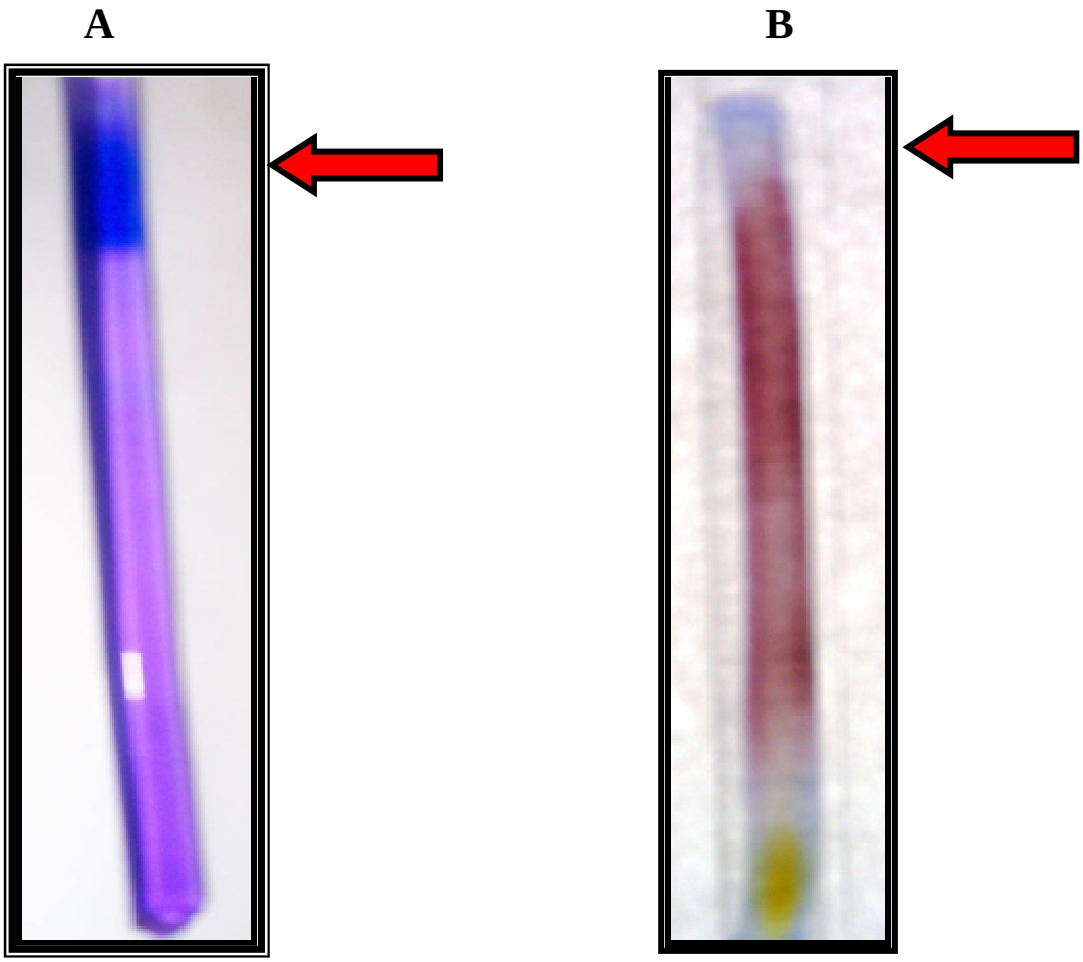
4.6 Characterization of α -amylase

4.6.1 α-amylase partial purity (Electrophoresis under Non- denaturized Conditions

The purity of the α-amylase produced by *B. stearothermophilus* M13 was initially identified by gel filtration Sephadex G-100 technique through obtaining a single sharp peak of activity matching the peak of protein. With the aim of confirming the result, another step was done described as protein electrophoresis under denaturized and non-denaturized conditions.

The electrophoresis of α-amylase purified by gel filtration, on polyacrylamide gel under non-denaturized conditions was done. Figure (4.8) showed the pattern of α-amylase electrophoresis , in which one single band was detected after staining the gel with Coomassie brilliant blue R-250 dye (figure 4.8-A) ,and one single band was visualized in the gel saturated with 1.5% starch grains ,then stained with the activity dye " iodine" (figure 4.8-B).It is clear that the band is localized near the upper part of the gel after about 3 .5 hours, which could be explained that the enzyme under non-denaturized condition has no enough counter-charge density (positively charged) at pH 6.8- 8.3 that could be driven towards the anode pole. The protein immigration on the gel depends on the net charge density of protein at a given pH and the configuration of it (Johanstone and Thorpe ,1987; Shi and Jackowski , 1998). The presence of a single band may indicate the homogeneity of enzyme . Lin *et al.*(1998) obtained two isoenzymes for α-amylase by electrophoresis depending on the activity staining ,then it found a single band for each isoenzyme using SDS-PAGE . Sometimes , it is confusing to determine clearly the single individual band using PAGE because of there are several possible explanations for the multiple forms (Isozymes) of amylases, including the purification procedures themselves, protease(s) hydrolysis carbohydrate content and protein aggregation during electrophoresis (Shih and Labbe,1995).

The result of this study is much similar to that of Pfueller and Elliott (1969) who reported that one single band for α-amylase was visualized by the activity staining (Iodine), using non-denaturized conditions for PAGE , near the upper part of gel .



Figure(4.8):Polyacrylamide Gel Electrophoresis "PAGE" (7.5%) for α- amylase purified from *B. stearo*thermophilus M13(amplified by S-splane programm).
A- Purified enzyme visualized by Coomassie brilliant blue(R-250) staining
B- Purified enzyme visualized by activity dying "lugol`s dye"
Red arrows indicated positions of the partially purified α-amylase band

4.6.2 Determination of α-amylase molecular weight (Mr)

The Mr of α-amylase was determined using two methods :

1- Gel filtration method

Sephadex G-100 column (2.6 x 35) was used to estimate the molecular weight (Mr) of α-amylase, as blue dextrane was used to determine the void volume of column .When the Ve/Vo was plotted against logarithm of the Mr, the Mr was estimated to be " 56234 " Dalton figure (4.14) in which that value is closer to what some previous studies were reported using gel filtration, Pfueller and Elliott(1969) determine the Mr of α-amylase produced by *B. stearothermophilus* using gel-filtration to be 53000 Dalton , while the Mr of thermophilic extracellular α-amylase produced by *B. licheniformis* was estimated to be 22500 Dalton (Saito ,1973) .Figure (4.9) showed the elution of blue dextrane, which has Mr of 2.6 x 10^6 Dalton, the presence of shoulder during the elution could be due to the degradation of it for its big molecular weight.

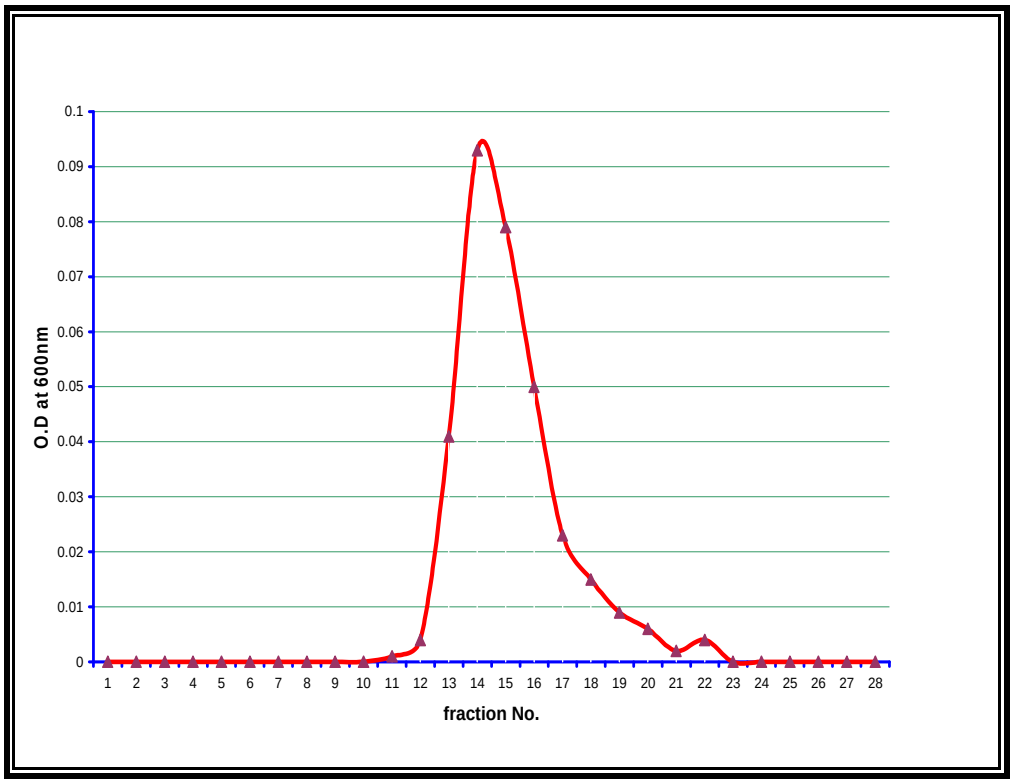


Figure (4.9): Sephadex G-100 gel filtration chromatography of Blue - dextrane, was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005 phosphate buffer (pH 7.5), the elution rate of 30 ml/hr, 5ml /frac.

Figure (4.10) showed the elution of Alkalane phosphatase which has Mr of 160000 Dalton, which is considered as a high Mr protein , one single peak was detected as well as small peak which could be explained as degradation of the main protein into small fractions .

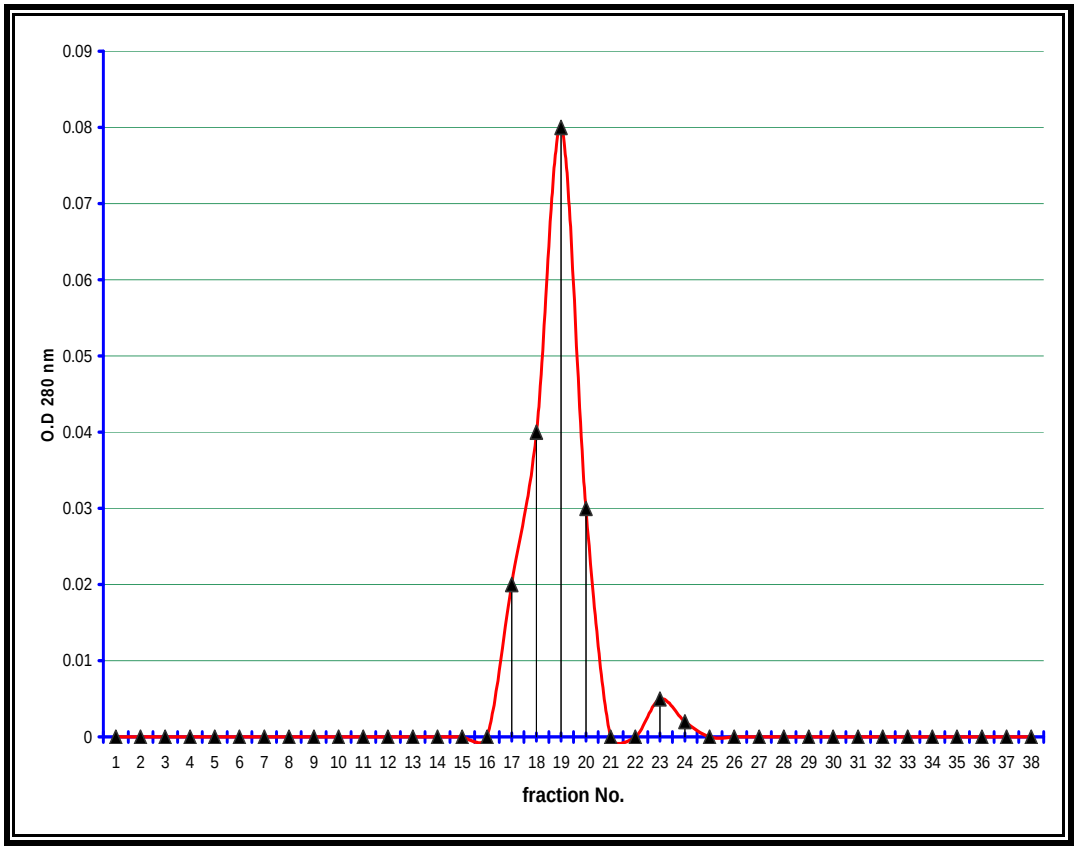


Figure (4.10) :Sephadex G-100 gel filtration of Alkalane Phosphatase , column was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005M phosphate buffer (pH 7.5), the elution rate of 30ml/hr, 5ml /frac.

In the figure (4.11), the chromatography of BSA, which has Mr of 76000 Dalton , one single peak was detected .

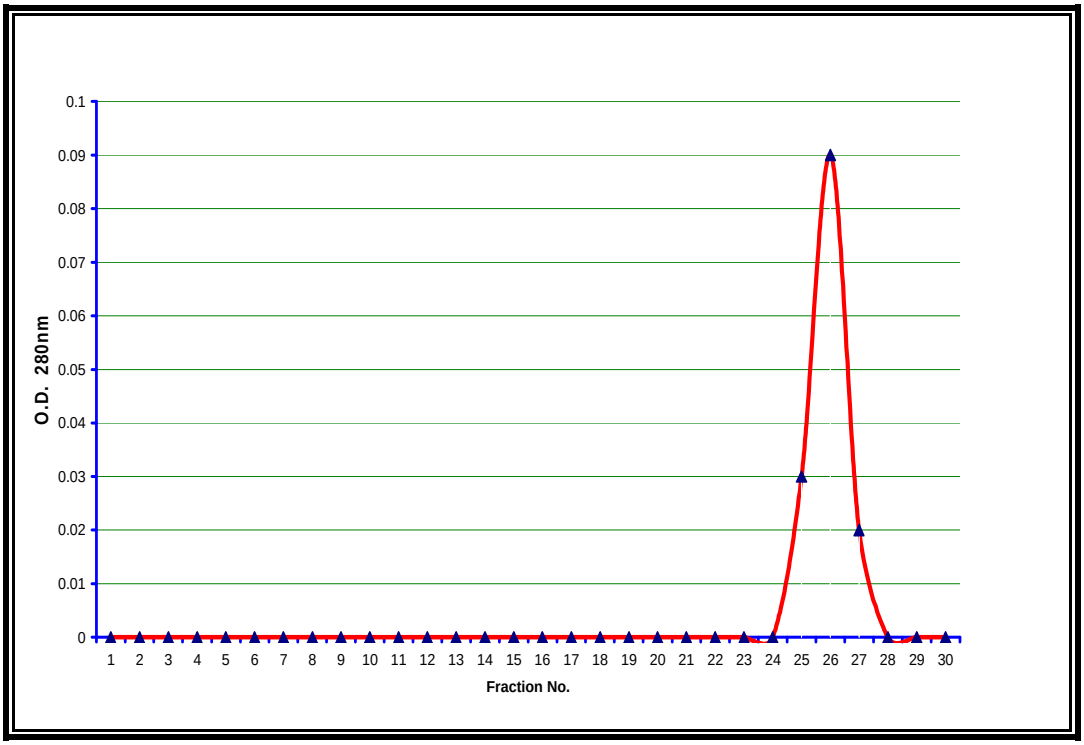


Figure (4.11): Sephadex G-100 gel filtration chromatography of BSA , column was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005M phosphate buffer (pH 7.0), the elution rate of 30ml/hr, 5ml /frac.

Figure (4.12) showed the chromatography of Lysozyme, which was considered as low Mr protein of 14400 Dalton . One single peak was detected

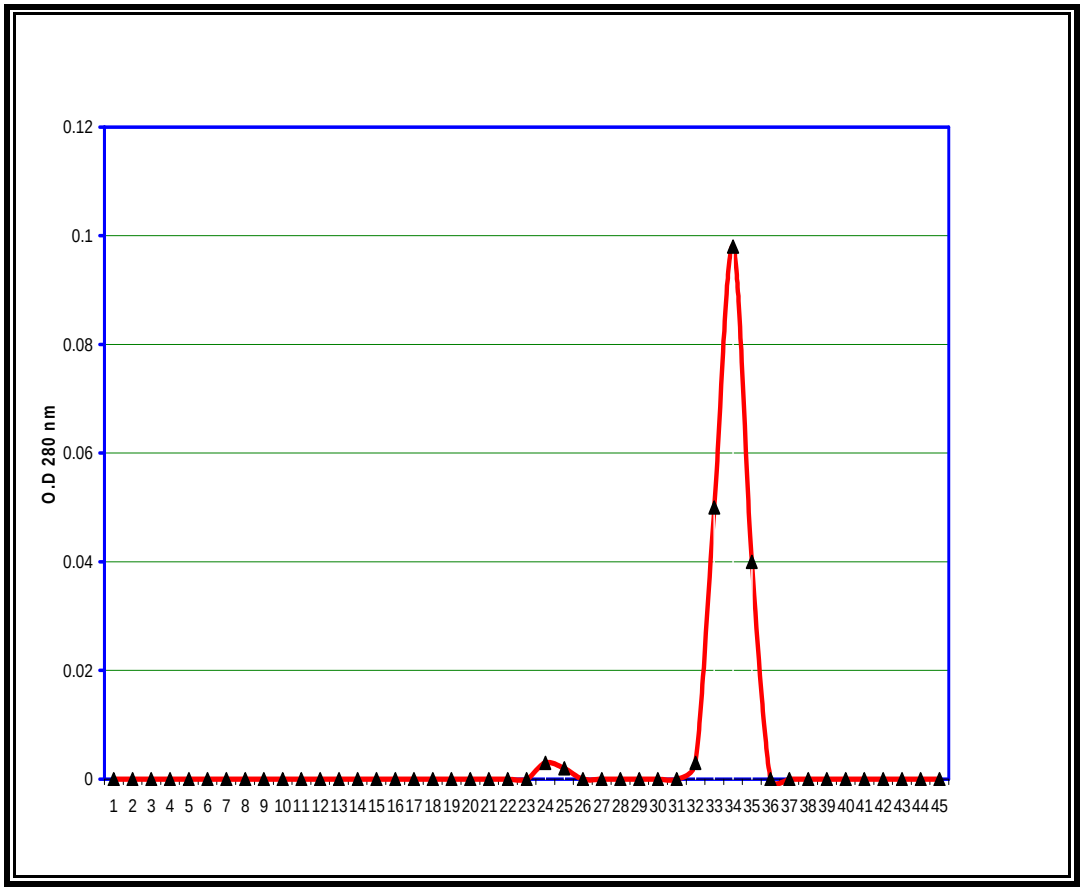


Figure:(4.12) Sephadex G-100 gel filtration chromatography of Lysozyme; column was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005M phosphate buffer (pH 7.0), the elution rate of 30ml/hr, 5ml /frac.

(Figure 4.13) showed the chromatography of Ovatransferrin which has Mr of 76000 Dalton , one single peak was detected .

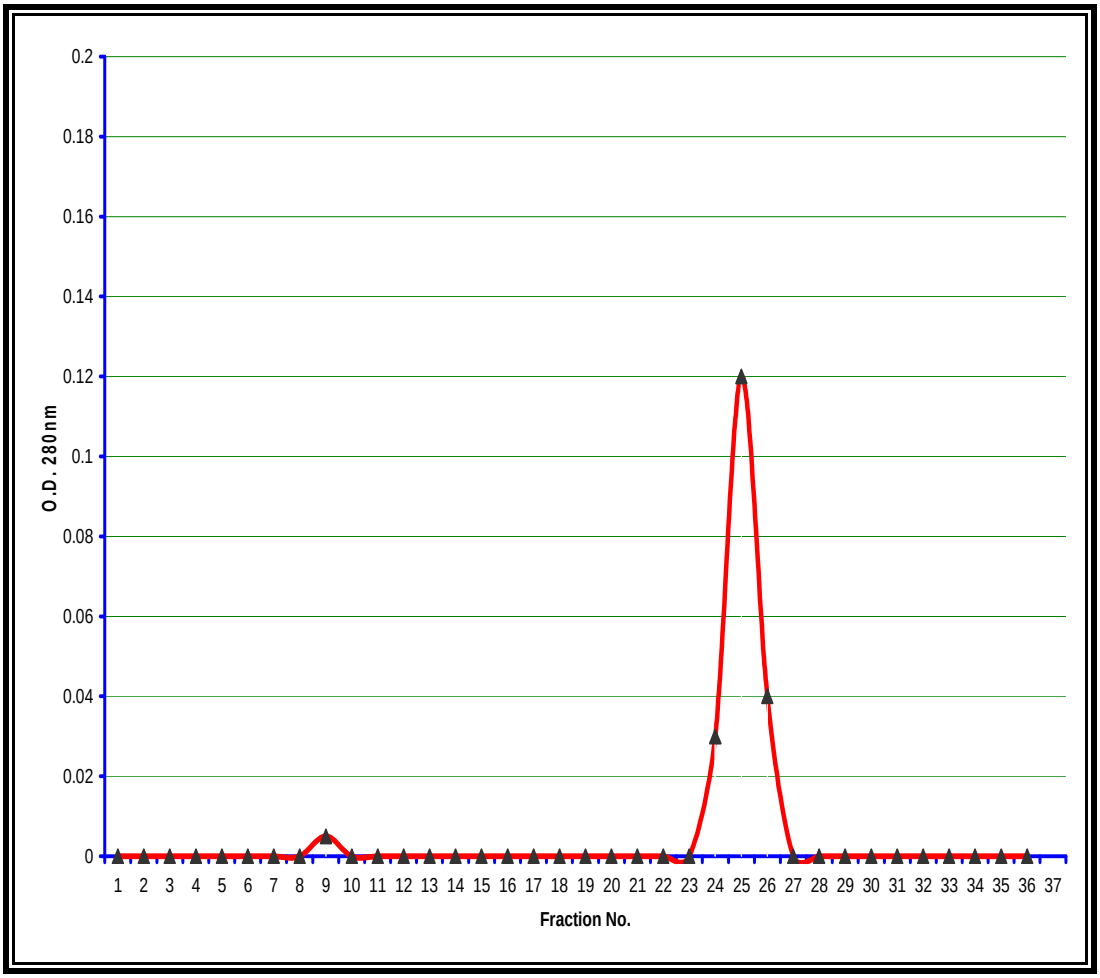


Figure (4.13): Sephadex G-100 gel filtration chromatography of Ovatrnsferrin; column applied in 1.3 X 35-cm Sephadex G-100. The column was equilibrated with 0.005M phosphate buffer (pH 7.5), the elution, at rate of 30ml/hr, was performed with the same buffer and 5-ml fractions were collected.

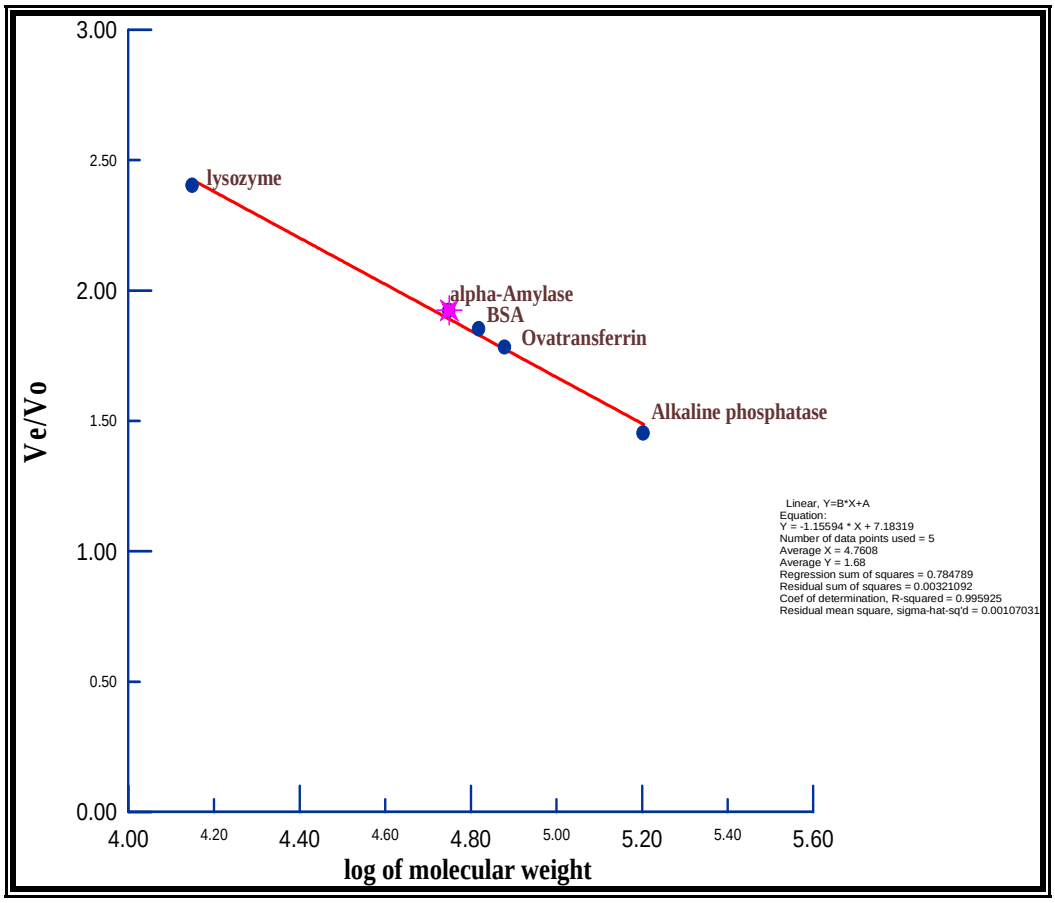


Figure (4.14): logarithms of α-amylase molecular weight as a function of the elution volume (V_e) and the ratios of the elution volume to the void volume (V_e/V_o)

2- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis" SDS-PAGE"

The Mr of α-amylase was determined by SDS-PAGE in which the Mr was determined based on known standard proteins , then the molecular weight was calculated using PhotoCaptMw and Photo impact softwares that were originally designed to help the biologist in the molecular weight calculations , the Mr was determined to be (55426 Da) figure(4-15 A,B).

Monomeric structure was revealed by this technique ,because of only one single band visualized , most studies mentioned that results , in which they were similar to those previously reported using SDS-PAGE method .

Shaw *et al.* (1995) showed that the α-amylase produced by *Thermus* sp. had Mr of (59000 Da)as a monomeric protein ,while Aiba *et al.*(1983) mentioned the Mr of α-amylase produced by *B. stearothermophilus* was 53000 Da. Igarashi *et al.*(1998) reported that the Mr of α-amylase produced by Alkaliphilic *Bacillus* sp. was 53000 Da . On other hand, *Xanthomonas campestris* produced α-amylase of Mr about 55000 (Abe *et al.*, 1994) . Bakhmatova *et al.*(1984) reported that three different variants of *B. subtilis* produced three different α-amylase in their $Mr_{(s)}$ 56000,57000 and 58000 Da.

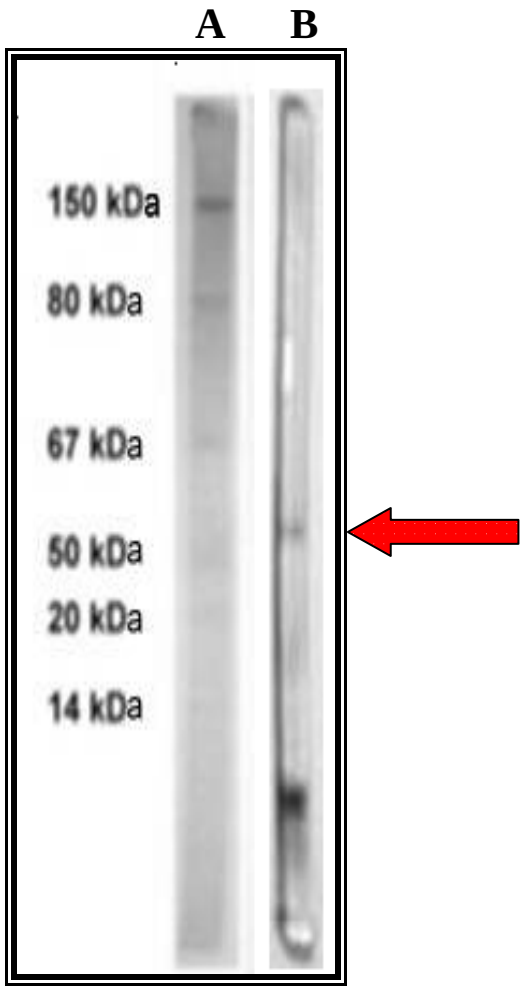


Figure (4.15 A): Estimation of the α-amylase Mr by SDS –PAGE using PhotCaptMr software (amplified by S-splane program).

A) Mr protein markers

B) α-amylase from *B. stearothermophilus* M13

Red arrow indicated α-amylase band position

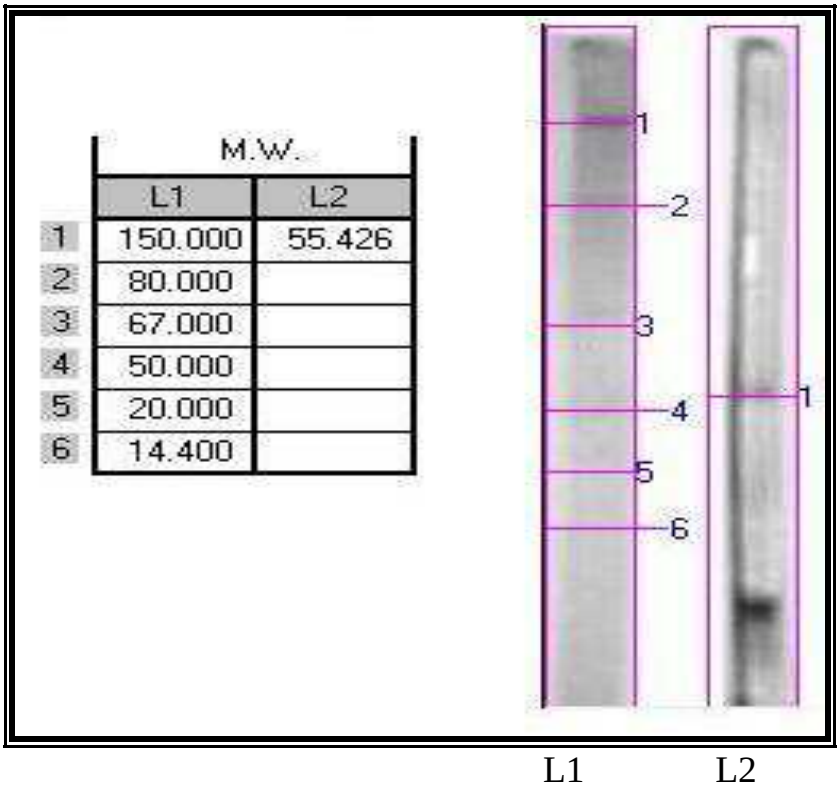


Figure (4.15 B):Calculation of α-amylase Mr processed by PhotoCaptMw version 10.01 and Photo impact softwares.

L1: standard proteins
L2: α-amylase band

4.6.3 Optimal pH for α-amylase activity

The optimal pH for α-amylase activity was studied in range of (5.0-8.5), figure (4.16) in which the optimal pH value for maximal activity was determined at value of 7.0, the activity was 0.2 U/ml , the enzyme displayed an activity curve over broad pH range , the decrease in the activity was at the extreme values of acidity (5.0-6.0) , alkalinity (8.0-8.5) which could be explained as the active sites on enzyme are frequently composed of ionizable

groups that must be in the proper ionic form in order to maintain the conformation of the active sites or change the configuration of the enzyme itself (Segel,1976). Concerning the results of the other studies, the optimal pH for α-amylase maximal activity produced by *B. stearothermophilus* was 6.9 which is similar to the results of Srivastava and Baruah(1986). Bakhmatova *et al.* (1983) indicted that the optimal pH for α-amylases produced by variants of *Bacillus subtilis* was within the acidic pH range(4.5-5.0) , while the optimal pH for α-amylase produced by *B. amyloliquefaciens* was at pH 6.0 (Kochhar and Dua 1982), which is similar to optimal pH for activity of that produced by *Clostridium perfringens* (Shih and Labbe ,1995) . The optimal pH for maximal activity of α-amylase produced by *Pyrococcus furiosus* was 5.5 (Dong *et al.*, 1997). Saito(1973) mentioned that the optimal pH for α-amylase produced by *B. licheniformis* was at 9.0 , which is similar to the findings of Krishnan and Chandra(1983) in activity of α-amylase from *B. licheniformis* was at pH of 9.0 ; also the activity of enzyme was reduced when Tris- hydrochloride and carbonate-bicarbonate buffers were used .In other studies, the optimal pH of α-amylase maximal activity produced by *Xanthomonas campestris* was 4.5 which is acidic (Abe *et al.*,1994), while the pH of maximal activity for α-amylase produced by Akaliphilic *Bacillus* was in alkalane range (8.0-8.5) (Igarashi *et al.*, 1998; Lin *et al.*, 1998) .

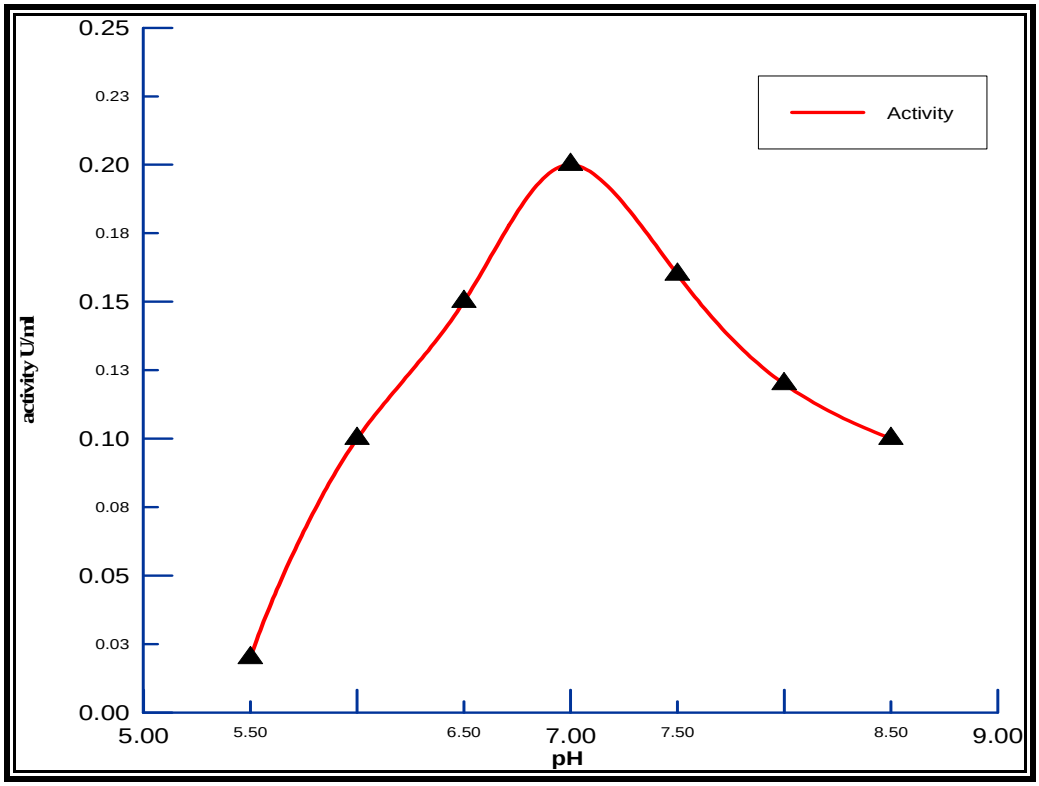


Figure (4.16) :Optimal pH for α-amylase activity

4.6.4 Effect of pH on α-amylase stability

With the aim of investigating The optimal pH for stability of α-amylase ,the enzyme was incubated in a buffer supplied with $CaCl_2$ 0.005 M (pH range 4-11) at 60°C for 30 min. The result (figure 4.17) showed the enzyme had excellent stability at pH range (6.5 – 8.0), while the stability was decreasing as it went toward the extreme acidity or alkalinity , by which the enzyme lost more than 50 % of its original activity at(pH 5.0 and pH 10.0) .While, it showed about (40-50 %)of the remaining activity at (pH 4.0- 10.5), which could be an advantage for utilizing the enzyme in some industrial purposes like detergents in which the pH is alkalane in washing conditions (Ito and Horikoshi ,2004).The effect of pH on enzyme stability could be explained in the formation of improper ionic form of enzyme or the active sites ,irreversible inactivation . The stability of enzyme depends on many

factors such as temperature, ionic strength ,chemical nature of buffer , concentration of various preservatives , concentration of metal ions , substrate or factors and enzyme concentration (Segel ,1976).

Concerning the results of other studies, the α-amylase produced by *Bacillus stearothermophilus* was stable at pH range (6.0-11) while the α-amylase produced by *B. amyloliquefaciens* was stable at pH (6-9.5) for 24 h. in the presence of calcium ion (Kochhar and Dua, 1983). α-amylase produced by *Bacillus licheniformis* was stable at pH rang (6- 11) after the incubation for 30min and 24 hr., and in another study it was stable at pH range (7.0-9.0) (Krishnan and Chandra, 1983) .

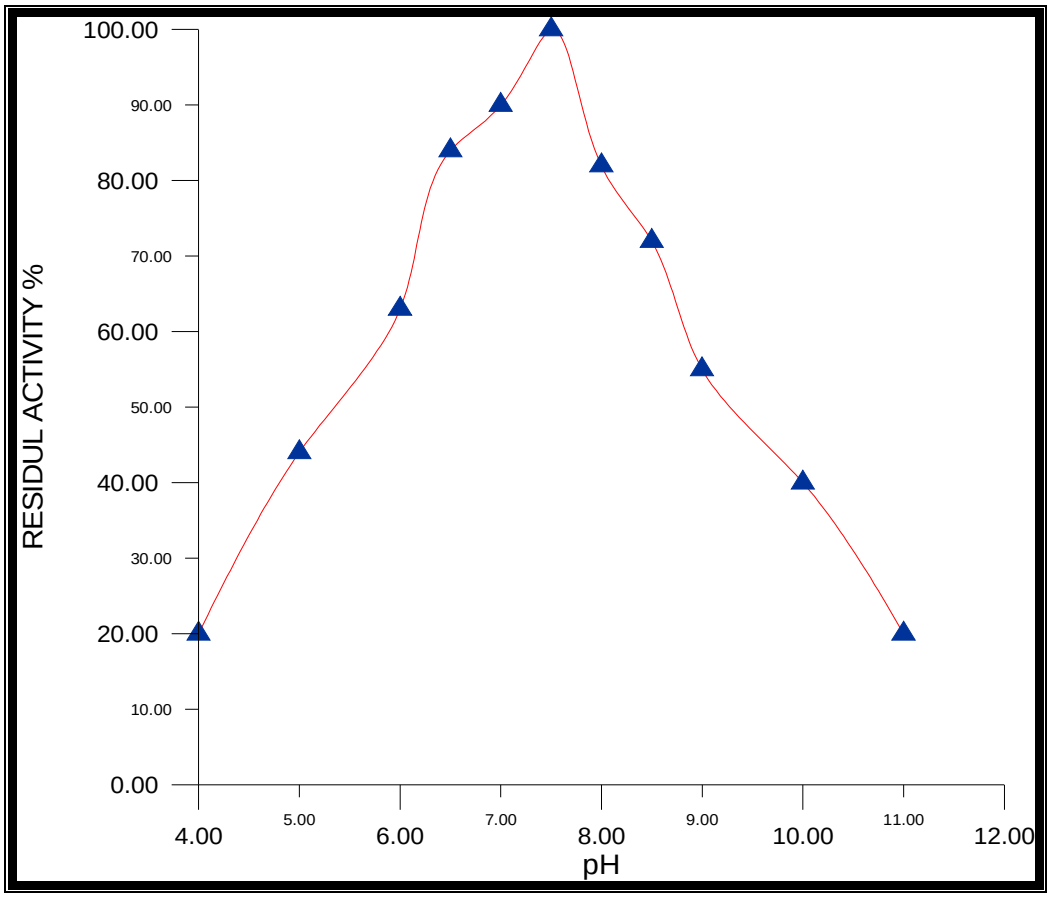


Figure (4.17): α-amylase stability at different pH values, supplied with 0.005 M $CaCl_2$

The α-amylase produced by *Clostridium perfringens* showed a relative stability at pH range (5.0-9.5) (Shih and Labbe, 1995). *Xanthomonas campestris* produced α-amylase which was stable at pH range (4.5-5.0) (Abe *et al.*, 1994). While α-amylase produced by alkliphilic *Bacillus* sp. had pH range of stability at (6.0-10). (Igarashi *et al.*, 1998).On other hand, α-amylase from another alkaliphilic *Bacillus* sp. had pH of stability at (8.5-9.0)(Lin *et al.*,1998).

4.6.5 Optimal temperature for α-amylase activity

The results in figure (4.18) showed that the maximal activity was at 60°C, while the activity decreased before and after 60°C. The increase in the temperature resulted in imparting more kinetic energy to the reactant molecules, resulting in more productive collisions per unit time ,but that should be within the intact and proper configuration of tertiary structure of enzyme in which the tertiary structure of an enzyme is maintained primarily by a large number of weak non-covalent bonds ,so if the molecule absorbs too much energy , the tertiary structure will disrupt and the enzyme will be denatured ,that is ,loss of catalytic activity (segel,1976).

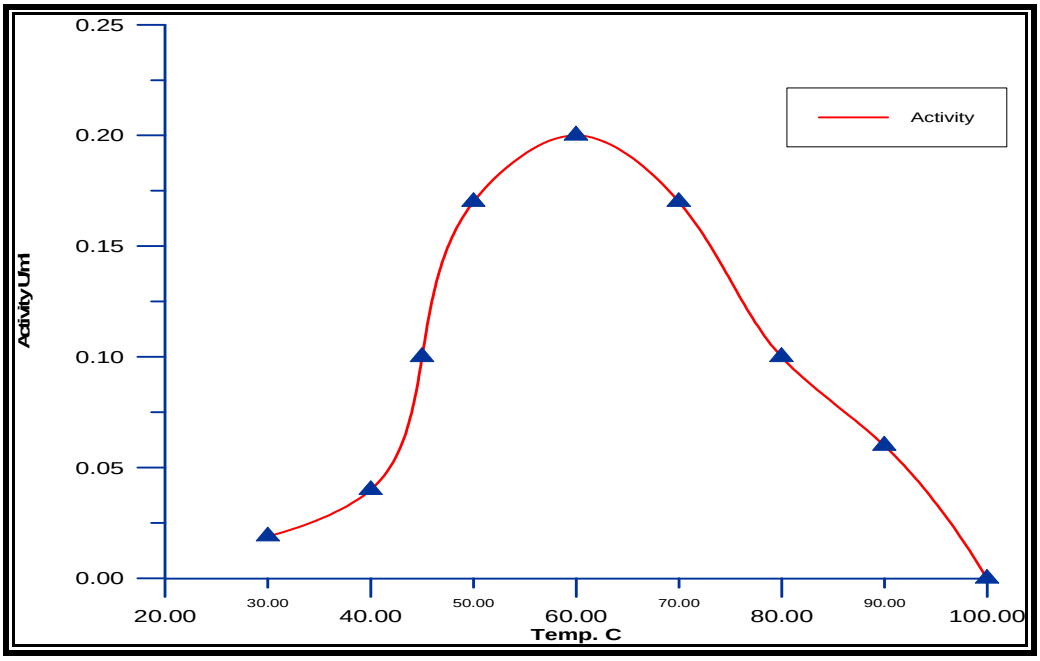


Figure (4.18): Optimal Temperature for α-amylase activity

Concerning the results of other studies, the optimal temperature for thermostable α-amylase activity produced by *Bacillus stearothermophilus* was at 40°C (Aiba *et al.*, 1983),while in another study it was at 82°C (Srivastava and Baruah, 1986).The maximal activity for α-amylase produced by *Bacillus licheniformis was* at 90°C (Krishnan and Chandra, 1983). Saito (1973)reported that the optimal temperature for the enzyme was at 76°C . α-amylase produced by *B. amyloliquefaciens* showed the maximal activity at temperature of 65°C. (Kochhar and Dua, 1983).On other hand, The optimal temperature for α-amylase produce by alkaliphilic and non-alkaliphilic *Bacillus* sp. was at 55°C(Igarashi *et al.*,1998; Teodoro and Martins, 2000) ,in another study, it was at 70°C (Lin *et al.* ,1998). The optimal temperature for α-amylase activity produced by *Clostridium perfringens* and *C. aceotbutylicum* was at 45°C. (Paquet *et al.*,1991;Shih and Labbe, 1995). The maximal activity for α-amylase of *Pyrococcus furiosus* was at 100°C (Laderman *et al.*, 1993;Dong *et al.*, 1997),while the optimal temperature for α-amylase activity produced by *thermos* sp. was at 70°C (Shaw *et al.*, 1995) .

4.6.6 Effect of temperature on α-amylase stability

Results (figure 4.19) revealed that the stability of α-amylase in the presence of $CaCl_2$ with 100 % of the remaining activity was up to 70 °C for 30 minutes ,then the stability decreased to be about 55% of the remaining activity at 90°C , and eliminated at 100°C. The presence of Ca^{2+} ions had a positive effect on the thermostability of other amylotic enzymes, in which it was supposed that the ions were acting as salt or ion bridges between two adjacent amino acids residues . The binding of Ca^{2+} ion was shown to increase the α-helical structure of α-amylase produced by alkaliphilic and thermophilic *Bacillus* sp..(Lin *et al.*, 1998), in the same aspect α-amylase produced by alkaliphilic *Bacillus* sp. retained 100 % of the original activity

after 60 min. of incubation at 45°C , and 3% of the remaining activity after treatment at 60°C for 60 min.(Igarashi *et al.*, 1998).*Xanthomonas campestris* produced α-amylase was stable up to 55°C for 30 min.(Abe *et al.*,1994).The α-amylase produced by *Clostridium perfringens* was stable in the presence of Ca^{2+} with 100% of remaining activity up to 45°C, and then the stability lowered (Shih and Labbe, 1995).While that produced by *C . acetobutylicum* retained about 30% of the remaining activity after treatment at 45°C for 60 min.(Paquet *et al.*, 1991).α-amylase produced by *Bacillus stearothermophilus* retained about 25% of the remaining activity at 90°C after 60 min. in the presence of Ca^{2} (Srivastava and Baruah,1986).While Aiba *et al.*, (1983) reported that the α-amylase retained about 60% of the remaining activity after treatment at 80°C for 60 min.

The α-amylase produced by *B. amyloliquefeaciens* retained 100% of the activity for 24 hr. at 65°C , then half life 0.5 hr. at 90 °C(Kochhar and Dua ,1982).The α-enzyme produced by *B. licheniformis* was 100% stable below 50°C and it lost the activity at 90°C in the absence of substrate (Krishnan and Chandra, 1983). While Saito(1973) reported that the α-amylase retained 100% of the original activity after treatment below 60°C. α-amylase produced by *Thermus* sp. retained about 20 % of the remaining activity after treatment at 80°C for 15min. ,the thermophilic trait for α-amylase could be used in the cake baking industry because the enzyme activity of the thermophilic nature ceases as the temperature approaches and passes through the temperature range of gelatinization (70-80 °C) ,this also provides a means to control dextrin formation . (Shaw *et al.*, 1995).

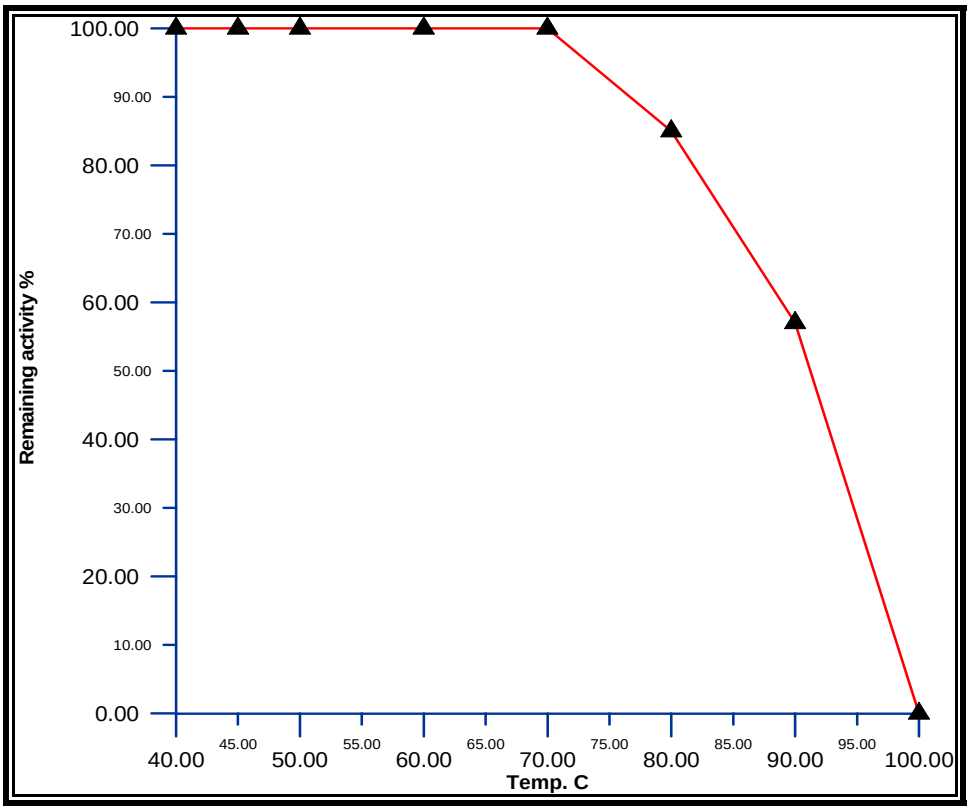


Figure (4.19): The effect of temperature on α-amylase stability in presence of 0.005 M $CaCl_2$ for 30 minutes

4.7 Immobilization of α-amylase

The immobilization of enzyme on insoluble supports provides a stabilization effect at elevated temperature and pH. The stabilization depends on the position of the support attachment to the protein molecule. (Taznov *et al.*, 2003) .Results (figure 4.20,4.21) showed the immobilized α-amylase by calcium alginate in which the remaining activity decreased , retaining about 40% of the remaining activity on 25th day after the immobilization , when it stored at 4°C . , the reasons behind the lowering of the activity along the storage period could be due to the nature of the alginate, buffering of the solution and Change in the configuration of enzyme along the period.

α-amylase was immobilized using cellulose fibers from bagasse where oxidized by periodic acid to obtain dialdehyde cellulose. Which they did react with amino groups of thermostable α-amylase. The immobilized α-amylase

from *B. licheniformis* was able to liquefy the starch in 10 cycles of reuse, in which the only 3 cycles of reuse of operation was worth better than the free enzyme at 40 °C and pH 7.0. (Varavinit *et al.*, 2002). Lectin concanavalin A (Con A), from jack bean (*Canavalia ensiformis*) was observed to form more extensive precipitates with enzymes,this was used as a matrix for immobilization of glycoamylase, in which they observed about 2.1 fold increases in the activity and another technique was applied ,it was termed a multiple- bioaffinity layering technique (Mislovicova *et al.*, 2000).Silica-bound enzyme is extremely stable at 40°C, the operating temperature of the pilot plant column, and in 30% dextrin solutions, the extrapolated half-life is several years. The measured half-lives ranged from 7.5 hr at 70°C to 519 hr at 55°C (Reilly, 1976).Glucoamylases were entrapped in cellulose triacetate fibers and immobilized glucoamylase had been by covalent attachment to organic and inorganic carriers, including low-cost magnetic support (Pieters and Bardeletti, 1992).

Calcium alginate and chitosane gel beads which exhibited high yield was used in immobilization of *Bacillus polymyaxa* in production of α-amylase (Rodziewicz and Rymowicz, 1999).Ivanova *et al.*(1995) used different techniques in the immobilization of growing cells *Bacillus licheniformis* producing thermostable α-amylase (Ca-Alginate, β-Carrageenan, Agar, and their combinations with polyethylene oxide) , adsorption on cut disks of polymerized polyethylene oxide , and fixation on formaldehyde activated acrylonitrile-acrylamide membranes , when an enzyme activity of 2750 U/mL culture medium was reached in the fifth repeated batch run with membrane-immobilized *Bacillus licheniformis* cells. While, Dobreva(1998) obtained the higher amylase yield (62% increase of the control) and operational stability (97% residual activity after 480 hr. repeated batch cultivation) was obtained using formaldehyde-activated polysulphone membranes.

Immobilization on paramagnetic polyacrolein beads was used with α-amylase by Varlan *et al.* (1996), in which they reported less of the activity compared to the control. Thermostable maltogenic amylase from *Bacillus stearothermophilus* for continuous was immobilized using bed column reactor and showed a remaining activity of 50% up to 20 days and gradually decreased to 40% over 37 days of continuous process (Kang *et al.*, 1997).

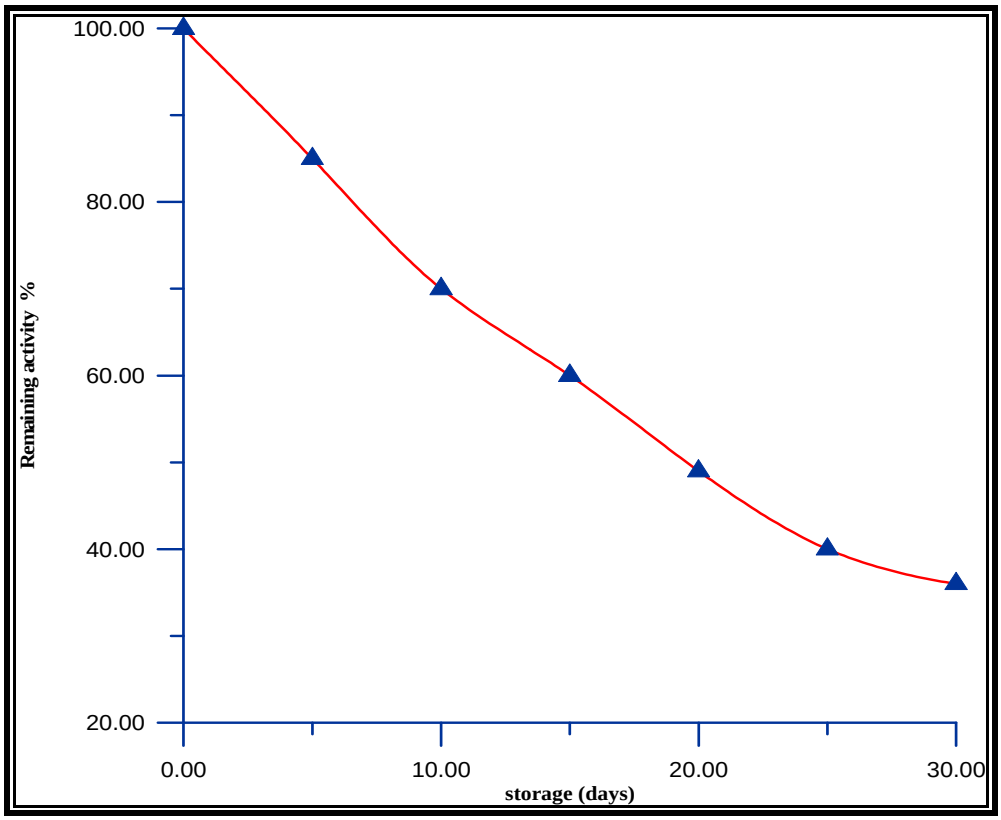


Figure (4.20): α-amylase activity immobilized onto Calcium alginate, storage at 4 °C .

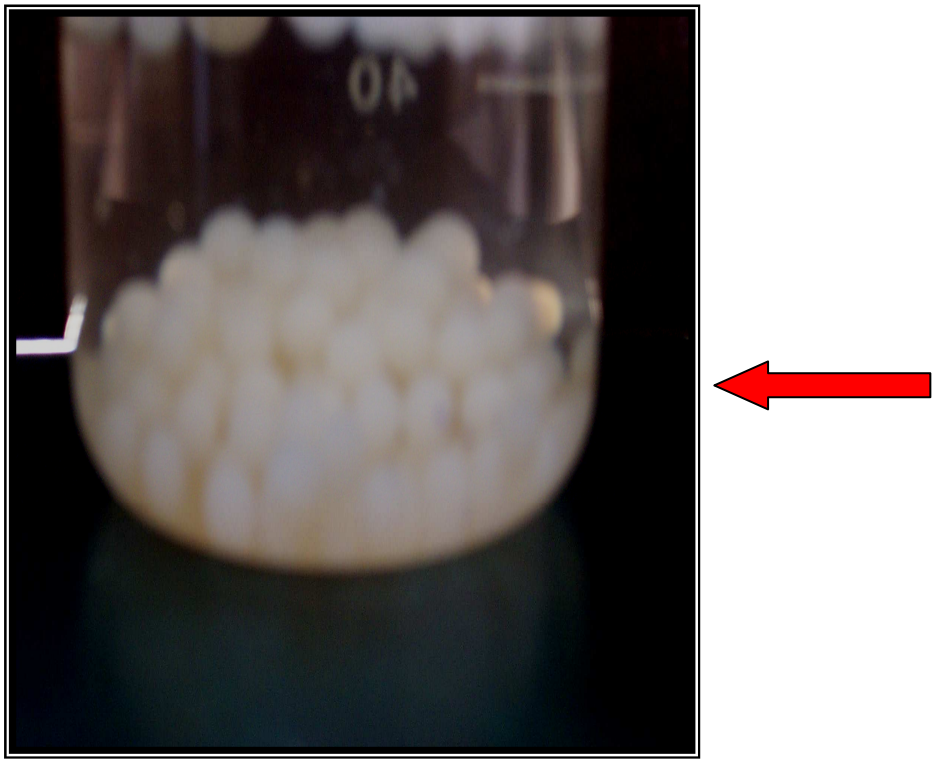


Figure (4.21): α-amylase immobilized onto Calcium alginate

4.8 Neutralization of α-amylase by its anti-amylase sera

Results in (figure 4.22) showed the remaining activity after treatment the α-amylase (purified by ion-exchange chromatography) is 62.6% compared with control. This could be interpreted that the anti-amylase serum could block about 37.4 % of the active sites where the binding should be happened, and it indicated that α-amylase is an immunogenic material for stimulation the immunity system and for availability the basic requirements for that ; foreignness , high molecular weight ,chemical complexity and degradability (Benjamini *et al.*, 2000)The cross- reaction occurred between the polyclonal antibody and the enzyme which caused the decreasing in the activity with 37.4%, because the α-amylase as a immunogenic protein could stimulate the formation of antibodies against the epitopes , but not all the active sites were epitopes ,that could explain why the activity was decreased with only 37.4% .

which could be used as a new approach to assay the activity and in the purification using the affinity method of purification based on specific monoclonal antibodies against α-amylase protein(s).

Yamaguchi *et al.* (1974a,b); Yoneda and Maruo, (1975) neutralize α-amylase activity with anti-amylase serum and conclude from the degree of neutralization the immunogenic alternation among different strain of *Bacillus subtilis* producing the α-amylase as a marker for the genetic analysis.

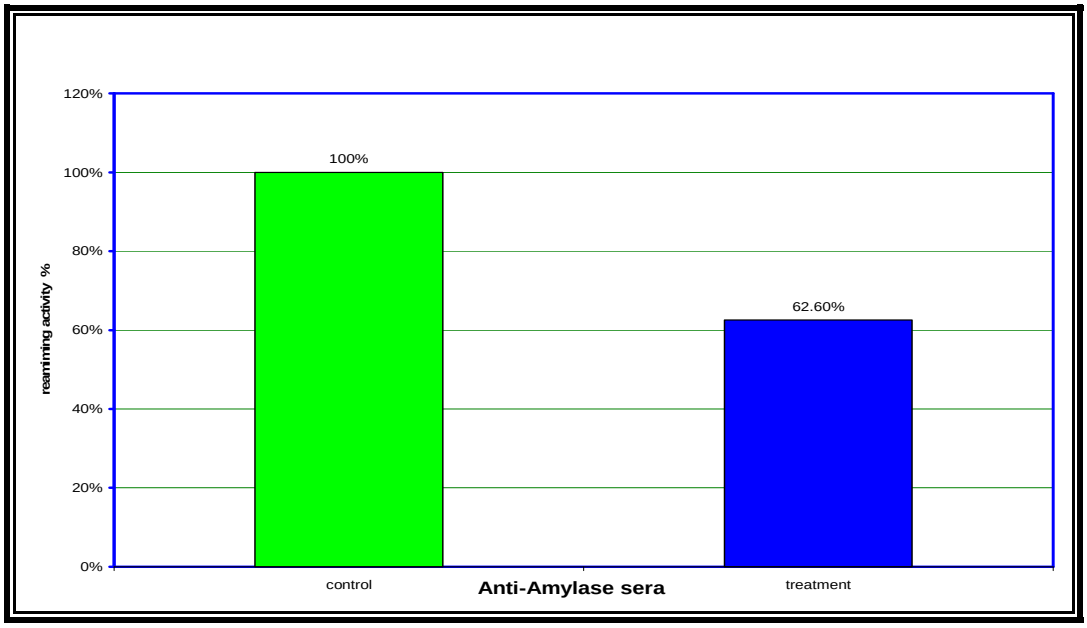


Figure (4.22): Neutralization of α-amylase by anti-sera

4.9 Ascending paper chromatography of α-amylase Products

The time course for α-amylase action on the soluble starch was investigated via paper chromatography to find out the mode of action on soluble starch.

The results in (figure 4.23) were obtained from different samples taken from the reaction mixture during intervals. After 45 min of the reaction starting , the final product was maltose and glucose and two unknown compounds in which it was difficult to identify them because the lack of possible matching standards compounds, while only maltose and unknown compound were formed after 5 and 10 min. The paper chromatography technique based on two phenomena molecules of different substances will

generally have different attractive adsorption forces to the cellulose fibers of which a paper is made and molecules of different constitutions of a mixture dissolved in a water- immiscible solvent will partition themselves in differerent degrees between this adsorbed water of the cellulose fibers and the solvent .(Budhiraja ,2004).

Satoh *et al.* (1993) investigated the products of α-amylase action on soluble starch through intervals, they could identify a series of oligosugares (G3, G4, G5 and G6) which among the products at the beginning of the reaction because the random action of α-amylase, finally, at the end products were identified as glucose and maltose .

Aiba *et.al,*.(1983) used a paper chromatography technique to verify the α-amylase by investigating the products of α-amylase action on soluble starch, which revealed the presence of glucose, maltose and oligosaccharides containing three, four , five and more glucose units.

A high-performance anion-exchange chromatography was used to identify the product of α-amylase action on soluble starch (Shaw *et al.,* 1995;Dong *et al.,* 1997) . Paquet *et al.*(1991) reported that the final products of α-amylase products by *C. acetobutylicum* were maltotriose, maltose and glucose which explained the mode of attacking the bonds .

Igarashi *et al.*(1998) used both TLC and HPLC techniques to investigate the final products of α-amylase action on the starch ,amylose ,amylopectin and glycogen .The results were similar to the present study ,but for hydrolysis of starch, the final products were (G1,G2 and G3).

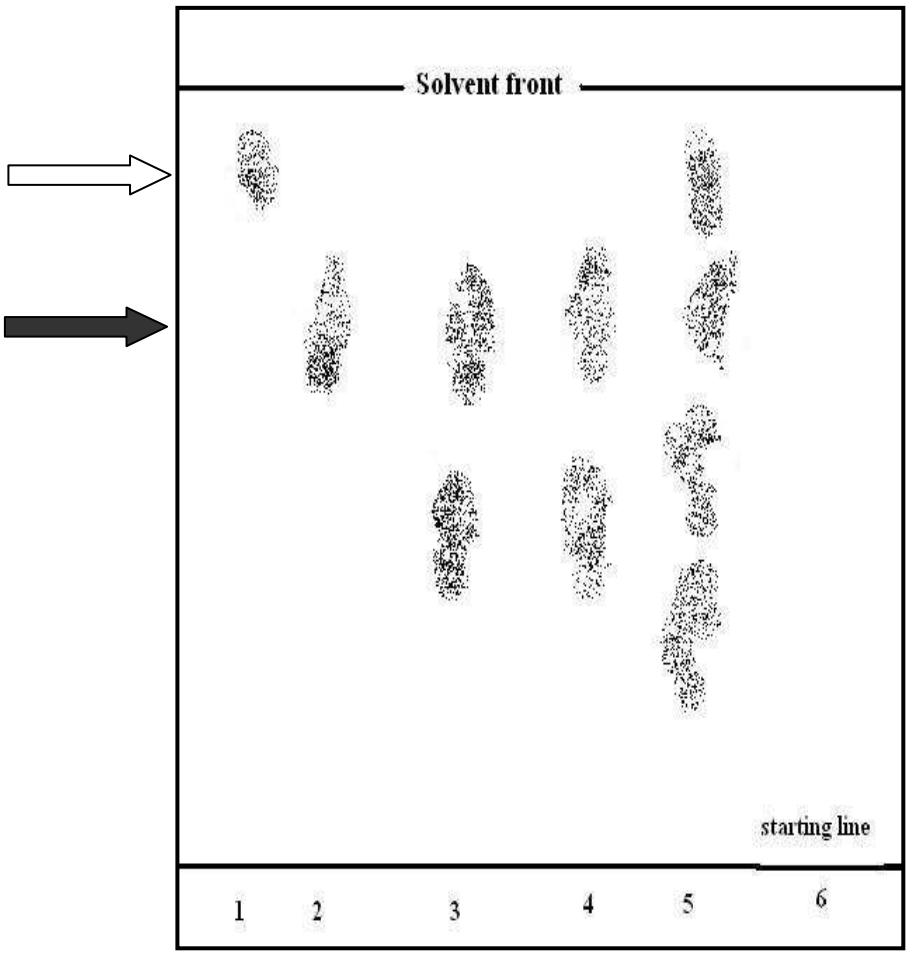


Figure (4.23): Ascending paper chromatograms of hydrolysis products by action of α- amylase on soluble corn starch
1. Standard glucose 2. Standard maltose
3. sample after 5min. of reaction 4. sample after 10 min. of reaction
5. sample after 45 min. of reaction 6. Control sample (D.W.) used in preparing standards sugars

4.10 Antibiotics susceptibility

The standard disk diffusion method was used to determine the susceptibility of *Bacillus stearothermophilus* M13 to several antibiotics. Results (table 4.6) showed Antibiotic susceptibility of *B. stearothermophilus*

M13 to six out of seven different antibiotics that represent a major group of Antibiotic families used nowadays around the world .

B. stearothermophilus M13 was susceptible to all studied antibiotics except for Aztreonam (ATM), in which it was resistant. These results could be interpreted in which the Ampicillin (Am) and Penicillin (P) belong to the penicillin family and the mechanism of resistance to this kind depends on the presence of β-lactamase (which is either chromosomally or plasmid encoded and most of these plasmids are self transmissible plasmids)(Prescott *et al.*,1990) or due to the lack of penicillin binding proteins (PBPs) and microorganisms may change their permeability to the drug(Malkawi and Youssef ,1996) , so the Bacteria may show no β-lactamase activity . On other hand , Aslim *et al.*(2002) reported that a few *Bacillus spp*. strains isolated from soil were resistant to antibiotics under study, they were sensitive to vancomycin, chloramphenicol, tetracyclane, gentamicin, erithromycin, cephalothin and ampicillin . Imanaka *et al.* (1981) isolated ten different plasmids for antibiotic resistance from thermophilic *Bacillus* sp. ,some of them were associated with resistances to the tetracyclane , erythromycin ,streptomycin and kanamycin .,*B. stearothermophilus* was sensitive to several antibiotics, except for tetracyclane. With the exception to the tetracyclane the antibiotic resistance generally was not associated with plasmid (Claus and Berkeley, 1986).Tanaka and Koshikawa (1977) isolated four types of plasmids from *Baciulls subtilis* with no resistance to the common antibiotics , such as chloramphinicol, tetracyclane, streptomycin, ampicillin and heavy metal ,$HgCl_2$ neither production of bacteriocins ,so the functions specified by the plasmids remained unknown. The sensitivity or resistance to different antibiotics was partially associated with the screening of plasmid in the *B. stearothermophilus* M13, the result of the primary screening for the presence of plasmid proved no plasmids of any kind .

Table (4.6): susceptibility of *Bacillus stearothermophilus* M13 to some antibiotics.

Antibiotic	Abbreviation	Susceptibility
Imipenem	IPM	Sensitive
Cefotaxime	CTX	Sensitive
Norfloxacin	NOR	Sensitive
Gentamicin	CN	Sensitive
Penicillin	P	Sensitive
Amipcilin	AM	Sensitive
Aztreonem	ATM	Resistant

4.11 Cloning of *B. stearothermophilus* M13 α-amylase gene in pBR*322* vector

Genomic DNA was isolated from this bacterium using the procedures of CTAB and salting out with some modifications in which the lysozyme final concentration increased to be 2 mg/ml. after that DNA electrophoresis was done using 0.4 and 0.9 % agarose, the result showed no detectable plasmids**.** To confirm the results, alkalane extraction procedure was done in an attempt to isolate the possible plasmid, if any. The result revealed no detectable plasmid of any size and kind (figure 4-24, lanes A and B**)** .

The chromosomal DNA band was very sharp which indicated the efficiency of the isolation and purification approaches and no degradation was happened due to the exonuclesaes activity , The purity ratio (OD_{260}/OD_{280}) was 1.8 that allowed for the next cloning steps figure(4.24) (Maniatis *et al.*,1982) .Then it was partially digested by *Hin*dIII (figure 4.25) .The partial digestion made the probability of obtaining the intact α-amylase gene(s) much higher than the complete digestion in cloning experiments (sambrook and Russell,2001). The restriction enzyme was frequently used in order to get the gene of α-amylase on intact DNA fragment to be able to express using of appropriate expression system (Aiba *et al.*, 1983;Rodriguez and Tait, 1983) .
The pBR *322* vector was purified from the host *E. coli* HB101 strain using CsCl/EtBr gradient ultracentrifugation as (figure 4.26) showed sharp band of covalently closed circular DNA plasmid. Because of plasmid DNA also behaved differently from *E.coli* DNA when centrifuged to equilibrium in cesium chloride gradients containing saturated quantities of ethidium bromide as intercalating dye which bind to lanear DNA was much more than to the plasmid and therefore bands at a higher density in cesium chloride gradients contained an intercalating agent(Maniatis *et al.*,1982), Then was digested with *Hin*dIII that made a single cut in tetracyclane gene so the cloning could be set up using insertional inactivation strategy .Then it was treated with calf intestine alkalane phosphatase to remove 5´ phosphates from both ends of the lanear DNA because in the construction of a chromosomal DNA gene bank ,it is desirable to optimize the formation of recombinant molecules as opposed to non-recombinant DNA molecules . This can be accomplished by either ligating a 10-20 fold excess of chromosomal DNA to the vector DNA (or)by pretreating the plasmid vector with alkalane phosphatase to prevent recircularization of digested plasmids or two or more digested plasmid in the reaction mixtures (Rodriguez and Tait,1983) .

The restricted fragments of *Bacillus stearothermophilus* M13 genome were ligated together using T4 ligase to ligate the cohesive ends of both insert and host DNA (figure 4-27,lane B,C,D and E)(figure4.28) , then the Amy⁻ *E. coli* MM 294 was transformed with a recombinant vector carrying the desired α-amylase gene .

3847 transformants were resistant to the ampicillin as a primary screening in which the cloning in pBR*322* using *Hin*dIII usually inactivates a gene necessary for the tetracyclane resistances ;only five clones out of those (0.13 %) were able to produce α-amylase tested by replica plating on the starch media after staining with lugol`s solution in a comparison with the control sample (figure 4-30,A-B).

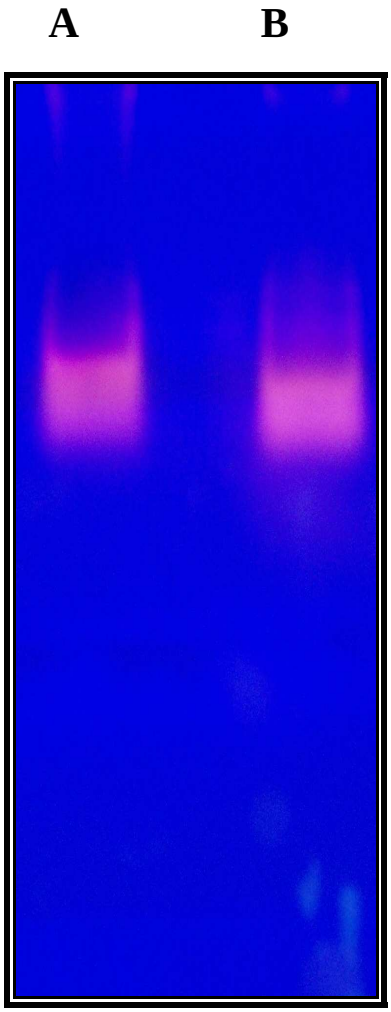
The five transformants *E.coli* MA 1,MA2,MA3,MA4 and MA5 showed α-amylase activity. The DNA of transformants was extracted and revealed harboring of hybrid plasmid termed as "pMA 322"; which revealed a molecular weight of 1790 bp (figure 4.28) that was calculated using PhotoCaptMw figure(4.29) as it was concluded from the literature that the length of amylase gene was about 1.8 Kbp . The missing 10 bases may be due to the technical calculation of the sharp accurate molecular weight during the study or may the gene be truncated in the promoter area in which the structural gene was intact as indicated by expression of the α-amylase gene of the cloned cells in the solid and liquid .In any way, sequencing should be done to determine which one happened .

restriction enzyme was frequently used in order to get the gene of α-amylase on intact DNA fragment be able to express using appropriate expression system (Aiba *et al.*, 1983;Rodriguez and Tait, 1983) .

The size of cloned fragment carrying the α-amylase gene was estimated of "1790bp ".It has been shown through many previous studies that α-amylase gene was isolated from *Bacillus* . spp. and cloned in different expression systems . Aiba *et al.*(1983) cloned the α-amylase gene in *Bacillus*

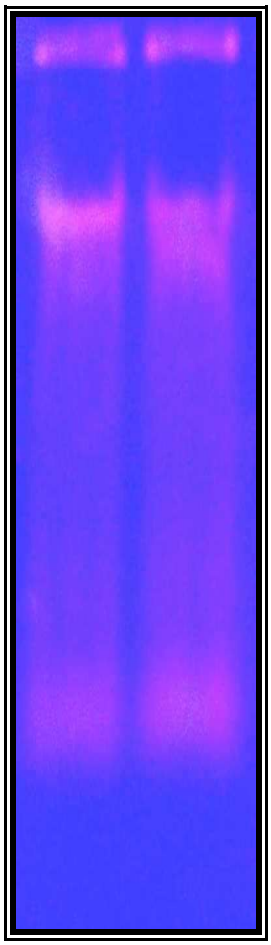
by the digestion of the pTB90 and the genomic DNA partially by HindIII, while Mielenz (1983) cloned the α- Amylase in *E.coli* and the α-amylase was expressed using pBR *322* as a vector , in which the *B. stearothermophilus* genomic DNA and the vector were cleaved with *Hin*dIII ; then ligated as ratio of 4: 1 , the cloned fragment was of 5.4 kp . It was confirmed by many similar studies that the structural gene for α-amylase enzyme *amy*E, and its regulatory gene, *amy*R are closely linked on the genetic map of the *B. subtilis* chromosome (Yamaguchi *et al.*, 1974b). α-amylase's promoter *amy*P contains an essential TGTG motif(-16 region) upstream of the -10 region (Martin and Chambliss,1998). A great deal of cloning has been done in many different microorganisms, mostly into *E.coli* which was applied as expression system for many sources of α-amylase gene. Satoh *et al.*(1993) cloned two α-amylase genes from *Streptococcus bovis* 148 chromosomal DNA in *E.coli* using pBR 322 vector ; They were expressed successfully in both forward and opposite orientation that could explain that the cloned fragment had its promoter that it is fully functional in *E.coli*. cloning α-amylase via cosmid was applied in some studies, Coronado *et al.*(2000) constructed *Halomonas meridiana* gene bank in broad-host rang cosmid pVK102 by partial digestion of genomic DNA with *Hind*III , then in vitro packing to infect the *E.coli.* HB101 ., Dong *et al.(*1997) cloned and a sequence of α-amylase 2.6 kb from *Pyrococcus furiosus* in *E.coli* . Cloning using PCR technique was applied for cloning α-amylase gene from hyperthermophilic archeabacterium *pyrococcus furiosus* using three primers the amplified fragments was inserted in *Pst* I site of pTV 118N and transform *E.coli* JM109 .The length of fragment was 5.3 kb. An α-amylase gene from *Bacillus* sp. strain TS-23 was cloned and expressed by using recombinant plasmid pTS917 in *E. coli*. SDS-polyacrylamide gel electrophoresis showed an apparent protein band with a mol. wt of approximately 65000. The amylase gene (*amy*A) consisted of an open reading frame of 1845 bp (Lin *et al.*, 1997)

Gene encoding the α-amylase of *Xanthomonas campestris* K-11151 was cloned into *E. coli* using pUC19 as a vector. An ORF of 1578 bp was deduced to be the α-amylase structural gene.(Abe *et al.*, 1996).



Figure(4-24): Agarose gel (0.9%) electrophoresis of *B. stearothermophilus* M13 chromosomal DNA :
Lane A: Genomic DNA of *B. stearothermophilus* M13 Isolated by salting out method.
Lane B: Genomic DNA of *B. stearothermophilus* M13 Isolated by Alkalane lysis method.
5V/cm, 90 min.

A B



Figure(4-25): Agarose gel (0.9%) electrophoresis of *B. stearothermophilus* M13 chromosomal DNA digested with *Hin*dIII , at 75V

Lane A: Genomic DNA of *B. stearothermophilus* M13 Isolated by salting out method partially digested by *Hin*dIII
Lane B: Genomic DNA of *B. stearothermophilus* M13 Isolated by alkalane lysis method partially digested by *Hin*dIII
5V/cm, 120 min.

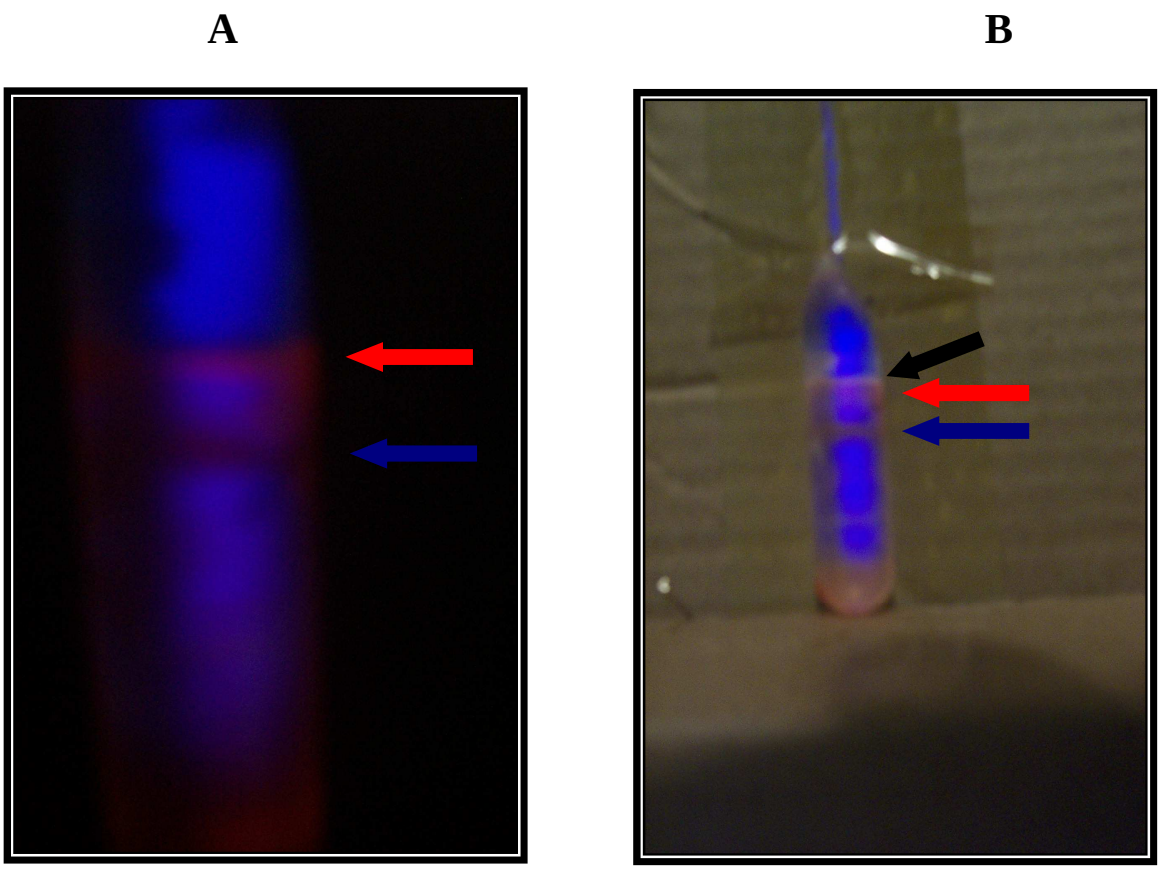


Figure (4- 26): Purification of the pBR *322* Vector by ultracentrifugation with CsCl/EtBr Gradients:

A: The CCC plasmid band as observed in dark room (blue Arrow)
B: The CCC plasmid band as observed in light room (blue Arrow)
Red Arrow indicated nicked circular or lanear chromosomal DNA
Black Arrow indicated proteins
45,000rpm,16hr

Figure (4-27) :Agarose gel (0.9%) electrophoresis of ligated DNA
Lane A: ligation reaction (control)
Lane B: ligation reaction, experiment 1
Lane C: ligation reaction, experiment 2
Lane D: ligation reaction, experiment 3
Lane E: ligation reaction, experiment 4

5V/Cm, 60min.

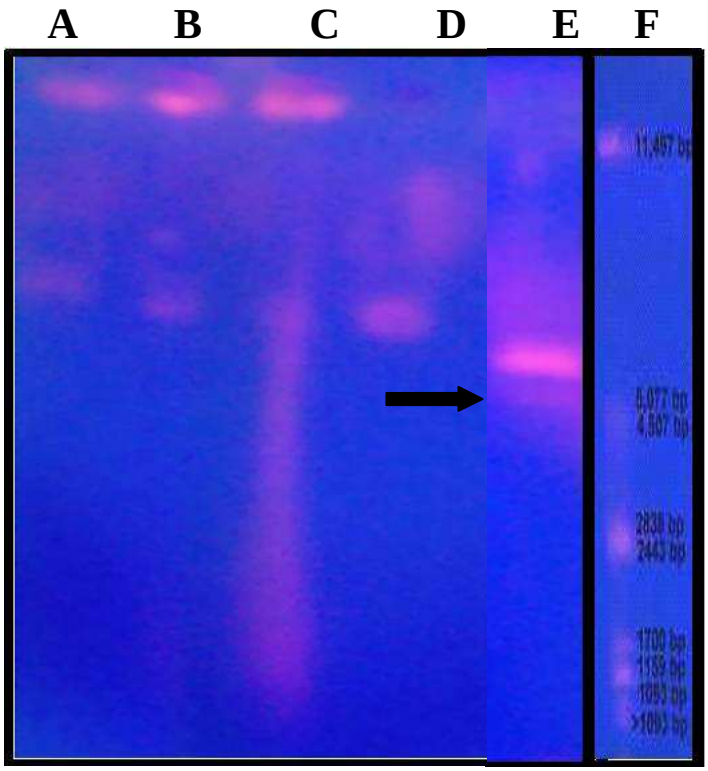


Figure (4-28): Agarose (0.9%) gel electrophoresis of :-

Lane A: ligation yield from experiment 2
Lane B: ligation yield from experiment 3
Lane C: partial digestion of *B. stearothermophilus* M13 genomic DNA
Lane D: Junk DNA
Lane E: Junk DNA
Lane F: (black Arrow) p*MA* 322 from the Transformant *E.coli* MM 294 producing recombinant α-Amylase

5V/Cm,120min.

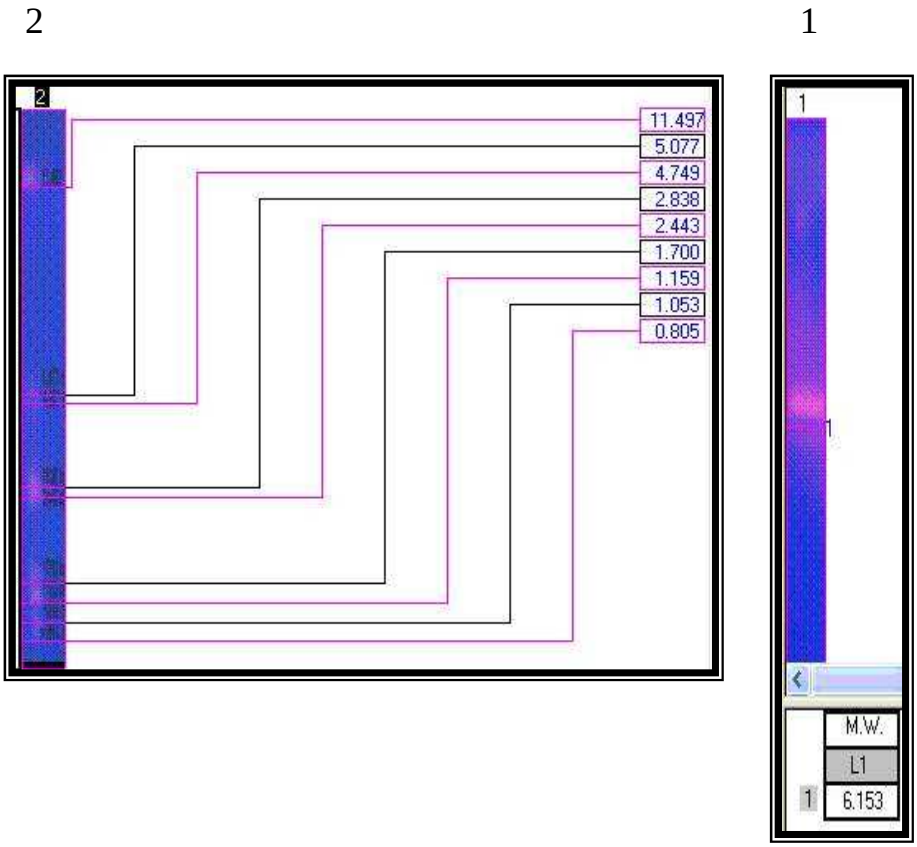


Figure (4-29): Estimation of pMA 322 molecular weight, using PhotoCapMw Software using agarose (0.9%) gel electrophoresis .

Lane 1: The hybrid pAM 322 extracted from *E.coli* AM1
Lane 2: molecular weight (standard markers) of λ DNA digested with *Pst*I
5V/Cm ,120 min.

4.12 Expression of recombinant α-amylase gene in the transformant *E. coli* harboring pMA *322*

The transformant *E.coli* MA1 harboring pMA 322 expressed successfully the cloned α-amylase on starch medium incubated at 37°C, as appeared in figure (4.30-B) in comparison with the negative control plate of *E.coli* MM294 Amy $^{-}$ fig(4.30-A).

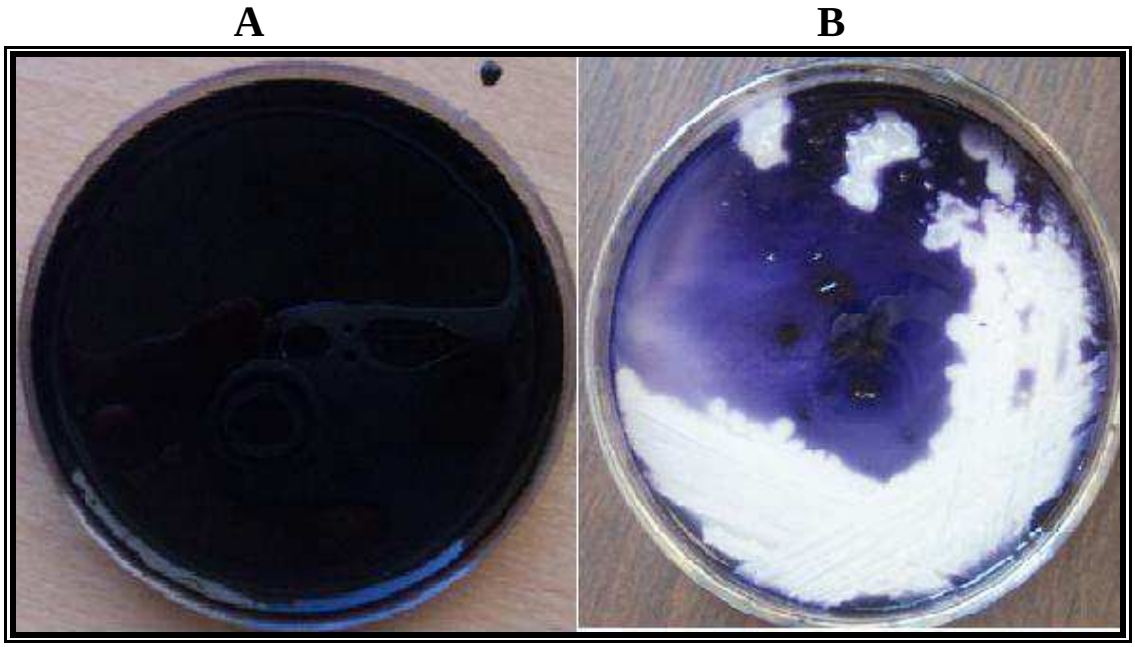


Figure (4.30) :Expression of α-amylase gene cloned into pBR *322* on solid medium supplied with starch , streaking , incubated for 24hrs at 37ºC

A: control , *E.coli* MM294, Amy $^{-}$
B: Transformant *E.coli* MA1 harboring pMA *322*

The specific activity was assayed for the crude extract of the five transformants *E.coli* MA1, MA2, MA3, MA4 and MA5 .The results in (Table 4-7) showed that the *E. coli* MA1 was the highest one in the α-amylase specific activity among the transformants , but it was less than that of *B. stearothermophilus* M13 which has specific activity 0.055 U/mg . This slight decrease in the activity could be attributed to many factors such as

physiological differences between *Bacillus* sp. and *E. coli* MM 294 ,and the transcription and translation mechanisms (Ali , 2003) or may be due to the gene dosage of α-amylase that encoded for it as a multicopy number gene in the original host "*B. stearothermophilus* M13 " and when the gene cloned in the new host ,it occurred as one or less copy than the original . It is a fact that increasing the number of copies of a gene is likely to increase the amount of the particular gene product that is manufactured by the host cell , which may be recompensed by cloning multiple tandem copies of a gene into vector (Lewin,1998)

This slight decrease in the specific activity could be favored to the recombinant α-amylase produced by the *E.coli* recognized by the absences of contaminating protease which was co-produced in the donor strain (Manonmani and Kunhi,1999).

One of the aims of the present study is to attempt cloning of α-amylase and to investigate the expression of it, regardless of the amount of the produced α-amylase, for which future studies should be directed to either optimize the production conditions of α-amylase by the *E.coli* MA1 , investigate the other reasons behind the lowering of the specific activity or to elevate α-amylase specific activity in a liquid medium or cloning many copies for α-amylase gene in the other vectors using *E. coli* or other microorganisms as suitable hosts .

Table (4-7): The specific activity of α-amylase produced by transformants *E.coli* MA isolates harboring plasmid pMA322 carrying recombinant α-amylase gene.

Transformant isolates	***E.coli* MA1**	***E.coli* MA2**	***E.coli* MA3**	***E.coli* MA4**	***E.coli* MA5**
Specific activity U/mg	0 .045	0.032	0.040	0.037	0.044

Conclusions

1. *Bacillus stearothermophilus* M13 produced a valuable amount of α-Amylase among the local thermophilic isolates , that could be used in many industrial applications.
2. The optimal conditions for α-Amylase production and by *Bacillus stearothermophilus* M13 were determined ;represented by TSM medium, at 55 ºC , late log and early stationary phase of growth .
3. α-amylase showed a high activity at pH 7.0 with stability at pH range (6.5-8.0), and a high activity at 70ºC with 100% of the remaining activity .while, at 90ºC showed 55% of the remaining activity .
4. The purification protocol included DEAE –sephadex Ion exchange and gel filtration gel were recommended.
5. Four possible forms (a, b, c and d) of enzyme were eluted from ion-exchange chromatography in which "b" showed the highest specific activity among them , and the overall activity may be due to it namely.
6. Molecular weight of α-amylase was" 56234 Da." when estimated by gel filtration and "55426 Da." when estimated by SDS- PAGE technique .
7. TLC technique was applied to verify the α-Amylase time-effect –products in which monosaccharide and disaccharides were among the products.
8. α-Amylase showed antigenic properties in vivo by decreasing the activity 37.4% for the partially purified α-amylase forms.
9. The DNA fragment that coded for α-Amylase was cloned into *E .coli* MM 294 using pBR*322* as a vector, in which the length of the cloned fragment was estimated of 1790bp approximately.
10. The expression of the recombinant fragment was detected in the transformants cells on a solid medium and liquid medium, which showed a

slight decrease in the specific activity in comparison with original organism with no accompanying proteases activity.

5.2 Recommendations

1. Studying the optimization of α-Amylase production by the cloned *E .coli* MA1 using the liquid media.
2. Conducting further molecular studies for α-Amylase regulation and secretion in the original *Bacillus stearothermophilus M13* and transfrormed (cloned) *E .coli* AM1.
3. Cloning the multicopy number for α-amylase gene in pBR322 or other suitable vectors.
4. Producing and purifying Monoclonal antibodies to detect the enzyme and more accurate assay of α-amylase activity.
5. Conducting more detailed studies concerning protein engineering of α-Amylase produced by the cloned *E. coli* to undergo the industrial processes within different conditions such as alkalinity stability and more thermophilic stability .
6. Studying the kinetics of α-Amylase form(s) (if any) in the new host .
7. Founding of a specialized center to enhance the production and to improve the proteins for future important industeries in Iraq.

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4. Results and Discussion

4.1 Isolation of *Bacillus* spp. producing α-amylase

Ninety six soil samples were collected from different places over Iraq (table 4.1).This showed that sixty nine isolates were thermophilic bacilli; sixty three (60.4%) isolates were α-amylase producing according to the clear zone around the growing colony on the 1.5% starch for 18 hours at 65°C (March and Larson, 1953). Twelve isolates were recognized by the higher production of the α-amylase on the solid media supplemented with 1.5% soluble starch as shown zin the table (4.1), in which the (Z/G) ratio was in the range (1.5 – 2.0) due to enzymatic hydrolysis of starch.

These isolates (12) that showed the highest productivity on the solid medium were chosen and named as (**M1, M9, M13, M23, M24, M26, M29 , M31 ,M36 ,M40 , M60** and **M69**) for further biochemical tests to select *B. stearothermophilus.* The primary screening for the organism that is able to produce an enzyme is considered one of the important steps for enzyme production(Maza *et al.*,1997;Teodoro and martins ,2000).

Table (4.1): Screening of thermophilic *Bacillus.* spp. isolates for their ability to produce α-amylase.

Isolate No.	Growth diameter (mm)	Halo (clear zone) diameter (mm)	Z / G ratio	Isolate No.	Growth diameter (mm)	Halo (clear zone) diameter (mm)	Z / G ratio
M1**	2	4	2	M50	3	4	1.33
M2	3	4	1.33	M51	4	5	1.25
M3	2	-	-	M52	4	-	-
M4	5	6	1.2	M53	5	-	-
M5	4	5	1.25	M54	5	6	1.2
M6	3	4	1.33	M55	3	4	1.33

M7	4	5	1.25	**M56**	4	5	1.25
M8	2	-	-	**M57**	3	4	1.33
M9*	2	3	1.5	**M58**			
M10	4	-	-	**M59**	3	4	1.33
M11	5	6	1.2	**M60***	2	3	1.5
M12	3	4	1.33	**M61**	4	-	-
M13**	2	4	2	**M62**	4	5	1.25
M14	2	3	1.33	**M63**	3	-	-
M15	5	7	1.4	**M64**	3	-	-
M16	3	4	1.33	**M65**	3	4	1.33
M17	2	-	-	**M66**	3	-	-
M18	2	-	-	**M67**			
M19	3	-	-	**M68**	3	4	1.33
M20	3	4	1.33	**M69***	2	3	1.5
M21	3	-	-	**M70**	2	-	-
M22	3	-	-	**M71**	3	4	1.33
M23**	3	6	2	**M72**	4	5	1.25
M24	3	5	1.66	**M73**	2	3	1.5
M25**	3	-	-	**M74**	2	-	-
M26	3	5	1.66	**M75**	3	5	1.25
M27	3	4	1.33	**M76**	3	4	1.33
M28	2	-	-	**M77**	5	6	1.2
M29**	2	4	2	**M78**	3	4	1.33
M30	2	-	-	**M79**	5	6	1.2
M31**	3	5	1.66	**M80**	3	-	-
M32	3	4	1.33	**M81**	2	-	-
M33	3	4	1.33	**M82**	2	-	-
M34	5	-	-	**M83**	3	4	1.33
M35	5	-	-	**M84**	4	5	1.25
M36*	2	3	1.5	**M85**	3	4	1.33
M37	3	-	-	**M86**	2	-	-
M38	5	6	1.2	**M87**	4	5	1.25

M39	4	-	-	**M88**	4	5	1.25
M40	2	3	1.5	**M89**	5	6	1.2
M41	3	-	-	**M90**	4	5	1.25
M42	3	-	-	**M91**	3	4	1.33
M43	3	4	1.3	**M92**	4	5	1.25
M44	3	4	1.3	**M93**	5	6	1.2
M45	4	5	1.25	**M94**	3	-	-
M46	3	-	-	**M95**	2	-	-
M47	2	-	-	**M96**	3	-	-
M48	2	-	-	**-**			
M49	3	4	1.3	**-**			

* **Thermophilic *Bacillus* sp., amylase producing**
**** *Bacillus stearothermophilus*, amylase producing**
H: halo, clear zone
G: Growth diameter
pH range of soil samples was (7.1-7.4);(slightly alkalane).

4.2 Identification of the isolates

Twelve isolates were subjected to morphological, microscopical , biochemical and physiological tests to identify *B. stearothermophilus* producing α-amylase, isolates were gram positive bacilli, negative on the Voges-Proskauer test at (pH 7.2), motile, central spores and predominantly unswollen cylindrical sporangia.The morphological and physiological characteristics for seven isolates of them showed that they were to be *Bacillus stearothermophilus* according to the Claus and Berkeley (1986) in which the growing colonies on the tryptic soya agar at 55 ^{o}C for 18 hours had the circular edges, irregular and dark rough surface have a diameter of (1.5-3.0 mm.) recognized by an unacceptable odor (Welker and Campbell ,1963) ,while on the nutrient starch agar and nutrient agar, colonies had a regular edge and a rough surface with (2-5mm.). The cells were examined by dying with gram stains; the results showed they are gram positive, spore forming in

which they had terminal spore causing the inflammation of the cells (figure4.1). Their growing in the nutrient broth appeared as fine granules causing the turbidity of the medium. The biochemical tests were done on these isolates to determine the species as pointed out in the table (4.2). The results were closely matching the properties of *B. stearothermophilus.* From these results , the bacteria were identified as *B. stearothermophilus* by the criteria of Bergey's Manual of Systematic Bacteriology, in which they were named as (*B. stearothermophilus* **M1, M13, M23, M24, M26, M29,** and **M31**).

Table (4.2):Biochemical and physiological tests for identification of *Bacillus stearothermophilus* producing α-amylase

Biochemical Tests		M1	M9	M13	M23	M24	M26	M29	M31	M36	M40	M60	M69
Starch hydrolysis		+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis		+	+	+	+	-	+	+	+	+	-	-	-
Growth in 7% NaCl		-	-	-	-	-	-	-	-	-	-	-	-
Aerobic growth		+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth		-	-	-	-	-	-	-	-	-	-	-	-
Growth at	40 °C	+	+	+	+	+	+	+	+	+	+	+	+
	50 °C	+	+	+	+	+	+	+	+	+	+	+	+
	60 °C	+	+	+	+	+	+	+	+	+	+	+	+
	65	+	+	+	+	+	+	+	+	-	+	-	+

°C												
Motility	+	+	+	+	+	+	+	+	-	-	-	+
V-P reaction	-	-	-	-	-	-	-	-	-	-	+	+
Citrate utilization	+	+	+	+	+	+	-	+	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	-	+	-	-
Nitrate to nitrite	+	+	+	-	+	+	-	-	+	-	-	+
Gas production	-	-	-	-	-	-	-	+	+	-	+	+
indol production	-	-	-	-	-	-	-	-	+	+	+	+

(+) positive result (-) negative results

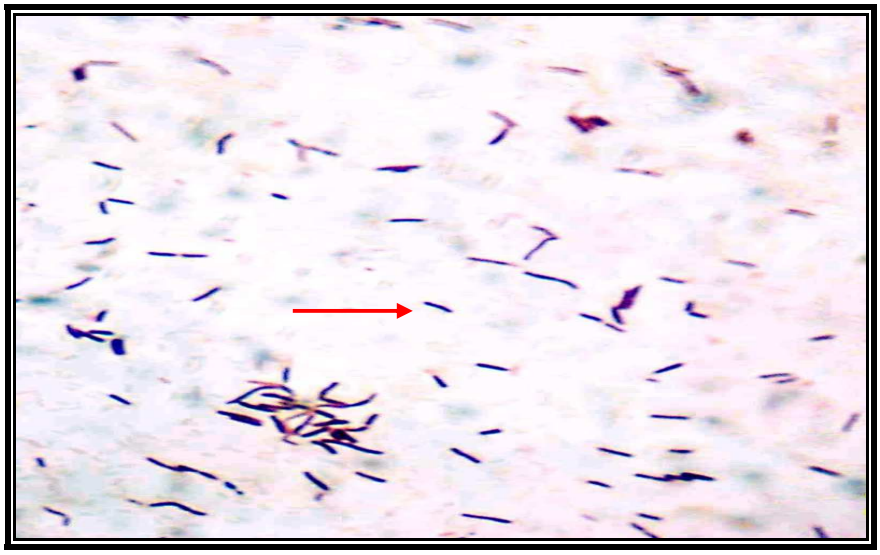


Figure (4.1): Microscopic field of *Bacillus stearothermophilus* M13 (red arrow)(growing on surface of nutrient Agar at 55°C for 24 hrs)stained with Gr.stain 1000X

4. 3 α-amylase Production in the submerged cultures

The seven isolates that have been chosen for the α-amylase production in the submerged cultures based on their production in the solid medium to determine the highest one in the production of α-amylase using N.B. medium supplemented with 1.5 % starch and to determine the optimal temperature for

the α-amylase activity assay. The results, shown table (4.3) for specific activity of α-amylase, produced by the seven selected isolates M1, M13, M23, M24, M25, M26, M29 and M31.That isolate M13 was the highest one among them ,which was 0.054 U/mg in the crude extract . While, the specific activity range for the others were (0.025-0.036) U/mg. when the assay done at 60°C . While the assay at 40°C was lower with about (20.3 %) as losing activity .According to those results, M13 was considered as the most efficient isolate for α-amylase production for this study and for accurate selection for the most efficient producer α-amylase. These isolates (7) were tested again to confirm the results. Then three subculture cycles were carried out for *B. stearothermophilus* M13 and revealed approximately constant specific activity values.

Bacillus stearothermophilus can produce large amounts of extracellular enzymes such as amylases and proteases, the α-amylase is highly thermostable and is desirable for the industrial applications (Mielenz , 1983 , Egelseer *et al.*, 1995).

Table (4.3) Quantitative screening of α-amylase production from *B. stearothermophilus* for in N.B medium supplied with 1.5 % starch ,incubated at 55ºC for 24 hrs.

Isolate No.	Specific Activity U/mg protein assayed at 40 °C	Specific Activity U/mg protein assayed at 60 °C
M1	0.012	0.03
M13	0.011	0.054
M23	0.01	0.028
M24	0.015	0.032
M26	0.012	0.036
M29	0.013	0.025
M31	0.012	0.032

4.4 Optimization of the α-amylase production in liquid culture.

4.4.1 The effect of media on α-amylase production

The seven isolates that have been chosen for being *Bacillus stearothermophilus* producing α-amylase were inoculated in three different liquid media SH, TSM and(N.B supplemented with 1.5 % potato starch), in which the pH was 7.0 for the all . According to the results shown table (4.4), the isolate M13 was the most efficient in the production of α-amylase using TSM medium in which the specific activity was 0.055 U/mg , and in order to confirm those results, a second screening was done for those isolates to confirm the specific activity using the same medium , the results were similar.

Table (4.4): α-amylase production from *B. stearothermophilus* isolates in different media, incubated at 55°C for 24 hrs , pH 7.0

Isolate No.	TSM Medium specific activity u/mg	N. B with (starch 1.5%) specific activity u/mg	SH Medium specific activity u/mg

M 1	0.039	0.03	0.025
M 13	0.055	0.054	0.037
M23	0.034	0.028	0.033
M24	0.037	0.032	0.028
M26	0.032	0.036	0.034
M29	0.031	0.025	0.021
M31	0.031	0.032	0.024

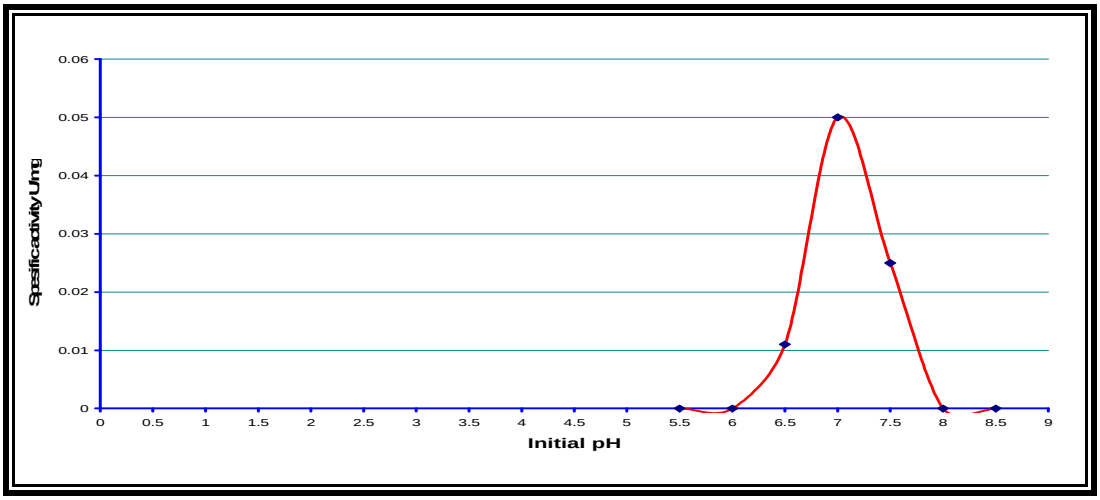
As table (4.4) illustrated that *B. stearothermophilus* M13 isolate was the most efficient one in the production of α-amylase in which the specific activity was 0.055 U/mg using the TSM medium. TSM medium was the best for the production of α-amylase among the tested media, which enhanced the production of α-amylase by the isolate *B. stearothermophilus* M13 .It was recommended by some previous studies (Saito and Yamamoto, 1975; Krishnan and Chandara,1983). The ability to support the production may be attributed to its composition, The composition and concentration of nutrients and factors in the media greatly affect the growth and production of extracellular amylases in bacteria (Srivastava and Baruah, 1986 ; Shih and Labbe,1995),yeast (Fossi *et al.*,2005)and fungus(Zangirolami *et al.*,2002) .The production of α-amylase could be affected by the amount and the nature of nitrogen source as trypton ; besides the variability of divalent cations Ca^{2+} ,Mg^{2+} that were tested for the effect production in which they slightly induced the production ,while the trivalent cations such as Fe^{+3},As^{+3} and Au^{+3} were inhibitors at certain morality concentrations (Srivastava and Baruah , 1986) .The form and quantity of carbon source in culture media is very important for both growth and production of α-amylase in *Bacillus* . Ajayi and Fagade (2006)revealed the presence of soluble potato starch as the sole carbon source as an inducer of the production of enzyme of *B.*

licheniformis because it had the α 1-4 glycosidic bond .While Aiyer (2004) reported that *B. licheniformis* could produce the enzyme in the absence of starch . On other hand, the ratio of C:N in the media could affect α-amylase production , Aiyer (2004) mentioned that the best ratio for production was "1" to be applied for *B. licheniformis* . While the ratio noticed in TSM was" 2 "this could be explained by the difference of the physiological and special requirements of α-amylase production between the *B. stearothermophilus* M13 and *B. licheniformis*.So, the TSM medium was the main medium applied during the present study, otherwise it indicated somewhere in the study.

4.4.2 The optimal initial pH for α-amylase production

To investigate the effect of initial pH medium on α-amylase production, *B. stearothermophilus* M13 was grown on TSM medium with different pH values .The results (figure 4.2) showed that initial pH for a higher production of α-amylase was close to optimal pH of bacterial growth which was (7.0), while there was no growth at pH values of < 6.0 and > 8.0 , in which we could suggest the higher production linked to the higher growth of the bacteria , that result was much similar to what Teodoro and Martins (2000) reported about the production of thermostable amylase by *Bacillus* sp. , Srivastava and Baruah,(1986) reported that the initial pH for the production of thermostable amylase by *B. stearothermophilus* was (6.9). The effect of the pH value on the enzyme production resulted from its role in the solubility of nutrients, ionization of the substrates and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme (Bull and Bushnell, 1972). Also, dissolved CO_2 in the medium affected the buffering capacity (Segel, 1976). The low pH reduced the solubility of CO_2 in the growth medium and limited its availability for assimilation by the anaplerotic enzyme pyruvate carboxylase (Maheshwari *et*

al., 2000). The research of Mazza and Ertola (1976) on the production of α-amylase by *B. subtilis* showed that the optimal pH was 7.2 using fermentor made him to attribute the influence of initial pH on enzyme formation, or on the release of enzyme from cells, and to consider strain differences as well as the influence of the components of the medium. It is clear that the optimal pH of α-amylase reproduction depends on the strain or the nature of the producer microorganism . Foosi *et al.*(2005) mentioned that the initial pH of media for amylase production by yeast was 4.5 . While, Lin *et al*,(1998) reported that optimal initial pH for the production by thermophilic and alkaliphilic *Bacillus* sp. was 8.5 . Furthermore, the pH affects the active sides on enzymes which are frequently composed of ionizable groups that must be in the proper ionic form in order to maintain the conformation of the active site (Segel, 1976) .



Figure(4.2):Optimal pH for amylase production by *B .stearothermophilus* M13 in TSM medium incubated at 55ºC for 18 hrs with shaking 120 rpm

4.4.3 Optimal temperature for α-amylase production

The results in (figure 4.3) showed that extracellular α-amylase activity increased with the temperature elevation from 20°C with specific activity of 0.01 U/mg to 60°C with 0.05 U/mg, in which the optimal temperature for the

production of α-amylase was 60 °C with activity 0.055 U/mg , then the activity will decrease .

Srivastava and Baruah (1986) reported the optimal temperature for production of thermostable amylase by *B. stearothermophilus.* was 50 ± 5°C. while Lin *et al.*, (1998) reported that the optimal temperature for the production of thermostable from thermophilic and alkaliphilic *Bacillus* sp. was 55°C which is also the optimum for growth. In another study on production of α-amylase by *B. licheniformis* SPT 27 was 37°C (Aiyer, 2004) .

Furthermore, Teodoro and Martins (2000) reported that the optimal temperature for growth and production α-amylase by *Bacillus* sp. were 50ºC . While these results are in contrast with the findings reported by Chandra *et al.(*1980)which reveled that the optimal temperature of growth for *B.licheniformis*CUM 305 was 30ºC , but it never produced the α-amylase at that temperature . In addition, Saito and Yamamoto (1975) studied a *B. licheniformis* which produced α-amylase at temperatures around 50°C and never produced the enzyme at temperatures lower than 45°C.

In general, the perish of enzyme activity with elevation or decreasing the temperature may be attributed to its effects on the growth of the microorganism and velocity of the enzymatic reactions inside the cells reflecting the properties (Cornish, 1979) .

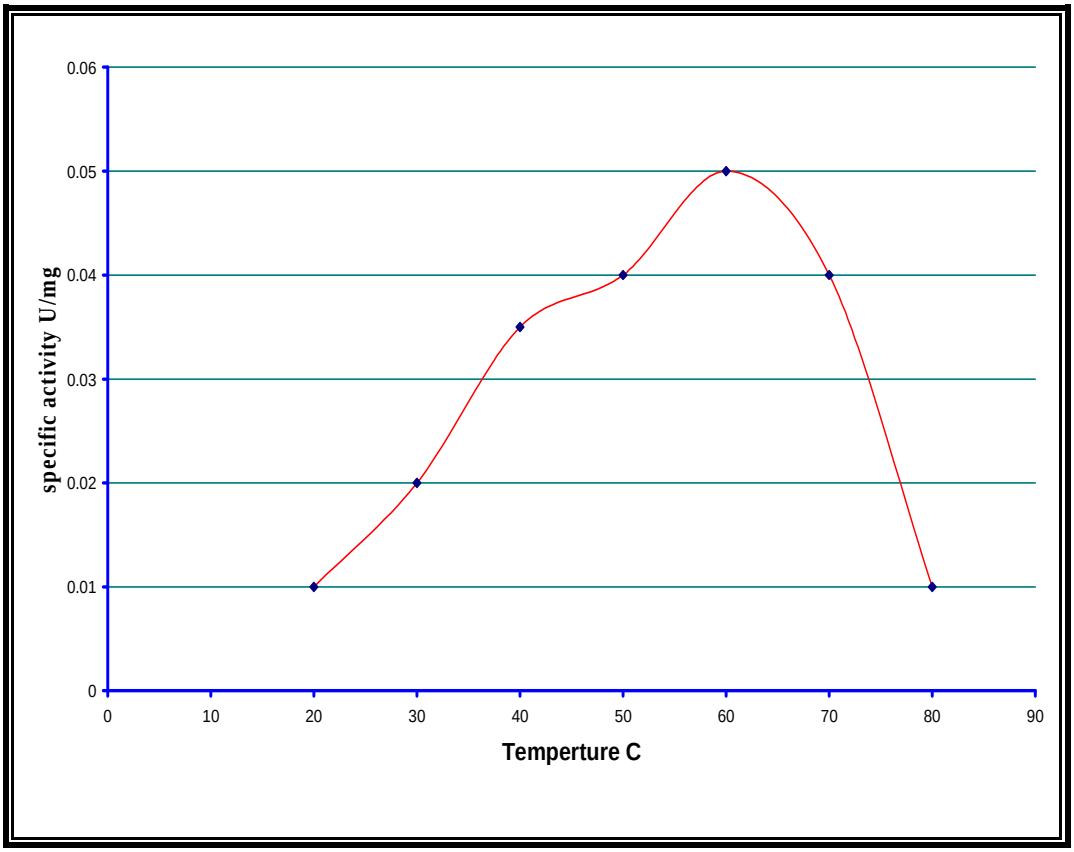


Figure (4.3): Optimal temperature for α-amylase production by *B. stearothermophilus* M13 , incubated at 55ºC ,pH =7,18 hrs with shaking 120rpm

As a fact large number of weak non-covalent bonds maintain the tertiary of the structure of an enzyme, when it absorbs too much energy the tertiary structure will disrupt, and the enzyme will be denaturized. temperature affects the production of enzyme through some aspects like O_2 concentration, medium moisture and enzymatic reactions inside the cell (Segel, 1976).

4.4.4 Optimal growth phase for α-amylase production

Measurements of the enzyme activity and cell growth of *B. stearothermophilus* M13 at a number of time intervals are shown in Figure (4.4). The addition of 10 mM calcium chloride to the liquid medium improved the growth and amylase production since the enzyme is known to be a calcium metalloenzyme as reported by Dauter *et al.*(1999).

The activity of α-amylase produced by *B. stearothermophilus* M13 during growth was investigated in the cell extracts at different time intervals which they covered the lag, log, stationary and the declane phases.

The results in (figure 4.4) showed that the growth entered the stationary phase at the age of about 18 h. in which the $O.D_{600nm}$ was 1.6 , while the growth at the late log had the higher α-amylase specific activity which was 0.05 U/mg than the early log and stationary phases .So it was concluded that the production of α-amylase depends on which phase the growth is passing through, making the highest production at the late log phase.

Teodoro and martins (2000) mentioned that the α-amylase produced by *Bacillus* sp. was at late log phase ,and in similar results Fossi *et al.*(2005) registered that the Amylase production by yeast was correlated to its biomass which was the highest at the late log phase .Concerning the *Bacillus stearothermophilus* , Srivastava and Baruah (1986) noticed that the amylase get the maximal production during post-exponential and stationary growth phases .

The termination of α-amylase production observed during the growth on starch might reflect the accumulation of glucose or maltose as repressing metabolite for α-amylase production. The other type of control over this enzyme is the graded production of the catabolic enzyme levels resulting from growth on other specific carbon sources which are independent of the presence of starch (Nagata *et al.*, 1974; Nicholson and Chambliss, 1985; Srivastava and Baruah ,1986; Haseltine *et al.*, 1996). On other hand, Zangirolami *et al.* (2002) set up a model for growth and α-amylase formation by *Aspergillus oryzae* , the specific rate of α-amylase production increased with the specific growth rate, α-amylase strongly repressed by residual glucose concentration .

Those results could be interpreted that the log phase is characterized by an increase in the biomass with highly active cells in most physiological activities which greatly affected enzyme production (Mischack *et al.*, 1985), while the elongation of fermentation time caused undesired effects on the production of α-amylase and its activity because environmental changes in media ,autolysis resulting in releasing a lot of material and intracellular lytic enzymes that could destroy the α-amylase (Lonsane and Ramesh ,1990) .

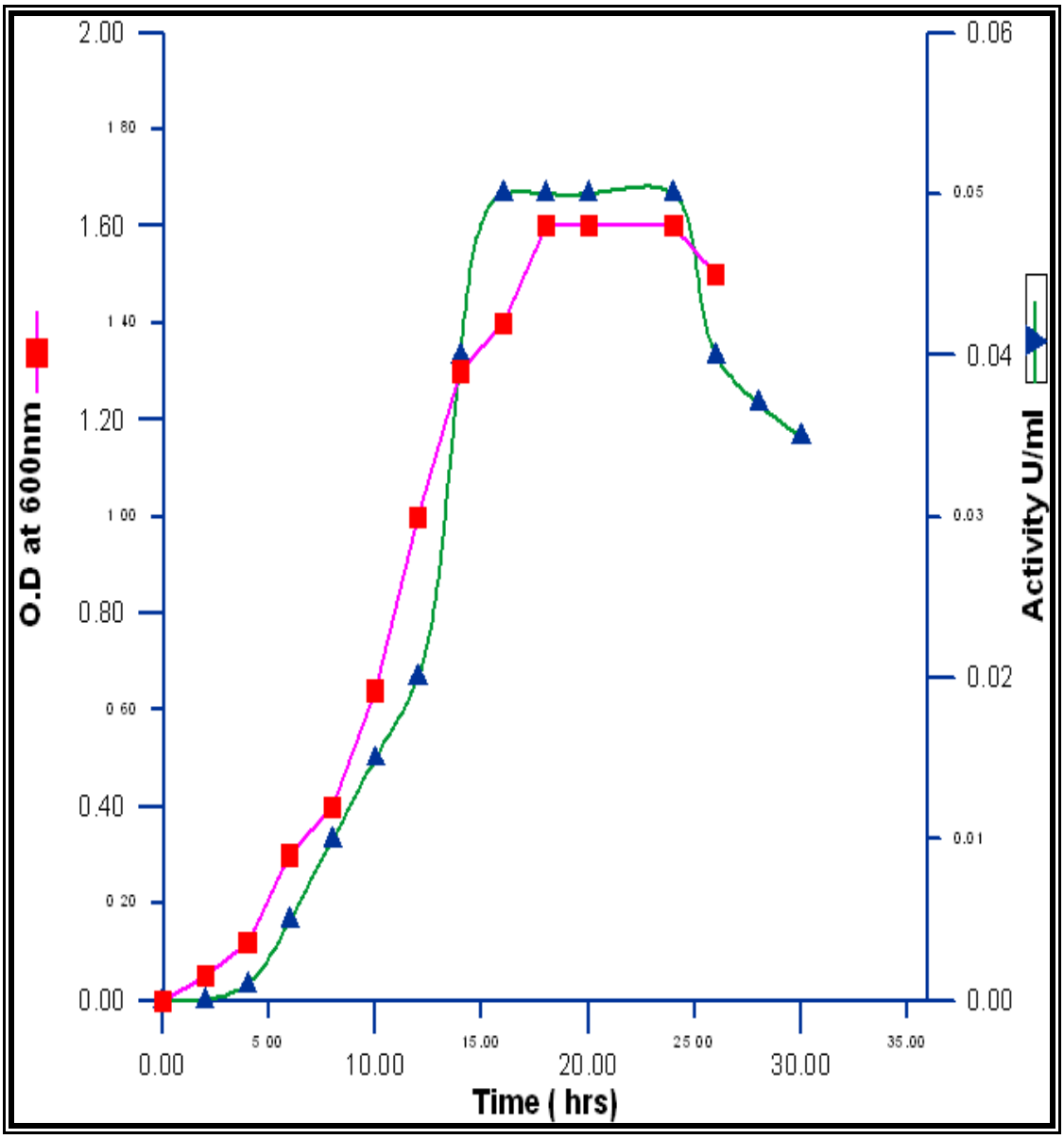


Figure: (4.4) growth curve and α-amylase production phase for the *B. stearothermophilus* M13 in TSM medium, incubated at 55°C , pH (7.0) with shaking 120 rpm

4.5 Purification of α-amylase of *B .stearothermophilus* M13

α-amylase applications in pharmaceutical ,industrial and clinical sectors requires high- purified enzyme or partial purified enzymes depending on the process taking into consideration the costs of each one related to that(Deutscher, 1990) .

4.5.1 Crude enzyme concentration by Ammonium sulfate

In order to concentrate on the crude α-amylase in culture supernatant and remove as much water and some protein molecules as possible, saturation ratio (40-80 %)was used to achieve that through two addition steps, the first step is increasing the saturation ratio from (0 - 40 %) ,which showed low specific activity of precipitated fraction of 0.002 U/mg .when (40-80)% saturation ratio was applied to the supernatant fraction , it achieved a higher specific activity to 0.051 U/mg , 0.92 purification folds with 57 % yield . From the results demonstrated above, approximately 96% of activity is within the (40-80) %. But, when we compare between the specific activity of crude to that precipitated by Ammonium sulfate , it was found the specific activity of crude is higher than resulted by precipitation ,so it is concluded that the Ammonium sulfate step is not properly recommended to this particularly stage of purification . Concerning the other studies, those results are in contrast with Sarivstava and Baruah (1987) who obtained specific activity of 210.6 U/mg for α-amylase produced by *B. stearothermophilus M13.* Through Ammonium sulfate precipitation ratio of (45-60)% ,while Aiba *et al.*(1983) obtained specific activity of 8 U/mg with 22% recovery from *B. stearothermophilus* and *B. subtilis* in successive steps using(30-45)% saturation ratio. While, Fossi *et al.* (2005) used 65% (w/v) sodium sulphate to achieve partial purification of α-amylase produced by yeast.

Furthermore, α-amylase produced by *Xanthomonas campestris* was precipitated by (45-70) % to achieve specific activity of 4.51 U/mg with 91% Recovery(Abe *et al.*, 1994) . In general, Ammonium sulfate is favored in precipitation step due to its high solubility, availability, being cheap and that it does not damage most enzymes (Volesky and Luong ,1985) .

The theory that explain the mechanism of salting out is combined with the old theory; salting-out occurs as a result of a decrease in the hydration of proteins in favor of the that of the ions of the neutral salt , the hydration layer surrounding the surface of the protein is transferred to the ions of the salt at a rate depending on the charge of the protein ,pH and temperature (Schutte, 2003) . And the recent theory is that a salt ion has a repulsive interaction with an image charge inside a low dielectric cavity (Zhou, 2005).

4.5.2 Ion –Exchange and Gel-filtration chromatography

The results in (table 4.5)showed that α-amylase purification could be interpreted that the first step after the centrifugation of culture extract contained the growth biomass , was to be concentrated, and dissolved in 20 ml of the distilled water containing 0.025 M of $CaCl_2$, then it was dialyzed (4.5 by 10-cm bags;10,000 kDa cutoff) against distilled water, phosphate buffer 0.005 M (pH7.0) supplied with 0.025 M $CaCl_2$ over four times . It achieved less specific activity to be compared with the crude with 57% yield ,0.92 folds of purification , but higher activity of 0.114 U/ml , which is considered as a step for the partial purification of some commercial important enzymes , in which it could be used at this step for further usages . Then anion ion-exchange chromatography using DEAE-Sephadex step was set up, initially it was equilibrated with the same buffer of dialysis 0.005 M phosphate buffer pH 7.0 . After applying the sample to the column, washing with the same buffer was run. the protein profile (figure 4.5)showed three peaks , but no activity was indicated for α-amylase , that could because of

those proteins have a similar charge to the anion (positively charged) or zero charge because when the pH ≤ pI for the enzyme , it will make the enzyme be marked with a positive charge . DEAE-sephadex is a weak anion exchanger with excellent flow properties and high capacity for proteins of most pI values with high resolution. The ion exchange functional group is diethylaminoethyl that remains charged and maintains consistently high capacities over the entire working range (LKB). Therefore, it could be concluded that α-amylase has been bounded onto the matrix of the exchanger, those protein fractions were washed out. then the elution was run using lanear salt gradient 0.005 M potassium phosphate – 1 M NaCl (pH 7.0) which could detect five peaks for protein and four main peaks for α-amylase activity figure (4.6), which marked as peak {***a,b,c*** and ***d*** } .Then the fractions for each single peak were collected with each other and concentrated using sucrose through dialysis bag , and dialyzed using the same procedure after the concentration by ammonium sulphate . The peak "***b***" showed the higher specific activity of 1.475 U/mg with 26.8 folds of purification and 73.75 % yield that made it as a good source for further purification among the others ,in which the peak "***a*****"** showed a specific activity of 0.416 U/mg with 7.56 folds of purification , peak "***c***" showed a specific activity of 0.20 U/mg with 3.63 folds of purification and peak "***d*****"** showed a specific activity of 0.043 U/mg with 0.78 folds of purification , that made an assumption of being participating to a large extent in the overall specific activity of the produced present α-amylase . The presence of four activity peaks for the α-amylase produced by *B. stearothermophilusM13* means that those could be forms (Isoenzymes) or " Isozymes" for the α-amylase which is in the first time a ,study showing such a number for *Bacillus stearothermophilus;* concerning the other studies of isoenzymes , Zhang *et al.*(1994) reported that three forms for α-amylase produced by *Bacillus* sp. with molecular weight

(Mr)110-140 kDa, two forms produced by *Bacillus* sp. with Mr of 150-42 kDa were purified by Lin *et al.*, (1998).

Furthermore, most of the studies showed a single form for α-amylase produced by *B. flavothermus* (Bolton *et al.*,, 1997) , *B. licheniformi* (Kim *et al.*, 1992; Ivanova *et al.*, 1993) . There are several possible explanations for being different Isozymes of α-amylase, they are either purification procedures due to protease(s) hydrolysis (Koch *et al.*, 1990; Pompeyo *et al.*, 1993), carbohydrate contents or protein aggregations (Spreinat *et al.*, 1990; Giraud *et al.*, 1993 ;) or deamination of α-amylase (Park *et al.*, 1991).

The fraction termed as (peak *b*) was collected together and concentrated by osmosis against sucrose crystals, then applied to Sepahdex G-100 gel filtration column in order to obtain more purified α-amylase . (G-100) is characterized by being easily prepared, long term stability and high speed flow rate and the ability to separate a broad range of the molecular weights for proteins . Sepahdex G-100 had a unique characteristic of being a combine between affinity chromatography and gel filtration properties on which it had a tendency to bind with α-amylase causing delaying in elution time of it (Scopes, 1987).

α-amylase was partially purified , Figure (4.7) showed two peaks for protein from fraction 25^{th} through fraction 30^{th} and matching approximately single peak for the α-amylase activity from fraction 27^{th} through 30^{th} which is an indicator for a partial purity of α-amylase after the gel filtration step, of specific activity 1.675 U/mg , folds of purification 30.45 and 50.25 % yield , which are considered as important results when compared to other studies . Shaw *et al.*(1995) obtained α-amylase purified from *Thermus* sp. of activity 2.22 U and 2.6 % yield using hydrophobic interaction chromatography step and followed by affinity chromatography onto corn starch .Paquet *et al.* (1991) purified the α-amylase from *Clostridium acetobutylicum* ATCC 824 using ion-exchange and gel filtration to get amylase with specific activity

57.7 U/mg , purification folds 22.2 and yield 23.2 % .while, shih and Labbe (1995) could purify the α-amyalse produced by *Clostridium perfringens* Type A using double filtration through DEAE-sephacryel achieving specific activity of 34.6 U/mg ,purification folds 20.4 and yield 11.3 %.α-amylase was purified from *B. stearothermophilus* using DEAE-Sephadex A 25 to get specific activity 100 U/mg, and 57 % yield(Aiba *et al.* ,1983).

Furthermore, α-amylase was purified from Alkaliphilic *Bacillus* sp. using DEAE –Toyopearl and CM-Toyopearl obtaining specific activity 5,009.3 U/mg and 35 % yield (Igarashi *et al.*, 1998). While, Lin *et al.*, (1998) used Sephacryel S-100 and HiTrap Q in a procedure to purify the α-amylase from Thermophilic alkaliphilic *Bacillus* sp. , they obtained a specific activity of 921.0 U/mg , purification folds of 708 .5 and yield 13.2 % ,Abe *et.al*,(1994) purified α-amylase from *Xanthomonas campestris* K-1151 by means of Cm- Toyopearl and phenyl-Toyopearl achieving a specific activity of 256 U/mg and 36% yield .

Table (4.5): purification steps of α-amylase produced by *B. stearothermophilus* M13 in TSM medium

Steps of purification	Activity U/ml	Volume ml	Protein conc. mg/ml	Specific activity U/mg	Total activity	Folds of purification	Yield %
Crude extract	0.04	100	.725	0.055	4	1	100
Ammonium sulphate precipitation 40-80%	0.114	20	2.2	0.051	2.28	0.92	57
Ion-exchange *a*	0.125	10	0.3	0.416	1.25	7.56	31.25
Ion-exchange *b*	0.295	10	0.2	1.475	2.95	26.8	73.75
Ion-exchange *c*	0.11	5	0.525	0.209	0.55	3.8	13.75
Ion-exchange *d*	0.08	10	1.825	0.043	0.8	0.78	20
Gel-filtration	0.201	10	0.12	1.675	2.01	30.45	50.25

a **stands for peak 1**
b **stands for peak 2**
c **stands for peak 3**
d **stands for peak 4**

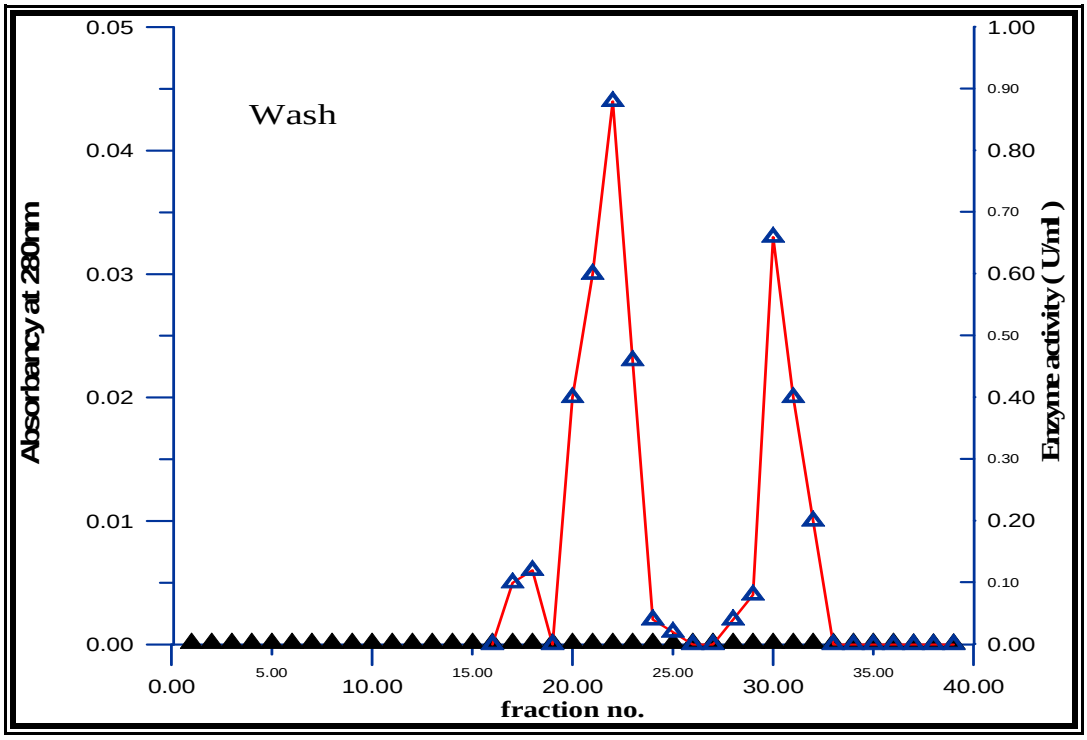
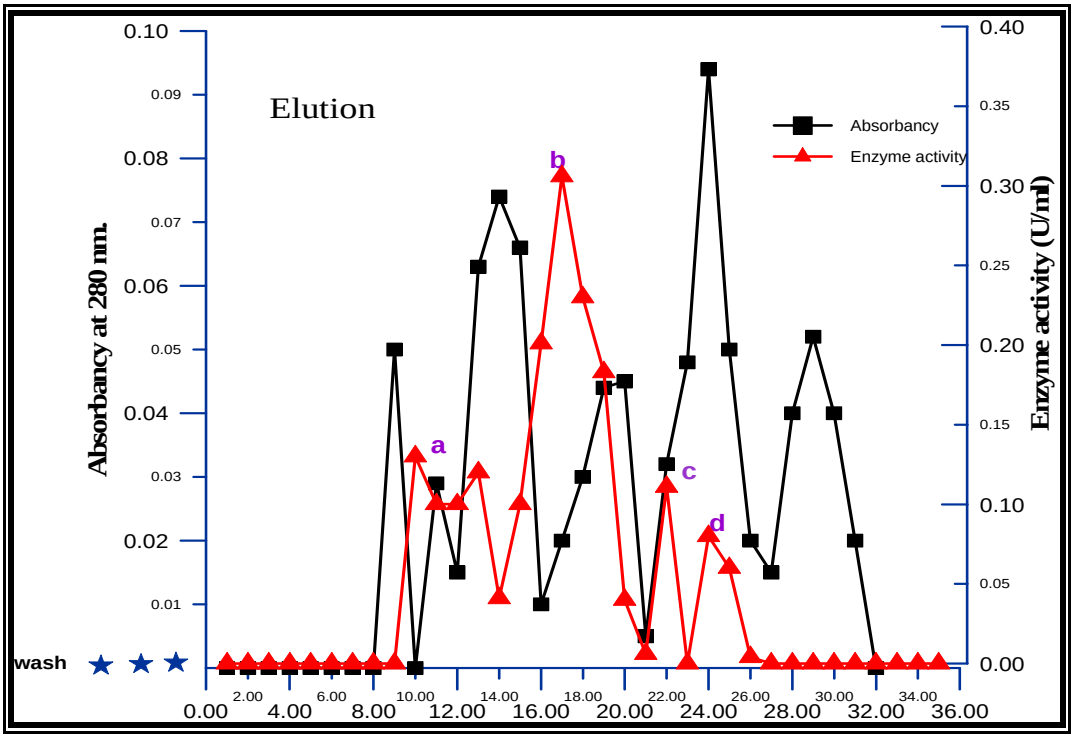


Figure (4. 5) : DEAE-Sephadex Ion- Exchange Chromatography column (2.5 X 35)cm .washing step with 0.005 M Phosphate Buffer pH 7.0 at flow rate of 30 ml/h. and 5ml /frac.



Figure(4.6):DAEA-Sephadex ion Exchange chromatography column (2.5 X35) cm.(Elution step) ,enzyme recovered with lanear salt gradient (0.005 M Phosphate Buffer – 1 M NaCl)pH 7.0 at flow rate 30 ml/h. 5ml/frac.

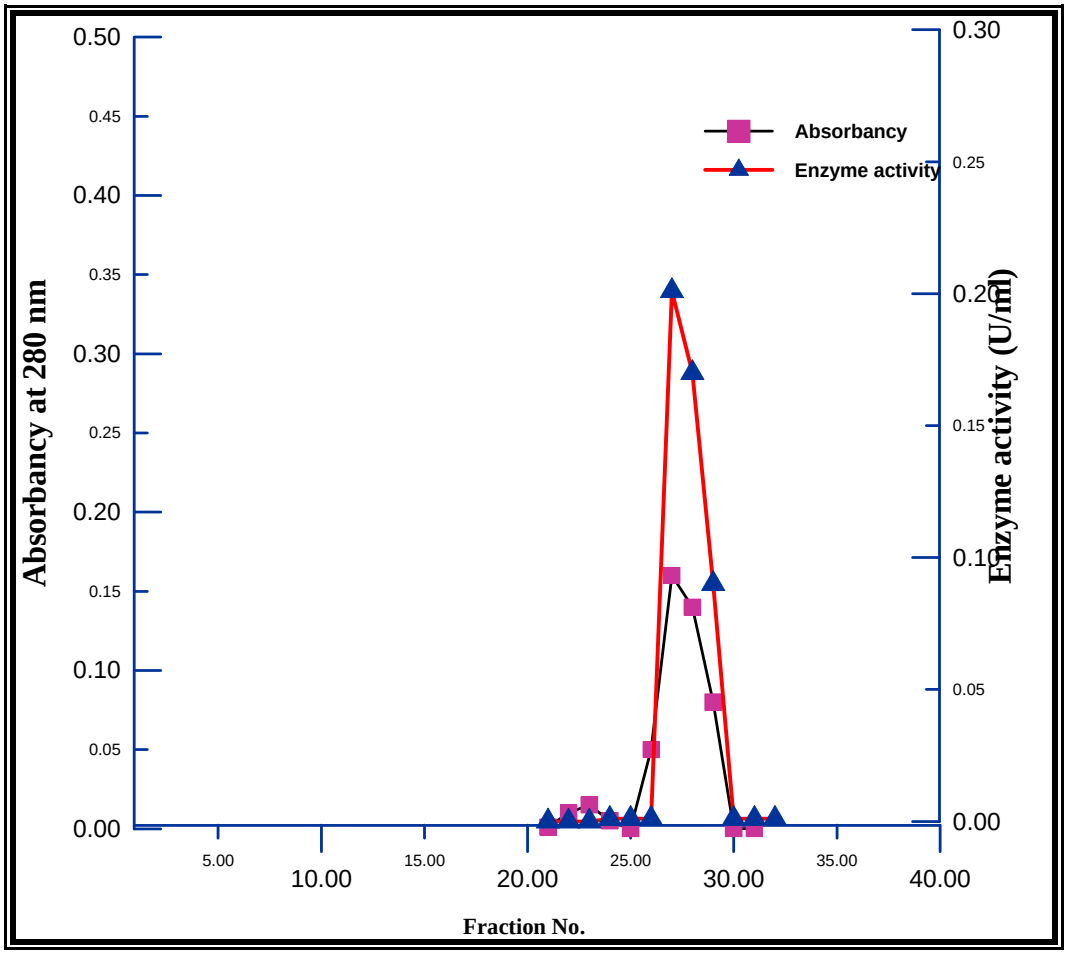


Figure :(4.7) Sephadex G-100 gel filtration, chromatography for active fraction from DEAE- Sephadex "peak *b* ", column was run onto 1.3 X 35-cm Sephadex G-100. The column was equilibrated with 0.005M phosphate buffer (pH 7.0), the elution at rate of 30 ml/hr, 5ml/ frac.

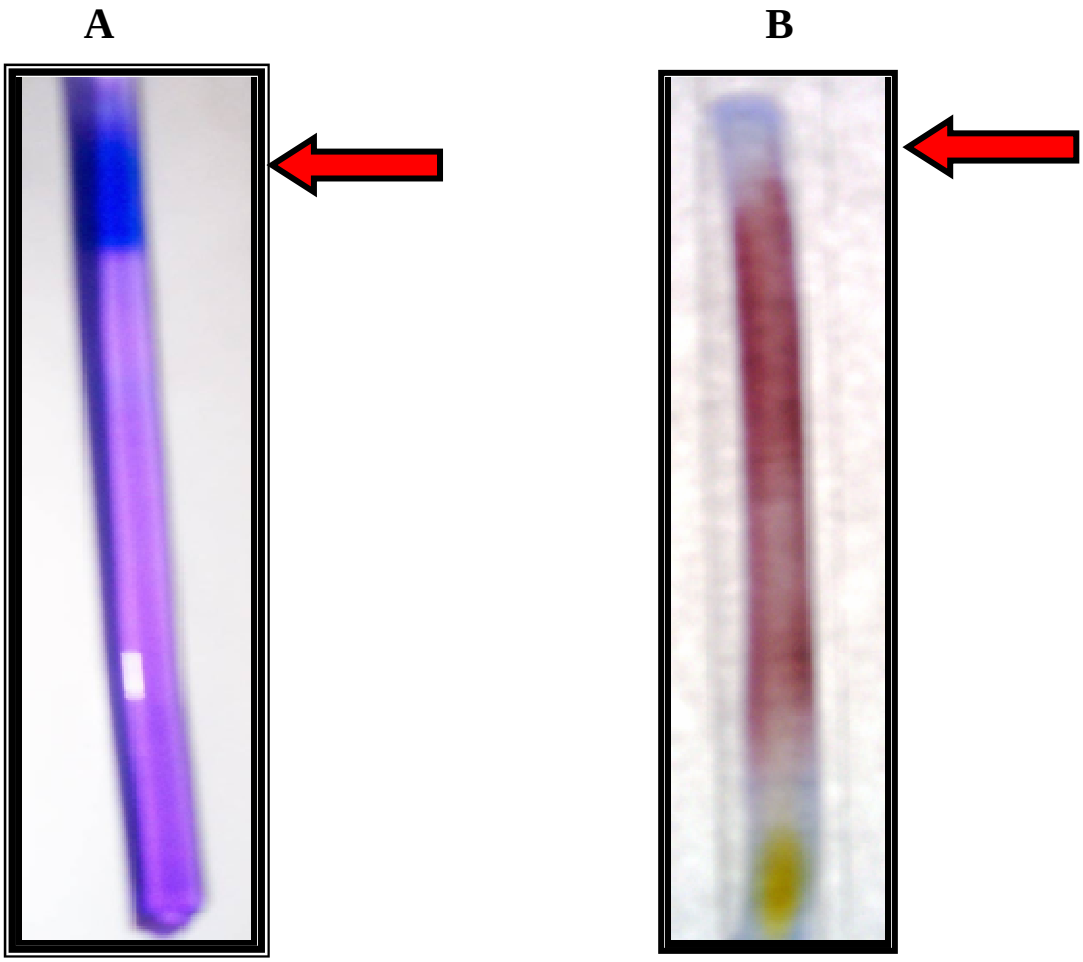
4.6 Characterization of α -amylase

4.6.1 α-amylase partial purity (Electrophoresis under Non- denaturized Conditions

The purity of the α-amylase produced by *B. stearothermophilus* M13 was initially identified by gel filtration Sephadex G-100 technique through obtaining a single sharp peak of activity matching the peak of protein. With the aim of confirming the result, another step was done described as protein electrophoresis under denaturized and non-denaturized conditions.

The electrophoresis of α-amylase purified by gel filtration, on polyacrylamide gel under non-denaturized conditions was done. Figure (4.8) showed the pattern of α-amylase electrophoresis , in which one single band was detected after staining the gel with Coomassie brilliant blue R-250 dye (figure 4.8-A) ,and one single band was visualized in the gel saturated with 1.5% starch grains ,then stained with the activity dye " iodine" (figure 4.8-B).It is clear that the band is localized near the upper part of the gel after about 3 .5 hours, which could be explained that the enzyme under non-denaturized condition has no enough counter-charge density (positively charged) at pH 6.8- 8.3 that could be driven towards the anode pole. The protein immigration on the gel depends on the net charge density of protein at a given pH and the configuration of it (Johanstone and Thorpe ,1987; Shi and Jackowski , 1998). The presence of a single band may indicate the homogeneity of enzyme . Lin *et al.*(1998) obtained two isoenzymes for α-amylase by electrophoresis depending on the activity staining ,then it found a single band for each isoenzyme using SDS-PAGE . Sometimes , it is confusing to determine clearly the single individual band using PAGE because of there are several possible explanations for the multiple forms (Isozymes) of amylases, including the purification procedures themselves, protease(s) hydrolysis carbohydrate content and protein aggregation during electrophoresis (Shih and Labbe,1995).

The result of this study is much similar to that of Pfueller and Elliott (1969) who reported that one single band for α-amylase was visualized by the activity staining (Iodine), using non-denaturized conditions for PAGE , near the upper part of gel .



Figure(4.8):Polyacrylamide Gel Electrophoresis "PAGE" (7.5%) for α- amylase purified from *B. stearo*thermophilus M13(amplified by S-splane programm).
A- Purified enzyme visualized by Coomassie brilliant blue(R-250) staining
B- Purified enzyme visualized by activity dying "lugol`s dye"
Red arrows indicated positions of the partially purified α-amylase band

4.6.2 Determination of α-amylase molecular weight (Mr)

The Mr of α-amylase was determined using two methods :

1- Gel filtration method

Sephadex G-100 column (2.6 x 35) was used to estimate the molecular weight (Mr) of α-amylase, as blue dextrane was used to determine the void volume of column .When the Ve/Vo was plotted against logarithm of the Mr, the Mr was estimated to be " 56234 " Dalton figure (4.14) in which that value is closer to what some previous studies were reported using gel filtration, Pfueller and Elliott(1969) determine the Mr of α-amylase produced by *B. stearothermophilus* using gel-filtration to be 53000 Dalton , while the Mr of thermophilic extracellular α-amylase produced by *B. licheniformis* was estimated to be 22500 Dalton (Saito ,1973) .Figure (4.9) showed the elution of blue dextrane, which has Mr of 2.6 x 10^6 Dalton, the presence of shoulder during the elution could be due to the degradation of it for its big molecular weight.

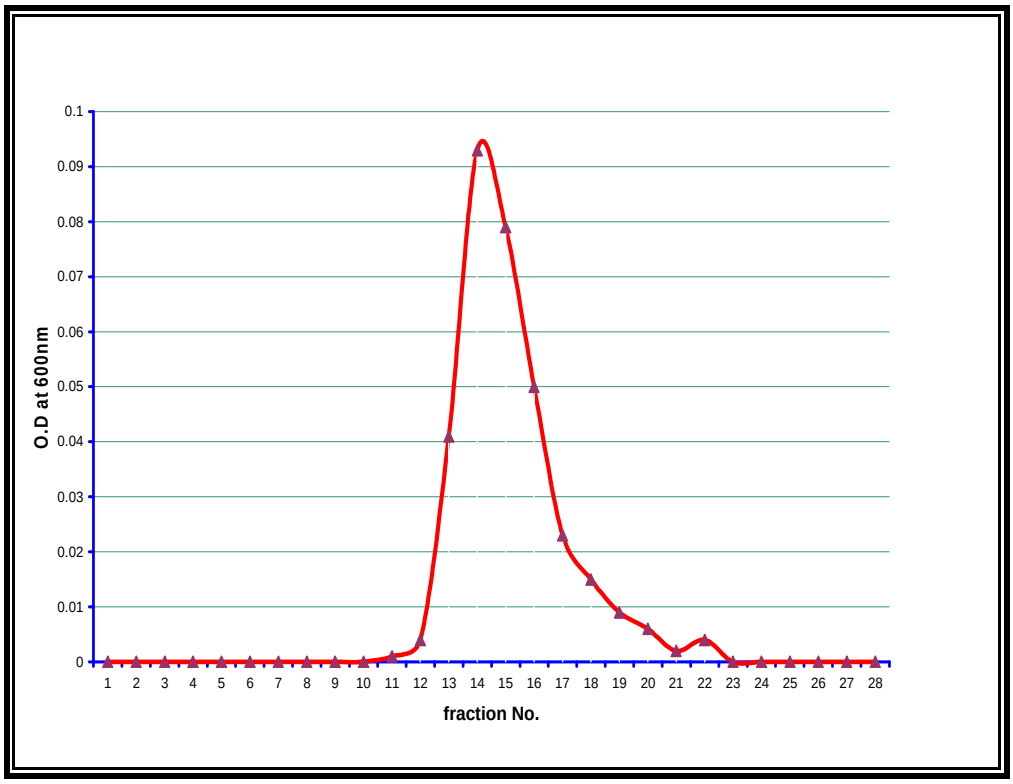


Figure (4.9): Sephadex G-100 gel filtration chromatography of Blue - dextrane, was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005 phosphate buffer (pH 7.5), the elution rate of 30 ml/hr, 5ml /frac.

Figure (4.10) showed the elution of Alkalane phosphatase which has Mr of 160000 Dalton, which is considered as a high Mr protein , one single peak was detected as well as small peak which could be explained as degradation of the main protein into small fractions .

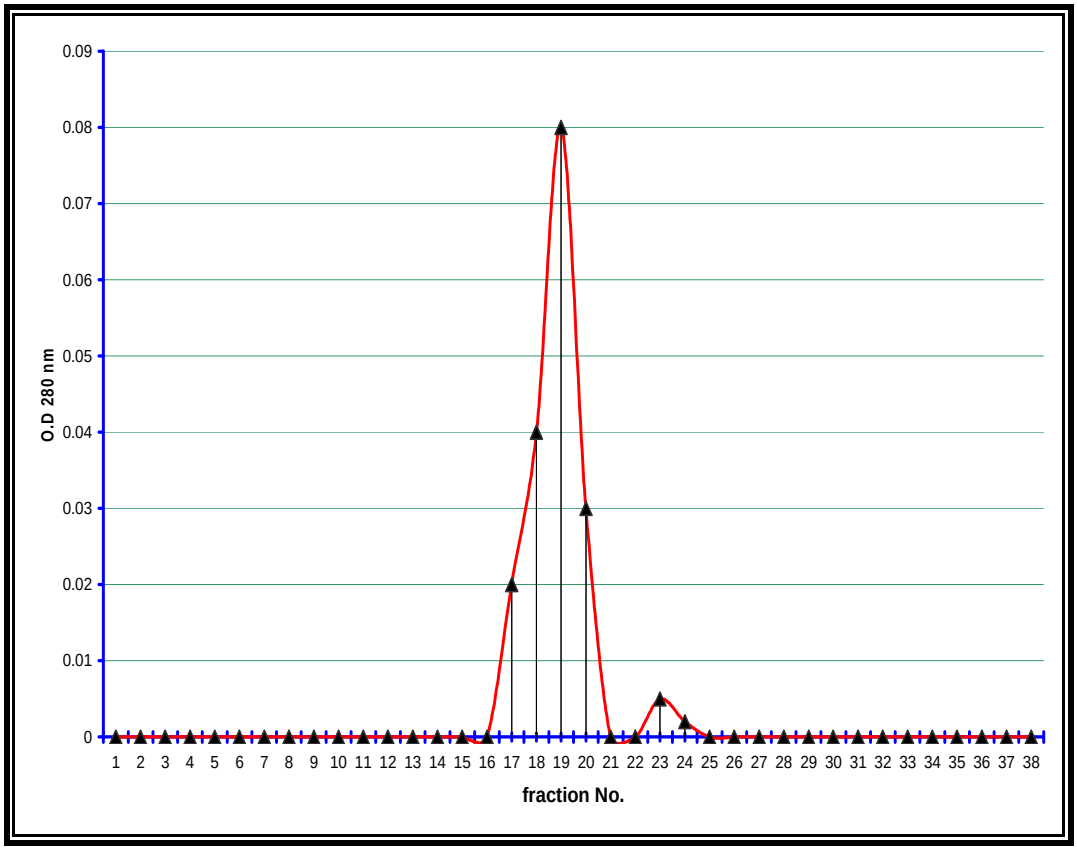


Figure (4.10) :Sephadex G-100 gel filtration of Alkalane Phosphatase , column was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005M phosphate buffer (pH 7.5), the elution rate of 30ml/hr, 5ml /frac.

In the figure (4.11), the chromatography of BSA, which has Mr of 76000 Dalton , one single peak was detected .

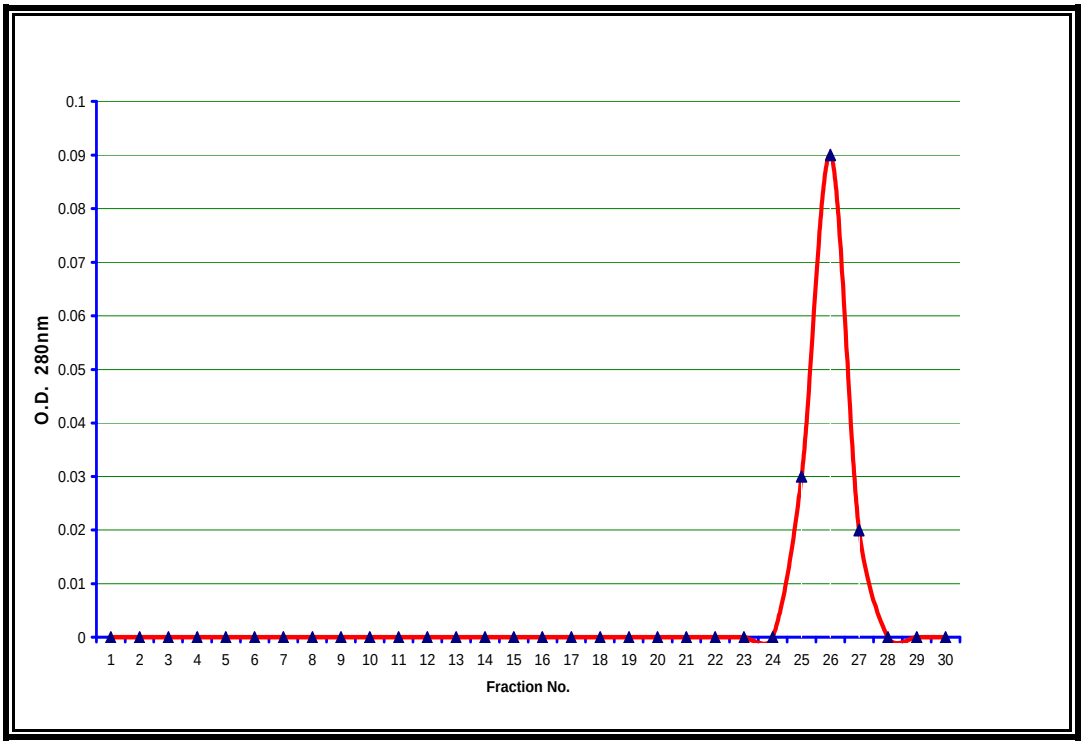


Figure (4.11): Sephadex G-100 gel filtration chromatography of BSA , column was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005M phosphate buffer (pH 7.0), the elution rate of 30ml/hr, 5ml /frac.

Figure (4.12) showed the chromatography of Lysozyme, which was considered as low Mr protein of 14400 Dalton . One single peak was detected

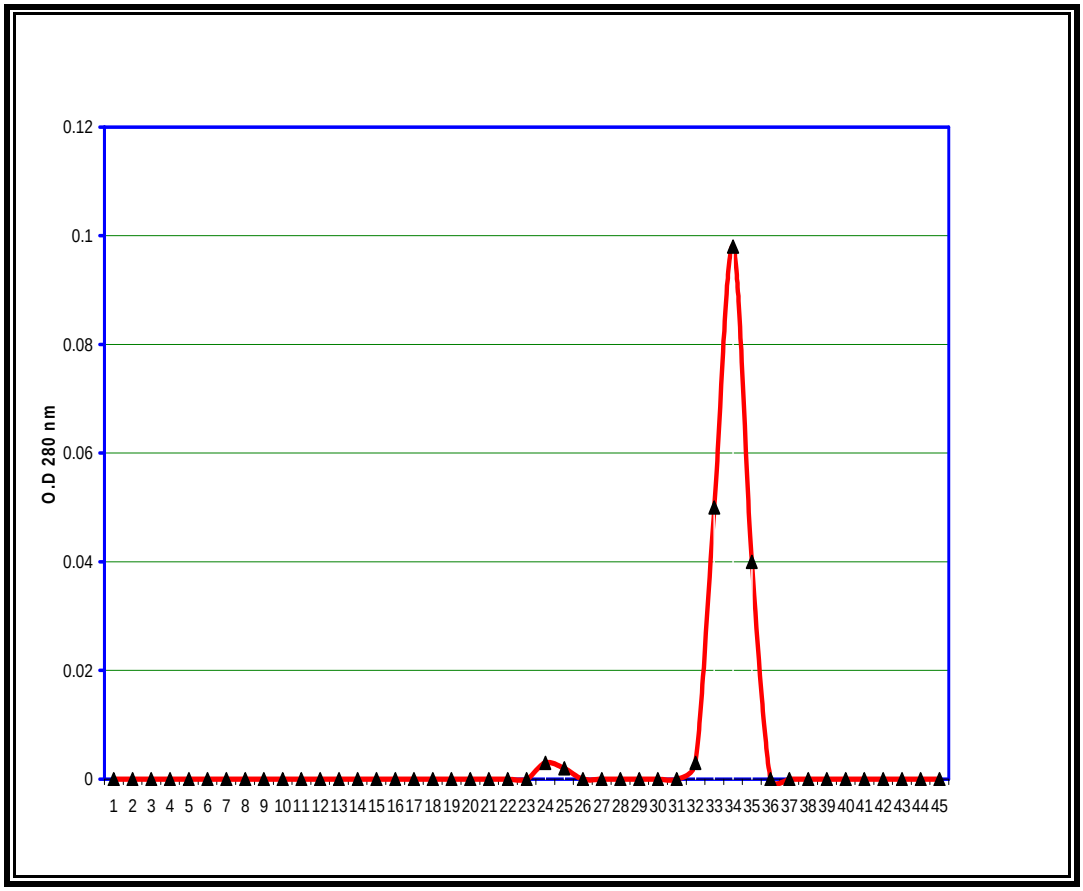


Figure:(4.12) Sephadex G-100 gel filtration chromatography of Lysozyme; column was applied in 1.3 X 35-cm Sephadex G-100 column. Equilibrated with 0.005M phosphate buffer (pH 7.0), the elution rate of 30ml/hr, 5ml /frac.

(Figure 4.13) showed the chromatography of Ovatransferrin which has Mr of 76000 Dalton , one single peak was detected .

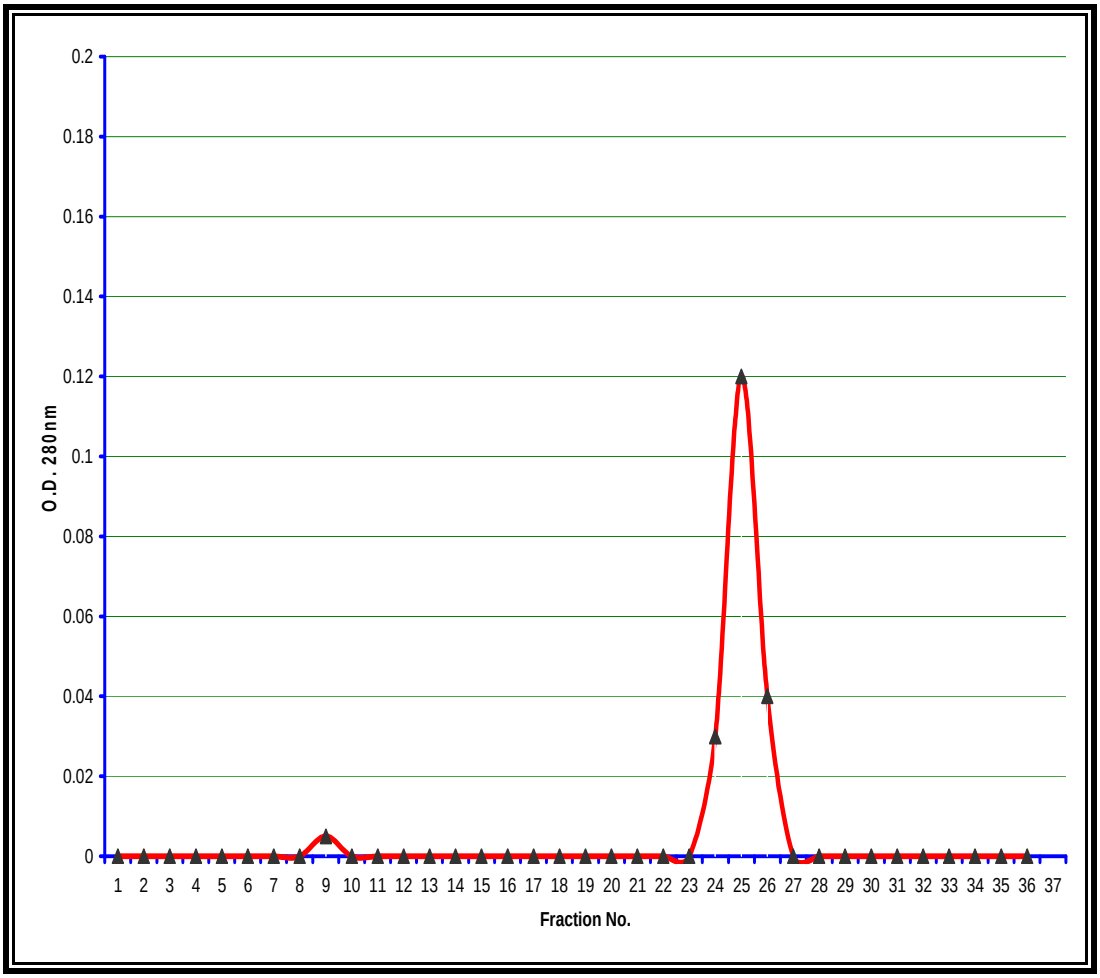


Figure (4.13): Sephadex G-100 gel filtration chromatography of Ovatrnsferrin; column applied in 1.3 X 35-cm Sephadex G-100. The column was equilibrated with 0.005M phosphate buffer (pH 7.5), the elution, at rate of 30ml/hr, was performed with the same buffer and 5-ml fractions were collected.

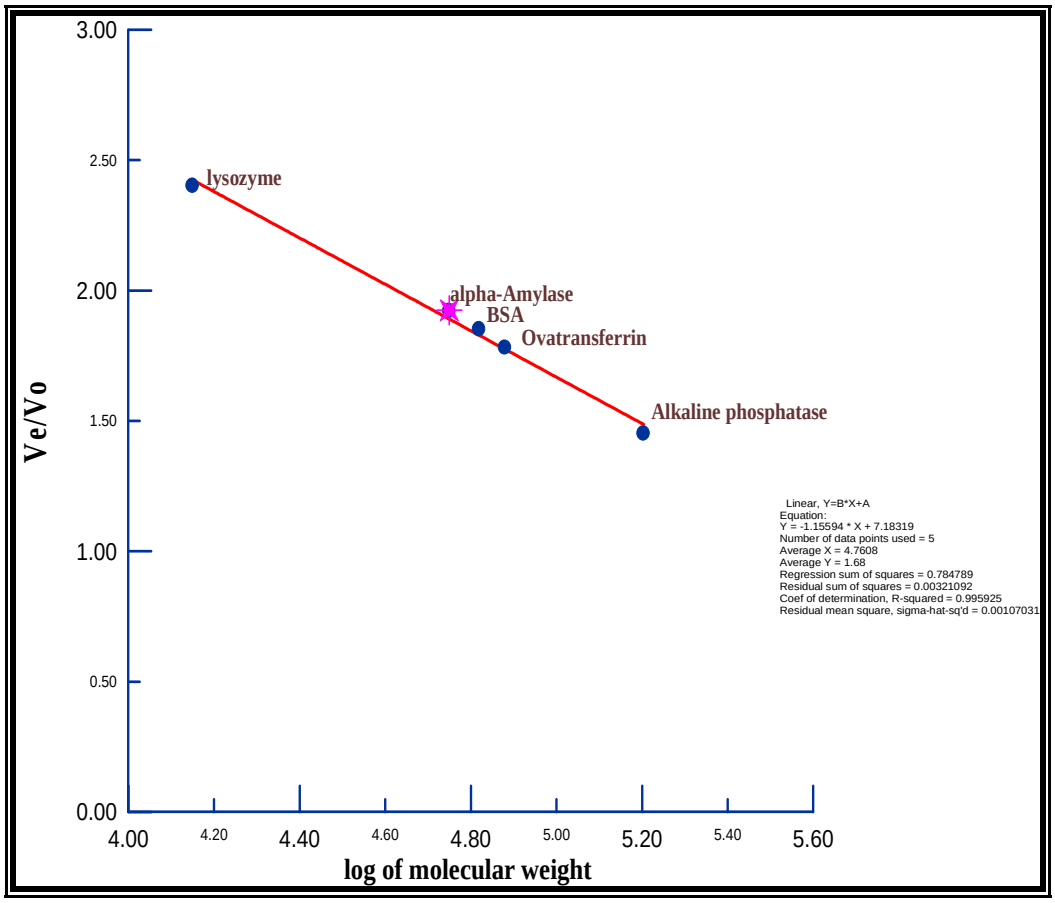


Figure (4.14): logarithms of α-amylase molecular weight as a function of the elution volume (V_e) and the ratios of the elution volume to the void volume (V_e/V_o)

2- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis" SDS-PAGE"

The Mr of α-amylase was determined by SDS-PAGE in which the Mr was determined based on known standard proteins , then the molecular weight was calculated using PhotoCaptMw and Photo impact softwares that were originally designed to help the biologist in the molecular weight calculations , the Mr was determined to be (55426 Da) figure(4-15 A,B).

Monomeric structure was revealed by this technique ,because of only one single band visualized , most studies mentioned that results , in which they were similar to those previously reported using SDS-PAGE method .

Shaw *et al.* (1995) showed that the α-amylase produced by *Thermus* sp. had Mr of (59000 Da)as a monomeric protein ,while Aiba *et al.*(1983) mentioned the Mr of α-amylase produced by *B. stearothermophilus* was 53000 Da. Igarashi *et al.*(1998) reported that the Mr of α-amylase produced by Alkaliphilic *Bacillus* sp. was 53000 Da . On other hand, *Xanthomonas campestris* produced α-amylase of Mr about 55000 (Abe *et al.*, 1994) . Bakhmatova *et al.*(1984) reported that three different variants of *B. subtilis* produced three different α-amylase in their $Mr_{(s)}$ 56000,57000 and 58000 Da.

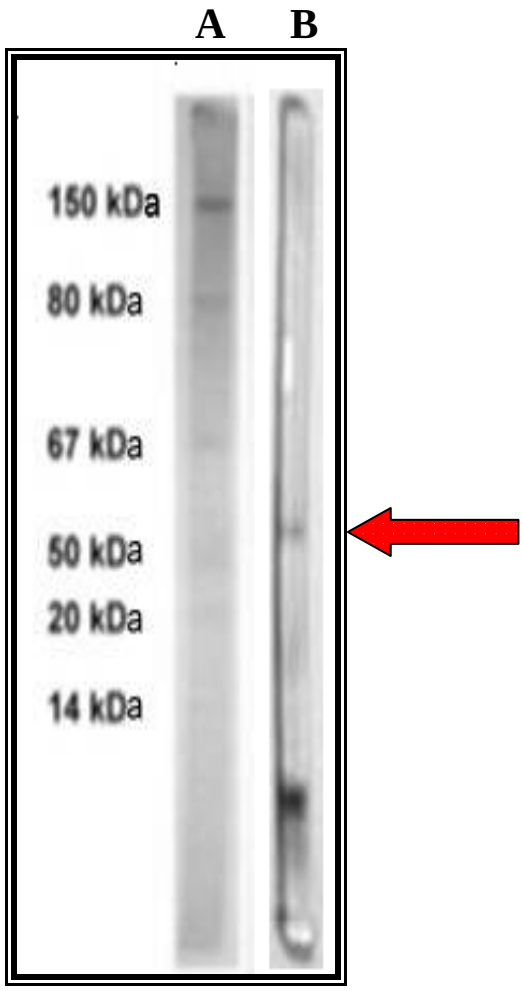


Figure (4.15 A): Estimation of the α-amylase Mr by SDS –PAGE using PhotCaptMr software (amplified by S-splane program).

A) Mr protein markers

B) α-amylase from *B. stearothermophilus* M13

Red arrow indicated α-amylase band position

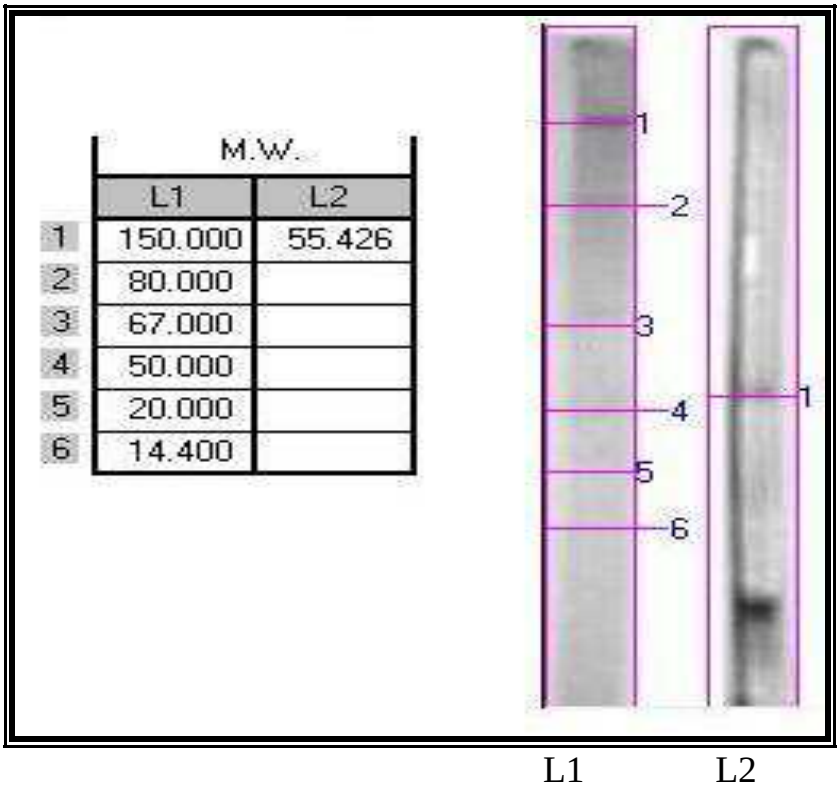


Figure (4.15 B):Calculation of α-amylase Mr processed by PhotoCaptMw version 10.01 and Photo impact softwares.

L1: standard proteins
L2: α-amylase band

4.6.3 Optimal pH for α-amylase activity

The optimal pH for α-amylase activity was studied in range of (5.0-8.5), figure (4.16) in which the optimal pH value for maximal activity was determined at value of 7.0, the activity was 0.2 U/ml , the enzyme displayed an activity curve over broad pH range , the decrease in the activity was at the extreme values of acidity (5.0-6.0) , alkalinity (8.0-8.5) which could be explained as the active sites on enzyme are frequently composed of ionizable

groups that must be in the proper ionic form in order to maintain the conformation of the active sites or change the configuration of the enzyme itself (Segel,1976). Concerning the results of the other studies, the optimal pH for α-amylase maximal activity produced by *B. stearothermophilus* was 6.9 which is similar to the results of Srivastava and Baruah(1986). Bakhmatova *et al.* (1983) indicted that the optimal pH for α-amylases produced by variants of *Bacillus subtilis* was within the acidic pH range(4.5-5.0) , while the optimal pH for α-amylase produced by *B. amyloliquefaciens* was at pH 6.0 (Kochhar and Dua 1982), which is similar to optimal pH for activity of that produced by *Clostridium perfringens* (Shih and Labbe ,1995) . The optimal pH for maximal activity of α-amylase produced by *Pyrococcus furiosus* was 5.5 (Dong *et al.*, 1997). Saito(1973) mentioned that the optimal pH for α-amylase produced by *B. licheniformis* was at 9.0 , which is similar to the findings of Krishnan and Chandra(1983) in activity of α-amylase from *B. licheniformis* was at pH of 9.0 ; also the activity of enzyme was reduced when Tris- hydrochloride and carbonate-bicarbonate buffers were used .In other studies, the optimal pH of α-amylase maximal activity produced by *Xanthomonas campestris* was 4.5 which is acidic (Abe *et al.*,1994), while the pH of maximal activity for α-amylase produced by Akaliphilic *Bacillus* was in alkalane range (8.0-8.5) (Igarashi *et al.*, 1998; Lin *et al.*, 1998) .

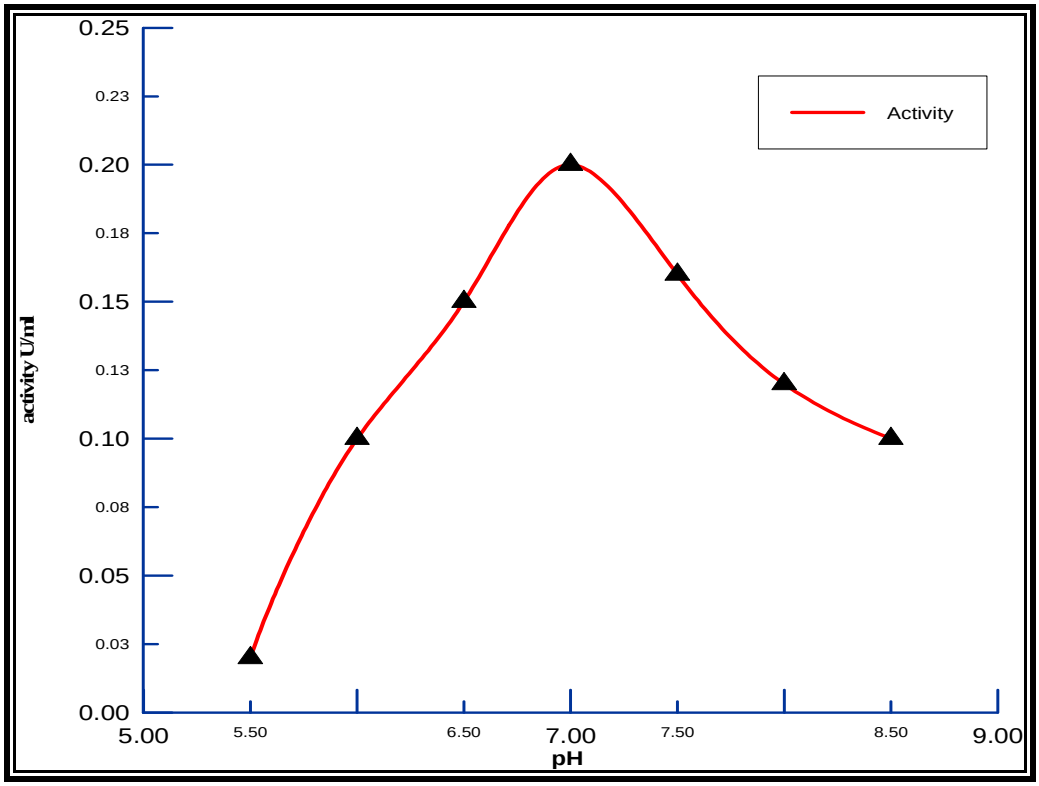


Figure (4.16) :Optimal pH for α-amylase activity

4.6.4 Effect of pH on α-amylase stability

With the aim of investigating The optimal pH for stability of α-amylase ,the enzyme was incubated in a buffer supplied with $CaCl_2$ 0.005 M (pH range 4-11) at 60°C for 30 min. The result (figure 4.17) showed the enzyme had excellent stability at pH range (6.5 – 8.0), while the stability was decreasing as it went toward the extreme acidity or alkalinity , by which the enzyme lost more than 50 % of its original activity at(pH 5.0 and pH 10.0) .While, it showed about (40-50 %)of the remaining activity at (pH 4.0- 10.5), which could be an advantage for utilizing the enzyme in some industrial purposes like detergents in which the pH is alkalane in washing conditions (Ito and Horikoshi ,2004).The effect of pH on enzyme stability could be explained in the formation of improper ionic form of enzyme or the active sites ,irreversible inactivation . The stability of enzyme depends on many

factors such as temperature, ionic strength ,chemical nature of buffer , concentration of various preservatives , concentration of metal ions , substrate or factors and enzyme concentration (Segel ,1976).

Concerning the results of other studies, the α-amylase produced by *Bacillus stearothermophilus* was stable at pH range (6.0-11) while the α-amylase produced by *B. amyloliquefaciens* was stable at pH (6-9.5) for 24 h. in the presence of calcium ion (Kochhar and Dua, 1983). α-amylase produced by *Bacillus licheniformis* was stable at pH rang (6- 11) after the incubation for 30min and 24 hr., and in another study it was stable at pH range (7.0-9.0) (Krishnan and Chandra, 1983) .

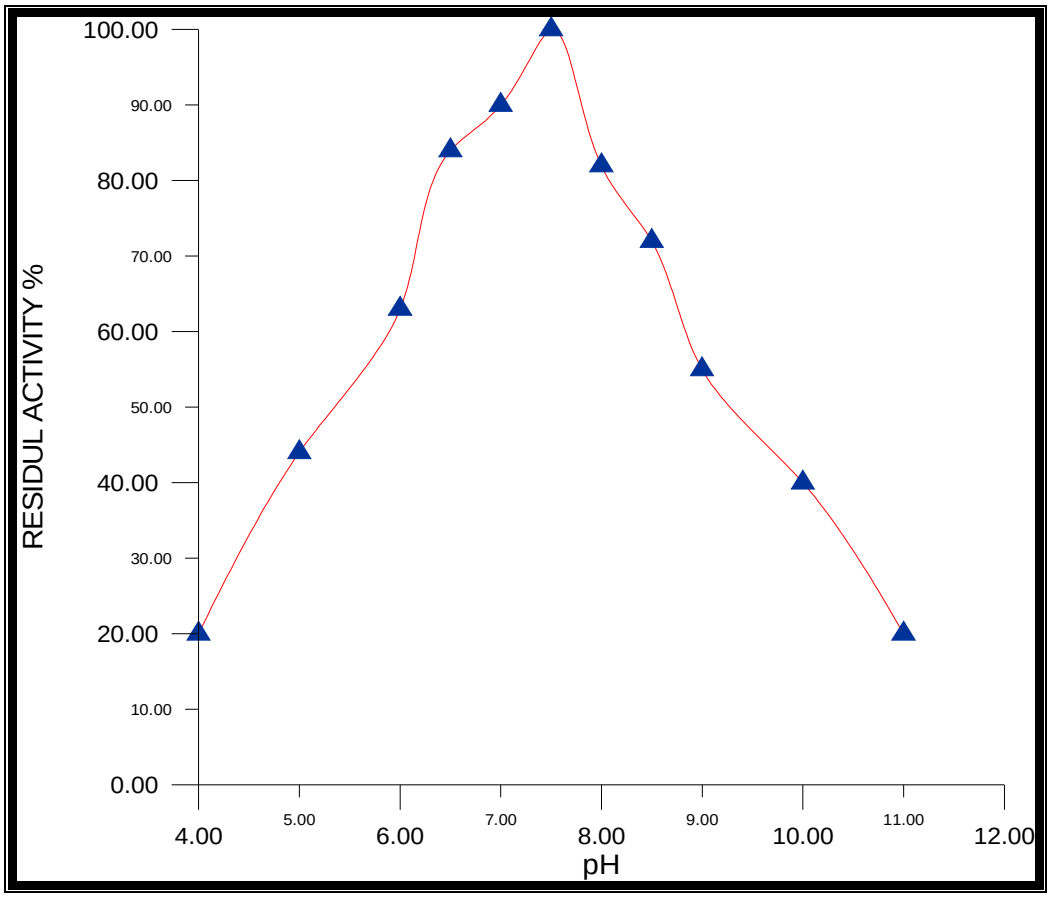


Figure (4.17): α-amylase stability at different pH values, supplied with 0.005 M $CaCl_2$

The α-amylase produced by *Clostridium perfringens* showed a relative stability at pH range (5.0-9.5) (Shih and Labbe, 1995). *Xanthomonas campestris* produced α-amylase which was stable at pH range (4.5-5.0) (Abe *et al.*, 1994). While α-amylase produced by alkliphilic *Bacillus* sp. had pH range of stability at (6.0-10). (Igarashi *et al.*, 1998).On other hand, α-amylase from another alkaliphilic *Bacillus* sp. had pH of stability at (8.5-9.0)(Lin *et al.*,1998).

4.6.5 Optimal temperature for α-amylase activity

The results in figure (4.18) showed that the maximal activity was at 60°C, while the activity decreased before and after 60°C. The increase in the temperature resulted in imparting more kinetic energy to the reactant molecules, resulting in more productive collisions per unit time ,but that should be within the intact and proper configuration of tertiary structure of enzyme in which the tertiary structure of an enzyme is maintained primarily by a large number of weak non-covalent bonds ,so if the molecule absorbs too much energy , the tertiary structure will disrupt and the enzyme will be denatured ,that is ,loss of catalytic activity (segel,1976).

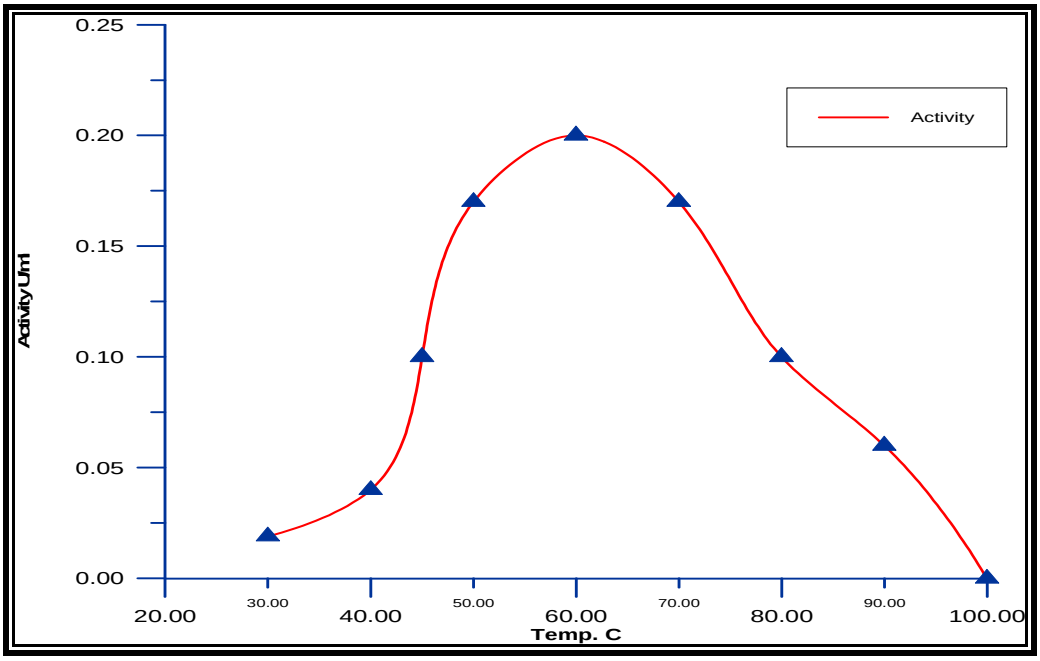


Figure (4.18): Optimal Temperature for α-amylase activity

Concerning the results of other studies, the optimal temperature for thermostable α-amylase activity produced by *Bacillus stearothermophilus* was at 40°C (Aiba *et al.,* 1983),while in another study it was at 82°C (Srivastava and Baruah, 1986).The maximal activity for α-amylase produced by *Bacillus licheniformis was* at 90°C (Krishnan and Chandra, 1983). Saito (1973)reported that the optimal temperature for the enzyme was at 76°C . α-amylase produced by *B. amyloliquefaciens* showed the maximal activity at temperature of 65°C. (Kochhar and Dua, 1983).On other hand, The optimal temperature for α-amylase produce by alkaliphilic and non-alkaliphilic *Bacillus* sp. was at 55°C(Igarashi *et al.*,1998; Teodoro and Martins, 2000) ,in another study, it was at 70°C (Lin *et al.* ,1998). The optimal temperature for α-amylase activity produced by *Clostridium perfringens* and *C. aceotbutylicum* was at 45°C. (Paquet *et al.*,1991;Shih and Labbe, 1995). The maximal activity for α-amylase of *Pyrococcus furiosus* was at 100°C (Laderman *et al.*, 1993;Dong *et al.*, 1997),while the optimal temperature for α-amylase activity produced by *thermos* sp. was at 70°C (Shaw *et al.,* 1995) .

4.6.6 Effect of temperature on α-amylase stability

Results (figure 4.19) revealed that the stability of α-amylase in the presence of $CaCl_2$ with 100 % of the remaining activity was up to 70 °C for 30 minutes ,then the stability decreased to be about 55% of the remaining activity at 90°C , and eliminated at 100°C. The presence of Ca^{2+} ions had a positive effect on the thermostability of other amylotic enzymes, in which it was supposed that the ions were acting as salt or ion bridges between two adjacent amino acids residues . The binding of Ca^{2+} ion was shown to increase the α-helical structure of α-amylase produced by alkaliphilic and thermophilic *Bacillus* sp..(Lin *et al.,* 1998), in the same aspect α-amylase produced by alkaliphilic *Bacillus* sp. retained 100 % of the original activity

after 60 min. of incubation at 45°C , and 3% of the remaining activity after treatment at 60°C for 60 min.(Igarashi *et al.*, 1998).*Xanthomonas campestris* produced α-amylase was stable up to 55°C for 30 min.(Abe *et al.*,1994).The α-amylase produced by *Clostridium perfringens* was stable in the presence of Ca^{2+} with 100% of remaining activity up to 45°C, and then the stability lowered (Shih and Labbe, 1995).While that produced by *C . acetobutylicum* retained about 30% of the remaining activity after treatment at 45°C for 60 min.(Paquet *et al.*, 1991).α-amylase produced by *Bacillus stearothermophilus* retained about 25% of the remaining activity at 90°C after 60 min. in the presence of Ca^{2} (Srivastava and Baruah,1986).While Aiba *et al.*, (1983) reported that the α-amylase retained about 60% of the remaining activity after treatment at 80°C for 60 min.

The α-amylase produced by *B. amyloliquefeaciens* retained 100% of the activity for 24 hr. at 65°C , then half life 0.5 hr. at 90 °C(Kochhar and Dua ,1982).The α-enzyme produced by *B. licheniformis* was 100% stable below 50°C and it lost the activity at 90°C in the absence of substrate (Krishnan and Chandra, 1983). While Saito(1973) reported that the α-amylase retained 100% of the original activity after treatment below 60°C. α-amylase produced by *Thermus* sp. retained about 20 % of the remaining activity after treatment at 80°C for 15min. ,the thermophilic trait for α-amylase could be used in the cake baking industry because the enzyme activity of the thermophilic nature ceases as the temperature approaches and passes through the temperature range of gelatinization (70-80 °C) ,this also provides a means to control dextrin formation . (Shaw *et al.*, 1995).

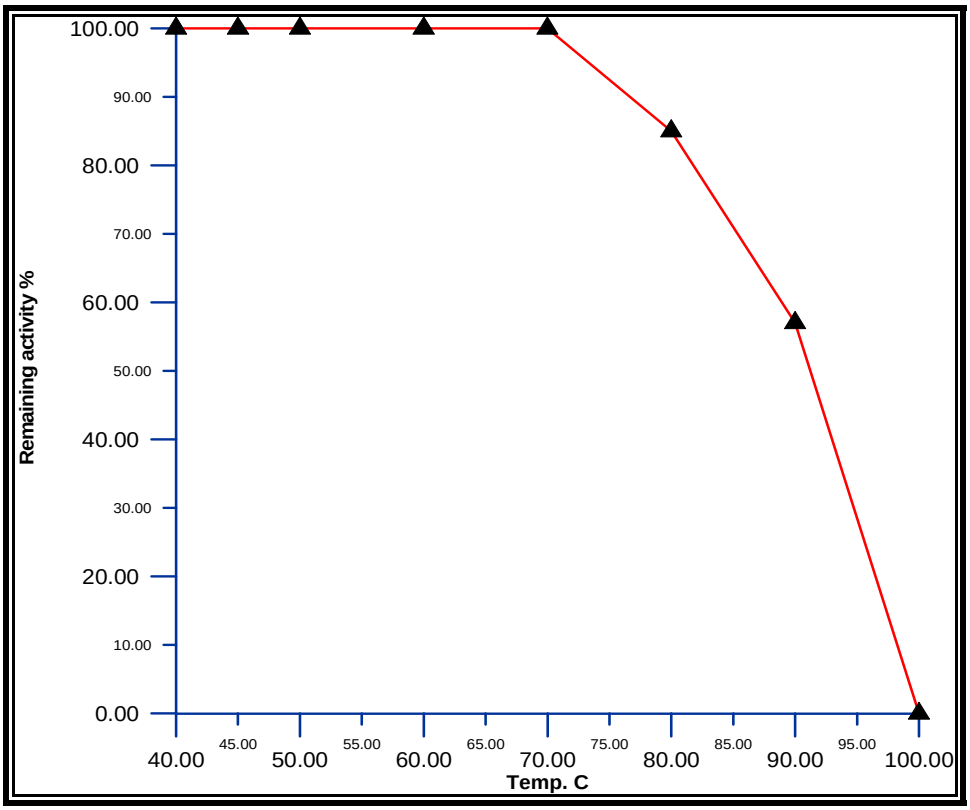


Figure (4.19): The effect of temperature on α-amylase stability in presence of 0.005 M $CaCl_2$ for 30 minutes

4.7 Immobilization of α-amylase

The immobilization of enzyme on insoluble supports provides a stabilization effect at elevated temperature and pH. The stabilization depends on the position of the support attachment to the protein molecule. (Taznov *et al.*, 2003) .Results (figure 4.20,4.21) showed the immobilized α-amylase by calcium alginate in which the remaining activity decreased , retaining about 40% of the remaining activity on 25th day after the immobilization , when it stored at 4°C . , the reasons behind the lowering of the activity along the storage period could be due to the nature of the alginate, buffering of the solution and Change in the configuration of enzyme along the period.

α-amylase was immobilized using cellulose fibers from bagasse where oxidized by periodic acid to obtain dialdehyde cellulose. Which they did react with amino groups of thermostable α-amylase. The immobilized α-amylase

from *B. licheniformis* was able to liquefy the starch in 10 cycles of reuse, in which the only 3 cycles of reuse of operation was worth better than the free enzyme at 40 °C and pH 7.0. (Varavinit *et al.*, 2002). Lectin concanavalin A (Con A), from jack bean (*Canavalia ensiformis*) was observed to form more extensive precipitates with enzymes,this was used as a matrix for immobilization of glycoamylase, in which they observed about 2.1 fold increases in the activity and another technique was applied ,it was termed a multiple- bioaffinity layering technique (Mislovicova *et al.*, 2000).Silica-bound enzyme is extremely stable at 40°C, the operating temperature of the pilot plant column, and in 30% dextrin solutions, the extrapolated half-life is several years. The measured half-lives ranged from 7.5 hr at 70°C to 519 hr at 55°C (Reilly, 1976).Glucoamylases were entrapped in cellulose triacetate fibers and immobilized glucoamylase had been by covalent attachment to organic and inorganic carriers, including low-cost magnetic support (Pieters and Bardeletti, 1992).

Calcium alginate and chitosane gel beads which exhibited high yield was used in immobilization of *Bacillus polymyaxa* in production of α-amylase (Rodziewicz and Rymowicz, 1999).Ivanova *et al.*(1995) used different techniques in the immobilization of growing cells *Bacillus licheniformis* producing thermostable α-amylase (Ca-Alginate, β-Carrageenan, Agar, and their combinations with polyethylene oxide) , adsorption on cut disks of polymerized polyethylene oxide , and fixation on formaldehyde activated acrylonitrile-acrylamide membranes , when an enzyme activity of 2750 U/mL culture medium was reached in the fifth repeated batch run with membrane-immobilized *Bacillus licheniformis* cells. While, Dobreva(1998) obtained the higher amylase yield (62% increase of the control) and operational stability (97% residual activity after 480 hr. repeated batch cultivation) was obtained using formaldehyde-activated polysulphone membranes.

Immobilization on paramagnetic polyacrolein beads was used with α-amylase by Varlan *et al.* (1996), in which they reported less of the activity compared to the control. Thermostable maltogenic amylase from *Bacillus stearothermophilus* for continuous was immobilized using bed column reactor and showed a remaining activity of 50% up to 20 days and gradually decreased to 40% over 37 days of continuous process (Kang *et al.*, 1997).

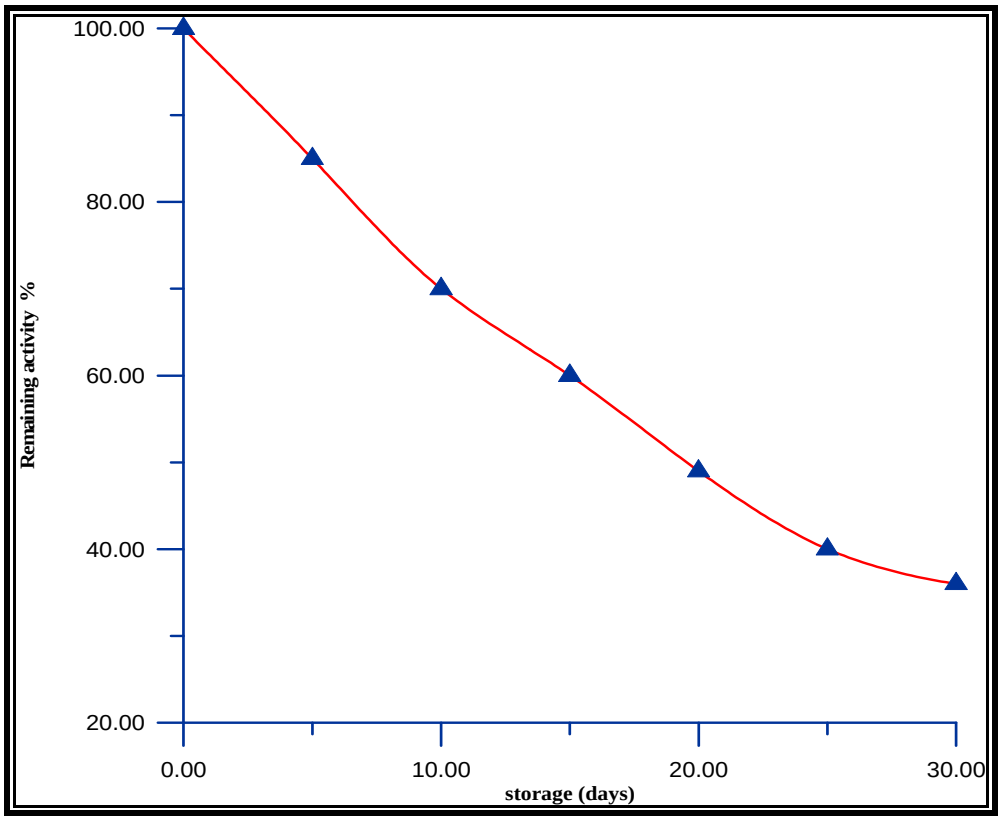


Figure (4.20): α-amylase activity immobilized onto Calcium alginate, storage at 4 °C .

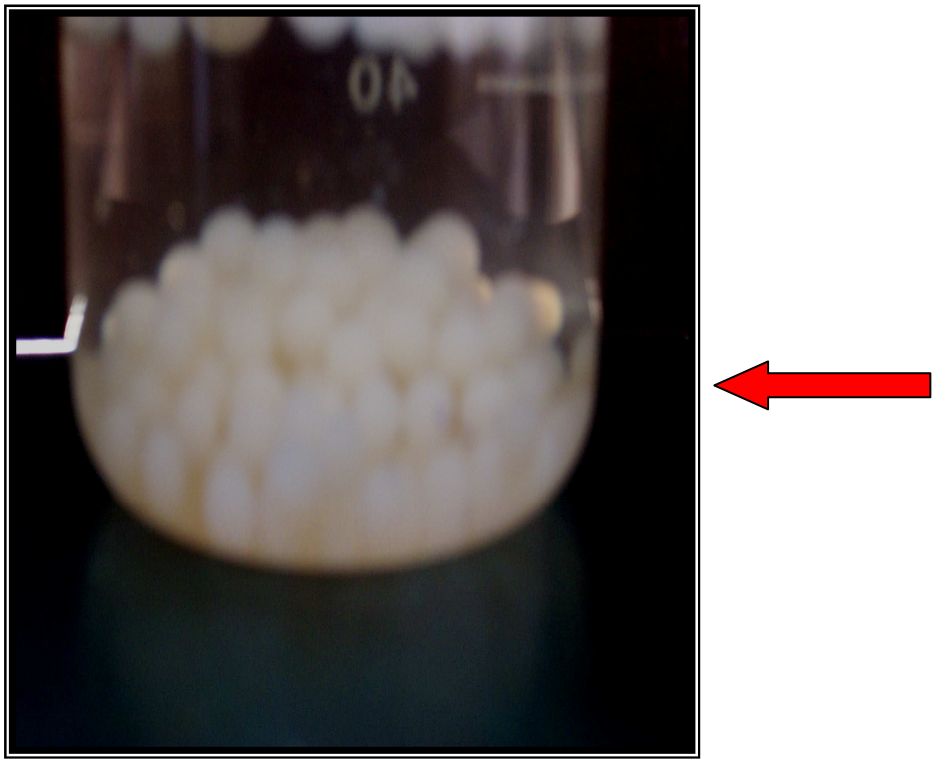


Figure (4.21): α-amylase immobilized onto Calcium alginate

4.8 Neutralization of α-amylase by its anti-amylase sera

Results in (figure 4.22) showed the remaining activity after treatment the α-amylase (purified by ion-exchange chromatography) is 62.6% compared with control. This could be interpreted that the anti-amylase serum could block about 37.4 % of the active sites where the binding should be happened, and it indicated that α-amylase is an immunogenic material for stimulation the immunity system and for availability the basic requirements for that ; foreignness , high molecular weight ,chemical complexity and degradability (Benjamini *et al.*, 2000)The cross- reaction occurred between the polyclonal antibody and the enzyme which caused the decreasing in the activity with 37.4%, because the α-amylase as a immunogenic protein could stimulate the formation of antibodies against the epitopes , but not all the active sites were epitopes ,that could explain why the activity was decreased with only 37.4% .

which could be used as a new approach to assay the activity and in the purification using the affinity method of purification based on specific monoclonal antibodies against α-amylase protein(s).

Yamaguchi *et al.* (1974a,b); Yoneda and Maruo, (1975) neutralize α-amylase activity with anti-amylase serum and conclude from the degree of neutralization the immunogenic alternation among different strain of *Bacillus subtilis* producing the α-amylase as a marker for the genetic analysis.

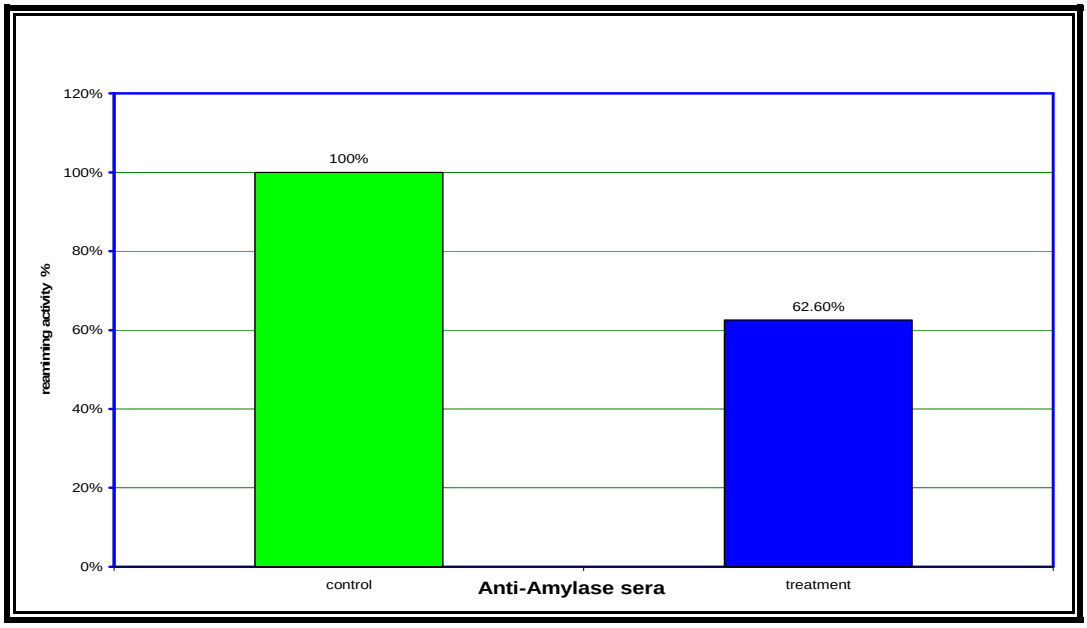


Figure (4.22): Neutralization of α-amylase by anti-sera

4.9 Ascending paper chromatography of α-amylase Products

The time course for α-amylase action on the soluble starch was investigated via paper chromatography to find out the mode of action on soluble starch.

The results in (figure 4.23) were obtained from different samples taken from the reaction mixture during intervals. After 45 min of the reaction starting , the final product was maltose and glucose and two unknown compounds in which it was difficult to identify them because the lack of possible matching standards compounds, while only maltose and unknown compound were formed after 5 and 10 min. The paper chromatography technique based on two phenomena molecules of different substances will

generally have different attractive adsorption forces to the cellulose fibers of which a paper is made and molecules of different constitutions of a mixture dissolved in a water- immiscible solvent will partition themselves in differerent degrees between this adsorbed water of the cellulose fibers and the solvent .(Budhiraja ,2004).

Satoh *et al.* (1993) investigated the products of α-amylase action on soluble starch through intervals, they could identify a series of oligosugares (G3, G4, G5 and G6) which among the products at the beginning of the reaction because the random action of α-amylase, finally, at the end products were identified as glucose and maltose .

Aiba *et.al,*.(1983) used a paper chromatography technique to verify the α-amylase by investigating the products of α-amylase action on soluble starch, which revealed the presence of glucose, maltose and oligosaccharides containing three, four , five and more glucose units.

A high-performance anion-exchange chromatography was used to identify the product of α-amylase action on soluble starch (Shaw *et al.*, 1995;Dong *et al.*, 1997) . Paquet *et al.*(1991) reported that the final products of α-amylase products by *C. acetobutylicum* were maltotriose, maltose and glucose which explained the mode of attacking the bonds .

Igarashi *et al.*(1998) used both TLC and HPLC techniques to investigate the final products of α-amylase action on the starch ,amylose ,amylopectin and glycogen .The results were similar to the present study ,but for hydrolysis of starch, the final products were (G1,G2 and G3).

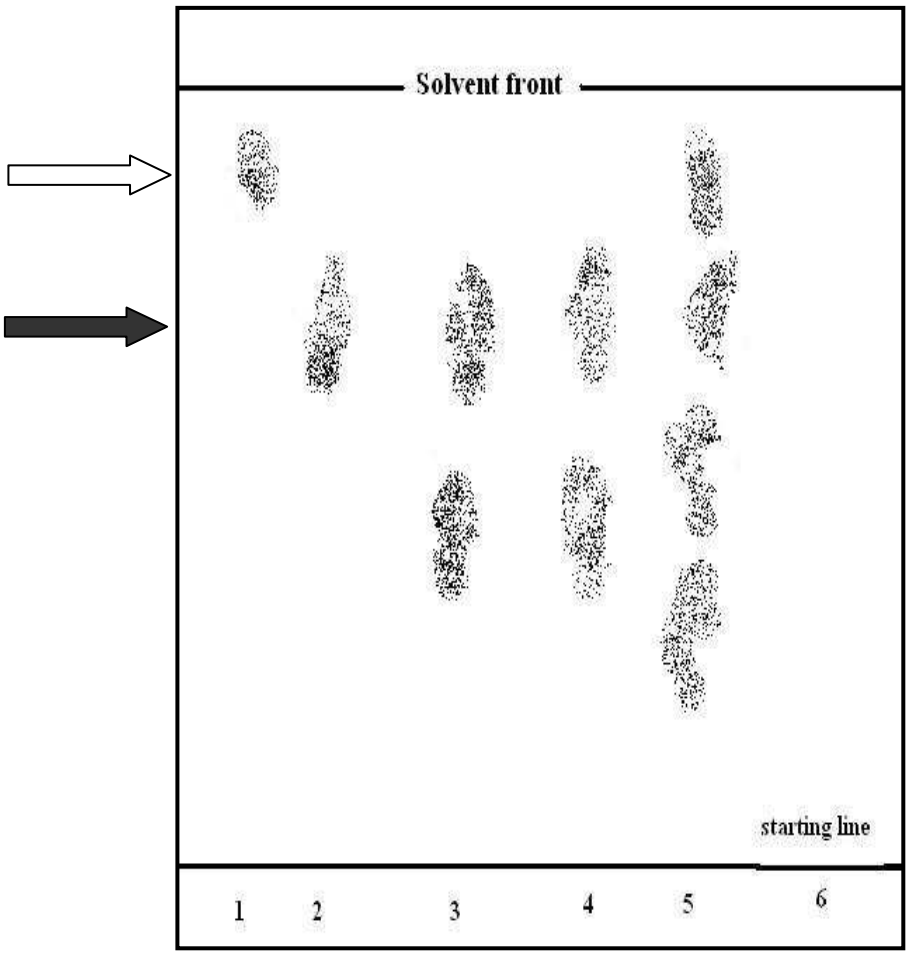


Figure (4.23): Ascending paper chromatograms of hydrolysis products by action of α- amylase on soluble corn starch
1. Standard glucose 2. Standard maltose
3. sample after 5min. of reaction 4. sample after 10 min. of reaction
5. sample after 45 min. of reaction 6. Control sample (D.W.) used in preparing standards sugars

4.10 Antibiotics susceptibility

The standard disk diffusion method was used to determine the susceptibility of *Bacillus stearothermophilus* M13 to several antibiotics. Results (table 4.6) showed Antibiotic susceptibility of *B. stearothermophilus*

M13 to six out of seven different antibiotics that represent a major group of Antibiotic families used nowadays around the world .

B. stearothermophilus M13 was susceptible to all studied antibiotics except for Aztreonam (ATM), in which it was resistant. These results could be interpreted in which the Ampicillin (Am) and Penicillin (P) belong to the penicillin family and the mechanism of resistance to this kind depends on the presence of β-lactamase (which is either chromosomally or plasmid encoded and most of these plasmids are self transmissible plasmids)(Prescott *et al.*,1990) or due to the lack of penicillin binding proteins (PBPs) and microorganisms may change their permeability to the drug(Malkawi and Youssef ,1996) , so the Bacteria may show no β-lactamase activity . On other hand , Aslim *et al.*(2002) reported that a few *Bacillus spp*. strains isolated from soil were resistant to antibiotics under study, they were sensitive to vancomycin, chloramphenicol, tetracyclane, gentamicin, erithromycin, cephalothin and ampicillin . Imanaka *et al.* (1981) isolated ten different plasmids for antibiotic resistance from thermophilic *Bacillus* sp. ,some of them were associated with resistances to the tetracyclane , erythromycin ,streptomycin and kanamycin .,*B. stearothermophilus* was sensitive to several antibiotics, except for tetracyclane. With the exception to the tetracyclane the antibiotic resistance generally was not associated with plasmid (Claus and Berkeley, 1986).Tanaka and Koshikawa (1977) isolated four types of plasmids from *Baciulls subtilis* with no resistance to the common antibiotics , such as chloramphinicol, tetracyclane, streptomycin, ampicillin and heavy metal ,$HgCl_2$ neither production of bacteriocins ,so the functions specified by the plasmids remained unknown. The sensitivity or resistance to different antibiotics was partially associated with the screening of plasmid in the *B. stearothermophilus* M13, the result of the primary screening for the presence of plasmid proved no plasmids of any kind .

Table (4.6): susceptibility of *Bacillus stearothermophilus* M13 to some antibiotics.

Antibiotic	Abbreviation	Susceptibility
Imipenem	**IPM**	**Sensitive**
Cefotaxime	**CTX**	Sensitive
Norfloxacin	**NOR**	Sensitive
Gentamicin	**CN**	Sensitive
Penicillin	**P**	Sensitive
Amipcilin **Aztreonem**	**AM** **ATM**	Sensitive **Resistant**

4.11 Cloning of *B. stearothermophilus* M13 α-amylase gene in pBR*322* vector

Genomic DNA was isolated from this bacterium using the procedures of CTAB and salting out with some modifications in which the lysozyme final concentration increased to be 2 mg/ml. after that DNA electrophoresis was done using 0.4 and 0.9 % agarose, the result showed no detectable plasmids**.** To confirm the results, alkalane extraction procedure was done in an attempt to isolate the possible plasmid, if any. The result revealed no detectable plasmid of any size and kind (figure 4-24, lanes A and B**)** .

The chromosomal DNA band was very sharp which indicated the efficiency of the isolation and purification approaches and no degradation was

happened due to the exonuclesaes activity , The purity ratio (OD_{260}/OD_{280}) was 1.8 that allowed for the next cloning steps figure(4.24) (Maniatis *et al.*,1982) .Then it was partially digested by *Hin*dIII (figure 4.25) .The partial digestion made the probability of obtaining the intact α-amylase gene(s) much higher than the complete digestion in cloning experiments (sambrook and Russell,2001). The restriction enzyme was frequently used in order to get the gene of α-amylase on intact DNA fragment to be able to express using of appropriate expression system (Aiba *et al.*, 1983;Rodriguez and Tait, 1983) .
The pBR *322* vector was purified from the host *E. coli* HB101 strain using CsCl/EtBr gradient ultracentrifugation as (figure 4.26) showed sharp band of covalently closed circular DNA plasmid. Because of plasmid DNA also behaved differently from *E.coli* DNA when centrifuged to equilibrium in cesium chloride gradients containing saturated quantities of ethidium bromide as intercalating dye which bind to lanear DNA was much more than to the plasmid and therefore bands at a higher density in cesium chloride gradients contained an intercalating agent(Maniatis *et al.*,1982), Then was digested with *Hin*dIII that made a single cut in tetracyclane gene so the cloning could be set up using insertional inactivation strategy .Then it was treated with calf intestine alkalane phosphatase to remove 5´ phosphates from both ends of the lanear DNA because in the construction of a chromosomal DNA gene bank ,it is desirable to optimize the formation of recombinant molecules as opposed to non-recombinant DNA molecules . This can be accomplished by either ligating a 10-20 fold excess of chromosomal DNA to the vector DNA (or)by pretreating the plasmid vector with alkalane phosphatase to prevent recircularization of digested plasmids or two or more digested plasmid in the reaction mixtures (Rodriguez and Tait,1983) .

The restricted fragments of *Bacillus stearothermophilus* M13 genome were ligated together using T4 ligase to ligate the cohesive ends of both insert and host DNA (figure 4-27,lane B,C,D and E)(figure4.28) , then the Amy^{-} *E.*

coli MM 294 was transformed with a recombinant vector carrying the desired α-amylase gene .

3847 transformants were resistant to the ampicillin as a primary screening in which the cloning in pBR*322* using *Hin*dIII usually inactivates a gene necessary for the tetracyclane resistances ;only five clones out of those (0.13 %) were able to produce α-amylase tested by replica plating on the starch media after staining with lugol`s solution in a comparison with the control sample (figure 4-30,A-B).

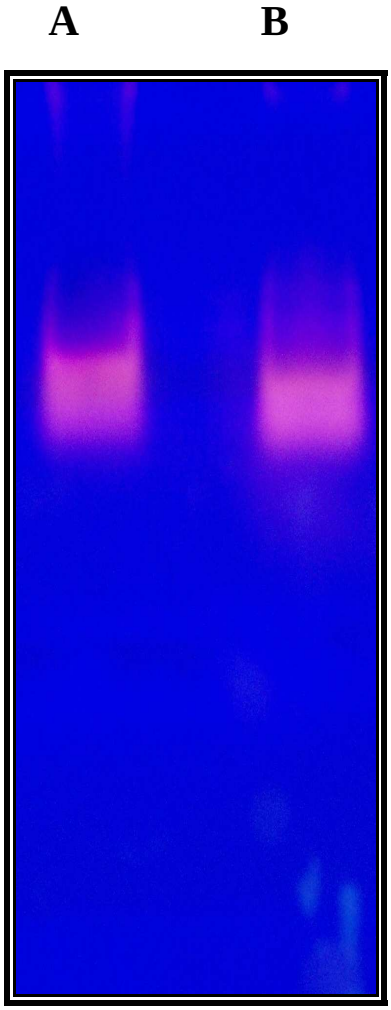
The five transformants *E.coli* MA 1,MA2,MA3,MA4 and MA5 showed α-amylase activity. The DNA of transformants was extracted and revealed harboring of hybrid plasmid termed as "pMA 322"; which revealed a molecular weight of 1790 bp (figure 4.28) that was calculated using PhotoCaptMw figure(4.29) as it was concluded from the literature that the length of amylase gene was about 1.8 Kbp . The missing 10 bases may be due to the technical calculation of the sharp accurate molecular weight during the study or may the gene be truncated in the promoter area in which the structural gene was intact as indicated by expression of the α-amylase gene of the cloned cells in the solid and liquid .In any way, sequencing should be done to determine which one happened .

restriction enzyme was frequently used in order to get the gene of α-amylase on intact DNA fragment be able to express using appropriate expression system (Aiba *et al.*, 1983;Rodriguez and Tait, 1983) .

The size of cloned fragment carrying the α-amylase gene was estimated of "1790bp ".It has been shown through many previous studies that α-amylase gene was isolated from *Bacillus* . spp. and cloned in different expression systems . Aiba *et al.*(1983) cloned the α-amylase gene in *Bacillus* by the digestion of the pTB90 and the genomic DNA partially by HindIII, while Mielenz (1983) cloned the α- Amylase in *E.coli* and the α-amylase was expressed using pBR *322* as a vector , in which the *B. stearothermophilus*

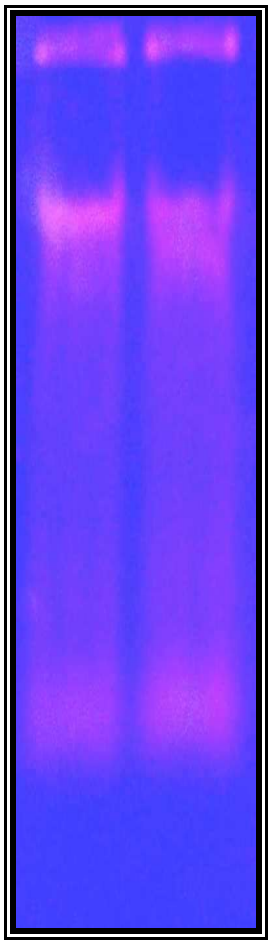
genomic DNA and the vector were cleaved with *Hin*dIII ; then ligated as ratio of 4: 1 , the cloned fragment was of 5.4 kp . It was confirmed by many similar studies that the structural gene for α-amylase enzyme *amy*E, and its regulatory gene, *amy*R are closely linked on the genetic map of the *B. subtilis* chromosome (Yamaguchi *et al.*, 1974b). α-amylase's promoter *amy*P contains an essential TGTG motif(-16 region) upstream of the -10 region (Martin and Chambliss,1998). A great deal of cloning has been done in many different microorganisms, mostly into *E.coli* which was applied as expression system for many sources of α-amylase gene. Satoh *et al.*(1993) cloned two α-amylase genes from *Streptococcus bovis* 148 chromosomal DNA in *E.coli* using pBR 322 vector ; They were expressed successfully in both forward and opposite orientation that could explain that the cloned fragment had its promoter that it is fully functional in *E.coli*. cloning α-amylase via cosmid was applied in some studies, Coronado *et al.*(2000) constructed *Halomonas meridiana* gene bank in broad-host rang cosmid pVK102 by partial digestion of genomic DNA with *Hind*III , then in vitro packing to infect the *E.coli.* HB101 ., Dong *et al.(*1997) cloned and a sequence of α-amylase 2.6 kb from *Pyrococcus furiosus* in *E.coli* . Cloning using PCR technique was applied for cloning α-amylase gene from hyperthermophilic archeabacterium *pyrococcus furiosus* using three primers the amplified fragments was inserted in *Pst* I site of pTV 118N and transform *E.coli* JM109 .The length of fragment was 5.3 kb. An α-amylase gene from *Bacillus* sp. strain TS-23 was cloned and expressed by using recombinant plasmid pTS917 in *E. coli*. SDS-polyacrylamide gel electrophoresis showed an apparent protein band with a mol. wt of approximately 65000. The amylase gene (*amy*A) consisted of an open reading frame of 1845 bp (Lin *et al.*, 1997)

Gene encoding the α-amylase of *Xanthomonas campestris* K-11151 was cloned into *E. coli* using pUC19 as a vector. An ORF of 1578 bp was deduced to be the α-amylase structural gene.(Abe *et al.*, 1996).



Figure(4-24): Agarose gel (0.9%) electrophoresis of *B. stearothermophilus* M13 chromosomal DNA :
Lane A: Genomic DNA of *B. stearothermophilus* M13 Isolated by salting out method.
Lane B: Genomic DNA of *B. stearothermophilus* M13 Isolated by Alkalane lysis method.
5V/cm, 90 min.

A B



Figure(4-25): Agarose gel (0.9%) electrophoresis of *B. stearothermophilus* M13 chromosomal DNA digested with *Hin*dIII , at 75V

Lane A: Genomic DNA of *B. stearothermophilus* M13 Isolated by salting out method partially digested by *Hin*dIII
Lane B: Genomic DNA of *B. stearothermophilus* M13 Isolated by alkalane lysis method partially digested by *Hin*dIII
5V/cm, 120 min.

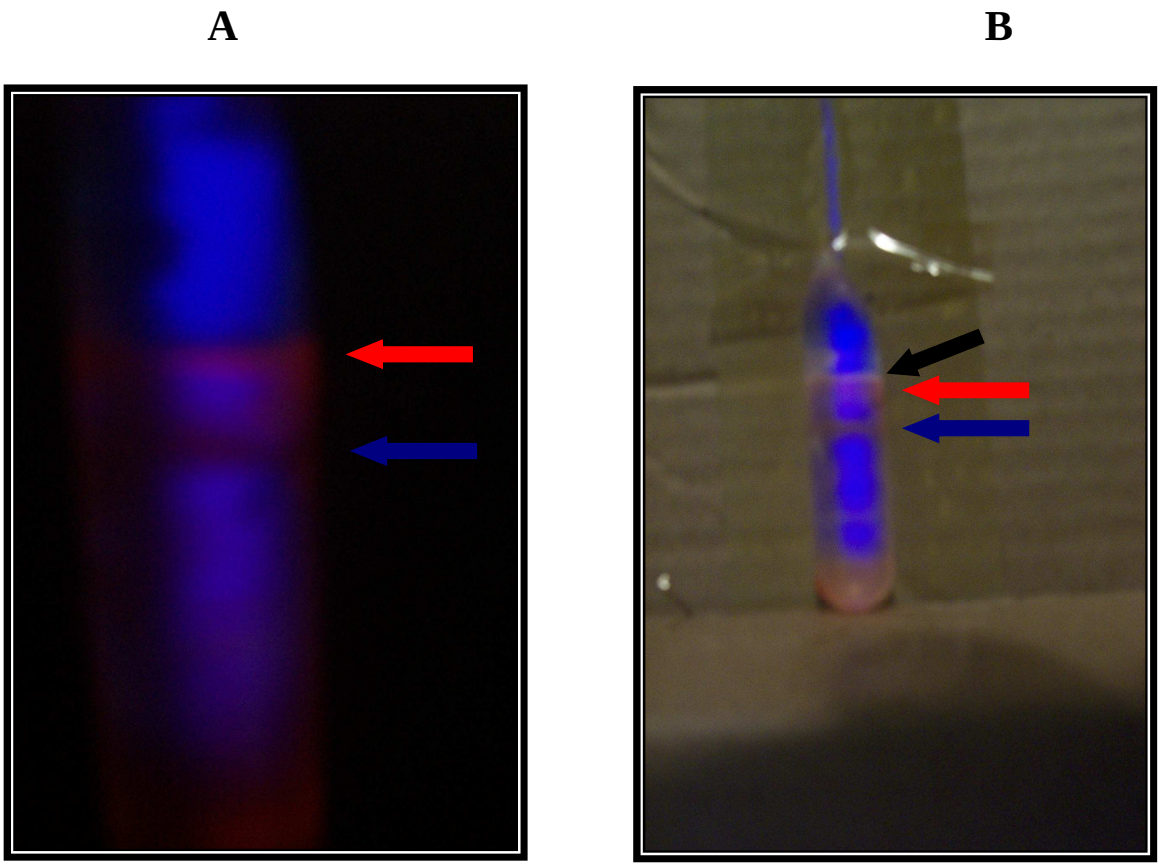


Figure (4- 26): Purification of the pBR *322* Vector by ultracentrifugation with CsCl/EtBr Gradients:

A: The CCC plasmid band as observed in dark room (blue Arrow)
B: The CCC plasmid band as observed in light room (blue Arrow)
Red Arrow indicated nicked circular or lanear chromosomal DNA
Black Arrow indicated proteins
45,000rpm,16hr

Figure (4-27) :Agarose gel (0.9%) electrophoresis of ligated DNA
Lane A: ligation reaction (control)
Lane B: ligation reaction, experiment 1
Lane C: ligation reaction, experiment 2
Lane D: ligation reaction, experiment 3
Lane E: ligation reaction, experiment 4

5V/Cm, 60min.

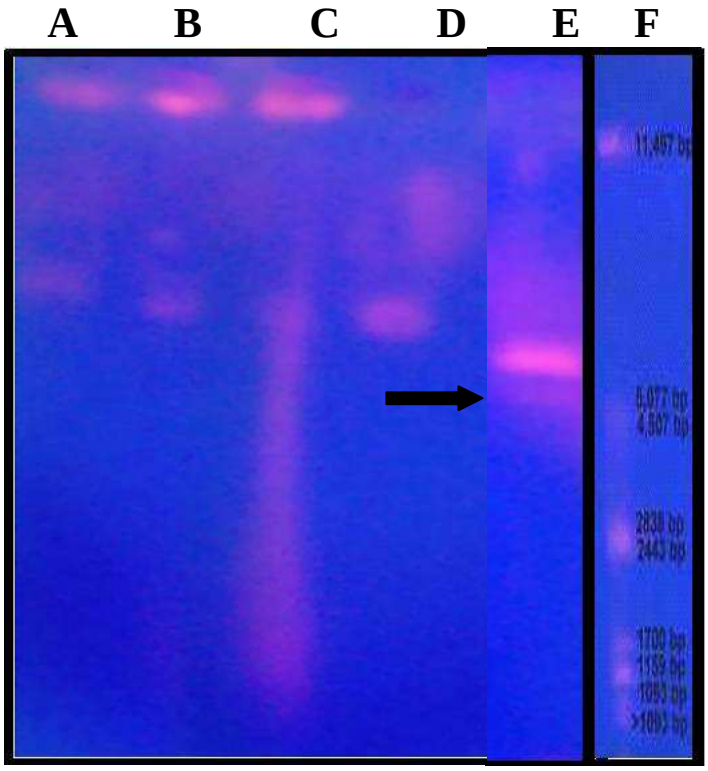


Figure (4-28): Agarose (0.9%) gel electrophoresis of :-

Lane A: ligation yield from experiment 2
Lane B: ligation yield from experiment 3
Lane C: partial digestion of *B. stearothermophilus* M13 genomic DNA
Lane D: Junk DNA
Lane E: Junk DNA
Lane F: (black Arrow) p*MA* 322 from the Transformant *E.coli* MM 294 producing recombinant α-Amylase

5V/Cm,120min.

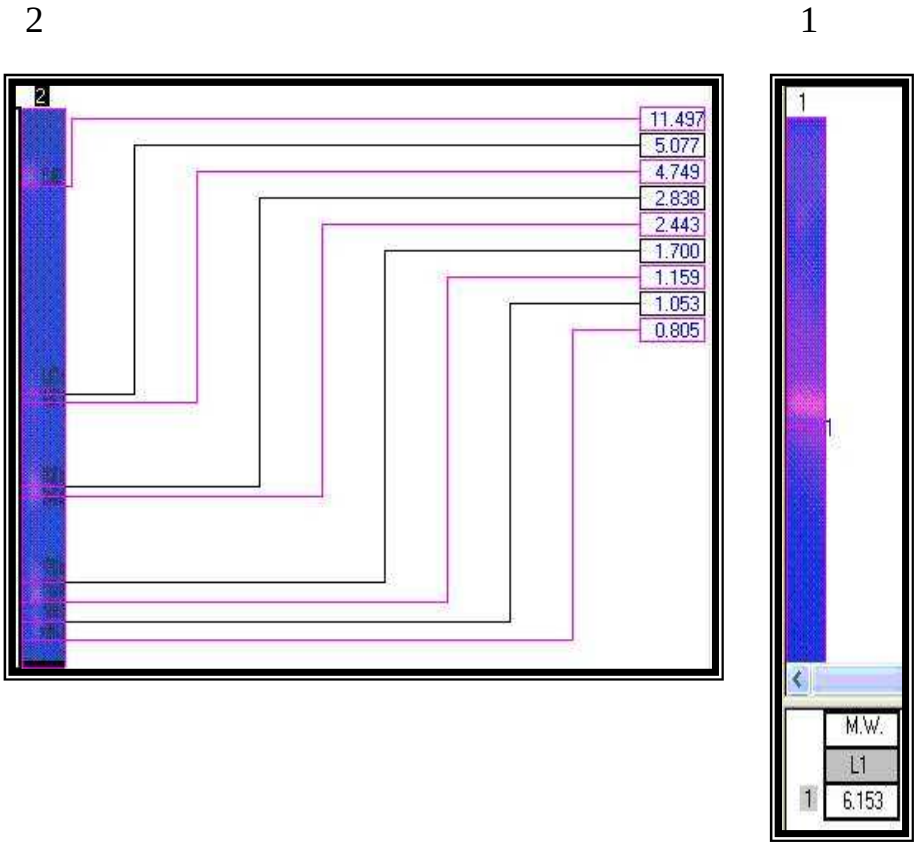


Figure (4-29): Estimation of pMA 322 molecular weight, using PhotoCapMw Software using agarose (0.9%) gel electrophoresis .

Lane 1: The hybrid pAM 322 extracted from *E.coli* AM1
Lane 2: molecular weight (standard markers) of λ DNA digested with *Pst*I
5V/Cm ,120 min.

4.12 Expression of recombinant α-amylase gene in the transformant *E. coli* harboring pMA *322*

The transformant *E.coli* MA1 harboring pMA 322 expressed successfully the cloned α-amylase on starch medium incubated at 37°C, as appeared in figure (4.30-B) in comparison with the negative control plate of *E.coli* MM294 Amy $^{-}$ fig(4.30-A).

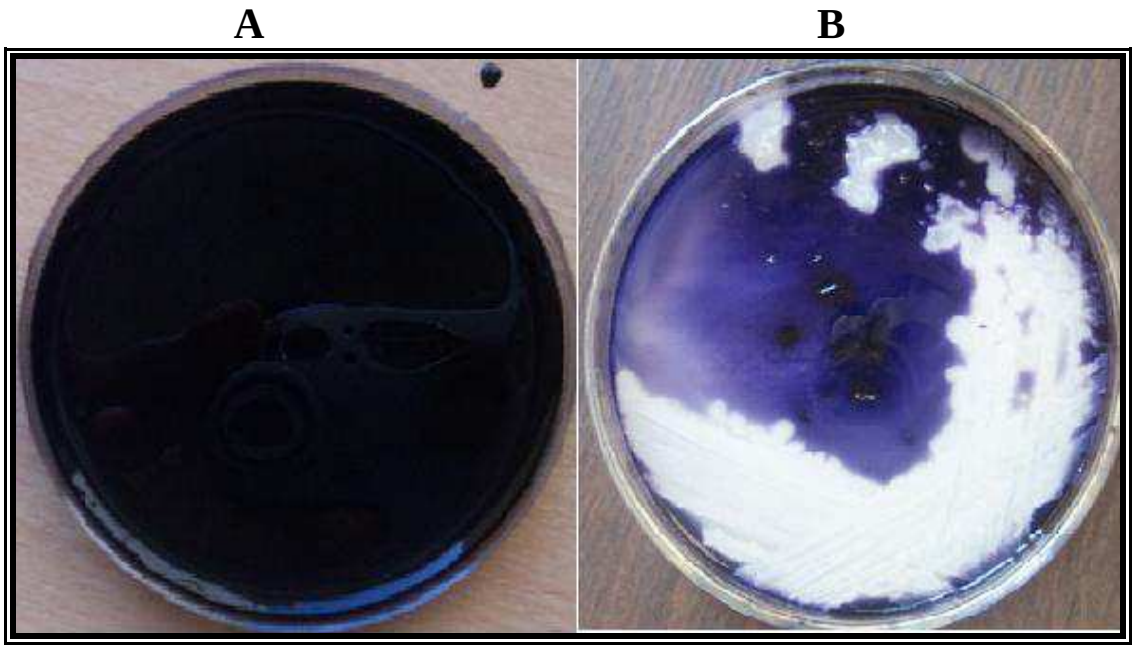


Figure (4.30) :Expression of α-amylase gene cloned into pBR *322* on solid medium supplied with starch , streaking , incubated for 24hrs at 37ºC

A: control , *E.coli* MM294, Amy $^{-}$
B: Transformant *E.coli* MA1 harboring pMA *322*

The specific activity was assayed for the crude extract of the five transformants *E.coli* MA1, MA2, MA3, MA4 and MA5 .The results in (Table 4-7) showed that the *E. coli* MA1 was the highest one in the α-amylase specific activity among the transformants , but it was less than that of *B. stearothermophilus* M13 which has specific activity 0.055 U/mg . This slight decrease in the activity could be attributed to many factors such as physiological differences between *Bacillus* sp. and *E. coli* MM 294 ,and the transcription and translation mechanisms (Ali , 2003) or may be due to the

gene dosage of α-amylase that encoded for it as a multicopy number gene in the original host "*B. stearothermophilus* M13 " and when the gene cloned in the new host ,it occurred as one or less copy than the original . It is a fact that increasing the number of copies of a gene is likely to increase the amount of the particular gene product that is manufactured by the host cell , which may be recompensed by cloning multiple tandem copies of a gene into vector (Lewin,1998)

This slight decrease in the specific activity could be favored to the recombinant α-amylase produced by the *E.coli* recognized by the absences of contaminating protease which was co-produced in the donor strain (Manonmani and Kunhi,1999).

One of the aims of the present study is to attempt cloning of α-amylase and to investigate the expression of it, regardless of the amount of the produced α-amylase, for which future studies should be directed to either optimize the production conditions of α-amylase by the *E.coli* MA1 , investigate the other reasons behind the lowering of the specific activity or to elevate α-amylase specific activity in a liquid medium or cloning many copies for α-amylase gene in the other vectors using *E. coli* or other microorganisms as suitable hosts .

Table (4-7): The specific activity of α-amylase produced by transformants *E.coli* MA isolates harboring plasmid pMA322 carrying recombinant α-amylase gene.

Transformant isolates	*E.coli* MA1	*E.coli* MA2	*E.coli* MA3	*E.coli* MA4	*E.coli* MA5
Specific activity U/mg	0 .045	0.032	0.040	0.037	0.044

Conclusions

1. *Bacillus stearothermophilus* M13 produced a valuable amount of α-Amylase among the local thermophilic isolates , that could be used in many industrial applications.
2. The optimal conditions for α-Amylase production and by *Bacillus stearothermophilus* M13 were determined ;represented by TSM medium, at 55 ºC , late log and early stationary phase of growth .
3. α-amylase showed a high activity at pH 7.0 with stability at pH range (6.5-8.0), and a high activity at 70ºC with 100% of the remaining activity .while, at 90ºC showed 55% of the remaining activity .
4. The purification protocol included DEAE –sephadex Ion exchange and gel filtration gel were recommended.
5. Four possible forms (a, b, c and d) of enzyme were eluted from ion-exchange chromatography in which “b” showed the highest specific activity among them , and the overall activity may be due to it namely.
6. Molecular weight of α-amylase was" 56234 Da.” when estimated by gel filtration and “55426 Da.” when estimated by SDS- PAGE technique .
7. TLC technique was applied to verify the α-Amylase time-effect – products in which monosaccharide and disaccharides were among the products.
8. α-Amylase showed antigenic properties in vivo by decreasing the activity 37.4% for the partially purified α-amylase forms.
9. The DNA fragment that coded for α-Amylase was cloned into *E .coli* MM 294 using pBR*322* as a vector, in which the length of the cloned fragment was estimated of 1790bp approximately.
10. The expression of the recombinant fragment was detected in the transformants cells on a solid medium and liquid medium, which

showed a slight decrease in the specific activity in comparison with original organism with no accompanying proteases activity.