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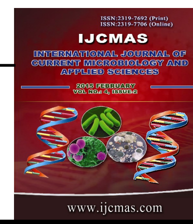

Original Research Article

Isolation of α- Amylase Producing *Bacillus subtilis* from Soil

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Bacillus subtilis,
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Soil,
Starch

A B S T R A C T

Bacillus subtilis is an aerobic, Gram-positive, endospore forming bacterium that has the ability to produce and secrete the hydrolyzing carbohydrate enzyme, α-amylase. α–Amylase is an enzyme that is used in various industries to rapidly degrade complex polysaccharides (e.g. starches) into smaller oligosaccharides. Starch is an abundant carbon source in nature, and α-amylase (1, 4-a-D-glucanohydrolase), which hydrolyzes a-1, 4-glucosidic linkage in starch-related molecules, is one of several enzymes involved in starch degradation. Amylase is the enzyme which breaks down starch into glucose molecules and commonly called as glycoside hydrolase enzymes. Amylases are among the most important industrial enzymes and also have great significance in Microbiology studies. In this paper, we screened soil bacteria and an isolate alkalophilic *Bacillus subtilis* was found to produce an alkaline α – amylase in different media *Bacillus subtilis* strain isolated from garden soil was tested for its abilities to hydrolyze the structural polysaccharides. The strain grows well at 37°c and the 2% starch concentration, with P^{H} near neutral. The enzyme activities were observed at 2% starch concentration. The present review was focused on bacterial amylase and this review assesses the following chapters: Amylase, Microorganisms and amylases, Physiology of amylases, Fermentation studies on bacterial amylase production and Commercial application of amylases**.**

Introduction

Enzymes were discovered in the second half of the nineteenth century, and since then have been extensively used in several industrial processes. Enzymes are known for their specifically, high efficiency and ability to work under mild conditions and provide a promising solution to these challenges. It is clear that enzyme technology can be used to develop a usable, more environmental friendly, economical competitive scouring process.

Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors. Environmental pollution is no longer accepted as inevitable in technological societies. Over the past century, there has been a tremendous

increase in awareness of the effects of pollution, and public pressure has influenced both industry and government. There is increasing demand to replace some traditional chemical processes with biotechnological processes involving microorganisms and enzymes such α-amylases ,xylanases , cellulases and mannanase, which not only provide an economically viable alternative but are also more environmentally friendly (Hoondal *et al., 2002*)

α -Amylases are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-D-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the *α* -amylases are metalloenzymes, which require calcium ions (Ca2+) for their activity, structural integrity and stability hydrolase group of enzymes. Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins forthe pharmaceutical industry. These enzymes account for about 30 % of the world's enzyme production. The *α* -amylase family can roughly be divided into two groups :the starch hydrolyzing enzymes and the starch modifying ,or trans glycosylating enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products,lower energy requirements and elimination of neutralization .

The α-amylase family, *i.e*. the clan GH-H of glycoside hydrolyses, is the largest family of glycoside hydrolases ,transferases and isomerases comprising nearly 30 different enzyme specificities .A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases : Endoamylases: cleave internal α-1,4 bonds resulting in anomeric product Exoamylases: cleave α-1,4 or α-1,6 bonds of the external glucose residues resulting in α- or β-anomeric.Debranching enzymes: hydrolyze α-1,6 bonds exclusively leaving long near polysaccharides, and Transferases: cleave α-1,4 glycosidic bond of the donor molecule and transfer part of the donar to a glycosidic accepter forming a new glycosidic bond.

Each application of α-amylase requires unique properties with respect to specificity, stability ,temperature and pH dependence (Hmidet, *et al*.,2010). The amylase exhibited activity at a wide range of pH and temperature, desirable characteristics which can lead to its application in detergents as additive and in textile desizing .Among bacteria, *Bacillus sp* is widely used for thermostable α-amylase production to meet industrial needs. They are known to be good producers of thermo stable α-amylase, and these have been widely used for commercial production of the enzyme for various applications.

Immobilized enzymes are becoming increasingly popular as reusable, Selective analytical chemical reagents in solid phase flow- through reactors, as membranes in sensors and as films in dry reagents kits. For industrial application, the immobilization of enzymes on solid support can offer several advantages, including repeated usage of enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bead reactors (Abdel-Naby, 1993).

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high

yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper .

Amylases are obtained from various origins like plant, animal, bacterial and fungal. Several researchers produces amylase enzyme using *Bacillus* sp. .There are about 3000 enzymes known today only few are industrially exploited. These are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectin's and cellulose .In the production of glucose syrup the α-amylase is used in the first step of enzymatic degradation yielding a mixture of glucose and fructose with high fructose content. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands because it is economical when produced in large quantities . Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors.

The industrial enzyme producers sell enzymes for a wide variety of applications. The estimated value of world market is presently about US$ 2.7 billion and is estimated to increase by 4% annually through 2012. Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) are the main industries, which use about 75% of industrially produced enzymes. Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market. An extra-cellular amylase, specifically raw starch digesting amylase has found important application in bioconversion of starches and starch-based substrates. The level of alpha amylase activity in various human body fluids is of clinical importance e.g. in diabetes, pancreatitis and cancer research, while plant and microbial alpha amylases are used as industrial enzymes. Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries. Although amylases can be derived from several sources, such as plants, animals and microorganisms, the enzymes from microbial sources generally meet industrial demands and had made significant contribution to the production of foods and beverages in the last three decades. The microbial amylases have almost completely replaced chemical hydrolysis of starch in starch processing industry.

Materials and Methods

Collection of soil sample

Soil sample was collected from arcot , kalavai and walajapet.

Isolation of organism

Starch agar is a selective medium was used for the isolation of *Bacillus subtilis* about 1.0gmof soil sample was serially diluted in sterile physiological saline and dilution was done upto 10^{-5} by thorough mixing . 0.1ml of sample from 10^{-3} dilution was spread on

sterile petridishes containing starch agar with the help of L-rod and the plates were incubated at 37°C for 24-48 hours. After incubation the plates were observed for the growth of bacteria.

Characterization of Bacterial isolates

The colonies grown on starch agar plates were subjected to various morphological, Cultural and biochemical tests for the identification of *Bacillus subtilis.*

Microbial reactions in litmus

Milk is an excellent medium for the growth of microorganisms because it contains the milk protein casein, the milk sugar lactose, vitamins, minerals and water. Litmus, a pH indicator is incorporated in the medium for the detection of production of acid or alkali and oxidation reduction activities. A variety of different chemical changes occur in milk, depending upong which milk ingredients are utilized by the bacteria. Once again, this is dependent upon the type of enymes that the organism is able to produce. Litmus milk medium consists of 10% powdered skim milk and the dye molecule litmus.

Confirmative test

The test organism was inoculated in starch agar plate and incubated at 37°C for 24 hours.

Determination of amylase activity

Al1 the *Bacillus subtilis* isolates were tested for amylase production by starch agar. Starch agar medium inoculated with the organism and subsequently flooded with iodine solution. After 72 hrs of growth the starch plate are flooded with the above iodine solution. Cleared zone are seen around amylase / producing colonies under blue background.

Barley preparation for sporulation

Barley of 25 gm were weighed and washed with distilled water for 3 times. Then it was dried for 30 minutes under aerated (avoid solar drying) conditions. These seeds was sterilized for 40 minutes at 151bs and kept for overnight under room temperature and once again sterilized for 30 minutes at 15 lbs on second day.

- Suspension of 2.5 m1 was added to sterilized barley.
- It was incubated at28^{0}C and spore formation observed after 72 hours of incubation.
- 100 ml of distilled water was *Bacillus subtilis* added into the conical flask containing barley with spores of *Bacillus subtilis*.
- The suspensions were prepared and the pellet (60 gm) of *Bacillus subtilis* spores was collected in clean beakers after the centrifugation.

Spore counting

Bacterial culture were inoculated into barley medium. Then the inoculated medium was incubated at room temperature 25-26^{0}C for 5 days. After incubation the spores were produced over barley as a black mat formed around it the barley with *Bacillus subtilis* spores were taken and weighed exactly one gram and dispensed in 100m1 of distilled water. Then the spore were counted funga1 culture by hemocytometer under microscope. The result were tabulated.

Immobilization

Preparation of sodium alginate

Preparation of sodium alginate (Appendix)

0.25M $CaCl_2$ preparation(Appendix)

Sodium alginate solution was mixed with breakers separately. 2ml of the above solution was pipette out by using sterile syringes. The solution was injected into the beaker containing CaCl2 solution as drop wise. The beads were prepared and stored in a refrigerator.

Effect of pH of the medium on the α-amylase production

3 inoculation loop of 72 hrs, old cultural inoculated into 200 ml of production media of various pH range 5, 6, 7 in 250 ml conical flask another set of production media is also inoculated with immobilized cells and free cells incubated in the incubator at 37^0C. at regular intervals 3 ml culture was withdrawn and used for α-amylase activity.

Effect of the temperature on the α-amylase activity

The culture supernatant of both immobilized cell and free cells was centrifuged and was incubated at various temperatures like 32^0C,37^0C,40^0C, mins. After the incubation period this crude enzyme used for α-amylase assay.

Effect of the substrate on α-amylase activity

3 inoculation loop of 72 hrs old 'culture was inoculated into 200 ml of production medium of various substrate concentrations in 250 ml flask. Immobilized cells are also inoculated into production media of different substrate concentration. The flask was incubated in incubator at37^0C. At regular intervals 3ml of culture was withdrawn & used for α amylase assay.

Enzyme preparation

Culture broth was centrifuged as 4500 for 10mints. The supernatant was separated and was assayed for α-Amylase activity in each case.

Enzyme assay

Amylase activity were assayed by measuring the amount of reducing sugars released from starch using dinitro salicylic acid method (Bernfeld 1995).

Estimation of glucose by dinitrosalicylic acid

Dinitro salicylic acid method is a simple and sensitive method.

Procedure

Pipette out 0.0 ml (blank) to 3.0 ml of sugar solution into into clean test tube. Make up the final volume to 3.0 ml with d.H_2O in all the tubes. To each tube add 3.0 ml of dinitrosalicylic acid reagent and cover the tube with aluminum foil. Place the tube in boiling water for 6 minutes and cool them in a containing tap water. Read the intensity at 575 nm (green filter). Plot a graph for series of the standard glucose solution

Calculation

Calculate the amount of reducing sugar present in the sample using standard graph.

Calculation

Estimation of glucose

The amount of glucose present per ml in o - amylase assay mixture was calculated from the standard graph.

Amount of glucose =

$$\frac{\text{OD of standard solution X Concentration of standard solution}}{\text{OD of unknown solution}}$$

The amount of glucose was expressed as µ gm / ml.

Calculation of enzyme activity

The Enzyme activity was calculates as

Concentration of the product produced/molecular weight of the product = 1/ incubation period
The enzyme activity is expressed as µ moles / ml/ min.

Results and Discussion

Isolates

Three different strains of *Bacillus subtilis* were isolated from various soil sample and were named as Arcot, Kalavai and Walajapet .

Isolation of *Bacillus subtilis*

Bacillus subtilis, isolated from soil was screened from maximum production of alpha amylase by observing clear zone of starch hydrolysis in petridishes as shown in the Fig-1**.**

Determination of amylase activity

Barely preparation for sporulation.

The bacterial colonies was obtained were inoculated on to barely for sporulation after 6 day spore counting was performed by heamocytometer under light microscope as shown in the Fig-2.

Immobilization

The bacterial colonies was obtained were inoculated on to barely for sporulation after 6 day spore counting was performed by heamocytometer under light microscope. Viable spore were immobilized on calcium alginate beads as shown in the Fig-3.

Effect of temperature on extra cellular α-amylase production

Assay of enzyme production was carried out at various temperature range 32°C 37° C, 40 ^{o}C, 60°C for 24 hours. It was found that both the free and immobilized cell of *Bacillus subtilis* showed the growth at 32° C, but there was less enzyme production however, the optimum temperature for enzyme production was recorded 37° C and 40° C for immobilized cell of *Bacillus subtilis.*

Effect of pH on extra cellular α- amylase production

The intial pH of medium was adjusted to variable pH range by adding the 0.1N HCL and enzyme purified was tested at the pH ranged between 5, 6, 7,8 the production of α amylase was found to be best at pH 5 for both free and immobilized cell of *Bacillus subtilis* below and above this pH, production of alpha amylase was significantly lower.
Extracellular α-amylase production and growth of *Bacillus subtilis* was maximal at pH and this result is in accordance with the work of Lin *at al*. (1998). The composition of cell wall and plasma membrane of microorganisms is known to be affected by the culture pH (Ellwood and Tempest , 1972). The change of the initial pH of the medium may be leads to change of the nature of the cell membrane and/or cell wall and hence affecting the α-amylase production and the growth of *Bacillus subtilis.* On the other hand, Mamo and Gessesse (1999) reported that high level of α-amylase at pH 5.0 and 6.0 and when the pH was increased low level of α-amylase was obtained.

α-amylase production and growth of

Bacillus subtilis increased after 24 to 48 h. After this period, the production of the enzyme decreased. The observed peaking and throughing of the production of extracellular enzymes can be attributed to (1) The products of action of one component inducing the synthesis of another, (2) Differential inhibition by products of substrate hydrolysis, (3) A decrease in growth was observed after 48 h of growth of *B. subtilis*. This probably resulted from cellular lysis, an observation previously reported of extracellular α-amylase by *B. subtilis* was growthing asscociated and this is agreement with other investigators (Bajpai and Bajpai, 1989, Stephenson *et al*., 1998; Riaz *et al*., 2003). The pH optimal for the free α-amylase of *B. subtilis* was found to occur at pH 7. These resutls are in accordance with those reported by Vihinen and Mantsala (1989), Hamitton *et al.* (1999), Saito (1973) and khoo *et al.* (1994). The immobilized-amylase showed optimum pH in the acidic range in comparison to the free enzyme. These effects may be dependent on the ionic environment around the active site of the enzyme bound to the carrier (Abdel-Naby, 1993). Similar shifts of pH optimal were reported by Sabhukhan *et al*. (1993) and Yoshida *et al*. (1989).

Table.1 Identification of isolates Morphological characteristics of the isolates

S.No	Morphological characteristics	Result
1	Gram's staining	G +Ve
2	Shape	Rods
3	Motility	Motile
4	Spore staining	Spore former

Table.2 Cultural characteristics of the isolate

S.No	Cultural characteristics	Observation
1	Nutrient agar	Pale yellow, small, Irregular opaque colony
2	Starch agar	Flat or slightly convex With irregular bodies

Fig.2 Isolation of B. subtilis by production of alpha amylase with clear zone of starch hydrolysis in petridishes

Fig.2 Barely preparation for sporulation

Fig.3 Immobilization on calcium alginate beads

Fig.4 Effect of pH on extra cellular α- amylase production

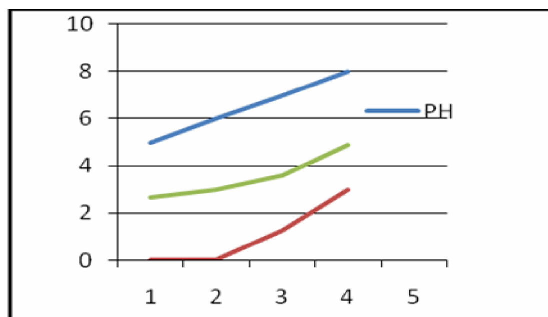
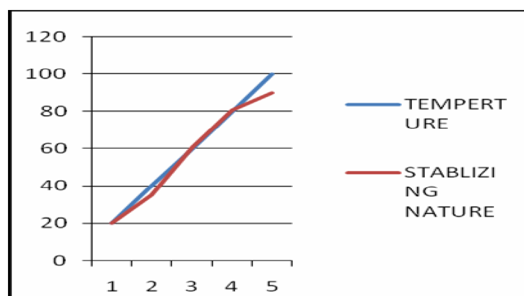


Fig.5 Effect of temperature on extra cellular α- amylase production



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