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Research Paper

Isolation and characterization of amylase producing *Bacillus* spp. from selected soil sample

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Abstract

Amylases are among the most important enzymes and are of great significance in present day industry. Starch degrading bacteria are most important for industries such as food, fermentation, textile and paper. Amylase is a hydrolytic enzyme and in recent years, interest in its microbial production has increased dramatically due to its wide spread use in food, textile, baking and detergent industries. The selected soil samples were obtained (10gm) from Roorkee, Haridware district, Uttarakhand state, India. The collected soil samples were labeled as banana field (A), potato field (B) and sugarcane field (C) respectively. Soil samples were then serially diluted in normal saline and plated on sterile nutrient agar plates. The colonies obtained from higher dilutions were subjected to Gram staining and starch hydrolysis test. All isolated colonies were screened by activity zone techniques with iodine solution. Out of 10 bacterial strains, only 5 bacterial colonies showed positive results for amylase production and out of 5, Bacterial strains, B3 showed widest diameter i.e. 8mm was selected for further study. Different Biochemical tests were used to confirmed the *Bacillus* sp. Isolated *Bacillus* sp. (B3) were chosen for amylase potential screening and it has shown maximum zone of clearance on starch agar plates. Bacterial colony yielding positive starch hydrolysis test (*Bacillus* sp. B3) were subjected to Amylase activity test. The amylase activity was also carried with respect to time, temperature and pH of the media in which it was inoculated. The amylase activity decrease from 0.981 to 0.215 U/ml as the incubation time increase from 24 to 72 hours at 35 ±2°C. The amylase activity shown at different temperature from 35 to 45°C has gradual decrease from 0.992 U/ml to 0.545 U/ml. The amylase activity recorded at different pH from 5 to 10. It shows maximum amylase activity at pH 7 (0.998 U/ml). There is increase in amylase activity at basic pH5 = 0.910 U/ml whereas decrease in acidic medium pH10 = 0.089 U/ml.

Keywords: Amylase activity, *Bacillus* sp., Incubation period, pH effect, Soil, Temperature effect.

Introduction

Amylase are hydrolyzing enzyme in function which causes hydrolysis of molecules. In biotechnology amylase are of the most important enzymes used[1]. They are widely distributed in microbial, plant and animal kingdoms[2]. They act by hydrolysing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved[3]. Enzymes are the biological catalyst, which initiate and accelerate thousands of biochemical reactions in living cells. Major sources of enzymes are the biological organism plants, animals, and microorganism. Microbial enzymes account the major volume. However, about less than 50 species are actually used to produce the entire microbial enzyme. The potential obviously exists to search for the species producing novel enzymes or enzymes with better properties and yield[4]. Enzymes produced by the organism are of two types Exoenzymes and Endoenzymes. Exoenzymes which are released from the cell and act on the substrates. These are mainly hydrolytic enzymes that degrade by the addition of high molecular

weight substrates (like polysaccharides, lipid and proteins) into small components (e.g. glucose) that can enter into the cell and are later assimilated. Enzymes required for the hydrolysis of cellulose, starch, pectin, lipid, casein and gelatin belong to the category of exoenzymes. Endoenzymes are utilized by the cell for further metabolic degradation of carbohydrates and are mainly responsible for synthesis. New protoplasmic requirements and production of cellular energy from assimilated materials and these enzymes function inside a cell.

Two example of such enzymes are maltose and lactose. Other examples belong to this category include carbohydrate fermentation, nitrate reduction, catalase, urease, IMVic test etc. α-amylase are exoenzymes that catalysis the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight product and such as glucose, maltose, and maltotroise units. α-amylase is a highly demanded industrial enzymes with extensive commercial applications in various sectors which is carried out with bacterial strain producing extracellular microbial α-amylase, isolated from soil. Amylase are among the most important enzymes that are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 30% of the world enzymes market. They have opened new frontiers of many commercial biotechnological processes including renewable energy, pharmaceuticals, saccharification or liquefication of starch, detergents industries, warp sizing of textiles, fibers, paper industries, food staffs, baking, classification of haze formed in beer or fruit juices and for pretreatment of animal feed to improve digestibility[5].

Moreover, microbial amylase have a broad spectrum of industrial applications as they are more stable with great genetic diversity, high enzymatic activity in a wide range of condition and (extreme pH, temperature, osmolarity, pressure etc) simple and cost effective production and easy manipulation to obtain enzymes of desired characteristics. Today a large number of microbial amylase completely replaced chemical hydrolysis of starch in starch processing industry. The spectrum of amylase applications has expanded in several fields such as clinical, medicinal and analytical chemistry. Starch is an important constituent of the human diet and for this purpose, is used chemically and enzymatically processed into a variety of different product such starch hydrolysates, glucose syrups, fructose maltodextrins derivatives or cyclodextrins used in food industry. Starch contributes greatly to the textual properties of many foods and is widely used in foods and industrial application as thickener, colloidal stabilizer gelling agent and water retention agent [6].

The α-amylase (α-1,4 glucan-4-glucanohydrolase) can be found in microorganism, plants and animals. α-amylase can be produced by different species of microorganism but for commercial applications α-amylase is derived from the genus *Bacillus* which is produced from *Bacillus licheniformis, B.stearothermophiles* and *B.amyloliquefaciens.* The production of microbial amylase from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrients requirements, incubation period, pH, temperature, metal ions and thermostability. Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability[7]. As enzymatic liquefaction and saccharification of starch are performed at high temperature (100-110°C). Thermostable amylolytic enzymes have been currently investigated to improve industrial process of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins. Beside, fungi mold species producing high level of amylase such as *Aspergillus niger, A. oryzas, Thermomyces lanuginosu and Pencillium expansum* in addition to many species of the genus *Mucor.*

Materials and Methods

Isolation of Amylase Producing Microorganisms

Soil samples were collected from different environment sources (Banana, Potato and Sugarcane field sample respectively). Serial dilution was made and was plated on nutrient agar by spreading 0.1ml of the diluted sample. Then the plates were kept for incubation at 37°C for overnight.

Screening for Amylase Activity (Starch Iodine Test)

Isolated colonies were picked up from each plate containing pure culture and streaked in straight lines in starch agar plates with starch as the only carbon source. After incubation at 37ºC for 24-48 hrs., individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is the

basis of the detection and screening of an amylolytic strain. The colonies which were showing zone of clearance in starch agar plates were maintained on to nutrient agar slants.

Morphological and Biochemical Characteristics
Gram staining, Indole Production, Methyl Red, Vogues Proskauer's, Citrate Utilization, Fermentation, Nitrate Reduction, Catalase, Oxidase, Urease, Hydrolysis of Casein, Hydrolysis Of Starch were carried out.

Enzyme production medium
Production medium contained (g/l) Bacteriological peptone 6.0gm, $MgSO_4.7H_20$ 0.5gm, KCL 0.5gm, Starch 10gm. 10 ml of medium was taken in a 100 ml conical flask. The flasks were sterilized in autoclave at 121^0C for 15 min and after cooling the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37^0C in shaker incubator for 24hr. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Amylase Assay
The enzyme activity was assayed following the method of Bernfeld 1955[6] using 3, 5- dinitrosalicylic acid.

Process Optimization for Amylase Production

pH: The effect of pH for amylase production was determined by culturing the bacterium in the production media with different pH. The experiment was carried out individually at various pH 5, 6, 7, 8, 9 and 10. The enzyme assay was carried out after 24 hours of incubation [8] .

Temperature: Temperature is an important role for the production of amylase. The effect of temperature on amylase production was studied by the incubating the culture media at various temperatures 35, 40, 45, along with arbitrary control at 37°C. The enzyme assay was carried out after 24 hours of incubation[9].

Incubation Period: The amylase production by the selected experimental microorganisms was determined by optimizing the media by adding different bacteria in the production media. The experiment was carried out individually at various incubation periods such as 24, 48 and 72 hrs. The enzyme assay was carried out after 24 hours of incubation[4] .

Results and Discussion

Amylase, producing organisms like fungi and bacteria are generally isolated from soil and most of the work is focused on the amylase. The present study deals with isolation of amylase producing bacteria from soil. This was performed by the serial dilution spread plate technique. Similar method has been used by[10,11]**.** Identification of selected *Bacillus* strain was identified on the basis of standard morphological and biochemical tests according to Bergey's Manual of determinative Bacteriology[12]**.** Isolated *Bacillus* strains were primarily screened for the production of amylase was done by starch agar plate method[13] . Then 10 bacterial isolates from soil were tested for production of amylase by the starch hydrolysis test.

Out of ten isolates, five bacteria showed the zone of clearance on starch agar media and among five, *Bacillus* sp. B3 showed the maximum zone of clearance on the starch agar medium i.e 8mm. So, B3 isolate was selected for the further study of amylase activity. Similar method has been used by [14] observed area of clearance 1.0mm on starch agar medium.

Different Biochemical tests such as Catalase Test, Fermentation Test, Nitrate Reduction Test , Urease Test , Citrate Utilization Test, MR-VP Test, Indole Production Test, Starch Hydrolysis Test and Casein Hydrolysis Test were used which confirmed the *Bacillus* sp. Isolated *Bacillus sp.* (B3) was chosen for enzyme (amylase) potential because, it has shown maximum zone of clearance on starch agar plates. *Bacillus* sp. (B3) was positive for Catalase, Starch Hydrolysis, Casein Hydrolysis, Sucrose and Dextrose Fermentation, Nitrate Reduction, Methyl Red, Citrate Utilization. While negative for Indol Production, Voges Proskauer, Urease. Culture B3 has fermented Sucrose, Dextrose, Lactose by acid production only no gas production was observed.

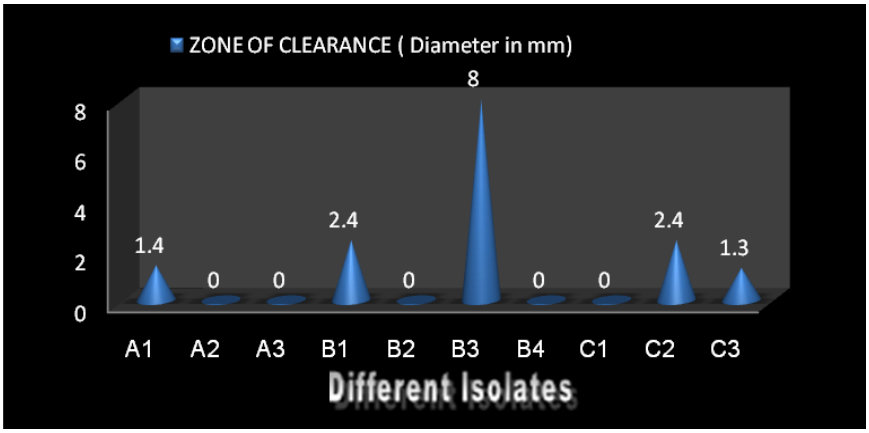

Figure 1: Qualitative screening of *Bacillus* sp. B3 for Amylolttic activity

The estimation of amylase activity was carried out according to the Dinitrosalicyclic acid Method of Bernfield, 1955[6] and Miller,1959[15]**.** Isolated *Bacillus* sp. B3 was found to be effective in releasing high amount of reducing sugars. The amylase activity decrease from 0.981 to 0.215 U/ml as the incubation time increase from 24 to 72 hours at 35 ±2°C. Similar work was done by [16] reported that *Bacillus* sp. showed the amylase production at 48-72 hours i.e. 0.04U/ml to 0.023 U/ml. respectively and reach maximum activity at 48 hours. While same work has been done by [17,18], reported that *Bacillus* sp. showed the amylase production at 24-72hours i.e. 0.908U/ml - 0.408U/ml and 0.780U/ml - 0.182U/ml respectively. For further optimization of enzyme production was carried out for pH and Temperature. Isolated *Bacillus* sp. B3 showed the amylase activity at different temperature from 35 to 45°C has gradual decrease from 0.992 U/ml to 0.545 U/ml. Similar work was done by [19] reported that the thermal stability of some alpha amylases were stable up to 50°C and some at 40°C after incubation for 15 min.

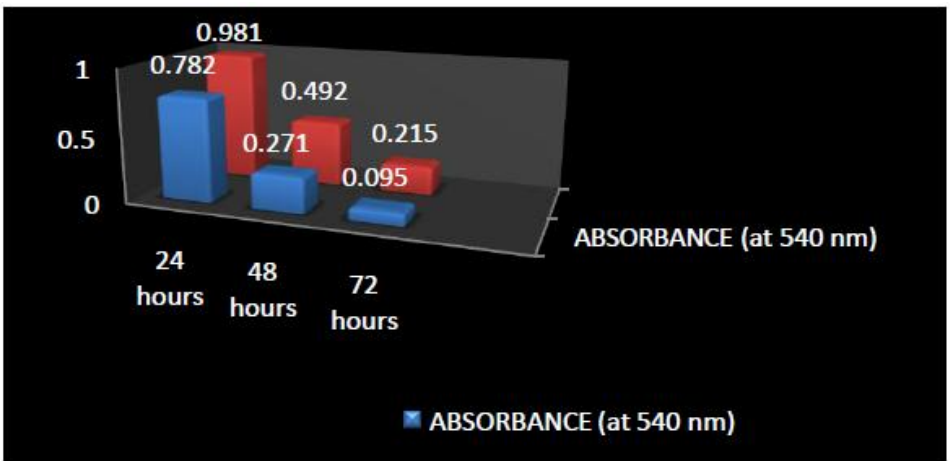

Figure 2: Quantitative screening of *Bacillus* sp. Amylolytic activity

While amylase activity recorded at different pH from 5 to 10. It shows maximum amylase activity at pH 7 (0.998 U/ml). There is increase in amylase activity at basic pH10 = 0.910 U/ml and decrease in acidic medium pH 5 = 0.089 U/ml. Similar work was done by[5] studied thermostable amylase producing *Bacillus species* that revealed an optimum enzyme activity at pH 8. But present strain B3 was found to be more competent for the production of amylase as compare to other. Similar work was done by[20]**.** In their study culture, morphological and metabolic characteristics of the bacterial isolates were studied. They isolated total 18 bacterial cultures from soil sample. Among bacterial isolates,14 showed the amylolytic activity and these were identified regarding to Bergey's Manual of systemic Bacteriology. They observed that the optimum pH for the growth of all culture was at pH 7 and the production of amylase was observed in the range of 0.045 to 1.35U/ml. Another study done by [21]

isolate amylase producing bacteria from the marine environment of Andaman and Nicobar Islands (india).

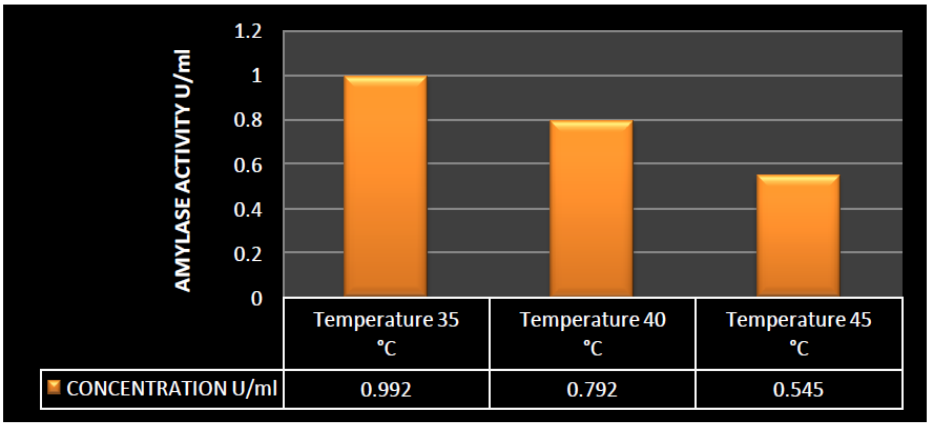

Figure 3: Amylase activity at different temperature

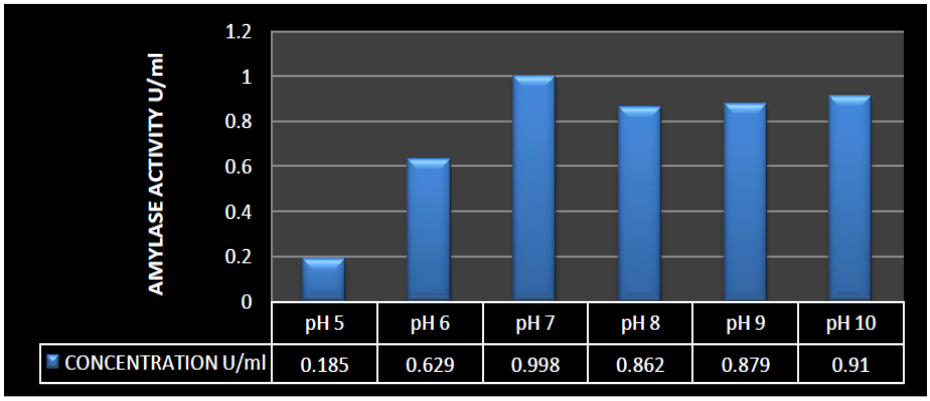

Figure 4: Amylase activity at different pH

Conclusion

The present study showed that the isolated amylolytic bacterial strain- B3 produced appreciable amount of amylase enzyme. Further, the study also revealed that amylase enzyme produced by the isolated amylolytic bacterial strain- B3 can be used for industrial application like starch modification with better efficiency with the increase in temperature.

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