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ORIGINAL RESEARCH ARTICLE

Amylase Production by *Bacillus* sp. Using Cassava as Substrate

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ABSTRACT

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. In the present study, the bacteria was isolated from the soil samples. The bacteria isolated was identified as *Bacillus* sp. based on Staining techniques, motility test, plating on selective media and biochemical tests. Amylase production by *Bacillus* sp. was detected by the disappearance of blue colour in the starch agar medium around the microbial colonies after incubation. Cassava was used as the substrates for the amylase production. Solid state fermentation was carried out for the production of amylase using *Bacillus* sp. The effect of different carbon and nitrogen source, Temperature and pH was determined on enzyme production by *Bacillus* sp. Amylase activity was determined by four methods such as DNSA method, Dextrinizing activity method, decrease in starch-iodine color intensity and Plate assay.

Key words: Amylase, *Bacillus* sp., Cassava, Optimization and Solid state fermentation.

1. INTRODUCTION

Microorganisms had significant contribution in production of various industrial enzymes. The global market for industrial enzymes estimated at $2 billion in 2010 and expected to rise at an average annual growth rate of 3.3%. These starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile, paper, pharmaceutical to sugar industries [1]. Conversion of starch into sugar, syrups and dextrins forms the major part of starch processing industry [2]. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits [3].

Nowadays, amylases (α-amylases, β- amylases and glucoamylases) represent one of the most important enzyme groups within the field of biotechnology [4]. α-amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase, endoamylase) is a classical calcium containing enzyme catalyze hydrolysis of starch and related carbohydrates by randomly cleaving internal α-D-(1-4) glycosidic linkage, yielding glucose, maltose, maltotriose, and other oligosaccharides [5]. It belongs to family thirteen in the classification of glycoside hydrolases. This family is the most varied of all glycoside hydrolase families, containing many enzymes able to catalyze various reactions, such as hydrolysis, transglycosylation, condensation and cyclization [6].

Amylase can be obtained from several fungi, yeast, bacteria and actinomycetes; however, enzyme from fungal and bacterial sources has dominated applications in industrial sectors [7]. The application of an amylase in industrial reactions depends on its unique characteristics, such as its action pattern, substrate specificity, major reaction products, optimal temperature, and optimal pH12. Bacterial α-amylase preferred for application in starch processing and textile industries due to its action at higher temperature (75- 105^0C) and neutral to alkaline pH13. Generally production of this enzyme has been carried out by submerged fermentation. Among the bacterial sources *Bacillus subtilis*, *Bacillus staerothermophilus, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus acidocaldarius,*

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Bifidobacterium bifidum and *Bifidobacterium acerans* are important species [8].

The α-amylases from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus stearothermophilus* are among the most widely studied amylases and are highly homologous with respect to primary and tertiary structure [9]. However, despite their structural similarities, these reported amylases vary significantly in their thermal stability. The thermal inactivation of *Bacillus* α-amylases has been suggested to involve a two-step process, with the first step of reversible unfolding, followed by an irreversible conformational change [10]. Calcium ions have also been implicated in mechanisms involving the thermal inactivation of the *Bacillus* α-amylases, whereby it has been proposed that the first step involves the reversible dissociation of calciumions from the native enzyme, followed by irreversible denaturation at high temperatures [11].

Amylase has a great deal of application in starch saccharification. The amylolytic enzymes find a wide spectrum of applications in food industry for production of glucose syrups, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices, solubilization and saccharification of starch for alcohol fermentation in brewing industries, and also find a wide range of application in baking, paper, textile and detergent industry [12]. In most cases the enzymatic process is inhibited by high substrate and product concentration and also instability of the enzyme under repetitive or prolonged use. The present study was carried out to evaluate the amylase activity of the amylolytic bacteria using cassava waste as a feed substrate. Pectinase production by different organisms in submerged state fermentation has received more attention and is found to be cost-prohibitive because of high cost of process engineering.

2. MATERIALS AND METHODS

2.1. Substrates used

Cassava was obtained from Salem. The substrates were ground into coarse powder with a blender.

2.2. Isolation and identification of *Bacillus* sp.

Pour plate technique was used for the isolation of *Bacillus* sp. from soil sample. Well grown bacterial colonies were picked and further purified by streaking. The isolated strains were maintained on Nutrient agar slants and stored in refrigerator at 4°C. Identification of the bacterial isolates was carried out by the routine bacteriological methods *i.e.*, By the colony morphology, preliminary tests like Gram staining, capsule staining, endospore staining, motility, catalase and oxidase, plating on selective medias and performing biochemical tests.

2.3. Solid state fermentation

Five gram of substrate (Cassava) was taken into a 250 ml (flask) Erlenmeyer flask and to this a bacterial amylase production containing (g/l) KH_2PO_4 – 1.4; NH_4NO_3 – 10; KCl – 0.5; $MgSO_4.7H_2O$ – 0.1; $FeSO_4.7H_2O$ – 0.01; starch – 20 gm; and distilled water was added to adjust the require moisture level. The contents of the flaks were mixed thoroughly and autoclaved at 121°C for 20 min. Solid state fermentation was carried at 30°C with substrate initial moisture content of 64% for 72 hours using 2 ml *Bacillus* sp. Suspension as inoculum. Studies were also performed to evaluate the influence of supplementation of substrate with different carbon sources such as glucose, maltose, sucrose, lactose (3% w/v) and nitrogen source such as peptone, casein, urea, yeast extract (3% w/v).

2.4. Optimization of culture condition

The effect of culture conditions the present study was carried out at different temperature (30, 40, 50 and 60°C), different pH (4, 5, 6 and 7), different carbon sources such as (glucose, maltose, lactose and sucrose) and nitrogen sources such as (peptone, casein, urea, yeast extract) were used to determine their effect on amylase production.

2.5. Enzyme extraction

22 ml of 0.1 M phosphate buffer (pH-6.5) was added to cultures, the mixture were shacked for 30 min at 19°C and 140 rpm on a rotary shaker. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was filtered through Whattman Number-1 filter paper and the filtrate was used as the crude enzyme preparation.

2.6. Assay of enzyme activity

2.6.1. DNSA method

Assay system for amylase activity was carried out by measuring the amount of reducing sugar according to the DNSA method. Amylase activity was determined by incubating a mixture of 1 ml of aliquot of each enzyme source and 1% soluble starch dissolved in 0.1 M phosphate buffer, at pH 7, at 55°C for 15 min. The reaction was stopped by adding 1 ml of 3, 5 Dinitrosalicylic acid, and then followed by boiling for 10 min. The final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that releasing 1μmol glucose equivalent per minute under the assay

conditions. Reducing sugar (Glucose or maltose) concentration was determined from a standard curve under same condition using glucose.

Calculation:

$$\text{Amylase activity (U/ml)} = \frac{\text{Microgram of glucose produced}}{\text{Volume of enzyme solution x Incubation time}}$$

2.6.2. Dextrinizing activity method

Amylase was assayed by the iodine method with slight modifications. Activity was estimated in a reaction mixture containing 1.0 ml of 0.15% soluble starch in 0.1M acetate buffer, pH 5.5, 200mM $CaCl_2$ and 1.0 ml enzyme and incubated at 65°C for 10 min. After incubation, 1.0 ml of 1 M acetic acid, 1.0 ml of 0.2% iodine, 2.0% KI solution and 15 ml of distilled water were added. The absorbance of the diluted solution was measured at 620 nm. A blank was prepared under the same condition by adding enzyme solution after the reaction had been stopped by the addition of 1.0M acetic acid. One unit of dextrinizing activity was defined as the amount of enzyme which hydrolyzes 1ml of starch/10minutes under the above conditions. Starch concentration was determined from a standard curve under the same assay condition using soluble starch.

Calculation:

$$\text{Amylase activity (U/ml)} = \frac{\text{Microgram of glucose produced}}{\text{Volume of enzyme solution x Incubation time}}$$

2.6.3. Decrease in starch-iodine color intensity

Starch forms a deep blue complex with iodine and with progressive hydrolysis of the starch, it changes to red dish brown. Several procedures have been described by various groups for the quantitative determination of amylase based on this property. This method determines the dextrinizing activity of amylase in terms of decrease in the iodine color reaction.

2.6.4. Plate assay

The plate assay was performed using agar plates amended with starch. The agar plates were prepared amended 2% of starch with 1.5% of agar. After agar solidification, around 10 mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with the culture filtrate and incubated at 37°C for overnight. 1% of iodine solution was over layered on the agar and the observation was made to see the hydrolytic zone around the well. The negative control was maintained by adding sterile water in the separate well.

2.7. Estimation of protein concentration

The protein concentration was estimated by the following the method of Lowry *et al.* [13] using Bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

Enzymes are substances present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it. There are three major sources of enzymes [14], that is derived from a variety of plants, e.g. pappain, animal enzymes – derived from animal glands, e.g. trypsin, pepsin and microbial enzymes – derived from micro organisms (fungal and bacterial) through the process of fermentation, e.g. amylase. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable [14].

Amylases are examples of hydrolases and function in the hydrolysis of molecules. Amylases are of the most important enzymes used in biotechnology [14]. Their use includes hydrolysis of starch to yield glucose syrup, amylase-rich flour and in the formation of dextrin during baking in food industries. Furthermore, in the textile industry, amylases are used for removal of starch sizing and as additives in detergents [15]. Cheap and readily available agricultural waste such as cassava peels, which presently constitutes a menace to solid waste management, may be a rich source of amylolytic bacteria [16]. Therefore, the main objectives of the study were to isolate and identify amylolytic bacteria from cassava waste dumpsites, and to perform partial characterization of the enzyme production and its properties with regard to the effect of temperature. In this present study, the bacterial isolate showing superior amylase production gave the following preliminary characterization: gram-positive spore forming bacilli, approximately one micron in length, with central spores usually smaller than the cell. Agar colonies were small, smooth, glistening, and round, or intermediate in size, flat, rough, and irregular, or sometimes rather large, spreading, and mucoid. The great majority of isolates fermented glucose and sucrose with the production of acid. Lactose was not fermented. Most of them liquefied gelatin completely within 72 hours. Provisionally, they all appear to fit into the *Bacillus* sp. group. The *Bacillus* sp. was isolated and characterized by Gram staining, motility test, selective medium and bio-chemical tests. Amylase activity was preliminarily checked by plating the *Bacillus cereus* on the Starch agar medium, the medium was flooded using iodine

indicator, clear zone around the colony indicated the presence of amylase activity.

Members of the genus *Bacillus* are heterogeneous and they are very versatile in their adaptability to the environment. There are various factors that influence the nature of their metabolic processes and enzymes produced [17]. A great deal of attention is being given to thermophilic and extremely thermophilic microorganisms and their enzymes [17]. *Bacillus* species produce a large variety of extra cellular enzymes, such as amylases, which have significant industrial importance [18]. In the same vein, bacterial enzymes are known to posses more thermostability than fungal amylases [19].

In this present study, the effect of temperature on amylase production by *Bacillus* sp. using cassava waste as substrate was carried out at various temperature *viz*., 30, 40, 50 and 60°C. The enzyme production was maximum at 60°C (3.9 U/ml) and minimum at 30°C (2.9 U/ml) (**Fig 1**). The effect of pH on amylase production by *Bacillus* sp. was carried out at various pH *viz*.,4, 5, 6 and 7 . The enzyme production was maximum at pH 7 (3.8 U/ml) and minimum at pH 4 (2.8 U/ml) (**Fig 2**).

In this present research, the effect of various carbon sources like Glucose, Maltose, Sucrose and Lactose was tested on amylase production by *Bacillus* sp. using cassava waste as substrate. The amylase production was high in the medium containing Glucose (4.2 U/ml) (**Fig 3**). The effect of nitrogen sources like Peptone, Yeast extract; Casein and Urea were tested for the amylase production. Among the various nitrogen sources tested, peptone (3.5 U/ml) maximum amylase production (Figure-4). Amylase activity was determined by Dinitrosalicylic acid assay (4.6 U/ml), Dextrinizing activity (9.2 U/ml), Decrease in starch-iodine color intensity, Plate assay (15 mm) (**Table 1**).

The influence of temperature on amylase production is related to the growth of the organism. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25 to 35°C. Optimum yields of α-amylase were achieved at 30 to 37°C for *Aspergillus oryzae* [20]. α-Amylase production has also been reported at 55°C by the Thermophilic fungus *Thermomonospora fusca* and at 50°C by *Thermomonospora lanuginosus* [21]. α-Amylase has been produced at a much wider range of temperature among the bacteria. Continuous production of amylase from *Bacillus amyloliquefaciens* at 36°C has been reported. However, temperatures as high as 80°C have been used for amylase production from the hyperthermophile *Thermococcus profundus*.

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. Most of the *Bacillus* strains used commercially for the production of bacterial α-amylases by SmF have an optimum pH between 6.0 and 7.0 for growth and enzyme production. This is also true of strains used in the production of the enzyme by SSF. In most cases the pH used is not specified excepting pH 4.2 in the case of *Aspergillus oryzae*, 8.0 in *Aspergillus oryzae* and 6.8 for *Bacillus amyloliquefaciens* [22].

The carbon sources as glucose and maltose have been utilized for the production of α-amylase. However, the use of starch remains promising and ubiquitous. A number of other non-conventional substrates as lactose, casitone, fructose, oilseed cakes [23] and starch processing waste water. Cui YQ *et al*. [24] have also been used for the production of α-amylase while the agro-processing by-product, wheat bran has been used for the economic production of α-amylase by SSF. The use of wheat bran in liquid surface fermentation (LSF) for the production of α-amylase from *Aspergillus fumigates* and from *Clavatia gigantea*, respectively, has also been reported. High α-amylase activities from *Aspergillus fumigatus* have also been reported using α-methyl-Dglycoside (a synthetic analogue of maltose) as substrate.

Organic nitrogen sources have been preferred for the production of α-amylase. Yeast extract has been used in the production of α-amylase from *Streptomyces* sp., *Bacillus* sp. and *Halomonas meridian* [25]. Yeast extract has also been used in conjunction with other nitrogen sources such as bactopeptone in the case of *Bacillus* sp., ammonium sulphate in the case of *Bacillus subtilis*, ammonium sulphate and casein for *C. gigantea* and soybean flour and meat extract for *Aspergillus oryzae*. Yeast extract increased the productivity of α-amylase by 156% in *Aspergillus oryzae* when used as an additional nitrogen source than when ammonia was used as a sole source.

Various other organic nitrogen sources have also been reported to support maximum α-amylase production by various bacteria and fungi. However, organic nitrogen sources *viz*. beef

extract peptone and com steep liquor supported maximum α-amylase production by bacterial strains soybean meal and amino acids by *Aspergillus oryzae* [26]. CSL has also been used for the economical and efficient production of α-amylase from a mutant of *Bacillus subtilis*. Apart from this, various inorganic salts such as ammonium sulphate for *Aspergillus oryzae* and *Aspergillus nidulans*, ammonium nitrate for *Aspergillus oryzae* and Vogel salts for *Aspergillus fumigatus* have been reported to support better α-amylase production in fungi [27].

Fig 1: Effect of temperature on amylase production by *Bacillus* sp.

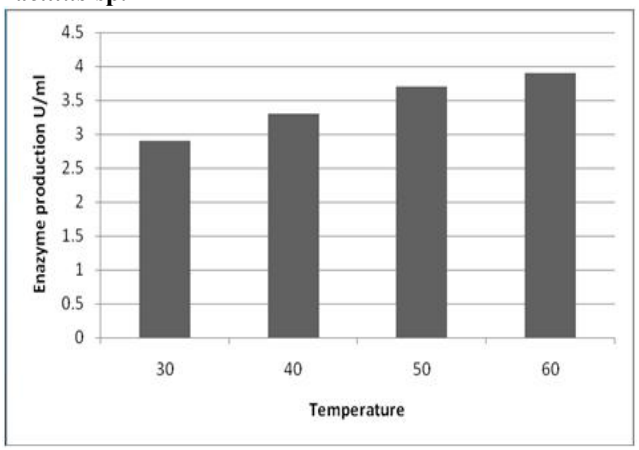

Fig 2: Effect of pH on amylase production by *Bacillus* sp.

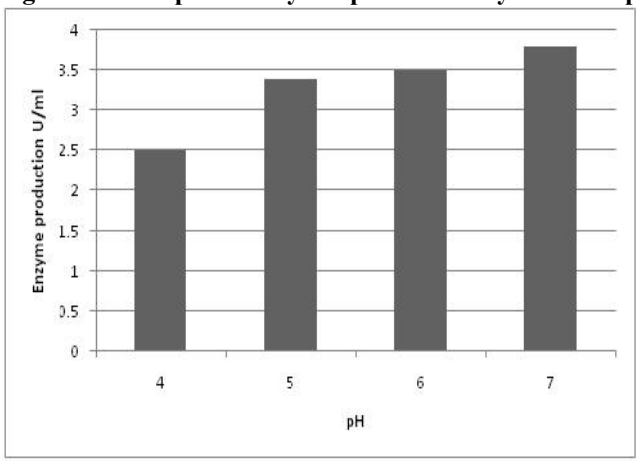

Fig 3: Effect of carbon sources on amylase production by *Bacillus* sp.

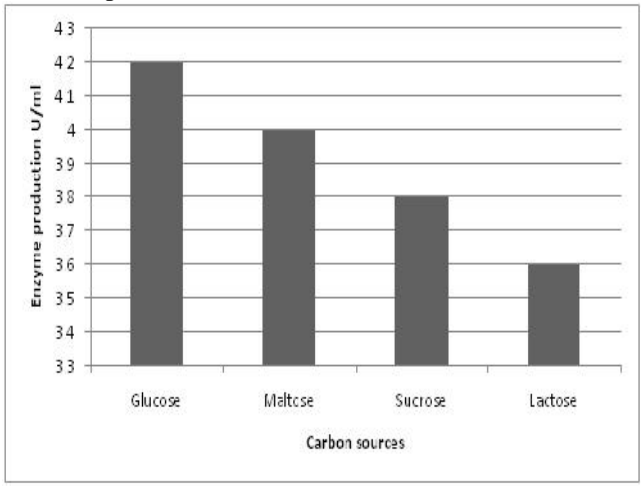

Fig 4: Effect of nitrogen sources on amylase production by *Bacillus* sp.

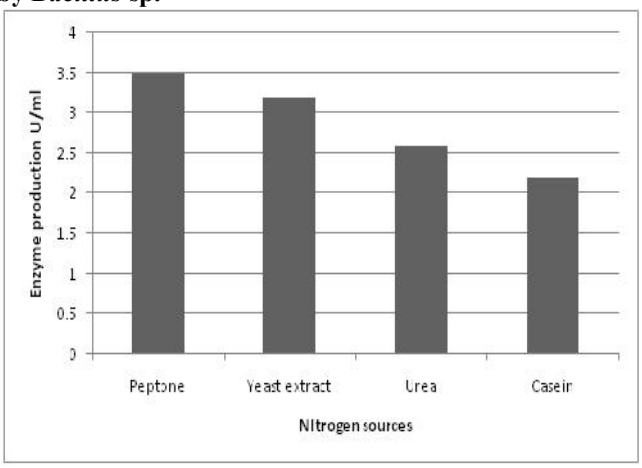

Table 1: Assay of amylase enzyme activity

S. No	Method	Results
1	Dinitro sacicyclic acid method (U/ml)	4.6
2	Dextrinizing activity (U/ml)	9.2
3	Plate assay (Diameter of zone in mm)	15

CONCLUSION

Research on amylase has progressed very rapidly over the last five decades and potential industrial applications of the enzyme especially in solid waste management have been identified. Major impediments to exploit the commercial potential of amylase are the yield, stability and cost of amylase production. Although microbes have been extensively studied by many researchers. The bacteria *Bacillus* sp. was used for the production of amylase and cassava wastes were used as a substrate.

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