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Amylase Production Using *Bacillus cereus* Isolated from a Vermicompost Site

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Abstract: *Bacillus cereus* strain isolated from a vermicompost site was tested for its abilities to hydrolyze the structural polysaccharides. The effect of different production parameters such as pH, temperature, carbon source, nitrogen source (Organic and inorganic) incubation time inoculum sizes and surfactants on amylase production by the isolated bacterial strain was studied. The enzyme production was assayed in submerged fermentation (SmF) condition. The maximum amylase production was observed with maltose (216±2.6 U/ml), yeast extract (163±3.6U/ml), ammonium sulphate (70±3.0U/ml), pH 7.0 (263±4.1), temperature 40°C (152±9.1), Tween-80 (165±2.8U/ml), inoculum size level 0.5% (132±2.0U/ml) and incubation time 48 hours (172.01± 0.56U/ml) in the production medium.

Key words: Amylase · *Bacillus cereus* · Optimization · Smf · Starch · Vermicompost

INTRODUCTION

Enzymes are capable to act as biocatalyst for a wide variety of chemical reactions. Although enzymes are produced from animal and plant sources, the microbial sources are generally the most suitable for commercial applications. The world market for enzymes remains in excess of $4500 million [1]. Amylase can be divided in three groups such as α-amylase, which cleave the bonds in the interior of the substrate (endoamylase); β-amylases, which act on the reducing extremities of the substrate (exoamylase); and amyloglucosidase, which liberates units of glucose from the non reducing end substrate molecules. Amylases have been reported to occur in microorganisms, although they are also found in plants and animals [2].

α -amylase as an enzyme that breaks the α [1-4] bonds of polysaccharides that have ten (or) more units of D-glucose united by α -1,4 glucosidic linkage. The attachment occurs in a non selective form (as endoenzyme) on different points of the chain simultaneously. So that the first hydrolysis products are oligosaccharides of 5 to 7 units of glucose α-amylases are calcium metallo enzymes completely unable to function in the absence of calcium. α-amylase and pullulanase are amylolytic enzymes of industrial importance particularly in the food and detergent industries [3].

The amylase producing bacteria (such as *Bacillus subtilis, B. cereus, B. licheniformis, B. amyloloquefaciens* and *B. megaterium*) and fungi (such as *A. niger, Penicillum, Cephalosporium, Neurospora* and *Rhizopus*) are major amylase producing microorganisms. Microorganisms such as yeasts, fungi, bacteria, actinomycetes and algae are effectively producing amylase [4].

The industrial use of enzymes often requires enzymatic reaction to be conducted at higher temperatures. Generally under those conditions productivity improved with less microbial contamination. Therefore, thermostable enzymes have been the heart of numerous studies involving in the elucidation of thermal denaturation mechanism and development of rational strategies for the enhancement of enzyme thermostability [5].

The present study was mainly focused on the production of amylase form *Bacillus cereus* by optimizing various parameters such as carbon sources, inorganic nitrogen sources, organic nitrogen sources, pH, temperature, substrate concentration, inoculum concentration, incubation time and surfactants.

MATERIALS AND METHODS

Collection of Sample: In the present study, soil sample was collected from vermicompost in Ayya Nadar Janaki Ammal College, Sivakasi, Tamilnadu, India.

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Isolation of Amylase Producing Microorganisms: Serial dilution was made from 10^{-1} to 10^{-9} 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.

Amylase Activity: The isolated bacterial strains were streaked on starch agar plates and the plates were incubated at 37°C for 48 hours. After incubation the plates were flooded with iodine solution, the amylolytic activity was confirmed by clear zone around the bacterial growth [6].

Morphological and Biochemical Characteristics: Gram staining, motility, indole production, methyl red, Voges Proskauer's, citrate utilization, triple sugar iron, nitrate reduction, catalase, oxidase, gelatin liquefaction, urease, hydrolysis of casein, hydrolysis of starch were carried out (KB009 H1 carbohydrate biochemical kit).

Amylase Enzyme Assay

Cell Separation: The cells from the culture were separated by centrifugation. Around 2ml of culture was taken in a sterile eppendorf tubes and were centrifuged at 6,600 rpm for 2 min in a cold room. The supernatant was transferred carefully into another test tube.

Amylase Assay: The enzyme activity was assayed following the method of Bernfeld [7] using 3, 5-dinitrosalicylic acid.

Protein Estimation of Crude Enzyme: The protein content of the crude enzyme was measured according to Lowry's (1951) method [8].

Medium Optimization for Amylase Production

Carbon Source: To identify the suitable carbon sources for amylase production by the *Bacillus cereus*. The following different carbon sources were tested such as glucose, sucrose, maltose, lactose, galactose, fructose and dextrose with sample concentration of 0.5% in the optimized carbon sources in production medium at 37°C [9].

Organic and Inorganic Nitrogen Sources: The amylase production by the selected bacterium was also optimized by supplementing different inorganic and inorganic nitrogen sources individually at the concentration of 0.5% such as potassium nitrate, ammonium sulphate, sodium nitrate, ammonium nitrate, ammonium chloride, casein, malt extract, peptone, urea, gelatin and yeast extract [10].

Effect of 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 and 9. The enzyme assay was carried out after 72 hours of incubation at 37°C [11].

Effect of 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 10, 20, 30, 40, 50, 60,70 and 80°C along with arbitrary control at 37°C [12].

Effect of Surfactants: To identify the surfactants facilitating amylase production, four different surfactants were used for experimentation. They were Tween-20, Tween-80, SDS (Sodium dodecyl sulphate) and PEG (Poly Ethylene Glycol). The surfactants were tested individually at the concentration of 0.2% in the optimized production medium [13].

Effect of Various Incubation Times on Amylase Production: 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 times such as 24, 48, 72, 96 and 120 hours. The enzyme assay was carried out individually after 72 hours of incubation [14].

Effect of Various Inoculum Concentrations on Amylase Production: The amylase production by the selected experimental microorganisms was determined by adding bacterium at different inoculum's concentrations such as 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5.0 % to test its ability to induce amylase production in the production medium [15].

RESULTS

Screening of Amylase Producing Bacteria from Vermicompost Site: The bacteria isolated from vermicompost soil were screened for amylase production on starch agar medium. From the soil samples 7 bacterial strains were isolated. But later during screening it was found that only 3 strains showed amylase activity. Later only one potential isolate was identified by standard morphological and biochemical characterization. After careful biochemical tests, it was confirmed that the isolate was *Bacillus cereus.*

Table 1: Effect of various carbon sources on amylase production

Carbon source (0.5%)	Specific activity (U/ml)
Glucose	44±1.0
Galactose	161±1.5
Maltose	216±2.6
Sucrose	160±2.5
Lactose	122±2.8
Fructose	107±1.7
Dextrose	117±1.0

Table 2: Effect of various inorganic nitrogen sources on amylase production

Nitrogen sources (0.5%)	Specific activity U/ml
Potassium nitrate	57±1.0
Ammonium sulphate	70±3.0
Sodium nitrate	47±1.2
Ammonium nitrate	57±1.2
Ammonium chloride	60±2.9

Table 3: Effect of organic nitrogen sources on amylase production

Organic nitrogen source (0.5%)	Specific activity (U/ml)
Casein	87±2.2
Malt extract	83±2.0
Peptone	79±2.7
Urea	80±2.0
Gelatin	64±1.7
Yeast extract	163±3.6

Table 4: Effect of various pHs on amylase production

pH	Specific activity U/ml
5	132±2.9
6	167±2.4
7	263±4.1
8	194±2.3
9	85±1.7
10	79±2.5

Table 5: Effect of various temperatures on amylase production

Temperature (°C)	Specific activity (U/ml)
10	84±0.6
20	95±1.3
30	107±1.8
40	152±1.9
50	117±1.2
60	90±1.5
70	71±0.9
80	27±0.7

Table 6: Effect of various surfactants on amylase production

Surfactants	Specific activity U/ml
Tween-20	132±2.0
Tween-80	165±2.8
SDS	110±1.0
PEG	90±1.0

Table 7: Effect of various incubation times on amylase production

Incubation time (hrs)	Specific activity (U/ml)
24	145.25±1.20
48	172.01±0.56
72	159.36±0.11
96	121.11±0.98
120	99.15±1.24

Table 8: Effect of various inoculum sizes on amylase production

Inoculum sizes (%)	Specific activity (U/ml)
0.5	132±2.00
1.0	90±1.80
1.5	110±1.00
2.0	51±2.80
3.0	131±0.36
4.0	99±0.58
5.0	165±2.80

Effect of Carbon Sources on Amylase Production: Table 1 shows the effect of carbon sources on amylase production after 48 hours of incubation period at 37°C. The maximum amylase production was recorded in maltose (216±2.6 U/ml) supplemented medium and minimum amylase production was recorded in glucose (44±1.0 U/ml).

Effect of Inorganic Nitrogen Sources on Amylase Production: Table 2 shows the effect of different kinds of inorganic nitrogen sources on amylase production after 48 hours of incubation period at 37°C. The maximum amount of enzyme production was observed in ammonium sulphate (70±3.0 U/ml) supplemented medium and minimum amount of amylase production was observed in sodium nitrate (47±1.2 U/ml) supplemented medium.

Effect of Organic Nitrogen Sources on Amylase Production: Table 3 shows the effect of different kinds of organic nitrogen sources on amylase production after 48 hours of incubation period at 37°C. The maximum amount of amylase production was observed in yeast extract (163±3.6 U/ml) with supplemented medium and minimum enzyme activity was observed in gelatin (64±1.7 U/ml).

Effect of pH on Amylases Production: Table 4 shows the effect of various pH on amylase production after 48 hours of incubation period at 37°C. The maximum amylase production was observed at pH 7.0 (263±4.1 U/ml) and minimum amount of amylase production was recorded at pH 10 (85±2 U/ml).

Effect of Temperature on Amylase Production: Table 5 shows the effect of various temperatures on amylase production. The maximum amylase production was obtained at 40°C (152±1.9 U/ml). Followed by this, 50°C temperature (117±1.8 U/ml) was the second best temperature on amylase production. On the other hand, the minimum amount of amylase production was observed at temperature 80°C (27±0.7 U/ml).

Effect of Surfactants on Amylase Production: Table 6 shows the effect of various surfactants on amylase production after 48 hours of incubation at 50°C. The maximum amount of enzyme was recorded in Tween-80 (165±2.8 U/ml) and minimum amount of amylase was observed in PEG (90±1.0 U/ml).

Effect of Incubation Time on Amylase Production: Table 7 illustrates the effect of different incubation times on amylase production. The maximum amount of amylase production was observed with 48 hours incubation time (172.01±0.56 U/ml). The minimum amount of amylase production was obtained with 120 hours incubation (99.15±1.24 U/ml).

Effect of Various Inoculum Sizes on Amylase Production: In the present study, the initial inoculum level has played an important role in amylase production by *Bacillus cereus*. The maximum amylase specific activity was registered at the 5% (165± 2.8U/ml) of inoculum level. On the other hand, the minimum amount of amylase production was observed at 2% of (51±2.8U/ml) inoculum level (Table 8).

DISCUSSION

The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. In our present study, the influence of maltose was more (216±2.6 U/ml) than the other carbon sources tested. Galactose was the second best supplementary carbon source (161±1.5 U/ml). Glucose gave the lowest amylase enzyme activity (44±1.0 U/ml). Rao and Sathyanarayana [16] reported that the different carbon sources have varied influence on the production of extracellular enzymes especially amylase strains. Ertan *et al.* [17] reported high amylase production by *Penicillium chrysogenum* under solid state fermentation in wheat bran supplied with galactose. These results are similar to the findings of Heseltine *et al.* [18] who observed that glucose represses the production of amylase in the hyperthermophilic archaeon *Sulfolobus solfataricus.* According to them glucose prevented amylase gene expression and not merely secretion of performed enzyme.

In the present study, ammonium sulphate was found to be the most suitable inorganic nitrogen source for *Bacillus cereus* and the enzyme activity observed was 70±3.0 U/ml. The lowest amylase production was observed in sodium nitrate (47±1.2 U/ml) supplied medium. Ramachandran *et al.* [19] reported that ammonium salts enhanced the enzyme activity. Sodium nitrate showed a negative influence, showing a steep decrease in α-amylase activity. Pederson and Nielson [20] also reported that nitrate was inferior to ammonia in α-amylase production. Ammonium sulfate, sodium nitrate and ammonium nitrate (inorganic nitrogen sources) inhibited the enzyme production by *P. chrysogenum* under SSF.

The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we used might stimulate or down modulate the production of enzymes. In the present study experiment on the effect of supplementary nitrogen sources on amylase production under SSF, showed that yeast extract was found to be a better nitrogen source for this isolate (163±3.6 U/ml).

Yeast extract is the best nitrogen source for amylase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components [21, 22] where it was found that the amylase production by *Aspergillus oryzae* under SSF of sugar cane bagasse was greatly influenced by organic nitrogen sources especially yeast extract. The amylase production by *A. oryzae* was also reported as high in yeast extract and casein [20]. Ramachandran *et al.* [19] reported that peptone gave an increase in enzyme yield in SSF using coconut oil cake as substrate. Yeast extract and peptone is favored for the growth and synthesis of amylase for *Bacillus sp*. [23].

The effect of initial pH on SSF of amylase showed that the pH range of 5-7 produced more amount of amylase and it was relatively high in pH 7.0 (263±4.1 U/ml) and pH 8 (194±2.3 U/ml). Above this level, the amylase production decreased, because the metabolic activities of microbes are very much responding to pH change.

Ellaiah *et al.* [24] stated that at high pH, the metabolic action of bacterium may be suppressed and thus it inhibits the enzyme production. Perez Roses and Perez

Guerra [22] reported that the amylase produced by *Aspergillus niger* increased with raise in pH to 6.0. A similar range of optimum 7 for amylase production was also noticed in *Bacillus sp.*

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation [25]. In the present study, the effect of temperature on amylase enzyme activity by SSF revealed that 40°C was optimum (152±1.9 U/ml) and at the tested higher temperatures, the enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability [26].

A similar result was reported by Francis *et al.* [27]. In contrast, low temperature values may reduce the metabolism of the microorganism [28] and consequently, the enzyme synthesis. Perez Roses and Perez Guerra [22] reported that the amylase production by *Aspergillus niger* under SSF with sugar cane bagasse has its optimum production at 30°C. Previously 45°C was reported as optimum temperature for amylase production by *Myceliophora thermophila* [29]. Surfactants in the fermentation medium are known to increase secretion of proteins by increasing the cell membrane permeability [30]. In the present study, the addition of Tween-80 increases the amylase production for *Bacillus cereus* (165±2.8U/ml). Arnesen *et al.* [31] reported that the addition of Tween-80 to the fermentation increased α-amylase production by 2 fold in *Thermomyces lanuginosus*. Surfactants such as SDS, cholic acid, Tween, etc, were reported to increase cell permeability, thereby enhancing enzyme yield.

The effect of incubation time on amylase production showed that 48 hours was the optimum duration for maximum amylase enzyme activity (172.01±0.56U/ml). Above this period the amylase enzyme activity started to decrease. This is because, the cells may reach the decline phase and displayed low amylase synthesis [32]. *Bacillus sp*. shows that the amylase production was detected from 48-72 hours and reached maximum activity at 48 hours (85µg/ml) by Prabakaran and Hewitt [33].

Since the carbon source represents the energetic source that is available for the growth of the microorganism, it could be that the enzyme production is growth associated and the presence of starch in the medium stimulated the increased production of the enzyme. Agger *et al.* [34] have reported that starch was the best inducer for α-amylase production in TAI strain of *Aspergillus nidulans* under SSF conditions. Srivastava and Baruah [35] reported that soluble starch has been found as the best substrate for the production of α-amylase by *Bacillus stearothermophilus.*

Inoculum concentration is other important factor that influences the production of metabolites under SSF [28]. An inoculums concentration higher than the optimum value may produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis [36]. On the other hand, lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium. This increases the necessary time to grow to an optimum number to consume the substrate and synthesize the desired product [6].

In the present study, the highest enzyme activity (165±2.8U/ml) was obtained at an inoculums level of 5% by *Bacillus cereus* under SSF. Kunamneni *et al.* [37] reported that the solid state fermentation of wheat bran by *Aspergillus niger,* wherein maximum amylase production was reported at 20% inoculums level. Ramachandran *et al.* [19] reported that *A. oryzae* showed increased enzyme production with the increase in inoculums size from the lowest value of 0.5ml and showed maximum enzyme activity at 2ml inoculums.

The above report stated the evidence for the production of amylase with substrate interactions of bacterial strains with simple and effective manner. More over this study gives us values as well as the microbial wealth of amylase producing bacteria which can be boon for the development of biotechnological processes.

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