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Research Paper

Production and optimization of alpha amylases using banana waste by *Bacillus licheniformis* DS3 under solid state fermentation

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Abstract

Amylases producing 10 bacterial strains were isolated from banana rhizosphere collected from Guntur, Andhra Pradesh, India, thereafter screened for amylase activity. Among them *Bacillus licheniformis* DS3 strain showed maximum zone of clearance in starch hydrolysis test was selected for this study. Identification of *Bacillus licheniformis* DS3 was confirmed by 16S rRNA sequencing analysis. In this context, different agro wastes like Banana peel, Potato peel, Orange peel, Rice husk, Maize straw and Sugarcane bagasse were tested for alpha amylase production. Among them banana peel containing the medium was found to be best for maximum amylase production. The various process parameters were optimized for Alpha amylases production including incubation period, temperature, pH concentrations and addition of different carbon and nitrogen sources. The strain *B. licheniformis* DS3 produced maximum amount of amylase after 48 h of incubation, 40º C temperatures at pH 7.0 with banana peel as the substrate. Considerable enzyme production (1140 U/ml) was observed by using banana peel as substrate. Addition of carbon and nitrogen sources in the banana peel medium greatly influenced the amylase the production. Maximum production was observed in starch (1430 U/ml) and yeast extract (1330 U/ml) used as carbon and nitrogen sources.

Keywords: Amylase, *Bacillus licheniformis,* Solid state fermentation, Banana peel.

Introduction

Amylases are most important enzymes in the present day taking approximately 25 to 30% in world enzyme market. Amylases have been reported to occur in many microorganisms, and are also found in plants and animals. There are two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. Amylases were divided into two categories, endo amylases and exo amylases. Alpha amylase ranks first in terms of commercial exploitation like textile industry, baking industry, brewing industry[1]. Microorganism used in α-Amylases and β-amylases production including *Bacillus subtilis, B. cereus, B. polymyxa, B. amyloliquefaciens, B. coagulans, B. subtilis, B. licheniformis, Bacillus steriothermophilu* and *Bacillus megaterium. Bacillus* strains such as *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known as good producers of alpha-amylase for various applications[2].

Bacillus are widely used for production of alpha-amylases and these bacteria need rich source of nutritional medium to achieve, different agro wastes usually considered a waste provide rich source of starch and nutrients for bacteria. The production of Amylase by microorganisms dependent on the fermentation media, either submerged or solid state fermentation. A number of *Bacillus* species reported to be the amylase activity and a few obtained from various agro waste [3-4]. Use of agro wastes like fruit peels banana peels, orange peels and vegetable wastes potato peels, maize straw, Rice husk and sugarcane bagasse for this amylase production is very low cost and easily available.

Recent studies have shown that Solid state fermentation is more efficient than submerged fermentation. Different factors influence the enzyme production including pH of the medium, temperature, incubation period, carbon source and nitrogen source and their concentrations are the main factors that influence greatly the growth and enzyme production[5-6-7].

Several attempts have been made to utilize agro wastes through ensilaging, and to eliminate or reduce the negative nutritional effects. It has been reported that sugars in banana peel contained 14.6% glucose and 56% sucrose [8]. It has also been reported that banana fruit stalk contained 56.8% total sugar, 27.0% starch, 4.65% reducing sugar and 4.3% protein on a dry weight basis, and *Bacillus subtilis* isolated from banana wastes, could produce α-amylase at significant levels compared to other strains recovered from the same source [9]. The average production of banana in Andhra Pradesh is 25.1 tonnes per hectare. Banana is the third most important crop after mango and citrus. Banana is very popular fruit with low price and high nutritive value. After consuming banana, the waste was thrown into garbage. Nearly 25-40% was being wasted while 2% was processed into bio based products and the remaining was used in raw form. However there is no much work on this banana peel waste was used for amylase production. Hence, the present study shows it is an important and inevitable to optimize the conditions for the production of higher level of amylase production in banana waste by solid state fermentation.

Materials and Methods

Isolation of Bacteria

One gram representative soil sample was suspended in 9 ml of sterile distilled water and shaken thoroughly for 10 minutes. Starch degrading microorganisms were isolated from collected samples by the serial dilution plate technique using Starch Agar Media (SAM). Serial dilutions up to 10^{-7} of each sample were prepared by using sterilized water [10]. Sample dilutions were plated (in triplicates) on the above solid medium. Then the plates were incubated at 35º C for 24 to 48 hours. Then the plates were flooded with 1% iodine reagent for 10 minutes. Colonies with good colourless halos around them were picked and maintained on starch agar slants at 4º C and further assessed for enzyme production in liquid medium. The preliminary characterization and identification of the isolate was made following Bergey's Manual of Determinative Bacteriology [11].

Preparation of substrates

Agro wastes like Banana peel, Orange peel, Sugarcane bagasse, Maize straw, Rice husk and Potato peel used as substrates was obtained from fruit market, Guntur, Andhra Pradesh. Substrates were chopped into small pieces. These pieces was spread on trays and oven dried at 70°C for 24 hours. After this was grind in mixer grinder, and stored in polythene bags at room temperature (25 ± 1°C).

Preparation of inoculum

The spores of *Bacillus licheniformis* DS 3 from pure culture were transferred aseptically to a 500 ml conical flask containing (100 ml/ g) of pre-sterilized inoculum medium containing 2.0 glucose, 0.3 yeast extract, 0.5 peptone, 1.5, NaCl, 1.1, $Na_2HPO_4.2H_2O$, 0.61, $NaH_2PO_4.2H_2O$, 0.3 KCl and 0.01 $MgSO_4.7H_2O$ in laminar air flow. The flasks were kept on rotatary shaker 200 rpm at 37°C for 24 h. The homogenous spore suspension (10^6-10^7 spores/ml) was used as inoculum.

Solid state fermentation

All the treatments were run in triplicate. The pH of the fresh chopped banana peel (70% moisture) was adjusted to pH 7 using 0.1-N hydrochloric acid (HCl) /0.1-N sodium hydroxide (NaOH) and sterilized in autoclave for 15 min at 121°C. After cooling, inoculum (5 ml) was added to each flask in the laminar chamber with the help of sterilized pipette. The flasks were then incubated at 35°C for 24 h without shaking in incubator. The SSF media flasks were gently shaken after every 12 h for uniform mixing of the substrate and microorganism.

Amylase extraction after solid state fermentation

Different substrates were mixed with 25 ml of ice cold phosphate buffer (20 mM, pH=7.0) for 30 minutes at 4°C in a rotary shaker at 200 rpm. The supernatant has been collected followed by centrifugation at 10,000 rpm for 15 min at 4°C and used for amylase assay.

Amylase assay

Alpha amylase activity of the extract is measured by DNS method[12]. In brief the reaction mixture containing 1% soluble starch, 20 mM phosphate buffer (pH=7) and fermented extract is taken and incubates at 37°C for 20 minutes followed by the addition of 3,5-Dinitrosalicylic Acid (DNS). The amount of the reducing sugar liberated during assay is estimated by measuring color development at 540 nm by using spectrophotometer. 1 Unit of amylase activity is defined as the amount of enzyme that liberates 1 micromole (µm) of maltose per minute under standard assay condition.

Amount of reducing sugar = Absorbance at 540 nm/ Slope of maltose standard

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Amount of reducing sugar x 1000}}{\text{Molecular weight of maltose x time}}$$

Optimization studies for amylase production

For the amylase production used as banana peel medium, under solid state fermentation various parameters influence including incubation period, effect of pH, effect of temperature, carbon and nitrogen sources. Composition of banana peel medium (Grams/100 ml): Banana peel- 8.0, Peptone-0.02, $MgSO_4$. $7H_2O$ -0.4, CaCl2. $2H_2O$- 0.1, KH_2PO_4 -0.4 and initial pH was adjusted to 7.0.

Effect of incubation period

Incubation period was determined by banana peel medium for amylase productions on different incubation periods (12, 24, 36, 48, 60 and 72 h) were maintained. It was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml banana peel medium and inoculated with 1% standard inoculum ($2.3x\ 10^{-6}$) for the tested bacterial isolate which incubated at 35º C on rotatary shaker at 200 rpm and further assayed for enzyme activity.

Effect of pH

8 % banana peel was used as a substrate. Substrate solution was prepared in sodium phosphate buffer at pH 6, 6.5, 7, 7.5, 8.0 and 8.5 in different test tubes. 0.5 ml each of diluted crude enzyme solution was added into buffer tubes. Then the mixture was incubated at room temperature for 15 min, reactions were terminated by adding 1 ml DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling the test tubes at room temperature, final volume was made to 12 ml with distilled water and the activity of enzymes was determined by using the spectrophotometer, absorbance at 540 nm.

Effect of temperature

1.5 ml of substrate (Banana peel medium) was taken into six different test tubes and 2 ml of phosphate buffer pH 7.0 was added in each test tubes. Tubes were marked with different temperature ranges 25, 30, 35, 40, 45 and 50º C. 0.5ml of diluted enzyme solution was added in each tube. Then tubes were incubated at specific temperature for 10 minutes. Reactions were terminated by adding 1 ml DNS reagent and the mixture incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12 ml with distilled water and the activity of enzymes were determined by using spectrophotometer at 540 nm.

Effect of carbon source

Different carbon sources were added to banana peel media at equivalent weight (1%). Various sources of carbon such as soluble starch, arabinose, fructose, maltose, glucose, lactose and sucrose were supplemented in growth media. Thereafter, amylase production was investigated. The inoculum was added in the medium and incubated at 35 °C for 48 hours under 200 rpm at room temperature. The activity of enzymes was determined by using spectrophotometer at 540 nm.

Effect of nitrogen source

The supplementation of additional nitrogen sources (either organic or inorganic) such as Ammonium sulphate, Beef extract, Peptone, Potassium chloride, Tryptone, L-Aspergine and yeast extract were used to determine the maximum enzyme activity. Therefore the amylase activity was tested by using spectrophotometer at 540 nm.

Statistical analysis

Three replicates were maintained for each treatment. Statistical analysis of the data was performed by using SPSS software (version 20). ANOVA two way and Duncan's multiple test was carried out and the results were considered to be significant at $P<0.05$.

Results and Discussion

For Solid State Fermentation several agro industrial wastes like Banana peel, Potato peel, Rice husk, Orange peel, Sugarcane bagasse and Maize straw were used as substrates. All the six were found to be best substrates as the α-amylase production. Whereas the enzyme production was maximum in banana peel was used as substrate and it found to be 1140 U/ml followed by Rice husk 1080 U/ml, Orange peel 970 U/ml, Potato peel 800 U/ml, Sugarcane bagasse 900 U/ml and Maize straw 750 U/ml (Table-1). Saxena *et al* [13] reported the amylase production with 5400 U/g using solid state fermentation by *Bacillus* species used as agro industrial wastes. Kokab *et al* [14] also reported that the amylase production in *B. subtilis* showed maximum production (9.06 IU/ml) was observed in solid state fermentation, Banana peel used as substrate.

Table 1: Screening of various substrates for the alpha amylase production

Substrates	Amylase production (U/ml)
Banana peel	1140
Potato peel	800
Orange peel	970
Rice husk	1080
Sugarcane bagasse	900
Maize straw	750

The present study for the solid state fermentation banana peel is used as the substrate for amylase production by *B. licheniformis* DS3. Various concentrations (2, 4, 6, 8 and 10 g) of banana peel were studied for α- amylase production. Maximum production was observed at 8% substrate concentration (Table-2). Amylase production was increased with increasing substrate concentration upto 8 grams maximum production 1140 U/ml. Similarly Shinde *et al*., [4] reported that the amylase production on solid state fermentation by wild type and mutant *B. licheniformis* from agro waste (Banana peel) as substrate showed the maximum enzyme activity of 103.4 U/ml. However, Hafeez *et al*., [15] have also reported that the seven substrates screened for solid state fermentation, banana peel showed the enzyme activity of 146.71 U/ml in *B. licheniformis.* The enzyme activity was decreased by the substrate concentration was increased upto 10%. The reduction in enzyme productivity at a high substrate concentration is more likely due to the high viscosity of the medium affecting the availability of oxygen concentration required for the microbial growth was reported by Agger *et al*., [16].

Table 2: Effect of substrate concentration on alpha amylase production

Substrate concentration (gr)	Amylase production U/ml
2	280
4	400
6	790
8	1140
10	890
12	660

Effect of incubation period

Amylase production was started with the initial period of 12 h of incubation period. Different incubation periods (12, 24, 36, 48, 60 and 72) effected the amylase activity and the maximum production (1130 U/ml) was observed in 48 hours. Further increase in incubation period the enzyme production was declined. Optimum incubation time for amylase production by *B. licheniformis* DS3 strain at 48 hours. Paul and Sumathy[17] reported that the production of amylase from banana peels with *B. subtilis* producing 1388.88 U/mi/min in 24 hours of incubation period under solid state fermentation.

Table 3: Effect of incubation period on amylase production

Fermentation periods (hours)	Amylase activity (U/ml)
12	660
24	890
36	1060
48	1130
60	700
72	540

Effect of pH

In our study the amylase production by *B. licheniformis* DS7 was found maximum 1130 U/ml at pH 7.0 (Table 4). Further increase in pH resulted decrease in the activity of amylase. However the pH of the fermentation medium was found to be optimum at 7.0 when pH is altered below or above the optimum activity is decreased. Unakal *et al*., [18] studied the α-amylase production by *Bacillus subtilis* in SSF of banana peel was used as substrate, maximum enzyme activity with 7.12 U/ml at pH 7.0.

Table 4: Effect of pH on amylase production

pH	Amylase activity (U/ml)
4.0	320
5.0	640
6.0	850
7.0	1130
8.0	870
9.0	450

Effect of Temperature

Results showed the effect of different temperatures on the production of amylase by *Bacillus licheniformis* DS7 strain under solid state fermentation. The maximum enzyme production 1140 U/ml was obtained at 40º C (Table 5). Amylase production was also observed in high temperatures at 45º C and 50º C respectively. Similar reports reveal the production of enzyme was greatly inhibited at 40º C, It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and hence enzyme formation was also prohibited[19-20].

Table 5: Effect of temperature on amylase production

Fermentation temperatures (ºC)	Amylase activity (U/ml)
25	660
30	860
35	1070
40	1140
45	700
50	560

Addition of carbon sources on amylase production

Amylase production was observed by using different sugars (1%) was added into the banana peel medium and this medium used as control. Addition of carbon source into the banana peel medium showed much variation (Table 6). Different carbon sources added to the production medium and Maximum enzyme production (1430) was observed in starch was used as carbon source. Relatively maximum enzyme production also observed in maltose and glucose added to the medium with the enzyme production of 1360 U/ml and 1300 U/ml respectively. Lowest enzyme production (1100 U/ml) was observed in sucrose containing the medium. Jadav *et al*.,[21] reported that amylase production 1.55 U/ml in *Bacillus subtilis* under solid state fermentation used as substrate of banana peel. Krishna and Chandrashekharan[22] who optimized solid state flask culture for production of α-amylase by *Bacillus subtilis* using banana stalk as a substrate.

Table 6: Effect of carbon sources on amylase production

Carbon source (1%)	Enzyme activity (U/ml)
Banana peel	1130
Banana peel + Glucose	1300
Banana peel + Sucrose	1100
Banana peel + Maltose	1360
Banana peel + Fructose	1200
Banana peel + Starch	1430
Banana peel + Arabinose	1280

Effect of nitrogen sources on Amylase production

Addition of different nitrogen sources were added into the banana peel medium and the maximum enzyme production (1330 U/ml) was observed in yeast extract containing the medium (Table 7). Less amount of amylases were found to be ammonium sulphate containing the medium. Among the nitrogen sources tested yeast extract medium showed the maximum amylase activity. Yeast extract has also been used in conjunction with other nitrogen sources such as bacto peptone in the case of *Bacillus* sp., ammonium sulphate in the case of *Bacillus subtilis*, ammonium sulphate was reported by Naidu *et al*.,[23].

Table 7: Effect of nitrogen sources on Amylase production

Nitrogen sources (0.5%)	Enzyme activity (U/ml)
Banana peel	1130
Banana peel + Ammonium sulphate	870
Banana peel + Peptone	1300
Banana peel + Tryptone	1260
Banana peel + Beef extract	900
Banana peel + Yeast extract	1330

Conclusion

There is no much information available in the production of amylases by solid state fermentation used as agro wastes like banana peels. The present work has been taken up with a view of exploring the possibilities of using a banana peel agro wastes as a starch substrate for the production of amylases by the tested *B. licheniformis* DS3 in solid state fermentation, which can hydrolyze starch to glucose. For the use of inexpensive substrates can economize the process of production. This strain *Bacillus licheniformis* DS3 was capable to produce the highest amylase enzyme after 48h as well as amylase after 48 h in the medium containing 8% banana peel starch waste. The maximum productivity of α-amylase (1140) was achieved by utilizing banana peel as the solid substrate with starch as an additional carbon source in 48 hours at temperature 40ºC and a pH of 7.0 and banana peel with yeast extract as best nitrogen supplement. At present there is a need for more efficient amylases from bacteria and fungi in various sectors a gaining importance in biopharmaceutical applications.

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Conflict of interest

The authors declare that there is no conflict of interest.

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