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OPTIMIZATION STUDIES ON ALPHA AMYLASE PRODUCTION BY *BACILLUS LICHENIFORMIS* DS3 AND *BACILLUS SUBTILIS* DS7 USING SUBMERGED FERMENTATION

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ABSTRACT

Microbial Amylases have several agricultural, clinical, industrial, and pharmaceutical applications. For the production of α-amylases 10 bacterial strains were isolated from banana field soils, Guntur district of Andhra Pradesh, India. From the preliminary studies, among the 10 strains the two strains DS3 and DS7 showed maximum amylase activity on starch hydrolysis test. Further these two strains *B. licheniformis* DS3 and *B. subtilis* DS7 were identified upto species level by 16 S rRNA sequencing analysis. These two strains were taken for optimization studies by using submerged fermentation. The effect of different incubation periods, pH, temperatures, metal ions, carbon and nitrogen sources was optimized. The amylase production was best in the conditions of 48 h of incubation, pH 7.0 and at 40^0 C temperatures. Maximum production (152 U/ml) was recorded in starch nutrient media. Addition of different carbon sources were added to the Starch broth media. Among them highest amylase productions (242.3 U/ml) and (211.4 U/ml) was observed in arabinose and lactose supplemented medium. Different nitrogen sources peptone, beef extract, ammonium sulphate, potassium chloride, Tryptone, L-Aspargine and yeast extract was assessed and peptone (148.3 U/ml) was found to be the ideal nitrogen source for amylase production. Metal ion $CaCl_2$ influenced the maximum amylase production.

KEYWORDS: Amylase, *Bacillus licheniformis*, *B. subtilis*, Di Nitro Salicylic acid (DNS), Starch Agar Medium (SAM).

1. INTRODUCTION

Microorganisms must be able to catalyse the chemical reactions efficiently. Through the action of regulatory enzymes, a number of metabolic pathways are coordinated to yield a interplay the many activities. Microoganisms are using as biotechnological sources of individually relevant enzymes and are stimulated by the exploration of extracellular enzyme activity. Most prominent enzymes like protease, cellulases and amylase were used in many industries. These enzymes widely utilized in brewing, detergent, textile, and food industries (Doss and Anand, 2012). Among the enzymes amylases are employed in the starch processing industries due to the hydrolytic nature, starch into simple sugars. Amylases were expanded into many fields, such as medicinal, clinical and analytical chemistry (khan and Jadav, 2011).

Alpha amylases were derived from a number of microbial sources such as bacteria and fungi. A number of bio-based products were obtained from Amylases. The huge industrial and market demands in the diversity of microbes as the source material for bio-based products is achieve every day. Also, amylases can be derived from other sources, such as microorganisms, plants and animals, because of the short growth period, biochemical diversity and the ease with which enzyme concentrations can be increased by nutritional, environmental conditions and genetic manipulation makes enzymes from microbial sources much better (Oliveira et al., 2007).

Hydrolytic enzymes like alpha amylase, lipase, Chitinase protease and Cellulases were obtained from microorganisms. However, various reports reveal that the bacillus species are of its ubiquitous nature, non fastidious nutritional requirements and high productivity of alpha amylases were organism choice (Ab*e et al*., 1988 and Zangirolami *et al*., 2002). The hydrolyzed products are widely used and applied in the food, paper, and textile industries (Nigam and Singh, 1955).

Amylase is majorly produced by bacterial species of *Bacillus* (Muralikrishna and Nirmala, 2005), and *B. subtilis* and *B. licheniformis* are among the species that have been widely studied (Nidhi et al., 2005). The most widely used source among *Bacillus* species, *B. amyloliquefaciens, B. megaterium* and *B. licheniformis* are extensively used for commercial production of the enzyme. Other *Bacillus* species which have been explored for production of the enzyme include *B. cereus* and *B. subtilis* to name a few from the available literature. Alpha Amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and

Bacillus amyloliquefaciens show promising potential in a number of industrial applications in processes such as food, fermentation, textiles (used as desizing agent) and paper industries (Konsoula et al., 2007, Coronado *et al*., 2000).

From the available literature *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus amyloliquefaciens* are known to be good producers of α-Amylase. Present study mainly focussed on amylase production by two *Bacillus* species, *B. licheniformis* DS3 and *B. subtilis* DS7. Industrially important enzymes have traditionally been obtained from submerged fermentation, because of the ease of handling and greater control of environmental factors such as incubation period, temperature, pH, metal ions, carbon and nitrogen sources. Hence, the aim of the present study is to isolate, identify and optimization for amylase producing bacterial species obtained from Agricultural field soils, Guntur district of Andhra Pradesh.

2. MATERIALS AND METHODS

2.1 Soil sample collection: Soil samples were collected from various Banana field soils in the vicinity of Guntur, Andhra Pradesh, India. From 3 to 4 cm depth with the help of sterile spatula, collected soil samples were transferred to sterile polythene bags and maintained in aseptic conditions for further studies.

2.2 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 (Sneath, 1986). Sample dilutions were plated (in triplicates) on the above solid medium. Then the plates were incubated at 35^0 C for 24 to 48 hours. After 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 4^0 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 (Holt *et al*., 1994). The Starch Agar medium used for the isolation of bacteria contained (Grams/Litre): Starch , 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 7.0.

2.3 Amylase production: For the production of amylase enzyme, starch broth medium initial pH was adjusted at 7.0 and 100 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker at 200 rpm at 45^0C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 x g for 10 minutes and cell free culture supernatant fluid used as enzyme source (crude enzyme).

2.4 Enzyme Assay: One ml of crude enzyme supernatant was taken in test tube and 1.0 ml of substrate (starch solution) was added in test tube. The test tubes were covered and incubate at 35°C for 15 minutes in water bath. Then 2.0 ml of DNS reagent was added in each test tube and the reaction was stopped by boiling the reaction mixture in water bath for 10 minutes. After cooling the test tubes at room temperature [RT], the absorbance (O.D) was measured at 540 nm by using spectrophotometer and the released sugar was determined from maltose standard curve (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme that released 1µmol reducing sugar equivalent to maltose per minute under the standard assay condition.

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

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

2.5 Optimization conditions for Alpha Amylase production

i). Effect of incubation period: Incubation period was determined by starch broth medium for amylase production on different incubation periods (12, 24, 36, 48, 60 and 72 h). It was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile starch broth medium and inoculated with 1% standard inoculum ($2.3x\ 10^{-6}$) for the tested bacterial isolate which incubated at 35^0 C on rotatary shaker at 200 rpm and further assayed for enzyme activity.

ii). Effect of pH: 1% Starch 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.

iii). Effect of temperature: 1.5 ml of substrate 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 50^0 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.

iv). Effect of carbon source: Different carbon sources were added to Starch broth 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.

v). 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.

vi). Effect of Metal ions

The effect of metal salts on α- amylase production was studied by adding different metal salts like $CaCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $CuSO_4$ and in the medium at 0, 0.1, 0.2, 0.3, 0.4 and 0.5% concentration. It was carried out in 250 ml of Erlenmeyer flasks containing 100 ml of sterile starch broth media and incubated the standard inoculum added. Production conditions were observed at 48 h of incubation, pH 7.0, and 37^0 C temperature.

3. 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 $P<0.05$.

4. RESULTS AND DISCUSSION

Ten out of 23 bacterial isolates possessed amylolytic activity on Starch Agar media. Among the 10 bacterial isolates, 2 were found to be higher amylase production on starch hydrolysis test. Based on morphological, cultural and biochemical characterization of the suspected colonies were identified as *Bacillus* species by following the Bergey's Manual of determinative bacteriology (Holt *et al*., 1994). We previously reported that the morphological cultural and biochemical characterization of *Bacillus* species (Silpa *et al*., 2018). The two strains *B. licheniformis* DS3 and *Bacillus subtilis* DS7 was identified by 16 S rRNA sequencing analysis, and the sequences were deposited in Gen bank (NCBI). Further these two strains were optimized and various physical parameters were affected by amylase production.

4.1 Effect of incubation period

The results of the incubation time reveal the enzyme activity of two isolated strains *B. licheniformis* DS3 and *B. subtilis* DS7. Samples were collected at regular intervals of 12 h periods. An increase in enzyme production from 12 h to 48 h and on further incubation the amylase production declined indicating 48 h as optimal incubation period for the isolated strains (Table- 1). The amylase activity by the isolated *Bacillus licheniformis* DS3 strain was observed at 48 h and the maximum amylase activity obtained was 88.3 U/ml. Similarly, Bole *et al.,* (2013) and Vishnu *et al.,* (2014) reported that the highest amylase production after 48h and 72h of incubation of *Bacillus* sp. and *Bacillus* sp. VS04, respectively. Nisha Kumari *et al*., (2017) studied the maximum amylase production occurs in *Bacillus cereus* after 36 h of incubation with the yield of 146.52 U. Gangadhran (2006) have also reported that the *B. amyloliquefaciens* showed maximum enzyme production at 72 h of incubation time.

Table. 1. Effect of incubation period on Amylase production by *Bacillus* sp.

Incubation period (Hours)	Amylase production (U/ml)	
	***Bacillus subtilis* DS 7**	***Bacillus licheniformis* DS 3**
12	21.3	29.6
24	32.3	40.3
36	59.3	49
48	71.6	88.3
60	53	59
72	34.3	38.6

*The overall model is significant, Data represent mean ± S.D: P<0.5.

4.2 Effect of pH

Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity. Amylase activity was observed in different pH levels (6.0, 6.5, 7.0, 7.5, 8.0, and 8.5). Optimum pH of 7.0 with a maximum enzyme activity (87.3 U/ml) was in *B. licheniformis* DS3. These results are shown in (Table-2). *Bacillus* species showed maximum amylase enzyme activity (11.0 U/ml) at pH 7.0 (Vidyalakshmi *et al*., 2009). Similarly Sankarlingam *et al*., (2012) reported that the *B. licheniformis* showed the maximum amylase activity at pH 7.0 under submerged fermentation. The enzyme activity was decreased at pH below 6.5 and above 7.0. Alpha amylase production was inactive in the acidic medium (Castro *et al*., 1993).

Table. 2. Effect of pH on Amylase production by *Bacillus* sp.

pH	Amylase production (U/ml)	
	***Bacillus subtilis* DS 7**	***Bacillus licheniformis* DS 3**
6.0	20	27
6.5	30.6	41.3
7.0	76.3	87.3
7.5	63.3	80.6
8.0	32.6	39.3
8.5	16.3	20.3

*The overall model is significant, Data represent mean ± S.D: P<0.5.

4.3 Effect of temperature

The isolated *B. licheniformis* DS3 and *B. subtilis* DS7 strains was tested in a wide range of temperatures ranging from 25^0C to 50^0C. In the present experiment with increase in temperature enzyme production increased up to a certain level (40^0 C) and upon further increase of temperature, production decreased. The results indicated that the *B. licheniformis* DS3 had potential to grow in all the tested incubation temperatures and produced amylase enzyme. The maximum growth was observed at 40^o C with amylase production of (93 U/ml) at 48 h as shown in (Table- 3). Kiran *et al*., 2005 reported that the enzyme synthesis by *Bacillus* species with an optimum temperature of 42^0C. Vidyalakshmi et al., (2009) also reported that the amylase production by *Bacillus* species showed maximum enzyme production at 35^0 C. Optimum temperature was observed for the production of alpha amylase by *B. subtilis* was also reported by Krishnan and chandrasekharan (1996).

Table. 3. Effect of temperature on Amylase production by *Bacillus* sp.

Temperature	Amylase production (U/ml)	
	***Bacillus subtilis* DS 7**	***Bacillus licheniformis* DS 3**
25^0 C	19.3	32
30^0 C	32.3	49
35^0 C	72.3	88.3
40^0 C	82	93
45^0 C	43	53.6
50^0 C	21	29.3

*The overall model is significant, Data represent mean ± S.D: P<0.5.

4.4 Effect of carbon sources

Several carbon substrates like Glucose, Arabinose, Fructose, Starch, Maltose, Lactose, and Sucrose were tested to evaluate the enzyme production by submerged fermentation. On supplementation of various carbon substrates maximum enzyme production was exhibited by Starch (1% w/v). Results showed different impact on enzyme production with different substrates. The maximum enzyme production obtained was 152 U/ml with 1% w/v Starch. Arabinose and Lactose when supplemented as additional carbon substrate to the medium has resulted in enhanced enzyme production of 242.3 U/ml and 211.4 U/ml respectively.

Various carbon sources effect of enzyme production by the isolated strains *B. licheniformis* DS3 and *B.subtilis* DS7 strains are represented in (Table- 4). This observation was supported by earlier studies of Anto *et al*., (2000) reported that the production of Amylase was activated by the presence of glucose, lactose and starch by *Bacillus* species. Bacillus sp. K-12 showed the maximum enzyme production at 1% starch was used as carbon source (Kiran *et al*., 2005). Aqueel and Umar (2010). Studied the effect of carbon source on alpha amylase by *Bacillus megaterium* showed maximum enzyme activity was obtained on 0.5% dextrose (1015 U/ml).

Table. 4. Effect of carbon sources on Amylase production by *Bacillus* sp.

Carbon sources (1%)	Amylase production (U/ml)	
	***Bacillus subtilis* DS 7**	***Bacillus licheniformis* DS 3**
Starch	123.3	152
Glucose	176.6	183.3
Maltose	181.6	200.3
Lactose	175.6	211.4
Arabinose	184.3	212.3
Fructose	192	78.6
Sucrose	210.3	127.3

*The overall model is significant, Data represent mean ± S.D: P<0.5.

4.5 Effect of nitrogen sources

Various nitrogen sources like Ammonium sulphate, Beef extract, Peptone, Potassium nitrate, Tryptone, L-Aspargine and yeast extract were tested to evaluate the enzyme production by submerged fermentation. On supplementation of different organic and inorganic nitrogen substrates maximum enzyme production (148.3) was exhibited by Peptone (0.5% w/v). Addition of Yeast extract and Beef extract enhanced the enzyme activity of 211.3 U/ml and 202.3 U/ml respectively. (Table-5). Lowest enzyme production was observed in tryptone containing the medium. Qader *et al.*, (2006) observed that the amylase production was maximum when yeast extract was used as a nitrogen source in *Bacillus* sp. AS-1. Presence of 1.5% nitrogen source peptone was utilised by *B. megaterium* showed the maximum enzyme activity Aqueel and Umar (2010).

Table. 5. Effect of nitrogen sources on Amylase production by *Bacillus* sp.

Nitrogen sources (0.5%)	Amylase production (U/ml)	
	***Bacillus subtilis* DS 7**	***Bacillus licheniformis* DS 3**
Peptone	116	148.3
Ammonium sulphate	172.3	194
Beef extract	192.6	202.3
Potassium chloride	178.6	185
Tryptone	174.6	170
L-Aspargine	173.3	187
Yeast extract	203	211.3

*The overall model is significant, Data represent mean ± S.D: P<0.5.

4.6 Effect of metal ions

Metal ions were considered to be the best ions for optimum growth of the bacteria as well as best inducer for amylase production. Different metal ions $CaCl_2$, $MgSo_4$, $MnSo_4$, $FeSo_4$ and $CuSo_4$ at 04% were studied for enzyme production (Table-6). Among them *B. licheniformis* DS3 in presence of $CaCl_2$ enhanced the enzyme production of (154.8 U/ml). Next to $CaCl_2$, maximum enzyme production was observed in $MgSo_4$ (128.4 U/ml) followed by $CuSo_4$ (104.2 U/ml) $MnSo_4$, (100.2 U/ml) and $FeSo_4$ (87.9 U/ml). These results are shown in (Table-6). Sharma and Vamil (2012) reported that the different heavy metals by *B. amyloliquefaciens* showed maximum enzyme activity in the presence of Ca^{2+} at 0.4% concentration.

Table. 6. Effect of metal ions on Amylase production by *Bacillus* sp.

Metal ions	Amylase production (U/ml)	
	***Bacillus subtilis* DS 7**	***Bacillus licheniformis* DS 3**
$CaCl_2$	126.6	154.8
$MgSO_4$	104	128.4
$MnSO_4$	76	100.2
Fe SO_4	102.3	87.9
Cu SO_4	99.3	104.2

*The overall model is significant, Data represent mean ± S.D: P<0.5.

5. CONCLUSION

From the results optimum conditions for maximum enzyme activity occurs in, incubation period 48 hours and pH 7.0 at 40^0C temperature, showed by bacillus species under submerged fermentation. Starch and Peptone was suitable carbon and nitrogen sources for maximum amylase activity. The present two strains *B. licheniformis* DS3 and *B. subtilis* DS7 showed the amylase production. When we compare the amylase enzyme production between these two strains, maximum amylase production was recorded by *B. licheniformis* DS3 and this strain may useful for further studies.

6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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