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ISOLATION, CHARACTERIZATION AND OPTIMIZATION OF AMYLASE ENZYME ACTIVITIES USING SUBMERGED FERMENTATION FROM *BACILLUS SP.*

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ABSTRACT
Amylase is an important digestive enzyme used in medical studies and has several industrial and commercial applications. Due to the enormous importance of this enzyme, this study was focused on optimization of culture conditions favoring the maximum enzyme production by *Bacillus sp.* using submerged fermentation. The optimum conditions for enzyme production were standardized using various parameters such as incubation period, pH and temperature. The production of enzymes was found maximum (28.42 U/mL) at 120 hrs after incubation. The optimum temperature and pH of the enzyme activity were found to be 40°C and 6 respectively. Supplementation of various carbon and nitrogen sources resulted in marginal increase in α-amylase production. Highest production was observed with starch (29.17 U/mL) and ammonium sulphate (21.90 U/mL), while amongst the minerals, magnesium sulphate (1mM) was found to induce the maximum production of amylase (37.33 U/mL).

INTRODUCTION

Amylases are the hydrolytic enzymes that catalyze the hydrolysis of starch into sugars by cleaving the α-1, 4 glucosidal linkage of complex polysaccharides. Amylases are present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Different forms of amylases like α-amylase (EC 3.2.1.1), α-amylase (EC 3.2.1.2) and α-Amylase (EC 3.2.1.3) Both α-amylase and β-amylase work on α-1,4 glycosidic linkage while γ-Amylase cleaves α-1,6 glycosidic linkages as well as the last α-1,4 glycosidic linkages. During the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit. Amylases are obtained from various origins like plant, animal, bacterial and fungal. Several researchers produced amylase enzyme using *Bacillus sp.* [Dharani, 2004; Zambare, 2010]. Amylases are the most important enzymes and have the great significance for biotechnology, constituting a class of industrial enzymes having approximately 25-30% of the world enzyme market [Azad *et al.*, 2009; Rajagopalan and Krishnan, 2008]. In the present day scenario, amylases have a great commercial value in biotechnological applications ranging from food, textile, paper and pulp, pharmaceuticals, baking and beverages, detergent and leather industries [Reddy *et al.*, 2003; Kar *et al.*, 2010].

Amylases production from several bacterial strains, have been reported through the use of submerged fermentation as well as solid state fermentation [Andualem, 2014; Teodoro and martins, 2000]. Optimization of various parameters and manipulation of media are one of the most important techniques used for the overproduction of enzymes in large quantities to meet industrial demands [Tanyildizi *et al.*, 2005]. A number of researchers have been reported findings to optimize culture condition for amylase production [Andualem, 2014; Kanimozi *et al.*, 2014; Sexena *et al.*, 2007]. The physical and chemical parameters like incubation period, pH, temperature, carbon source, nitrogen source, minerals *etc.* of microbial fermentation process play great role in enzyme production.

The *Bacillus sp.* isolated from soil is considered as an ideal source for the production of bulk extracellular amylase for industrial applications [Riaz *et al.*, *2003*]. Therefore, the aim was to evaluate extracellular amylase by *Bacillus sp.* under

various culture parameters in an attempt to standardize an industrially applicable *Bacillus sp.* as a source of amylase and optimize its extracellular amylase secretion conditions.

MATERIALS AND METHODS

Study area and collection of sample

The study area was peri-urban area around Meerut Institute of Engineering and Technology located on NH-58 Meerut-Delhi Bypass, Meerut and Uttar Pradesh, India. The soil sample was collected from M.I.E.T. ground during the period of November, 2007.

Microorganism and screening conditions

Bacillus sp. isolated from soil showed true potential in extracellular amylase secretion. The sample was processed using serial dilution and spread plate technique. The *Bacillus* isolates were tested for amylase activity by employing zone clearing technique (Arikan, 2008) using starch agar medium. The inoculated plates were incubated at 37ºC for two days. After incubation, the zone of hydrolysis of starch was detected by flooding the plates with 1 % iodine solution. The development of blue colour indicated the presence of starch, while the areas around the hydrolytic bacteria appeared clear.

Identification of *Bacillus sp.*

The isolates which exhibited clear zone of hydrolysis on starch agar medium was further inoculated into nutrient broth for enrichment and subjected to conventional method of identification. The one isolate was found to be *Bacillus sp.* from the biochemical test results. Biochemical profiling of the positive isolates were listed in Table 1.

Preparation of seed culture

The potent *Bacillus isolate* was cultured in starch agar plates at 37ºC to obtain better colony of each isolate. Fifty milliliter of inoculums medium containing nutrient broth 13 g/L, pH 7.4 ± 0.2 was transferred to a 100 mL conical flask and cotton plugged. It was sterilized in an Autoclave at 15 lbs/inch2 pressure at 121ºC for 20 min. After cooling to room temperature, Single colonies from each plate were aseptically transferred to it. The flask was incubated overnight at 37ºC and 150 rpm in a rotary shaking incubator.

Production of enzyme in shake flask cultures

The enzyme production was carried out in the basal Asgher *et al.* (2007) medium containing 0.1% KH_2PO_4,0.25% Na_2HPO_4, 0.1% NaCl, 0.2% $(NH_4)_2SO_4$,0.005% $MgSO_4.7H_2O$, 0.005% $CaCl_2$, 0.2% tryptone and 1% soluble starch. 1 mL of 24 hours grown inoculums were cultivated in 250 ml Erlenmeyer flasks containing 100 mL of medium with an initial pH 7.0. The cultures were shaken at 150 rpm in a orbital shaker incubator at 37ºC. After 5 days, the fermented broth was centrifuged in a refrigerated centrifuge machine at 10000 rpm for 20 minutes at 4ºC and cell free supernatant was used for determining the amylase activity.

Enzyme assay and characterization

Amylase activity was determined according to Gomes *et al.* (2001). 1% soluble starch dissolved in 0.05 M Sodium phosphate buffer pH 7. The reaction mixture containing 1.8 mL substrate solution and 0.2 mL suitably diluted enzyme solution was incubated at 50ºC for 10 min. The reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS). The reducing sugar released was determined by the method of Miller (1959). The absorbance was measured at 540 nm by spectrophotometer. The enzyme activity was calculated from standard curve of glucose. One unit of enzymes activity was defined as the amount of enzyme that releases 1μ mole of reducing sugar as glucose per minute under the standard assay conditions. Stock solution and solution for enzyme assay was prepared from 1% or 1000 mg/100 mL glucose.

Optimization conditions of amylases enzyme

Different *Bacillus* species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain variability. Optimization of the process parameters is needed for improved production of enzyme to make the process cost effective [Vijayalakshmi *et al.*, 2012; Subramaniyam and Vimala 2012; Andualem 2014]. Flasks containing 50 ml basal medium were observed with different parameter like at different incubation period (0 to 168 hrs.), carbon sources (Glucose, maltose, galactose, starch, fructose, sucrose, mannitol, lactose), nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulfate, casein, peptone, tryptone, urea and yeast extract), minerals ($MnCl_2$, $CuSO_4$, $CaCl_2$, $FeSO_4$, $MgSO_4$, $ZnCl_2$, $FeCl_3$, $CoCl_3$) , pH (1-8) and temperature (0-80ºC) respectively.

RESULTS AND DISCUSSION

The genus *Bacillus* produces a large variety of extracellular enzymes, of which amylases are of particularly considerable industrial importance [Swain *et al.*, 2006]. Present study deals with the production condition, optimization and partial characterization of crude extracellular amylase produced by *Bacillus sp.*, was able to hydrolyze starch showing zone of hydrolysis around the colonies on agar medium supplemented with soluble starch (Fig. 1).

Optimization of cultural condition for amylase production

To enhance the production of enzyme, various parameters associated with the production of amylase were studied in the medium used for the enzyme production. Optimization of culture conditions is very important for maximum microbial

Table 1: Biochemical tests for *Bacillus sp.*

S.No.	Biochemical tests	Results
1.	Gram's reactions	Gram positive, rod
2.	Indole test	Positive
3.	Methyl red test	Negative
4.	Voges-proskauer test	Positive
5.	Citrate utilization test	Positive
6.	Starch hydrolysis	Positive
7.	Catalase test	Negative
8.	Oxidase test	Positive
9.	Urease test	Positive
10.	Test for H_2S production	Positive
11	Fermentation tests	
a.	Glucose	Acid
b.	Fructose	Acid
c.	Lactose	Acid
d.	Sucrose	Acid

Table 2: Effects of different culture conditions on amylase activity (U/ml)

Culture condition	Amylase activity (U/ml) (Mean ± SE)	Relative activity (%)	CD at 5%
Incubation period (hours)			
0	0.00±0.00		0.86
24	12.19±0.54		
48	13.41±0.39		
72	14.09±0.16		
96	24.13±0.71		
120	28.42±0.10		
144	13.00±0.50		
168	10.23±0.74		
Carbon source			
Control	18.17±0.25	0	0.66
Galactose	22.71±0.27	24.98	
Glucose	20.77±0.30	14.30	
Starch	29.17±0.49	60.53	
Lactose	21.93±0.73	20.69	
Fructose	21.42±0.43	17.88	
Sucrose	19.20±0.10	5.66	
Maltose	23.99±0.07	32.03	
Mannitol	20.40±0.17	12.27	
Nitrogen source			
Control	11.14±0.12	0	0.66
Casein	12.88±0.28	15.61	
Ammonium nitrate	14.30±0.30	28.36	
Ammonium sulfate	21.90±0.56	96.58	
Ammonium chloride	17.45±0.31	56.64	
Peptone	12.53±0.46	12.47	
Yeast extract	19.13±0.72	71.72	
Urea	15.48±0.20	38.95	
Tryptone	13.70±0.10	22.98	
Mineral			
Control	19.57±0.21	0	0.77
$MnCl_2$	20.43±0.21	4.39	
$CuSO_4$	20.80±0.10	6.28	
$CaCl_2$	32.60±0.56	66.58	
$FeSO_4$	24.37±0.45	24.52	
$MgSO_4$	37.33±0.24	90.75	
$ZnCl_2$	23.23±0.32	18.70	
$FeCl_2$	28.38±0.36	45.01	
$CoCl_2$	21.63±0.84	10.52	
pH			
1	10.88±0.39		0.96
2	12.16±0.37		
3	12.67±0.57		
4	14.23±0.25		
5	21.17±1.04		
6	29.13±0.25		
7	17.39±0.52		
8	13.87±0.23		
Temperature			
0	0.00±0.00		2.84
10	0.00±0.00		
20	14.23±0.25		
30	17.35±0.48		
40	29.73±0.37		
50	24.52±0.19		
60	19.57±4.99		
70	15.43±0.21		
80	0.00±0.00		

growth and enzyme production by microorganisms [Kathiresan and Manivannan, 2006].

Incubation Period

Optimum amylase enzyme activity (28.42 U/mL) was observed at 120 hrs. Followed by 96 hrs. (24.13 U) and the lowest production was at 168 hrs (10.23 U/mL) as depicted in (Table 2 and Fig. 2). The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme

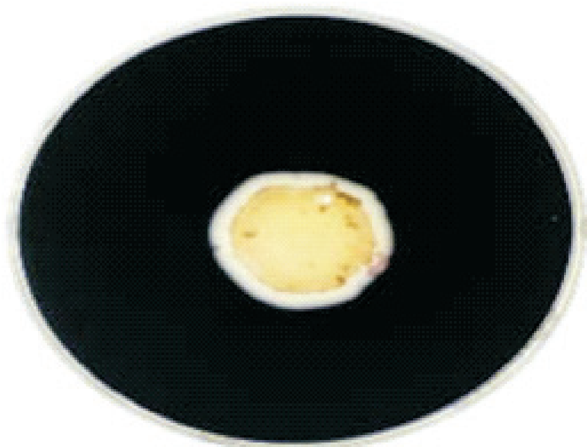

Figure 1: Amylase production on starch agar plate after iodine flooding (grown at 37°C)

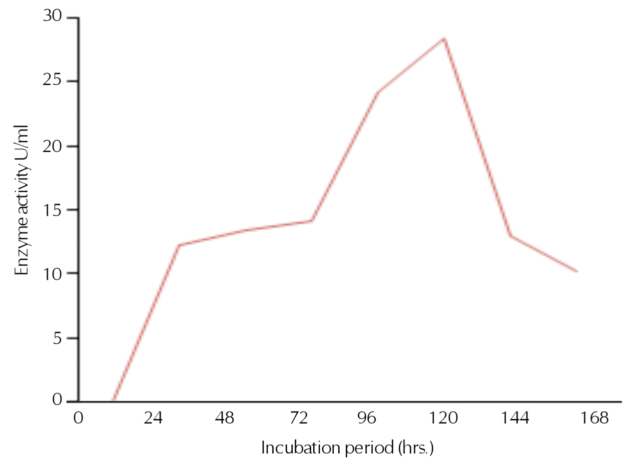

Figure 2: Effect of Incubation period on enzyme activity

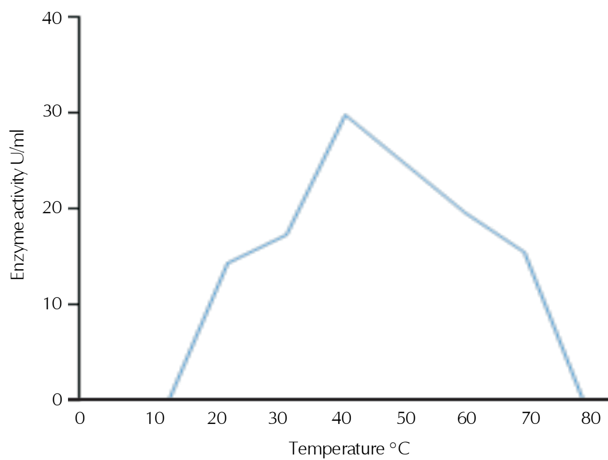

Figure 3: Effect of Temperature on Enzyme Activity

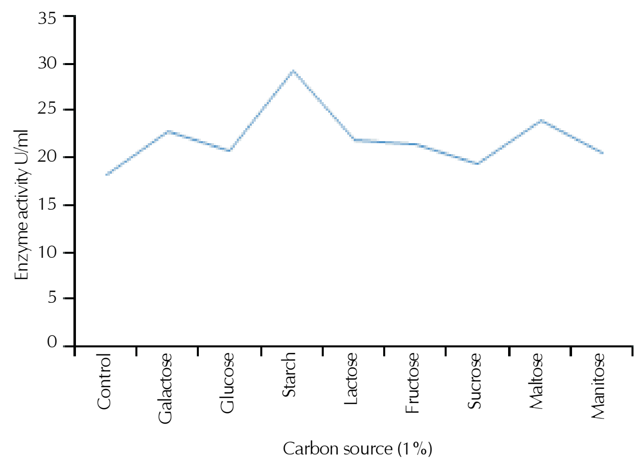

Figure 4: Effect of carbon source on Enzyme Activity

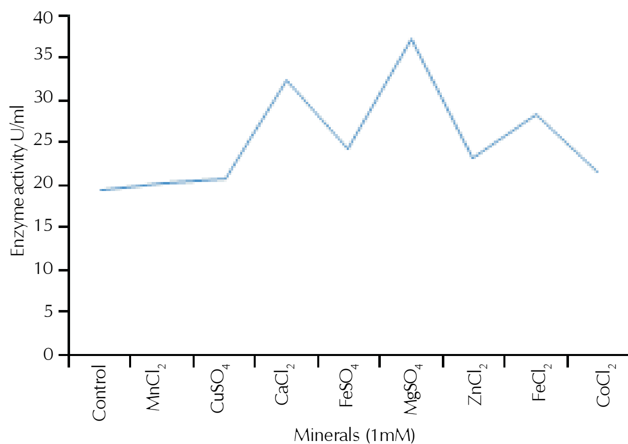

Figure 5: Effects of Minerals on Enzyme Actiity

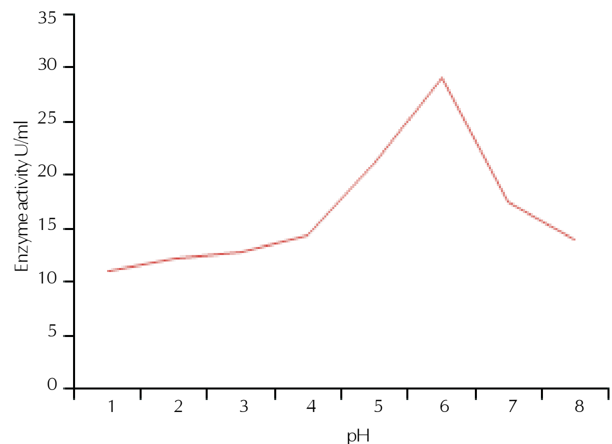

Figure 6: Effect of pH on Enzyme Activity

production. Shafique *et al.* (2009) reported that the maximum α-amylase production was obtained at 72h of incubation for the two strains. Beyond this period, the α-amylase enzyme production started to decrease. It might be due to the deficiency of nutrients, accumulation of toxic substances and proteolysis of α-amylase as interpreted by many workers.

Effect of temperature on amylase activity and stability

The enzyme activity was recorded between temperatures 10°C to 80°C. The optimum temperature for the enzyme reaction

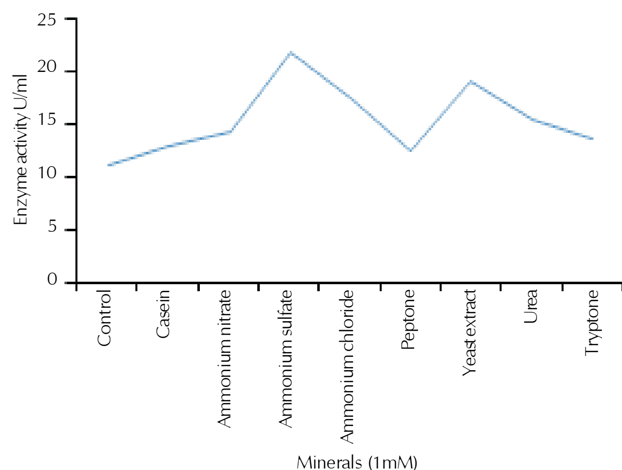

Figure 7: Effect of Nirigen Source on Enzyme Activity

was 40ºC, (Table 2, Fig. 3) at which Maximum amylase activity (29.73 U/mL) was observed and followed by 50ºC (24.52 U/mL) respectively. The amylase production was ceased by strain at low (10ºC) as well as high temperature (80ºC). The similar findings reported by other workers [Nusrat and Rahman, 2007; Kanimozhi, 2014]. Bacterial amylases are produced at a much wider range of temperature 30ºC -50ºC, α-amylase production was optimum at temperature 37ºC by the *Bacillus amyloliquefaciens* [Ashwini *et al.,* 2011].

Effect of sugars

The effect of carbon source on the activity of α-amylase was measured by incorporating 1% of various carbon sources such as starch, maltose, glucose and lactose and sucrose, galactose, fructose, mannitol. Amongst the all carbon sources used, enzyme production was recorded maximum on starch (29.17 U) followed by maltose (23.99) (Fig. 4). The least production of amylase was observed on sucrose and mannitol respectively (19.20 and 20.40 U/mL). These results are similar to the findings of Sexana *et al.* (2007) and Sumrin *et al.* (2011) where α-amylase production was optimum on starch used as the carbon source.

Minerals

Among minerals, $MgSO_4$ (1mM) was found to induce the maximum production of enzyme (37.33 U/ml) followed by $CaCl_2$ (32.60 U) (Fig. 5). Addition of $MnCl_2$, $ZnCl_2$, $FeCl_3$, $CoCl_3$ resulted in decreased amylase level which reached less than 25 U/ml. The least production of amylase was observed in basal medium supplemented with $MnCl_2$ (20.43 U), while in the absence of minerals, amylase production was 19.57 U/ml. In case of *Bacillus cereus* MTCE 1305, Anto *et al.*, (2006) reported that Fe^{+2}; Zn^{+2}, Co^{+2} were not effective in increasing the production of amylase. The findings of Sodhi *et al.* (2005) supported the results where $LiSO_4$ (20 mM) and $MgSO_4$ (1 mM) increased amylase production by *Bacillus sp.* I-3.

Effect of pH on enzyme activity

The production of amylase recorded with change in pH from 1.0 to 8.0. (Fig-6) Maximum amylase level (29.13 U/mL) was observed at pH 6.0 followed by pH 5 (21.17 U/mL). Least production was observed at pH 1.0 (10.88 U/mL). Most of the earlier studies revealed an optimum pH range suitable between 6.0 and 7.0 for the growth of bacterial strains and amylase enzyme production [Gupta *et al.,* 2003; Nusrat and Rahman 2007].

Nitrogen Source

The inorganic source, ammonium sulphate was found to induce the maximum production of amylase (21.9 U/ml) followed by yeast extract (19.13 U/ml). Least amylase production was observed on Peptone (12.53) and Casein (12.88) respectively (Fig. 7). Earlier results of Gangadharan *et al.* (2006) and Saxena *et al.* (2007) revealed that *Bacillus sp.* grew better and produced a high level of amylase in the medium containing ammonium sulphate. However amylase production by *Bacillus sp.* was lower when casein was used as a nitrogen source. It has been reported that yeast extract also served as good organic nitrogen source for α-amylase synthesis from *Bacillus amyloliquefaciens* [Sharma *et al.,* 2012].

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