International Journal of Applied Biotechnology and Biochemistry.
ISSN 2248-9886 Volume 2, Number 4 (2012) pp. 341-354

http://www.ripublication.com/ijabb.htm

Effect of C:N Ratio on Amylase Production by Bacteria Isolated from Soil

Jyoti Mittal and Priya Goel*

Amity Institute of Biotechnology, Amity University,
Sector 125 Noida 201303 Uttar Pradesh, India.
*Email id: jmittal@amity.edu, pgoel3@amity.edu**
*Corresponding author *pgoel3@amity.edu*

Abstract

The nature and amount of **c**arbon and nitrogen sources in culture media play an important role affecting the growth and production of extracellular enzymes by bacteria. In the present study, three new amylase producers B1, B2 and B3 were isolated from soil and identified as *Bacillus* species and gram positive rods. High enzyme titres were obtained after 32 h in starch supplemented media though media without starch also favoured low amounts of enzyme production. It has been found that 1% (w/v) soluble starch and 1% peptone worked best carbon and nitrogen source respectively for amylase production and a C:N ratio of 1:1 (for strain B1 and B3) and 1:0.5 (for strain B2) provided best optimum conditions for amylase production.

Keywords: Bacteria, Soil, Amylase, Carbon, Starch, Nitrogen, C:N ratio

INTRODUCTION

Microorganisms have enormous metabolic diversity, different group are capable of producing different metabolic products depending upon their habitat and genetic makeup. By careful, judicious and rationalized screening and selection of high yielding strains of microorganisms, several products of microbial origin have been produced on industrial scale. One important area where microbes have been used as potential source is the production of desirable enzymes, owing to their high yield and stability in a wide range of temperature and pH (1, 2, 3). It includes amylases, proteases, lipases, esterases, cellulases, xylanases, etc. A number of starch degrading enzymes that are used in production of industrially important products, belongs to Amylase family and comprise about 30% of world's enzymes.

Amylase primarily converts starch into sugars. This enzyme catalyses the random endo-hydrolysis of the α-1, 4 glycosidic bonds between successive glucose units in starch and related substrates, releasing short oligosaccharides, limit dextrins and glucose as end products. Production of extracellular amylase has been demonstrated in bacteria, fungi and yeast. At present, amylases are produced on commercial scale from fungi, bacteria and yeast; Fungal amylases are obtained from *Aspergillus niger* (4) and A. *oryzae* (5, 6) and Bacterial amylases from *Bacillus subtilis* (7, 8), B. *amyloliquifacians* (9, 10) and B. *licheniformis* (2. 11) and Yeast amylases from *Cryptococcus flavus* (12), *Schwanniomyces alluvius, Saccharomyces cereviasiae, S. diastaticus* and *C. albidus* (13). Microbial amylases particularly of bacterial origin are used in different industries including food, fermentation, textile, paper industries and in several other areas like detergent preparation, medicine, laundry, milling and effluent treatment (9, 14).

With advances in biotechnology, the interest and demand for enzymes with novel physiological/physical properties is increasing day by day. Hence, considerable efforts are being put in for discovery of microorganisms from varied environments viz. soil for production of enzymes. As the applications and demand of microbial amylase are increasing, it is necessary to isolate and screen high yielding strains of microbes to meet these demands and also maximize enzyme production from them. Carbon and Nitrogen sources tend to have a large impact on the growth and metabolism of microbes. Hence, manipulating the nature and relative ratio of carbon and nitrogen sources plays an important role in the production of extracellular enzymes (15).

Keeping in mind the above points, the present study was undertaken to isolate new amylase producing strains from Balawala soil in Dehradun district of U.P. The yield was produced in sufficient amounts which were confirmed by percentage blue value and by identification of products obtained by starch hydrolysis at 37°C and pH 7.0. Attempts were made to increase the production of enzyme by optimizing the nature and relative concentration of different carbon and nitrogen sources.

MATERIALS AND METHODS

Sample collection: Soil samples were collected from neighbouring fields of Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences and Research, Balawala, Dehradun city of U.P. Samples were collected from six different localities. 100 grams of soil sample was collected from soil surface to a maximum depth of 10 cm, in sterilized polythene bags from each site and pooled together.

Screening and Isolation of Amylase-producing Bacteria from Sample

Preparation of Soil Suspension: The soil samples were filtered through a sieve (1.8 mm mesh size) to remove the large sized debris or pieces of vegetation. One gram of soil suspension was added to 10 ml of sterilized saline water and mixed well to dispense the microbial flora in saline water. The contents were allowed to sediment and the supernatant was collected in sterilized test tubes. The suspension was further diluted to obtain different dilutions ranging from 10^{-2} to 10^{-6}.

Plating: One ml of each of dilution were spread on starch agar plate having starch as a sole source of carbon and energy plates were incubated at 37°C for 24 h to 48 h. Plates were searched for bacterial colonies showing clear zone of starch hydrolysis around them. Starch hydrolysis was further confirmed by iodine test, which gives blue black colour due to the formation of starch iodine complex.

Starch agar medium: Bacterial strains were isolated by spread plate culture technique on starch agar media with the following composition:

Peptone	:	6.0 g
$MgSO_4.7H_20$	:	0.5 g
NaCl	:	0.5 g
Starch	:	10.0 g
pH	:	7.0
Water	:	1000 ml

Pure Culture Isolations: Colonies showing starch hydrolysis zone around them were picked up with the help of inoculating needle and transferred to the middle of starch agar plate. Plates were incubated at 37±1°C for 24 h. Isolated strains were transferred to starch agar slants and stored as mother working cultures in the culture bank of Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences and Research, Balawala, Dehradun.

Maintenance and Production Medium: The cultures were maintained on agar slants of the following medium composition:

Peptone	:	0.6 g
MgSO4.7H20	:	0.5 g
NaCl	:	0.5 g
Starch	:	10 g
pH	:	7.0
Water	:	1000 ml

Production of Amylase: The culture was harvested from slants in 10 ml of distilled water by pouring 10 ml of sterilized distilled water in the slant in aseptic conditions followed by gentle shaking. The cell suspension was poured in to separate sterilized test tube according to the respective mark. 50 ml of production medium was taken in 250 ml of Ehlrenmeyer flask and inoculated with 1% inoculum of production media, the pH was adjusted to 7.0. The flasks were kept on rotary shaker at 37±1°C for 48-72 h at 180 rpm.

Analytical procedures: The amylase saccharolytic activity was determined by Dinitrosalicylic acid method (16). In order to measure amylase activity and residual sugar, 5 ml of broth from shake flasks after 72 h of incubation was taken and centrifuged at 10000 rpm for 15 minutes. The supernatant was decanted and used to study amylase activity.

Estimation of amylase activity: The clear supernatant of culture obtained at the end of incubation was used as a crude enzyme. The enzyme activity was measured in terms of development of brown colour due to conversion of starch into glucose units.

0.5 ml of 1% soluble starch was taken in clean test tube and 0.2 ml of crude enzyme was added, the contents were thoroughly mixed and the tubes were incubated at 37±1°C for 10 minutes. 1.0 ml of 3,5-Dinitrosalicylic reagent was added to all the tubes, followed by another step of boiling for 10 minutes to develop brown colour. Optical density was measured at 540 nm with UV-VIS spectrophotometric-119, Systronics, against glucose as standard.

The unit of enzyme is defined as the amount of enzyme that catalyses the liberation of 1 µmol of reducing sugar equivalent to D-glucose per minute under the optimal assay conditions.

Calculation: Enzyme units/ml from standard curve was calculated using the following formula:

$$\frac{O.D.of\ unknown}{slope\ of\ curve} X \frac{Test\ sample}{Incubation\ time}$$

Effect of starch on amylase production: Effect of starch on enzyme production on all three selected strains was performed in order to compare the enzyme production with starch- and without starch- containing medium and O.D. was measured at different hours of incubation viz. 4h, 8h, 12h, 28h, 32h.

Effect of different starch concentrations: The effect of different starch concentrations viz. 0.5%, 1%, 1.5%, 2.0% and 3.0% were studied on the rate of enzyme production and analysed by the method given in point 4.

Effect of different carbon (soluble sugar) sources: Effect of starch, mannitol, lactose, dextrose and xylose on the production of enzyme was also studied by incorporating these carbon sources (1% w/v) in the production medium as a sole source of carbon and energy. 50ml of medium was taken in 250 ml of flask after sterilization and inoculated with 1% of cell suspension. The flask was thereafter, incubated at 37±1°C for 72 h on rotary shaker and analysed following the same methods as given in point 4.

Effect of different nitrogen sources: Various inorganic and organic nitrogen sources were studied for their effect on enzyme production. Organic nitrogen sources include peptones, tryptone, casein, yeast extract and Inorganic sources include ammonium oxalate, ammonium sulphate, ammonium chloride, potassium nitrate, ammonium acetate. 1% concentration of each nitrogen source was incorporated separately in basal media and analysed.

Effect of C:N ratio on amylase production: For C:N ratio, potato starch and peptone was used as carbon and nitrogen sources respectively. 1% soluble starch was taken constantly while the amount of peptone was varied to attain the desired C:N

ratio. The activity of amylase was recorded after 72 h. The C:N ratio used were 5.0, 2.0, 1.0, 0.5, 0.3.

Identification of isolates: Morphological characterization of the bacteria isolated was done by the standard gram staining method (17). Biochemical characterization of the isolates was done by the standard oxidase test and IMViC test i.e. Indole test, Methyl Red test, Voges-Proskauer test and Citrate utilization test.

RESULTS

Screening and Isolation of amylase producers: Single colonies of pure culture were obtained serially diluting the soil sample taken from Balawala district. The bacterial colonies were able to grow on starch agar media using starch as the sole source of carbon. Individual colonies were screened for their ability of starch hydrolysis. Around 12 of them were isolated (Plate 1) and their potential was measured by pinpoint inoculation of these strains on the centre of the plate containing starch agar media. Depending upon hydrolysis zone on starch agar media, 3 strains showing highest zone of hydrolysis were selected for further studies and named B1, B2, B3 respectively (Plates 2 and 3).

Plate 1. Different amylase producers isolated from soil

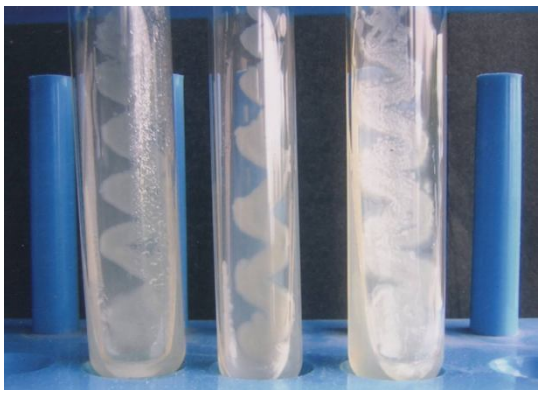

Plate 2. The three selected amylase producers

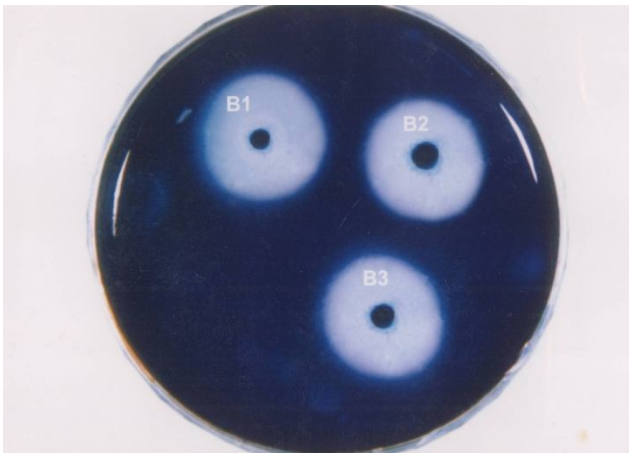

Plate 3. Starch hydrolysis zones by selected strains

Estimation of amylase activity

Effect of starch on amylase production: High enzyme titres were obtained in the starch supplemented production media though some amounts were also detected in media without starch. It was also found that with increase in duration of incubation, enzyme production increases in both with- starch and without- starch containing media upto 32 h for all the three strains (Table 1) after which it starts declining.

Table 1. Effect of Starch on Amylase production (EU/ml) by *Bacillus* sp. (Temperature 37°C, pH 7.0)

Time	Strain 1		Strain 2		Strain 3	
	With starch (1%)	**Without starch**	**With starch (1%)**	**Without starch**	**With starch (1%)**	**Without starch**
4 h	46.8	0.15	20.80	1.25	17.06	2.33
8 h	52.0	3.05	21.90	3.23	26.04	5.20
12 h	75.4	3.95	35.00	9.51	34.12	5.74
24 h	92.8	10.40	197.91	62.40	43.46	8.62
28 h	111.3	16.34	218.00	98..40	143.49	9.87
32 h	**117.2**	**18.31**	**230.90**	**159.80**	**147.44**	**32.50**

*Data represents average of three trials

Effect of different starch concentrations: After incorporation of starch in basal media at the concentration between 0.5 to 3.0%, it was observed that enzyme production was increased from 0.5 to 1.5% using all the three strains. Enzyme production was recorded in decreasing manner at 2.0 and 3.0% starch concentration. Maximum activity was obtained at concentration of 1.0% starch (Figure 3, Table 2). Further increase in the concentration of starch upto 3% resulted in gradual decrease in

the enzyme production. Thus, in the present study, maximum enzyme yield was obtained when starch concentration in the medium was 1%.

Table 2. Effect of different starch concentration on amylase activity by *Bacillus* sp. (Incubation time 72 h, Temperature 37°C, pH 7.0)

Starch concentration (% w/v)	**Strain B1**	**Strain B2**	**Strain B3**
	E.U. (U/ml)	**E.U. (U/ml)**	**E.U. (U/ml)**
0.5	112.06	126.01	163.25
1.0	**168.46**	**233.80**	**181.39**
1.5	140.44	145.40	178.33
2.0	95.00	84.59	131.46
3.0	35.00	58.54	109.50

*Data represents average of three trials

Effect of different carbon sources: In the present investigation, starch was found out to be the best source for amylase production in comparison to others viz. Glucose, Maltose, Mannitol, Lactose, Xylose. Xylose was found to be the second best carbon source for amylase production by strain B1 which is producing 157.68 U/ml of the enzyme, while a very poor carbon source for strain B2 and B3 which was just producing 127.06 and 125.77 U/ml of amylase. However glucose and lactose served as a good source of carbon for all the three strains (Table 3) after incubation of 72 h at 37°C and pH 7.0. With maltose, enzyme production was slightly lesser than Lactose in strains B1 and B3 and slightly higher in strain B2. Data also shows that mannitol, a five carbon sugar was a very poor source of carbon, leading to poor enzyme production, showing similar trends in all the three strains (Figure 4). Out of the three strains, using mannitol as a sole source of carbon, maximum amylase activity was shown in strain B2 with 14.90 U/ml of enzyme.

Table 3. Effect of different carbon source on amylase activity by *Bacillus* sp. (Incubation time 72 h, Temperature 37°C, pH 7.0)

Starch concentration (1%w/v)	**Strain B1**	**Strain B2**	**Strain B3**
	E.U. (U/ml)	**E.U. (U/ml)**	**E.U. (U/ml)**
Glucose	132.09	170.43	164.77
Maltose	86.02	139.81	110.72
Mannitol	10.77	14.90	6.91
Xylose	157.68	127.06	125.77
Lactose	116.73	128.54	121.44
Starch	**184.62**	**181.48**	**189.40**

*Data represents average of three trials

Effect of different nitrogen sources: By using soluble starch with different nitrogen sources, it was observed that more amylase was produced when organic nitrogen compounds were used.

Organic Nitrogen sources: Among different organic nitrogen sources tested, peptone was considered to be the best nitrogen source among tryptone, casein, yeast extract and gelatin (Table 4). Tryptone and Yeast extract also proved to be moderately good source of both amylase production and growth for all the three strains after incubation for 72 h at pH 7.0, temperature 37±1°C (Table 5, Figure 6). Casein and Gelatin showed lower enzyme production as compared to other nitrogen sources which was found to be less as compared to other nitrogen sources.

Table 4. Effect of different organic nitrogen sources on amylase activity by *Bacillus* sp. (Incubation time 72 h, Temperature 37°C, pH 7.0)

Inorganic nitrogen source Concentration (1% w/v)	**Strain B1**	**Strain B2**	**Strain B3**
	E.U. (U/ml)	**E.U. (U/ml)**	**E.U. (U/ml)**
Peptone	**75.25**	**82.25**	**129.48**
Trypyone	64.47	45.61	112.06
Casein	59.26	12.75	88.18
Yeast extract	58.90	55.13	114.22
Gelatin	39.51	16.34	72.37

*Data represents average of three trials

Table 5. Effect of different inorganic nitrogen source on amylase activity by *Bacillus* sp. (Incubation time 72 h, Temperature 37°C, pH 7.0)

Inorganic Nitrogen source Concentration (1% w/v)	**Strain B1**	**Strain B2**	**Strain B3**
	E.U. (U/ml)	**E.U. (U/ml)**	**E.U. (U/ml)**
Ammonium oxalate	29.45	42.92	39.33
Ammonium sulphate	**42.39**	**102.01**	43.82
Ammonium chloride	33.59	**38..25**	**82.25**
Potassium nitrate	29.64	51.10	65.37
Ammonium acetate	20.30	53.51	36.04

*Data represents average of three trials

Inorganic Nitrogen sources: Maximum amylase yield was gained using ammonium sulphate containing medium by strain B1 and B2 (Table 5), while ammonium chloride proved best for strain B3. Least amylase yield was obtained in medium containing ammonium acetate by strain B1 and B3 and ammonium chloride by strain B2. Potassium nitrate and ammonium oxalate gave almost similar results after

incorporation at a concentration of 1% (w/v) in basal medium without any other nitrogen source for B1.

Effect of C:N ratio on amylase production: The production media was optimized using different C:N ratio in order to get maximum yield of amylase using potato soluble starch and peptone as carbon and nitrogen sources respectively. Maximum enzyme yield was obtained when C:N ratio was 1:0 in case of strain B1 and B3 (Table 6) as compared to strain B2, where best optimum C:N ratio is 0.5. Very low C:N ratio i.e. 0.3 and very high i.e. nearly 5 gave less yield of amylase production for all the three strains.

Table 6. Effect of different C:N ratio on amylase activity by *Bacillus* sp. (Incubation time 72 h, Temperature 37°C, pH 7.0)

C:N ratio	Strain B1	Strain B2	Strain B3
	E.U. (U/ml)	E.U. (U/ml)	E.U. (U/ml)
0.3	48.31	51.00	72.55
0.5	49.56	**120.30**	97.52
1.0	**71.10**	102.99	**104.70**
2	36.81	71.65	72.55
5	36.09	29.01	49.02

*Data represents average of three trials

Identification of isolates: Staining, colony morphology and different biochemical tests concluded that all the three isolates B1, B2 and B3 closely resembled *Bacillus* sp. (Table 7). B1, B2 and B3 were Gram positive rods while the cell arrangement was different for all the three strains. Strain B1 is encapsulated and B2 and B3 were capsulated, the isolates were compared for different biochemical attributes and the results were compared with Bergey's manual of systematic Bacteriology (18). On the basis of above morphological and Biochemical tests, isolates B1, B2 and B3 were found to belong to *Bacillus* sp.

Table 7. Biochemical and Morphological Characterization of isolates

Characteristic	Strain B1	Strain B2	Strain B3
Gram reaction	+	+	+
Cell shape	Rods	Rods	Rods
Capsule	-	+	+
Colony morphology	Small, Cream to white in colour with regular margin	Mucoid, cream in colour with regular margin	Mucoid, Large white in colour with regular margin
Cells arrangement	Chains (Streptobacilli)	Cluster	Single

Starch hydrolysis	+	+	+
Indole test	-	+	+
Methyl Red	+	-	+
Voges Proskauer	-	+	-
Citrate	+	+	-
Oxidase	-	-	-

DISCUSSION

With the recent advancements in biotechnology, the application of amylase production has extended to fields of clinical, medical, chemical, analytical applications, as well as food, textile, laundry, bakery and distillery. To meet these demands, more and more production of this extracellular enzyme is required. Hence, it has become necessary to find newer and better options to provide best optimum culture conditions to bacteria, which favours more enzyme production.

In the present study, bacteria were isolated from soil and tried upon with different culture conditions, having varying carbon and nitrogen ratios, to find the C:N ratio which will best support the enzyme production. Amylolytic strains of bacteria hydrolyze the starch present in their surrounding to give clear zone of hydrolysis around them while non-amylolytic ones show blue-black zone around them because of the formation of starch iodine complex. Individual colonies of bacteria colonies grown on starch agar media were screened for their ability of starch hydrolysis and the three strains showing highest zone of hydrolysis were selected for further studies, comparable results have also been reported during isolation of bacteria from soil (1, 2, 19, 20). Starch supplemented the production of enzyme; the results are similar to those conducted on *B. subtilis* (21) and *Bacillus* SPT27 (15), though media containing no starch also supported little enzyme production, as has also been seen earlier in case of *Bacillus* sp. BKL20 (3) and *B. subtilis* (22), which suggests some paraconstitutive production of amylase (23).

Morphological and Biochemical tests concluded that all the three isolates B1, B2 and B3 belonged to *Bacillus* sp. and were gram positive rods.

Increase in duration of incubation increased enzyme production, in both with-starch and without- starch containing media, upto 32 h for all the three strains after which it starts declining (24, 25) though some previous studies have found maximum enzyme production at 72 h incubation by *B. subtilis* (19) and at 120 h by thermostable bacteria isolated from a Himalayan glacier (1).

In the present study, best results were obtained with 1% starch concentration in the culture medium at temperature 37°C and pH 7.0. These findings corroborated with those of previous workers (26, 21, 27, 15). Others, however, have found intensive enzyme production in 0.05% starch by *Bacillus* sp. BKL20 (3) and *Bacillus subtilis* (22) and in 4% starch-containing medium (24). Starch favoured enzyme production better than glucose, maltose, mannitol, lactose, xylose as has also obtained from experiments done on *B. subtilis* (28, 29, 21) and on *Bacillus* sp. (25, 26), though

sucrose has also been reported the best amylase producing medium (15). On the contrary, no effect of the presence of starch in the culture medium has been found on amylase production by *Bacillus* sp. BKL20 (3). Best growth of *Bacillus* sp. has been found in medium containing lactose and poorest in medium readily metabolizable substrates, particularly, monosaccharides such as glucose and fructose (23, 30). This may be related to the latter's catabolic repression action on amylase production. This, infact, prevents the wastage of bacteria's energy by synthesizing extracellular polysaccharide hydrolyzing enzymes required for growth which are already available in the culture medium. Maximum amylase yield by *Azotobacter chroococcum* has earlier been reported in sucrose containing medium and the least in mannitol (31).

Five different organic- and five inorganic nitrogen sources were used for enzyme production and it was observed that greater enzyme productivity was presented when organic nitrogen compounds were used with soluble starch, the same has been found earlier (31, 24), though, inorganic nitrogen sources have also been found as effective as organic ones (19); some studies have found that inorganic nitrogen sources, particularly ammonium salts increased enzyme yield better than organic nitrogen sources (28). Among different organic nitrogen sources tested, peptone was considered the best, which agrees with the studies done on *B. subtilis* (28, 24, 21) and *Bacillus* sp. BKL20 (3), though tryptone (19) and yeast extract have also been found the best for amylase production by *A. chroococcum* (31), by *Bacillus* sp. (29) and by *Microbacterium foliorum* and *Bacillus cereus* (1). Casein and Gelatin showed lower enzyme production as compared to other nitrogen sources which was found to be less as compared to other nitrogen sources. The same pattern was seen earlier in a study done on *Bacillus* SPT 27 (15).

Among various inorganic nitrogen sources, ammonium sulphate proved best for strains B1 and B2 and ammonium chloride for B3; ammonium acetate containing medium gave least results with strains B1 and B3 and ammonium chloride for B2. Previous studies have found varied effects of various sources on amylase yield by different species of bacteria viz. ammonium nitrate gave maximum amylase yield (28), potassium nitrate containing medium gave negligible amylase yield (32), ammonium acetate and ammonium nitrate were proved best nitrogen sources for growth and enzyme production (33), while another study (24) found inhibitory effect of all inorganic sources on amylase production.

Excessively high or low amounts of nitrogen inhibited enzyme production, hence it is essentially required to justify the relative ratio of carbon and nitrogen in the culture medium for best results. Potato soluble starch and peptone were used as carbon and nitrogen sources, respectively, and maximum enzyme yield was obtained when C:N ratio was 1:0 for strain B1 and B3 and 0.5 for B2. Very low or high C:N ratios gave less yield of amylase production for all the three strains. Results are similar to those in studies conducted on *Bacillus* SPT27 (15).

CONCLUSION

The present study investigates the effect of various carbon and nitrogen sources, in terms of their nature and relative amount, on the production of extracellular enzyme

α-amylase by bacteria belonging to Bacillus sp. isolated from soil of northern region of India. 1% starch and 1% peptone proved best for enzyme production when used as sole source of carbon and nitrogen respectively and C:N ratio of 1:1 and 1:0.5 in the culture medium provided best optimum condition for amylase production.

REFERENCES

[1] Kuddus, M., Roohi, S. and Ahmad, I. Z., 2012, Cold-active extracellular a-amylase production from novel bacteria Microbacterium foliorum GA2 and Bacillus cereus GA6 isolated from Gangotri glacier, Western Himalaya, J. Genet. Eng. Biotechnol.., http://dx.doi.org/10.1016/j.jgeb.2012.03.002

[2] Alkando, A. A., and Ibrahim, H. M., 2011, "A potential new isolate for the production of a thermostable extracellular α- amylase," J. Bacteriol. Res., 3 (8), pp. 129-137.

[3] Kubrak, O. I., Storey, J. M., Storey, K.B., and Lushchak, V.I., 2010, "Production and properties of α-amylase from Bacillus sp. BKL20" Can. J. Microbiol., 56, pp.279-288

[4] Djekrif-Dakhmouche, S., Gheribi-Aoulmi, Z., Meraihi, Z., and Bennamoun, L., 2006, "Application of a statistical design to the optimization of culture medium for _-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder," J. Food Process. Eng. 73, pp. 190–197.

[5] Kammoun, R., Naili, B., and Bejar, S., 2008, "Application of a statistical design to the optimization of parameters and culture medium for alphaamylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product)," Bioresour. Technol. 99, pp. 5602-5609.

[6] Francis, F., Sabu, A., Nampoothiri, K. M., Ramachandran, S., Ghosh, S., Szakacs, G., and Pandey, A., 2003, "Use of response surface methodology for optimizing process parameters for the production of _-amylase by *Aspergillus oryzae*,". Biochem. Eng. J. 15, pp. 107–115.

[7] Asgher, M., Asad, M. J., Rahman, S. U., and Legge, R. L., 2007, "A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing," J. Food Process. Eng., 79, pp. 950 - 955.

[8] Baysal, Z., Uyar, F., and Aytekin, C., 2003, "Solid state fermentation for production of _-amylase by a thermotolerant *Bacillus subtilis* from hot spring water", Process Biochem., 38, pp. 1665-1668.

[9] Haq, I., Ali, S., Javed, M. M., Hameed, U., Saleem, A., Adnan F., and Qadeer. M. A., 2010, "Production of alpha amylase from a randomly induced mutant strain of Bacillus Amyloliquefaciens and its application as a desizer in textile industry," Pak. J. Bot., 42(1), pp. 473-484.

[10] Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K.M., Sukumaran, R.K., Pandey, A., 2008, "Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*," Bioresour. Technol. 99, pp. 4597-4602.

[11] Hmidet, N., El-Hadj Ali, N., Haddar, A., Kanoun, S., Alya, S., Nasri, M., 2009, "Alkaline proteases and thermostable _-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive" Biochem. Eng. J. 47, pp. 71–79.

[12] Wanderley, K. J., Torres, F. A., Moraes, L. M., and Ulhoa, C. J., 2004, "Biochemical characterization of alpha-amylase from the yeast *Cryptococcus flavus,*" FEMS Microbiol. Lett., 231, pp. 165-169.

[13] Fossi, B. T., Tavea, F., Jiwoua, C., and Ndjouenkeu, R., 2009, "Screening and phenotypic characterization of thermostable amylases producing yeasts and bacteria strains from some Cameroonian soils," Afr. J. Microbiol. Res.., 3(9), pp. 504-514

[14] De Souza, P. M., and De Oliveira e Magalhães, P., 2010, "Application of microbial _-amylase in industry – A review", Braz. J. Microbiol., 41, pp. 850-861.

[15] Aiyer, D. P. V., 2004, "Effect of C:N ratio on α-amylase production by Bacillus licheniformis SPT 27", Afr. J. Biotechnol., 4, pp. 519-522

[16] Bernfeld, P., 1955, Amylases: α and β. In: Methods In Enzymology, eds, Colowick, S.P., and Kaplan, N.O., Academic Press, New York, pp. 149-158

[17] Mackie, and McCartney, 2008, Practical Medical Microbiology, 14th ed. Churchill Livingstone, Chap. 41, pp. 695-720.

[18] Demirkan, E., 2011 "Production, purification, and characterization of α-amylase by Bacillus subtilis and its mutant derivates," Turk. J. Biol. 35, pp. 705-712

[19] Mishra, S., and Behera, N., 2008, Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes, Afr. J. Biotechnol., 7(18), pp. 3326-3331

[20] El-Banna, T. E., Abd-Aziz, A. A, Abou-Dobara, M. I., Ibrahim, R. I., 2007, "Production and immobilization of alpha-amylase from Bacillus subtilis," Pak. J. Biol. Sci., 10(12), pp. 2039-2047

[21] Konsula, Z., and Liakopoulou-Kyriakides, M., 2004, Hydrolysis of starches by the action of an a-amylase from Bacillus subtilis, Process Biochem., 39, pp. 1745–1749

[22] Suman, S., and Ramesh, K., 2010, "Production of a thermostable extracellular amylase from thermophilic *Bacillus* species" J. Pharm. Sci. Res., 2(2), pp. 149-154

[23] Sudharhsan, S., Senthilkumar, S., and Senthilkumar, K., 2007, "Physical and nutritional factors affecting the production of amylase from species of bacillus isolated from spoiled food waste" Afr. J. Biotechnol., 6(4), pp. 430-435

[24] Varalakshmi, K. N., Kumudini, B. S., Nandini, B. N., Solomon, J. D., Mahesh, B., Suhas, R., Kavitha, A. P., 2008, "Characterization of Alpha Amylase from Bacillus sp.//isolated from paddy seeds," J. Appl. Biosci., 1(2), pp. 46 – 53

[25] Mendu, D. R., Ratnam, B. V. V., Purnima, A., and Ayyanna, C., 2005, "Affinity chromatography of α-amylase from *Bacillus licheniformis,"* Enzyme Microb. Technol., 37, pp. 712-717

[26] Rameshkumar, A., and Sivasudha, T. 2011, "Optimization of Nutritional Constitute for Enhanced Alpha amylase Production Using by Solid State Fermentation Technology," Int. J. Microbiol. Res., 2(2), pp. 143-148

[27] Ashwini, K., Kumar, G., Karthik, L., and Rao, B. K. V., 2011, "Optimization, production and partial purification of extracellular α-amylase from *Bacillus* sp. *Marini*." Arch. Appl. Sci. Res., 3(1), pp. 33-42

[28] Teodoro, S., and Martins, L., 2000, "Culture conditions for the production of thermostable amylase by *Bacillus* sp.," Braz. J. Microbiol., 31, 298-302.

[29] Gutiérrez-Rojas, I., Torres-Geraldo, A. B., Moreno-Sarmiento, N., 2011, "Optimising carbon and nitrogen sources for Azotobacter chroococcum growth," Afr. J. Biotechnol., 10(15), pp. 2951-2958

[30] Aditi, A., Boora, K. S., and Chaudhary, K., 2004, "Production of extracellular a-amylase by thermophilic Bacillus sp.", Asian J of Microbiol. Biotech. Envt.Sci., 3, pp. 391-395.

[31] Ray, R. C., 2004, "Extracellular amylase production by fungi Botrydiplodia theobromae and Rhizopus oryzae grown on cassava starch residue," J. Environ. Biol., 25, pp. 489-495.

[32] Aneja, K. R., 2003, Experiments in Microbiology Plant Pathology and Biotechnology, *New Age International (P) Ltd., Publishers*, New Delhi, Fourth Edition.