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A study on partial purification and characterization of extracellular amylases from *Bacillus subtilis*

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ABSTRACT

Soil collected from kitchen waste decomposing plots of the restaurant, Lucknow was enrinched in laboratory by adding chopped potatoes to enhance the Amylase producing microorganisms. The bacterial isolate isolated from the enriched soil was screened for amylase production, and the isolate showing maximum hydrolysis identified as Bacillus subtilis. The production was carried out by submerged fermentation. Maximum Amylase activity (0.0115 U/ml/min) was obtained at 37°C after 72 h of incubation. The enzyme was purified using ammonium sulphate precipitation and showed a molecular weight of 65Kda by SDS- PAGE. The enzyme was relatively stable between pH 7 to pH 9 and at temperature ranging from 37°C to 50°C. Moreover, activity was enhanced by using metal ions such as Ca^{2+}, Mg^{2+} and Zn^{2+}. The decline in activity was observed by adding SDS and EDTA.

Key Words: Amylase; *Bacillus subtilis;* SDS- PAGE.

INTRODUCTION

Enzymes are among the most important products obtained for human needs through plants, animals and microbial source. Nowadays, the use of enzymes in industrial sector is increasing due to increase of industries especially in food, beverages, textile, leather and paper industries. Amylases are the enzymes that break down starch or glycogen. The Amylases can be derived from several sources such as plants, animals and microbes. The major advantages of using microorganisms for production of Amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzyme of desired characteristics [Vidyalakshmi, R. *et al*., 2009]. Amylases have been derived from several fungi, yeast, bacteria and actinomycetes but members of the genus *Bacillus* are heterogeneous and they are very versatile

in their adaptability to the environment. Amylases have been purified earlier from various *Bacillus species* such as *Bacillus megaterium* [Oyeleke, S. B. *et al*., 2010] from *Bacillus subtilis* [Riaz, N. *et al*., 2003], from *Bacillus licheniformis* SPT 27 [Aiyer, P. V. D., 2004].

The enzyme of Amylase family have a great significance due to its wide area of potential application. Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of starch molecules producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non reducing end successively resulting in short end products [Reddy, N. S. *et al*., 2003]. The production of microbial amylases from bacteria is dependent on type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermostability [Haq, I. *et al*., 2010].

Spectrum of application of amylase has widened in many sectors such as food, textile, baking and detergent industries. Besides its use in the saccharification or liquefication of starch, the enzyme is also used for the warp sizing of textile fibres, the clarification of haze formed in beer or fruit juices and for pretreatment of animal feed to improve digestibility [Nusrat, A and Rahman, S. R., 2008].

This study reports the partial purification and characterization of extracellular amylases from *Bacillus subtilis.*

MATERIALS AND METHODS

Microorganism:
Soil sample was collected from the kitchen waste decomposing plots of Rinku's Restaurant, Gomti Nagar, Lucknow and it was enriched under laboratory conditions by adding chopped potatoes for 1 month. Serial dilution method was performed by adding 0.5 gm of soil to 5 ml sterile saline. A series of dilution of the suspension from 10^{-1} to 10^{-5} were done. 50 µl of the soil suspension were pipetted and lawn into nutrient agar. All the plates were incubated at 37°C for 24 hrs. Nutrient agar supplemented with 1% starch was used for screening of purified culture for amylase production. The isolated pure strains were screened for the production of extracellular amylase production using starch agar as described by [Suman, S and Ramesh, K., 2010] The pure cultures were centrally streaked on starch agar plates and incubated for 3 days at 37°C. After incubation gram's iodine was overlayered on the plates, a clear zone of hydrolysis was observed. The culture showing maximum zone of hydrolysis was selected for further studies and was identified by performing and comparing various staining and biochemical tests according to the Bergey's manual cited in the book of [Aneja, K. R., 2003].

Fermentation Medium:
The inoculum was prepared by inoculating the loopful of strain in to nutrient broth and it was incubated in shaker for 24 hrs. 100µl of this 24 hr old inoculum was transferred aseptically to 100 ml production medium (g/l) containing: peptone 5; yeast extract 5; KH_2PO_4 1; $MgSO_4$ 0.2; $CaCO_3$ 0.2; NaCl 5; starch 10; distilled water 1000 ml and incubated in shaker for 72 hrs. The

effect of metal ions (Ca^{2+}, Mg^{2+} and Zn^{2+}), inhibitors (SDS and EDTA), different temperature and various pH values on the amylase production were also studied.

Purification of Amylases:
After 72 hrs of growth, the culture was centrifuged at 5,000 rpm for 5 min at 4°C and the cell free supernatant was used for used. Ammonium sulphate was added to the supernatant to 70% saturation at 4°C and the mixture was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet so obtained was dissolved in 100mM Tris buffer (pH 7). The enzyme was dialysed in same buffer overnight at 4°C.

Enzyme assay and estimation of proteins:
Activity of enzyme (crude and purified) was assayed with DNS method using starch as substrate in 100mM Tris buffer (pH 7) and the activity was calculated using maltose as standard. Activity of purified enzyme was also assayed at various pH values (5, 7, 9, 11), at different temperatures (4°C, 16°C, 37°C and 50°C). Effect of metal ion such as Ca^{2+}, Mg^{2+} and Zn^{2+} and inhibitors namely SDS and EDTA were also studied on enzyme activity using 0.5% of metal ions and inhibitors. Protein concentration (crude and purified) was measured with Lowry's method using BSA as standard.

Determination of molecular weight:
The molecular weight of purified enzyme was determined by SDS- PAGE using BSA as standard.

RESULT AND DISCUSSION

The bacterial strains (MJR 1101, MJR 1102, MJR 1103, MJR 1104 and MJR 1105) enriched in laboratory were screened for amylase activity (Figure 1 and Table 1).

Table 1: Screening of purified cultures

S.NO.	CULTURE	CHARACTER
1	MJR1101	-
2	**MJR1102**	**+++**
3	MJR1103	+
4	MJR1104	- -
5	MJR1105	++

Table 2: Staining and Biochemical activity of MJR1102

S.NO	TEST	RESULT
1.	Gram Staining	+ve,*Strepto Bacillus*
2.	Endospore Staining	+ve
3.	Catalase Test	+ve
4.	Mannitol Test	+ve
5.	VP Test	+ve

The strain i.e., MJR 1102 showing the maximum zone of hydrolysis was selected for identification. The strain was Gram +ve, Endospore +ve, Catalase +ve, Mannitol +ve and VP +ve (Table 2). The bacterial isolate was identified as *Bacillus subtilis.*

Figure 1: Amylase screening results

This strain was stable at pH between 7 to 9 (Figure 2) and temperature between 37°C and 50°C.

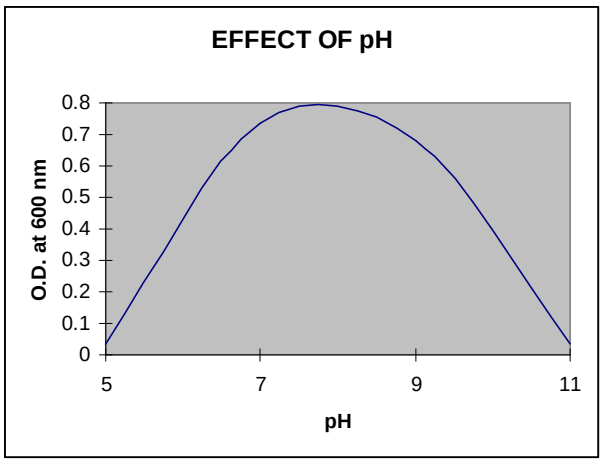

Figure 2: Effect of pH on growth.

The maximum amylase production was obtained after 72 hr of incubation at pH 7 and 37°C.

DNS assay of crude and purified enzyme:

Standard Graph:

In order to get the concentration of enzyme in enzyme sample, a standard graph with known concentration of a standard (Maltose) was plotted as shown in Table 3 and Figure 3

TABLE 3: DNS ASSAY

MALTOSE (in ml)	DISTILLED WATER (in ml)	DNS (in ml)	CONC. OF MALTOSE (mg/ml)		O.D AT 540nm
0.0	1.0	1	0.05	BOIL FOR 15 MINUTES AT 100°C	0.00
0.1	0.9	1	0.10		0.01
0.2	0.8	1	0.15		0.06
0.3	0.7	1	0.2		0.12
0.4	0.6	1	0.25		0.16
0.5	0.5	1	0.3		0.20
0.6	0.4	1	0.35		0.26
0.7	0.3	1	0.4		0.29
0.8	0.2	1	0.45		0.34
0.9	0.1	1	0.5		0.37
1.0	0.0	1	0.55		0.41

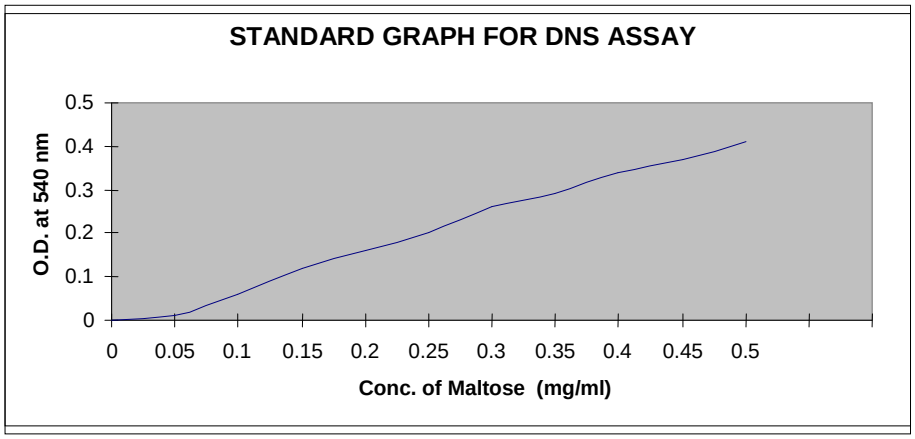

Figure 3: DNS ASSAY

DNS assay of crude and purified enzyme:

Concentration of enzyme in crude and purified sample were calculated by reacting the enzyme with 1% starch & comparing the resultant O.D with standard graph. The Table 4 and Figure 4 shows the concentration of enzyme in crude and purified sample were 0.018U/ml/min and 0.0115U/ml/min respectively.

Table 4: DNS assay of crude and purified enzymes

S No.	ENZYME (in ml)	1% STARCH (in ml)		DNS (in ml)		O.D AT 540 nm	ACTIVITY (U/ml/min)
BLANK	0	0	INCUBATED AT 37°C FOR 15 MINUTES	1	BOIL FOR 15 MINUTES AT 100°C	0.0	0.0
CRUDE EXTRACT	0.5	0.5		1		0.55	0.018
SALT PRECIPITATION	0.5	0.5		1		0.30	0.016
DIALYSIS	0.5	0.5		1		0.20	0.0115

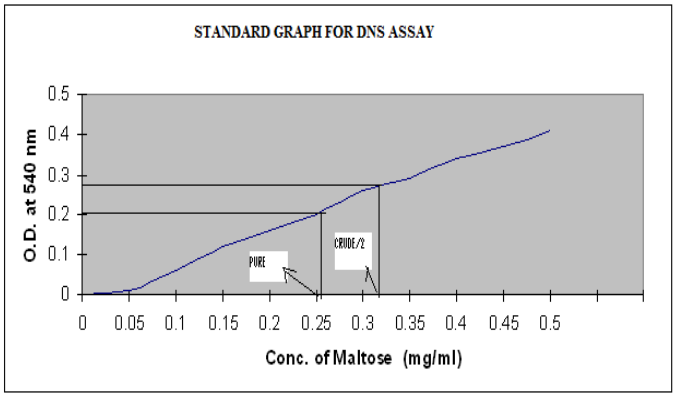

Figure 4: DNS assay of crude and purified enzymes

Effect of various temperatures i.e., 4°C, 20°C, 37°C and 50°C were studied on enzyme activity, and at 37°C enzyme activity was found to be maximum (Figure 5)

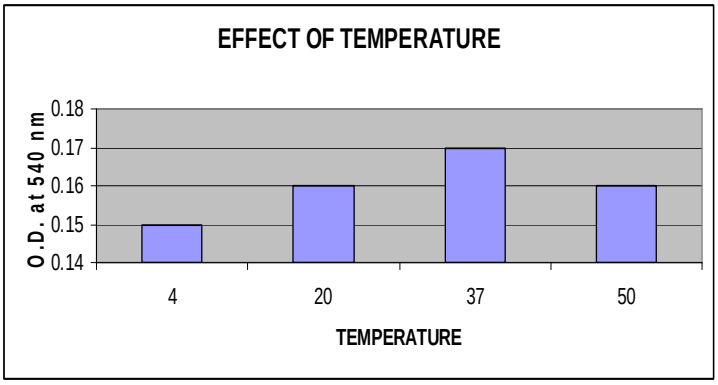

Figure 5: Effect of temperature on enzyme activity

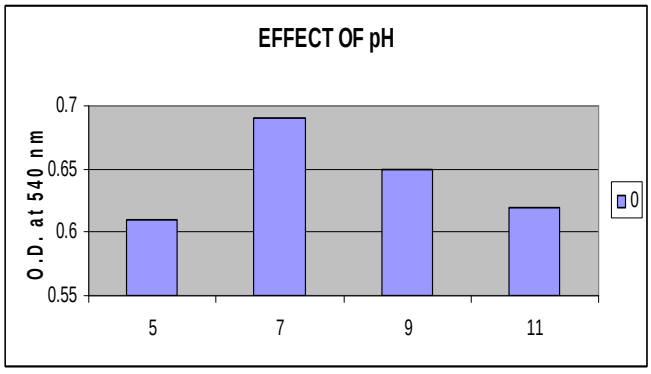

Figure 6: Effect of pH on enzyme activity

Enzyme activity of purified enzyme was calculated at different pH and the maximum activity was observed at pH 7 (Figure 6)

Effect of activators was studied on purified enzyme, it observed that Calcium enhances the maximum enzyme activity (Figure 7) and effect of inhibitors was studied on purified enzyme, it observed that SDS decreases the maximum enzyme activity (Figure 8).

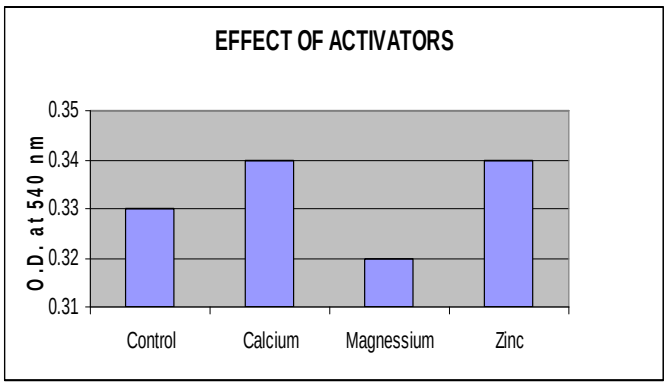

Figure 7: Effect of activators

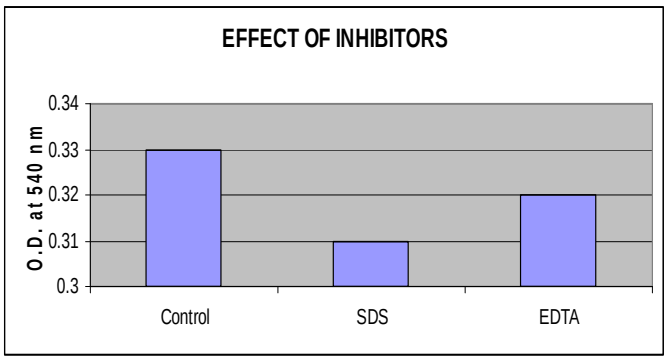

Figure 8: Effect of inhibitors

Concentration of protein in crude and purified enzyme were calculated by reacting the enzyme with lowry's reagent & comparing the resultant O.D with standard graph. Table 5 and Figure 9 shows the concentration of protien in crude and purified enzyme was 0.228 mg/ml and 0.18 mg/ml respectively.

Table 5: Protein estimation of crude and purified enzyme

S. No.	ENZYME (IN ml)	DISTILLED WATER (IN ml)	REAGENT C (IN ml)	INCUBATED AT ROOM TEMP. FOR 15 MINUTES	REAGENT D (IN ml)	INCUBATED FOR 30 MINUTES IN DARK	O.D.	CONC. OF PROTEIN (mg/ml)
BLANK	0.0	1	5		0.5		0.0	0.0
CRUDE EXTRACT	0.5	0.5	5		0.5		1.65	33.6
SALT PRECIPITATION	0.5	0.5	5		0.5		0.79	0.228
DIALYSIS	0.5	0.5	5		0.5		0.62	0.18

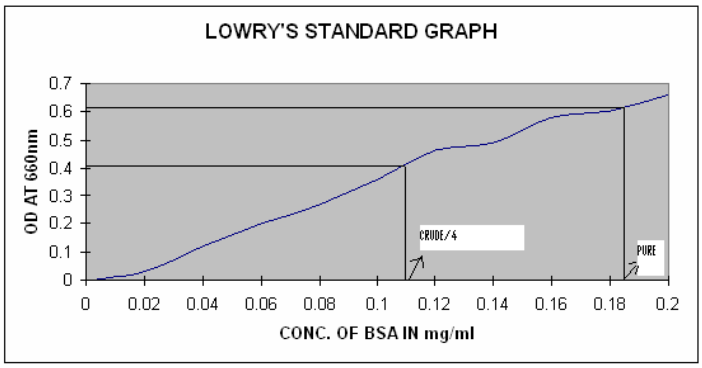

Figure 9: Protein estimation of crude and purified enzyme

Purification Chart

The Table 6 below shows the complete purification chart of the enzyme purification carried out in this experiment.

Table 6: Purification chart

S NO.	VOLUME (in ml)	PROTEIN (mg/ml)	ACTIVITY (U/ml/min)	TOTAL PROTEIN	TOTAL ACTIVITY	SPECIFIC ACTIVITY	FOLD PURIF.	YIELD
CRUDE EXTRACT	70	0.48	0.0528	33.6	3.696	0.11	-	-
SALT PPT	10	0.228	0.018	2.28	0.18	0.07	0.63	4.8%
AFTER DIALYSIS	10	0.18	0.0115	1.8	0.115	0.06	0.54	3.11%

Below is the destained SDS PAGE gel showing the clear bands of the purified enzyme in wells: 3, 6 and 7 whereas, BSA was loaded in wells: 1 and 5. By comparing the bands of purified enzyme and BSA, the molecular weight of Purified enzyme was 65 KDa (approx) (Figure 10).

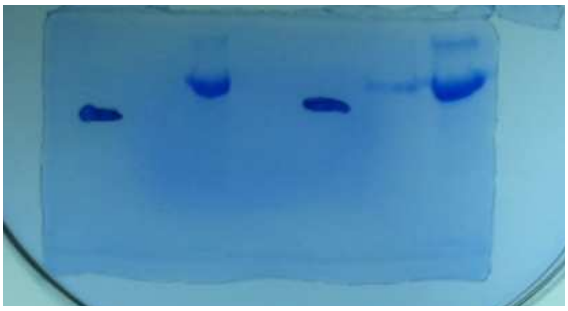

Figure 10: Molecular weight determination by SDS- PAGE.

DISCUSSION

Screening of purified cultures was done on MAM (Minimal agar media) supplemented with 1% starch the cultures growing in MAM were flooded with iodine solution and the zone of hydrolysis were obtained in the plates showing starch hydrolysis similar method has been used earlier by [Suman, S. and Ramesh, K., 2010] in order to screen the microorganisms for amylase production.

The bacterial species was identified by the help of various physical characteristics, staining & biochemical activities as done earlier by [Oyeleke, S. B. *et al.*, 2010].

Submerged fermentation for production of alpha amylase was done earlier by [Riaz, N. *et al.*, 2003].

Partial purification of the crude amylase was done by ammonium sulphate precipitation & dialysis similar techniques have been used earlier by [Yandri. *et al.*, 2010].

The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent, using maltose as standard and the enzyme activity of purified enzyme was calculated out to be 0.0115 U/ml/min, previously the enzyme activity was 1338 U/ml/min by [Aiyer, P. V. D., 2004].

Protein concentration was measured by Lowry's method [Lowry. *et al.*, 1951] using bovine serum albumin as standard and the amount of protien in purified sample was calculated out to be 0.18 mg/ml, previously the concentrations of protein was 10.13mg/ml by [Niaz, M. *et al.*, 2010] have been reported.

The purified enzyme was characterized for the effects of temperatures, pH, activators and inhibitors, 37°C was found to be the optimum temperature , pH 7 as optimum pH, Ca/Mg/Zn as good enhancers, SDS and EDTA as inhibitors: earlier also purified enzymes have been characterized for the effect of temperature, pH , activators and inhibitors by [Kubrak, O. I. *et al.*, 2010] and [Srivastava, R. A. K.,1987].

Molecular weight of the purified enzyme was determined by SDS PAGE and a single band was observed slightly parallel to the band of the marker (BSA 66 KDa), it was said to be 65KDa (approx.), molecular weights of the purified enzymes have been determined earlier and have been reported to be 56 KDa by [Liu, X. D & Xu, Y., 2007]; [Aajedi, R. H. *et al.*, 2005] determined the molecular weight was purified enzyme which was 59KDa, molecular weight of purified enzyme have also been determined by [Haq, I., *et al.*, 2010] and it was 55KDa, [Ahmadi, A *et al.*, 2010] determined the molecular weights of the purified enzymes resulting in a single band with an apparent molecular weight of 66 KDa.

CONCLUSION

Finally based on the above study it can be concluded that bacterial species can be a good source for the production of a very important enzyme amylase being used industrially.
Amylases purified here was found to be stable in a pH range of 7 to 9 and temperature range of 37°C to 50°C. The activity was found to be enhanced under the influence of cations such as Ca^{2+} and Zn^{2+} and retarded under the influence of anions such as EDTA and SDS. The activity of the amylases purified here is comparable to the activities of the amylases purified earlier by various researchers.

The molecular weight was determined by SDS- PAGE and a single band was observed after staining and destaining procedures giving indication of purity of the amylases.

Further work includes further purification of the enzymes in order to attain more higher specific activity. The purification has to be carried out with further purification processes including chromatography techniques such as affinity chromatography, Ion exchange chromatography and HPLC

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