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A STUDY ON PARTIAL PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR ALKALINE AMYLASES FROM *Bacillus megaterium* BY SOLID STATE FERMENTATION

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ABSTRACT : A highly alkaline amylase producing *Bacillus megaterium* was isolated from enriched soil under lab conditions. In order to enrich the soil for alkaline amylase producing Bacteria, soil was supplied with raw potatoes (substrate) and water at pH 10. Soil was left for 1month. After serial dilution five different colonies were obtained and were screened for amylase production. The *culture* MJS1105 showing maximum hydrolysis zone was subjected to solid state fermentation using wheat bran as substrate. The enzyme was purified using ammonium sulphate precipitation followed by dialysis. Total activity of **7.056 U** was obtained at 37ºC after 96 hours of incubation and was found to have a molecular weight of 63KDa (approx.) by SDS – PAGE. The enzyme was relatively stable between pH 5 – pH 13 and at temperature ranging from 32ºC to 50ºC. Moreover, activity was enhanced by using metal ions such as Ca^{2+}, Mg^{2+} and Na^{2+}. The decline in activity was observed by adding SDS and EDTA.

Key Words: Amylases, *Bacillus megaterium,* Hydrolysis, SDS- PAGE**.**

Abbreviations: SDS –PAGE: Sodium dodacyl sulphate polyacrylamide gel electrophoresis; ml= mililiter; min= minute; EDTA= ethylene diamine tetracaetic acid;Ca^{2+} = calcium; Mg^{2+} = magnesium**.**

INTRODUCTION

Amylases from various fungal and bacterial species have been studied in a great detail and they have been found to be a very good source for amylases production. Because of the time required for the generation of fungal strains they have been studied a bit lesser, but because of the reasonable generation time of the Bacterial species especially the Genus *Bacillus* has been explored to a large extent. Generally amylases purified from the microbial systems have found a large range of applications in the in starch saccharification and in the textile, food, brewing and distilling industries (Pandey *et al.,* 2000). Irrespective of the wide range of applications their are some limitations of the amylases one major being their ineffectiveness in the detergent industry. It has been shown in earlier research that alkaliphilic amylases can be a good source for the detergent industry. Alkaliphiles are extremophiles and have been studied throughout the world because of their known higher metabolic activities. Alkaline amylases studied in this study have been deciphered as a replacement to the normal amylases which have been found to be ineffective during their applications in the detergent industry (McTigue *et al.,* 1995). Keeping in mind the very first paper on the alkaliphiles by Horikoshi , 1971 and the recent activities on alkaline amylases (Kudo *et al.* 1990; Takagi et al. 1996); (Aygan, A. *et al.* 2008)., the present study was designed to evaluate the inhabitance of Bacterial species in the soil sample enriched with starchy material under alkaline conditions, to decipher some cheaper substrates for the production of alkaline amylases by solid state fermentation and also to extract, purify and characterize the extra cellular alkaline amylases.

METHODOLOGY

ENRICHMENT OF SOIL SAMPLE:

In order to enrich the soil for the alkaline amylase producing microbes, 100gm of potato was placed in a beaker containing soil. The soil was moistened with water of pH 10. Beaker was left for 1month. During the enrichment process the soil was moistened with water of pH 10 several times.

ISOLATION AND PURIFICATION OF BACTERIA FROM ENRICHED SOIL:

Bacterial samples were isolated from the enriched soil by serial dilution agar plating method on Nutrient agar media (pH 10). The isolates obtained were differentiated on the basis of colony morphology from the mixed culture plates and named as MJS 1101, MJS 1102, MJS 1103, MJS 1104, MJS 1105. The isolates were then purified by the help of quadrant streaking on Nutrient agar media(pH 10), and the purity was cross checked by Gram's staining procedure.

SCREENING OF THE PURIFIED CULTURES FOR AMYLASE PRODUCTION:

The purified cultures were screened for their potential of alkaline amylases production on Minimal agar media (MAM) supplemented with 1% starch prepared at pH 10. The cultures were streaked centrally on the solidified MAM plates and incubated at 37oC for 72 hours. Subsequently flooded with iodine solution. zone of clearance around the microbial growth indicated the production of amylase. On the basis of the area of clearance, the strain MJS1105out of the five bacterial isolates was selected for further studies.

IDENTIFICATION OF THE CULTURE MJS1105:

The isolate MJS1105 was identified by the help of various staining (Gram Staining, Endospore staining) and bichemical activities (Mannitol Test, , Catalase Test and voges proskaurer test.) based on the key of Bergey's manual given in the book of K R Aneja.

PRODUCTION OF ALKALINE AMYLASES:

Amylase was produced on lab scale by Solid State Fermentation using wheat bran as a starchy material. Wheat bran was moistened with a mineral salts media (sodium hydrogen phosphate-0.2gm, disodium hydrogen phosphate-0.6gm, sodium chloride- 0.5gm, ammonium chloride- 0.2gm, magnesium sulphate-0.01gm, glucose- 0.8gm, potassium chloride- 0.1gm, distilled water-100ml) maintained at pH 10 and inoculated with 1 ml of 24 hours old broth of culture MJS1105. Fermentation was carried out at 37 ^{o}C for 96 hours.

PURIFICATION OF ENZYME:

Extracellularly secreated enzyme was rcovered in 100ml of 100 mM Tris buffer (pH 10) by adding 100ml of the buffer to the flask containing fermented media the flask was shaken (150 rpm) for one hour. The suspension was filtered through whatman filter paper No. 1 at 4 ^{o}C. The filterate was transferred in centrifuge tubes and the spun at 5000 rpm for five minutes at 4 ^{o}C. The supernatant was collected in a chilled beaker and treated as crude enzyme. The crude enzyme was purified by ammonium sulphate precipitation upto 70 % saturation , the precipitate was resuspended in 10ml of 100 mM Tris buffer, pH 10, which was dialysed in the same buffer for 24 hour, with changes in buffer made every 2 hour. The dialysed sample was treated as purified extracellular alkaline amylase and assayed for the calculation of enzyme activity.

PROTEIN ESTIMATION

Concentration of protein in crude and purified extracts was determined by Lowry's method of protein estimation wherein 0.5 ml of enzyme was made upto 1ml with distilled water and reacted with Lowry's reagent C and D as per the protocol given by Lowry *et al*., 1951. The absorbance (660nm) obtained after the reaction was compared with the standard graph plotted by reacting a standard protein (BSA; 0.02-0.2 mg/ ml) with Lowry's reagents and the concentration of protein in crude and purified extracts was obtained.

ENZYME ASSAY

Amylase activity in crude and purified enzyme was assayed (DNS method of Miller.,1959) by reacting 0.5 ml of enzyme to 0.5 ml soluble starch (1% v/v dissolved in 100mM Tris Buffer pH 10) and incubating the reaction mixture at 37ºC for 15 min. The reaction was stopped by the addition of 1 mL of 3,5-dinitrosalicylic acid reagent. Reaction was further completed by boiling the tubes for 15 minutes and absorbance was read at 540nm. The absorbance obtained was compared with a standard graph plotted between concentration of maltose (0.05 to 0.5 mg/ml) and absorbance obtained after reaction of maltose with DNS reagent and thereby mg/ml maltose released after reaction of enzyme and substrate was calculated . This concentration was used to get the enzyme activity in U/ml/min. One unit enzyme was defined as micromoles of maltose released per ml enzyme used per minute.

CHARACTERIZATION OF THE PURIFIED ENZYME:

The purified enzyme was further characterized for the effect of pH (5, 7, 9, 11 and 13), Temperature(at 4ºC, 32ºC, 37ºC and 50ºC), activators (Calcium, Magnesium and Sodium) and inhibitors (SDS and EDTA).

For studying the effect of pH enzyme substrate reaction was carried out at various pH (5, 7, 9, 11 and 13) by adjusting the pH of substrate at different pH ranges as stated earlier. Activity was calculated by DNS method of Miller .,1959 as described earlier.

Enzyme was characterized for the effect of temperature by incubating the enzyme substrate mixture at different temperatures (4ºC, 32ºC, 37ºC and 50ºC) and the activity was calculated by the DNS method of Miller ., 1959.

Enzyme was studied for the effect of activators (Calcium, Magnesium and Sodium) on the activity wherein the enzyme substrate mixture was incubated along with 0.2 ml of cations (mg/ml). Activity was calculated by DNS method of Miller *et al*., 1959.

Similarly the enzyme was studied for the effect of inhibitors (SDS and EDTA) and activity was calculated.

MOLECULAR WEIGHT DETERMINATION BY SDS-PAGE

SDS-PAGE was carried out in 12.5 % resolving gel and 4.5 % stacking gel for determination of molecular mass as per the method of Laemmli., 1970. Protein bands were detected by destaining the gel in a methanol-acetic acid-water solution (4:1:5) after a staining process with 0.2% Coomassie brilliant Blue R250.

RESULTS

STRAIN SELECTION AND SCREENING:

Bacterial cultures were isolated from enriched soil by serial dilution on Nutrient Agar medium. Five cultures. were isolated from soil. They were named as MJS 1101, MJS1102, MJS1103, MJS1104 and MJS1105. The cultures were screened for the production of alkaline amylases on minimal agar media supplemented with 1% starch maintained at pH 10.

All the isolates were tested for amylase production by starch hydrolysis test on MAM plates. When minimal agar medium was inoculated with the organism and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylase. On the basis of the area of clearance, one isolate (MJS1105) out of five bacterial isolates was selected for further studies on alkaline amylase production. Table 1 below shows the ranking of cultures on the basis of visualization of the zone of hydrolysis. Figure 1 below shows the culture MJS1105 after starch hydrolysis test.

Table 1: Ranking on the basis of zone of hydrolysis.

S.NO.	CULTURE	CHARACTER
1	MJS1101	--
2	MJS1102	++
3	MJS1103	-
4	MJS1104	+
5	MJS1105	+++

NOTE: +++ (intense decolourization) ++ (moderate decolourization)
+ (slight decolourization) - (no decolonization)

IDENTIFICATION OF THE CULTURE MJS1105:

The culture showing maximum zone during starch hydrolysis test was identified by performing by performing various staining and biochemical activities namely Gram's staining, Endospore Test, Catalase Test, Mannitol fermentation Test, Voges Proskaurer Test based on the **Bergey's manual.** Table 2 below shows the staining and biochemical characteristics of the culture MJS1105.

Table 2: Staining and Biochemical activities for *Bacillus* Sp.

S.No	Biochemical Tests	Response
1.	Gram's staining	Positive
2.	Mannitol Test	Positive
3.	Endospore Test	Positive
4.	Catalase Test	Positive
5.	Voges Proskaurer	Negative

By comparing the above obtained results of the staining and biochemical activities of the culture with the key of Bergey's manual given in the book of Aneja, K. R., 2003 the culture was tentatively identified as *Bacillus megaterium.*

PROTEIN ESTIMATION:

Concentration of protein in crude and purified enzyme was estimated by Lowry's method (Lowry *et al*., 1951) using bovine serum albumin as standard and the amount of protein in purified sample was calculated out to be 0.42mg/ml. Table 3 below shows the concentration of protein estimated throughout the recovery procedure. Figure 1 below shows the standard graph and concentration of protein in crude and purified enzyme.

Table 3: Protein estimation by Lowry's method.

S. No.	ENZYME (in ml)	DISTILLED WATER (in ml)	REAGENT C (in ml)	INCUBATED AT ROOM TEMP. FOR 15 MINS	REAGENT D (in ml)	INCUBATED FOR 30 MINS IN DARK	O.D.	CONC. OF PROTEIN (mg/ml)
BLANK	O	1	5		0.5		0.0	0.0
CRUDE EXTRACT	0.5	0.5	5		0.5		1.9	0.45
SALT PRECIPITATION	0.5	0.5	5		0.5		1.70	0.42
DIALYSIS	0.5	0.5	5		0.5		1.71	0.42

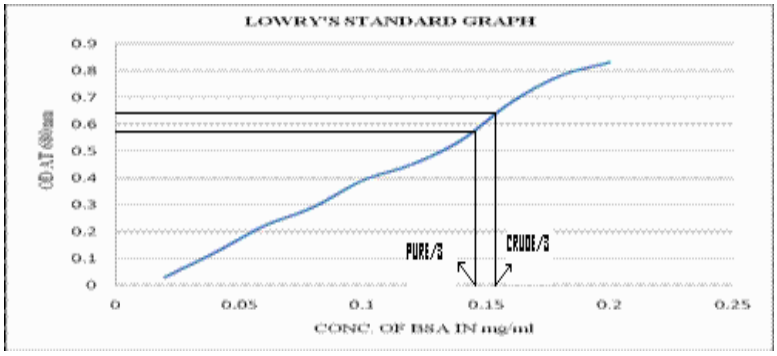

Figure 1: Protein estimation of crude and purified enzyme.

ENZYME ASSAY:

Enzyme activity was determined by DNS method of Miller., 1959, using maltose as standard, the enzyme activity of purified enzyme was calculated as 0.7056 U/ml/min. Table 4 below shows enzyme activity calculated throughout the recovery procedure. Fig 2 below shows the standard graph showing the mg/ml maltose released for crude and purified enzyme.

Table 4: DNS Assay of crude and purified enzymes.

S No.	ENZYMES (in ml)	1% STARCH (in ml)	INCUBATED AT 37°C FOR15 MINS	DNS (in ml)	BOIL FOR 15 MINS AT 100°C	O.D AT 540 nm	ACTIVITY (U/ml/min)
BLANK	0	0		1		0.0	0.000
CRUDE EXTRACT	0.5	0.5		1		1.04	0.0576
SALT PRECIPITATION.	0.5	0.5		1		0.78	0.0451
DIALYSIS	0.5	0.5		1		1.22	0.7056

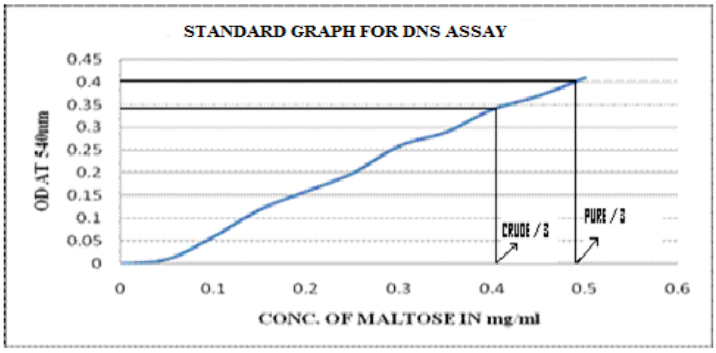

Figure 2: DNS Assay of crude and purified enzymes.

Table 5 below shows the purification chart for the enzyme purified in the present study.

Table 5: Purification chart.

S NO.	VOLUME (in ml)	PROTEIN (mg/ml)	ACTIVITY (U/ml/min)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY(U/min)	SPECIFIC ACTIVITY (U/mg)	FOLD PURIFICATION	YIELD (%)
CRUDE EXTRACT	70	0.45	0.0576	31.5	4.032	0.128	1	100
SALT PRECIPITATION	10	0.42	0.0451	4.2	0.451	0.107	0.835	11.185
AFTER DIALYSIS	10	0.42	0.7056	4.2	7.056	1.68	13.125	175

CHARACTERIZATION OF ENZYME

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

Effect of various temperatures i.e., 4°C, 32°C, 37°C and 50°C was studied on enzyme activity, and it was found that the optimum temperature for the activity of the alkaline amylases purified here ranges between 32 ºC to 37ºC and also the enzyme is able to survive a bit at higher temperature that is 50°C. Figure 3 below shows the results discussed above.

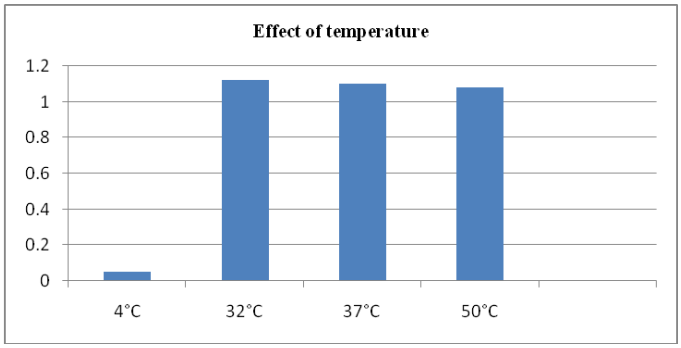

Figure 3: Effect of temperature on enzyme activity.

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 as shown in Table 6 and Figure 4.

Table 6: Effect of pH on enzyme activity

PURIFIED ENZYME (in ml)	1% STARCH (in ml)	DIFFERENT pH	INCUBATED AT 37°C FOR15 MINS	DNS (in ml)	BOIL FOR 15 MINS AT 100°C	O.D AT 540nm	ACTIVITY (U/ml/min)
-	-	7		1		0.0	0.0
0.5	0.5	5		1		0.0	0.00
0.5	0.5	7		1		1.05	0.057
0.5	0.5	9		1		1.01	0.053
0.5	0.5	11		1		1.14	0.066
0.5	0.5	13		1		0.87	0.048

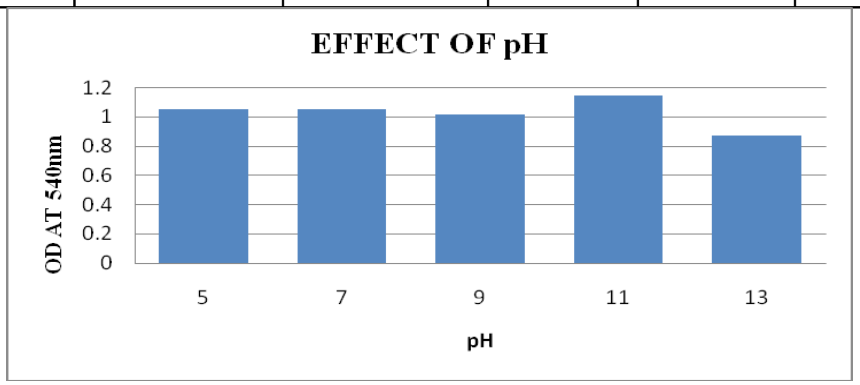

Figure 4: Effect of pH on enzyme activity.

EFFECT OF ACTIVATORS

Effect of activators was studied on purified enzyme, it was observed that Calcium enhances the enzyme activity maximally as shown in Figure 5 below.

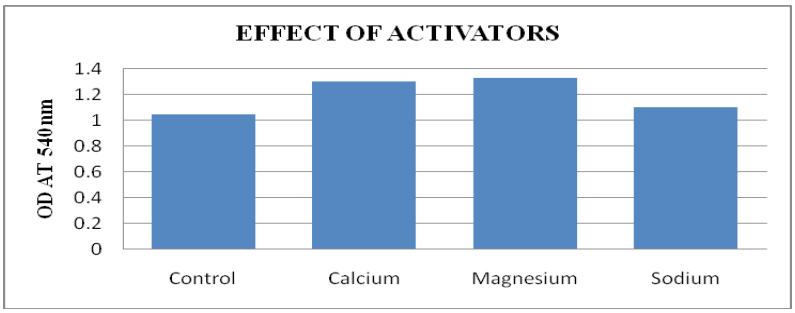

Figure 5: Effect of activators

EFFECT OF INHIBITORS

Effect of inhibitors was studied on purified enzyme and it was observed that SDS as well as EDTA act as inhibitors as shown below in Figure 6.

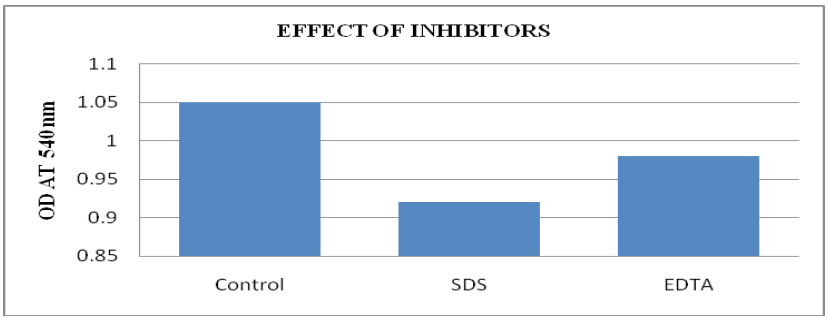

Figure 6: Effect of inhibitors.

MOLECULAR WEIGHT DETERMINATION BY SDS PAGE:

Molecular weight of the purified enzyme was determined by SDS PAGE wherein wells 3, 4 and 5 were loaded with purified enzyme, whereas BSA (marker 66 KDa) was loaded in wells 1, 2, 9 and 10 as shown in Figure 7 and as can be seen from the figure below a single band was observed slightly parallel to the band of the marker (BSA 66 KDa), thus it was said to 63 KDa (approx).

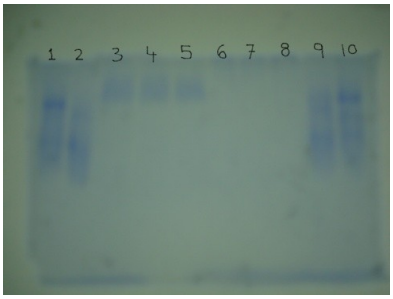

Figure 7: SDS- PAGE

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 Behal, A. *et al.,* 2005, in order to screen the microorganisms for amylase production.

Alkaline Amylases were produced by solid state fermentation using wheat bran as a starchy substrate as done earlier by Sivaramakrishnan, S. *et al.,* 2006 and Suganthi, R., *et al.,* 2011.

Partial purification of the crude amylase was done by ammonium sulphate precipitation & dialysis similar techniques have been used earlier by Farouk Al-Quadan *et al.,* 2009.

The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent, using maltose as standard and the specific activity of purified enzyme was calculated out to be 1.68 previously maximum amylases with specific activities of 2487 has been calculated by Farouk Al-Quadan *et al.,* 2009. The lesser specific activity in this study is because of the lesser purification because of the unavailability of resources in our laboratory as we used only salt precipitation and dialysis but all the articles in literature perform further purification steps.

The purified enzyme was characterized for the effects of temperatures, pH , activators and inhibitors, 32°C was found to be the optimum temperature , pH 11 as optimum pH for enzyme purified after solid state fermentation and Ca/Mg/Na 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 Kim *et al*., 1995., the alkaline amylases purified by them was also stable at 50 ºC, at pH 10.5, was enhanced by metal ions such as Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ag^{+}, Zn^{2+}, and Fe^{2+}, and was inhibited by EDTA and 1 mM phenylmethylsulfonyl fluoride.

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 63 KDa (approx.), molecular weights of the purified enzymes have been determined earlier and have been reported to be 66 KDa by Aygan, A. *et al.* 2008.

CONCLUSION

Finally based on the above study it can be concluded that *Bacillus Megaterium* can be a good source for the production of a very important alkaline amylase enzyme being used industrially. Based on the present research it can be said that solid state fermentation can be utilized for the large scale production of alkaline amylases and also can be a bit cheaper as the source being deciphered in the present study wheat bran costs nearly nothing. Alkaline amylases purified here can be a good source for the treatment of waste water systems which have a alkaline pH. Alkaline amylases can also be a good replacement of the neutral amylases which are a bit ineffective on their applications in the detergents.

Alkaline amylases purified here was found to be stable in a pH range of 5 to 13 by both submerged and solid state fermentation and temperature range of 32°C to 50°C. The activity was found to be enhanced under the influence of cations such as Ca^{2+} and Mg^{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 alkaline 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 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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