ENZYMIC STUDIES OF α-AMYLASE FROM SUGARCANE LEAVES (*Saccharum officinarum* L.)

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Abstract

In this research, α-amylase enzyme [EC 3.2.1.1] was extracted from sugarcane leaves (*Saccharum officinarum* L.) and the α-amylase activity was measured by Nelson-Somogyi method. The optimum pH and the optimum temperature were found to be 5.0 and 50°C respectively. The protein content of the enzyme was determined by using Biuret method. The purification of α-amylase extracted from sugarcane leaves was carried out by using ammonium sulphate precipitation method and Sephadex G-100 gel chromatographic method. The specific activity (the relative purity of the enzyme) increased about 7 folds from crude to final purification step. The homogeneity of the purified α-amylase was confirmed by non-sodium dodecyl sulphate polyacrylamide gel electrophoresis (non SDS-PAGE). The purified α-amylase enzyme showed a single band on non SDS-PAGE. An estimated molecular weight of purified α-amylase from sugarcane leaves sample was found as 57543 Dalton. In this research, immobilization of purified α-amylase was carried out on oxycellulose support. Then the optimum pH, optimum temperature and storage stability of immobilized α-amylase were determined. The pH and temperature profile of free and immobilized α-amylase enzyme were very similar in nature. There were the same optimum pH 5.0 and optimum temperature 50°C. In the case of storage stability, the immobilized enzyme was more stable than free enzyme. During 15 days storage time at 4°C free α-amylase enzyme lost 49.39 % of original activity, whereas immobilized one lost only 4.56 % of original activity.

Keywords: α-amylase, sugarcane leaves, Nelson-Somogyi method, Biuret method, non SDS-PAGE

Introduction

Sugarcane

Sugarcane or Sugar cane (*Saccharum*) (Figure 1) is a genus of 6 to 37 species (depending on taxonomic interpretation) of tall perennial grasses (family Poaceae, tribe Andropogoneae) native to warm temperate to tropical

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regions of the Old World. All of the sugarcane species interbreed, and the major commercial cultivars are complex hybrids (Sharp, 2008).

Scientific Classification of Sugarcane

The Scientific classification of sugarcane is as follow:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Liliopsida
Order	:	Poales
Family	:	Poaceae
Genus	:	*Saccharum* Linn.
Species	:	*S.officinarum*
Myanmar name	:	Kyan Net

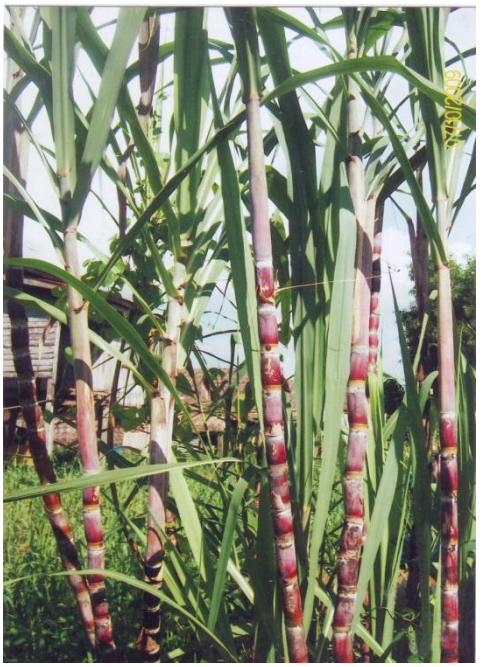

Figure 1: Photograph of sugarcane (*Saccharum officinarum* L.)(Kyan-Net)

α-Amylases are digestive enzymes which hydrolyze glycosidic bonds in starch to glucose, maltose, malt triose and dextrin. They have diverse applications in a wide variety of industries such as food, textile, paper,

detergent as representing approximately 30 % of the world enzyme production (Yamamoto, 1988). Many sweets, bakery products and even ice cream are based on sugars digested with the help of amylase.

Materials and Methods

Sugarcane leaves were collected from Kyauktan Township, Yangon Region. Firstly, α-amylase enzyme was extracted from sugarcane leaves by centrifugation method. Sugarcane leaves (50 g) were homogenized in 500 mL of 0.1 M acetate buffers (pH 5.0) containing 1mM $CaCl_2$ with a blender (Miyagi, 1990). The crude homogenate was then filtered through bleached cotton wool to remove gross particulate matter and the filtrate was centrifuged at 5,000 rpm for 30 min. After centrifugation, the supernatant (500 mL) was collected and the crude enzyme solution was kept in refrigerator at 4 °C. The α-amylase activity was measured by Nelson-Somogyi method. The protein content of the enzyme was determined by using Biuret method. Then standard calibration curve was constructed by using bovine serum albumin protein solution. In this curve, the straight line passed through the origin, therefore Beer's Law was obeyed. The purification of α-amylase from sugarcane leaves (*Saccharum officinarum* L.) was carried out by using ammonium sulphate precipitation method and Sephadex G-100 gel chromatographic method. The pH stability and thermostability of the α-amylase enzyme were also studied at various incubation times.. In this research, determination of molecular weight of the purified α-amylase was carried out by using non SDS-PAGE electrophoresis technique in European Molecular Biology Laboratory, Heidelberg, Germany. Before electrophoresis, purifications of the crude α-amylase Sephacryl S-200 and sephadex G-75 chromatographic technique were carried out. The immobilization of purified α-amylase was carried out on oxycellulose support. Then the optimum pH, optimum temperature and storage stability of immobilized α-amylase were determined.

Results and Discussion

Extraction of α-Amylase from the Sugarcane Leaves

α-Amylase [EC 3.2.1.1] is ubiquitous in living cells, and is important for the use of polysaccharides. In higher plants, α-amylase have been isolated from barley, oats, rye, wheat, soybeans, broad beans, rice and sorghum and extensive studies have been done on their properties and structures.

Amylases have wide scale application ranging from textile to effluent treatment.α-Amylase is an important industrial enzyme (Godfrey and Reichelt, 1983). Plant α-amylases are sensitive to acid and lose their activities by incubation at pH 3.7. Commercial amylase preparations used in human foods are obtained from grains, such as barely, wheat, rye, oats, maize, sorghum and rice.

In the present research, α-amylase enzyme was isolated from Kyan-Net leaves from Kyauktan Township, Yangon Region. The α-amylase was extracted from sugarcane leaves by centrifugation method. Then, α-amylase activity was determined by spectrophotometric method.

α-Amylase Activity of Crude Enzyme Solution from Sugarcane Leaves

α-Amylase activity was confirmed by using iodine staining method (Harper, 1977). The blue colour was developed by soluble starch with iodine solution. An adsorption complex of starch and iodine was appeared rather than a definited compound. When the blue colour disappeared, this shows the decomposition of starch polymer by enzyme action.

In the present research, a blank solution containing the mixture of starch and distilled water showed deep blue colour with iodine solution, whereas solution mixture containing enzyme and starch solutions showed no color with iodine solution. Therefore, the α-amylase enzyme isolated from the sugarcane leaves hydrolyzed the starch by breaking down the α-glucosidic bond.

Wavelength of Maximum Absorption of Arsenomolybdate Chromogenic Compound by Using Nelson-Somogyi Method

Nelson-Somogyi method is one of the several widely used method for determination of reducing sugar by measuring the absorbance of

arsenomolybdate chromogenic compound formed from reduction by sugar (Slater, 1986). In this research, the absorption spectrum of arsennomolybdate chromogenic compound was recorded in the range form 600-900 nm and the wavelength of maximum absorption was found at 750 nm. Therefore, the absorbance of arsenomolybdate chromogenic compound formed from reduction by maltose was measured at this wavelength by using Nelson-Somogyi method.

Purification of α-Amylase

The α-amylase enzyme was purified by ammonium sulphate precipitation method and gel filtration on Sephadex G-100. Salting out with ammonium sulphate used as an early step in a purification protocol, can be a highly effective method of separating proteins. Ammonium sulphate precipitation method was chosen for salt fractionations because of its high solubility in water, lack of toxicity, cheapness, lack of harmful effects on enzyme activity. In this work, crude α-amylase was firstly purified by ammonium sulphate precipitation method and then the enzyme protein precipitate was purified by gel filtration on Sephadex G-100. Sephadex types (G 10 - G 100) are available in different particle size grades, viz., superfine, fine medium and coarse (Wiseman, 1985). Each Sephadex G-type has a different molecular weight over which molecules can be fractionated. Sephadex G-100 superfine is a new kind of gel filtration medium which combines a highly porous gel structure with excellent chemical and physical stability (Azarkan *et al*., 2003). Sephadex is preswollen and ready to use in both analytical and preparation applications.

In this research, Sephadex G-100 was used in glass column (2.0 × 25 cm) that will fractionate proteins in the molecular weight range of 3000 to 60,000. Sephadex G-100 was used and its parameters are bead diameter 40-120 μm.

Table 1 shows the relationship among protein absorbance at 280 nm, the α-amylase activity and fraction numbers. Protein content was estimated by measuring the absorbance at 280nm. Figure 2 shows purification of α-amylase enzyme by Sephadex G-100 gel filtration chromatography. The fractions of highest activity (34-38 fractions) were pooled.

Molecular Weight of the Purified α-Amylase Enzyme

The molecular weight of a protein may be determined by a variety of methods (Murray, 1993). The most accurate molecular weights are those obtained from sequence studies, a procedure hardly suitable in routine studies. Usually molecular weights are obtained by methods involving the analytical ultracentrifuge and other methods: gel filtration, osmotic pressure, light scattering, electron microscopy, etc., have been employed. Molecular weights are best determined when both the band of interest and the standards appear as sharp, narrow bands so that there is no mistake as to where to measure the migration distance (Hames, 1981).

Table 1: Relationship among Protein Absorbance at 280 nm, the α-Amylase Activity and Fraction Numbers

Fraction No.	Protein absorbance at 280 nm	α-Amylase activity (μmol min^{-1} mL^{-1})
1	0.000	5.73
3	0.000	5.21
5	0.000	4.07
7	0.000	6.89
9	0.000	2.31
11	0.000	5.67
13	0.000	5.47
15	0.001	2.18
17	0.231	3.14
19	0.145	2.79
21	0.147	4.46
23	0.154	5.55
25	0.139	4.99
27	0.188	6.20
29	0.251	5.53
31	0.717	5.51
33	1.078	3.58
35	1.339	5.43

Fraction No.	**Protein absorbance at 280 nm**	**α-Amylase activity (μmol min^{-1} mL^{-1})**
37	1.079	7.97
39	0.665	3.87
41	0.145	2.90
43	0.145	3.11
45	0.008	4.89
47	0.175	4.50
49	0.055	3.19
51	0.008	3.59
53	0.039	3.05
55	0.008	3.69
57	0.039	3.93
59	0.018	4.42
61	0.008	3.18
63	0.000	1.94
65	0.000	3.28
67	0.000	2.94
69	0.000	3.28
71	0.000	3.74
73	0.000	2.16
75	0.000	1.12
77	0.000	1.29
79	0.000	1.20
81	0.000	0.26

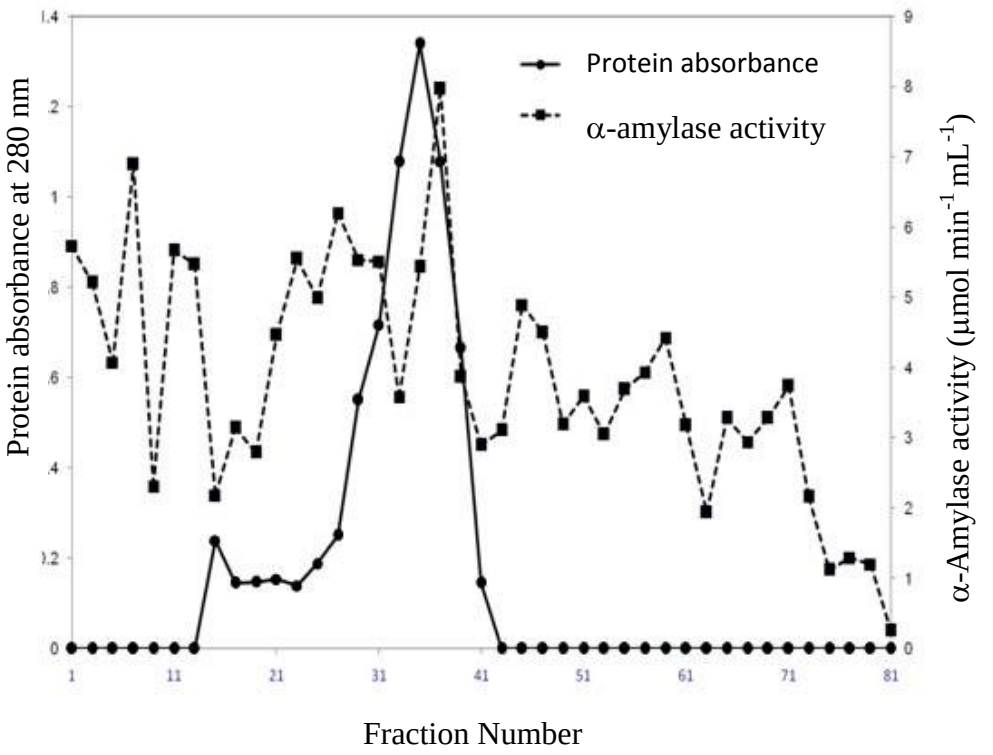

Figure 2: Purification of α-amylase enzyme by Sephadex G-100 gel filtration chromatography

In this research, proteins from the high molecular weight calibration kit: (pharmacia) thyroglobulin (667,500), ferritin (439,000), urease trimer (230,000), β-glactosidase (139,000) and fructose-6-phosphate kinase (66,500) were used for molecular weight determination by non-SDS-PAGE. The use of polyacrylamide gel electrophoresis for determining protein molecular weight has become a routine laboratory technique (Phamacia Inc, 1987). The molecular weight of a protein under investigation was determined by comparing its electrophoretic mobility with that of protein standards of known molecular weights.

Figure 3 shows photograph of non-sodium dodecyl sulphate polyarcylamide gel-electrophoresis. Table 2 shows relationship between log of molecular weight of standard marker proteins and relative mobility (R_f)

values obtained from non-SDS-PAGE. Figure 4 shows plot of relative mobility (R_f) obtained from non-SDS-PAGE as a function of log of molecular weight of high molecular weight (HMW) marker proteins.

The homogeneity of the purified α-amylase was confirmed by non sodium dodecyl sulphate polyacrylamide gel electrophoresis (non-SDS-PAGE). The purified α-amylase enzyme showed a single band on non-SDS-PAGE where the molecular weight of purified α-amylase was located near the standard protein (mol. wt 66,500(Figure 3). An estimated molecular weight of purified α-amylase from sugarcane leaves sample was found as 57543 Dalton.

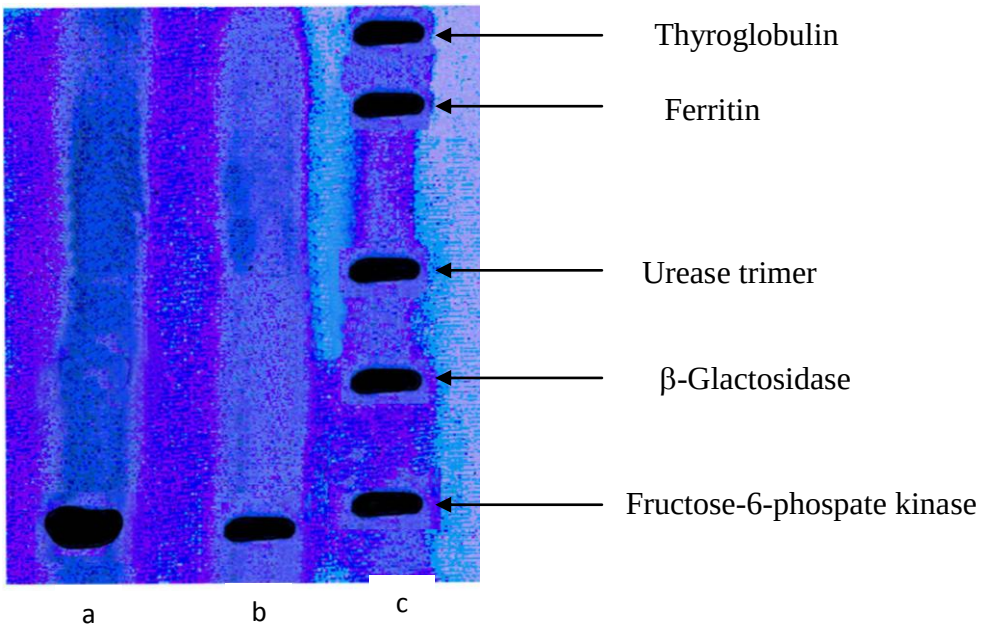

Figure 3: Photograph of non-sodium dodecyl sulphate polyacrylamide gel-electrophoresis

Lane a. Purified α-amylase fraction obtained from Sephacryl S 200

Lane b. Purified α -amylase fraction obtained after successive purification with Sephacryl S-200 and Sephadex G-75

Lane c. High molecular weight marker proteins

Table 2: Relationship between Log of Molecular Weight of Standard Marker Proteins and Relative Mobility (R_f) Values Obtained from non-SDS-PAGE

No.	Standard HMW marker proteins	MW (Dalton)	Log of MW	R_f
1	Thyroglobulin	667500	5.8244	0.04
2	Ferritin	439000	5.6425	0.18
3	Urease trimer	230000	5.3617	0.45
4	β-Glactosidase	139000	5.1430	0.65
5	Fructose-6-phosphate kinase	66500	4.8228	0.87

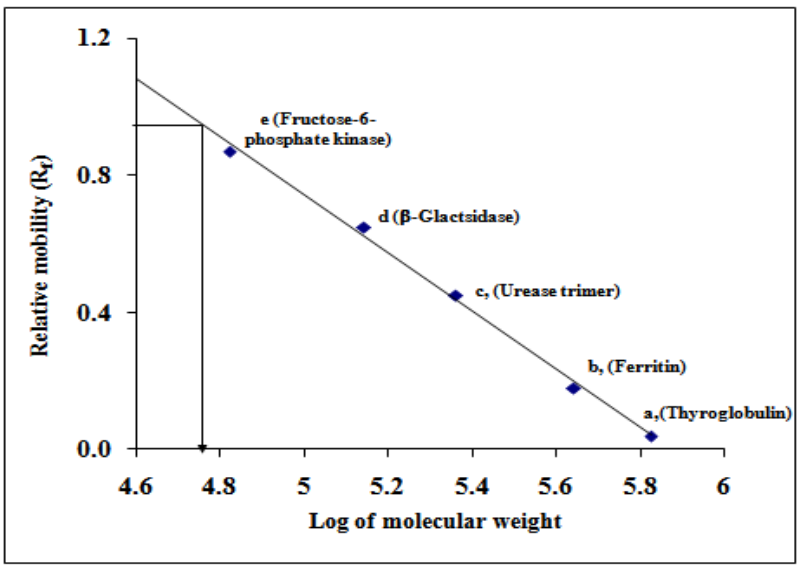

Figure 4: Plot of relative mobility (R_f) obtained from non-SDS-PAGE as a function of log of molecular weight of high molecular weight (HMW) marker proteins

Comparison of Enzymic Properties (pH, Temperature and Storage Stability) of Free and Immobilized α-Amylase

In the presence work, α-amylase was immobilized on oxycellulose as support (Figure 5). Enzyme properties of immobilized α-amylase were studied by determining the effect of pH, temperature and storage stability. The pH and temperature profile of free and immobilized α-amylase enzyme was very similar in nature. There are the same optimum pH 5.0 (Table 3 and Figure 6) and optimum temperature 50 °C (Table 4 and Figure 7). However, in the case of storage stability, the immobilized enzyme was more stable than free enzyme. During 15days storage time at 4 °C free α-amylase enzyme lost 49.39 % of original activity, whereas immobilized one lost 4.56 % of original activity after storage for 15 days at 4 °C.

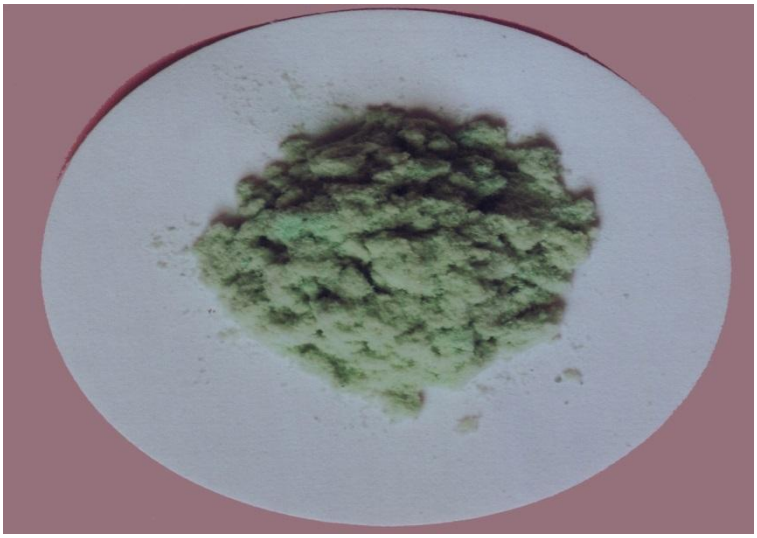

Figure 5: Photograph of immobilized α-amylase enzyme

Table 3: Relationship between α-Amylase Activity and pH of the Solution for Free and Immobilized Enzyme

pH	Free enzyme		Immobilized enzyme	
	Absorbance at 750 nm	α-amylase activity (μmol min^{-1} mL^{-1})	Absorbance at 750 nm	α-amylase activity (μmol min^{-1} mL^{-1})
3.6	1.632	1.511	1.467	1.359
4.0	1.750	1.631	1.593	1.475
4.5	1.800	1.667	1.513	1.401
5.0	1.854	1.761	1.735	1.607
5.5	1.764	1.634	1.578	1.462
6.0	1.629	1.564	1.499	1.388
7.0	1.564	1.449	1.355	1.255
8.0	1.468	1.360	1.214	1.124

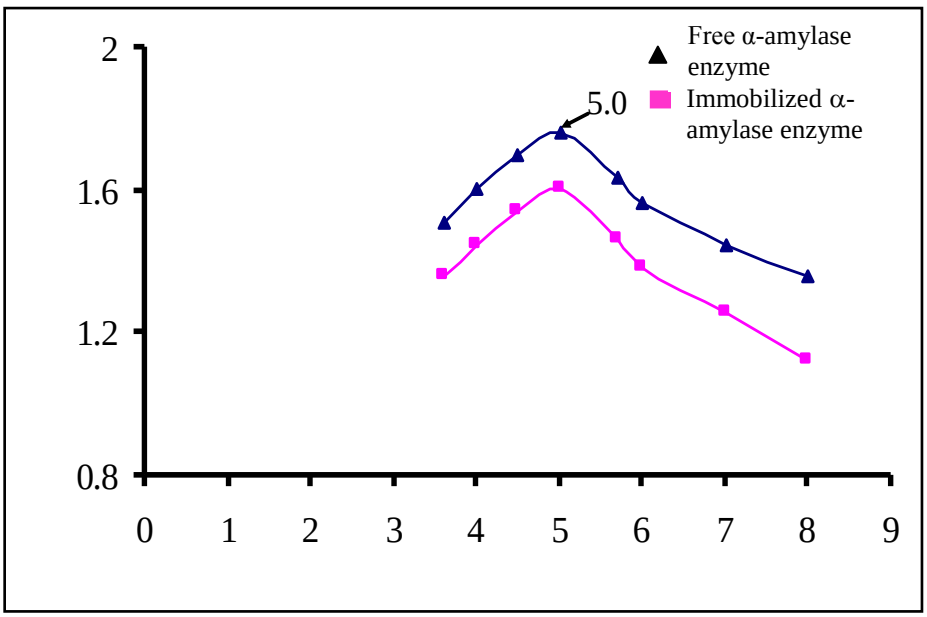

Figure 6: Plot of free and immobilized α-amylase activity as a function of solution

Table 4: Relationship between α-Amylase Activity and Temperature of the Solution for Free and Immobilized Enzyme at pH 5.0

Temperature (°C)	Free enzyme		Immobilized enzyme	
	Absorbance at 750 nm	α-Amylase activity (μmol min^{-1} mL^{-1})	Absorbance at 750 nm	α-Amylase activity (μmol min^{-1} mL^{-1})
30	0.738	0.683	0.618	0.572
35	0823	0.762	0.705	0.653
40	0.976	0.904	0.878	0.813
45	1.156	1.074	1.139	1.055
50	1.287	1.192	1.225	1.135
55	1.102	1.020	1.062	0.984
60	0.885	0.736	0.675	0.625
65	0.769	0.712	0.568	0.526
70	0.637	0.590	0.509	0.472
75	0.629	0.583	0.405	0.375

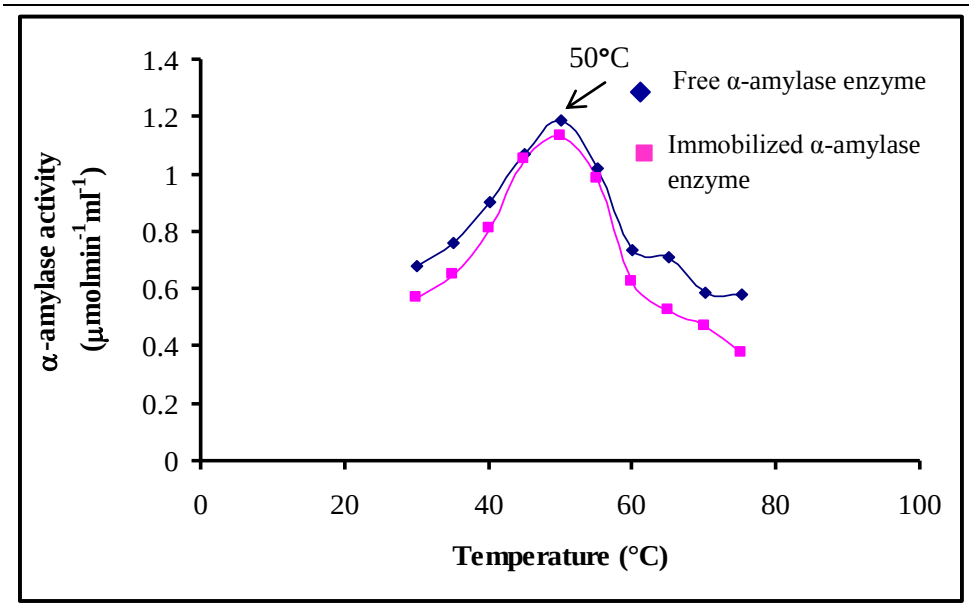

Figure 7: Plot of free and immobilized α-amylase activity as a function of temperature of solution

Conclusion

In the extraction of α-amylase from sugarcane leaves, centrifugation method was used. Nelson-Somogyi method was used to determine the amount of maltose, that is formed by the hydrolysis of α-amylase on starch. The protein content of the enzyme was determined by using Biuret method.

Further purification of α-amylase from sugargane leaves (*Saccharum officinarum* L.) was carried out by using ammonium sulphate precipitation method and Sephadex G-100 gel chromatographic method. The specific activity (the relative purity of the enzyme) increased about 7 folds from crude to final purification step.

The homogeneity of the purified α-amylase was confirmed by non sodium dodecyl sulphate polyacrylamide gel electrophoresis (non-SDS-PAGE). The purified α-amylase enzyme showed a single band on non-SDS-PAGE. An estimated molecular weight of purified α-amylase from sugarcane leaves sample was found as 57543 Dalton.

The pH and temperature profile of free and immobilized α-amylase enzyme were very similar in nature. There were the same optimum pH 5.0 and optimum temperature 50 °C. However, in the case of storage stability, the immobilized enzyme was more stable than free enzyme. During 15 days storage time at 4°C free α-amylase enzyme lost 49.39 % of original activity, whereas immobilized one lost only 4.56 % of original activity. Sugarcane leaves are one of the agricultural wastes. So, the problems related at their disposal and pollution can be solved by the extraction of an industrial enzyme,α-amylase from the sugarcane leaves waste.

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References

Azarkan, M., Moussaoui, A.E., Ven Wuytswinkel, D., Dehon, G., and Looze, Y. (2003). "Fractionation and Purification of the Enzymes Stored in the Latex of Carica Papaya".*J. Chromatogr B*, 790, pp.229-238

Godfrey, T. and Reichelt. J. (1983), Industrial Enzymology. New York: Macmillan Inc., pp.15-17

Hames, B.D. (1981)."*An Introduction to Polyacrylamide Gel Electrophoresis*, *Gel Electrophoresis of proteins*: *A Practical Approach*". Oxford:IRL Press, pp. 52-55

Happer, H.A. (1977). Review of Physiology Chemistry. New York: John Wiley and Sons Inc., pp. 509-519

Miyagi, M., Oku, H. and Chinen, I. (1990). "Purification and Action Pattern on Soluble Starch of α-Amylase from Sugarcane Leaves". *J. Argic. Biol. Chem.*,vol. 54, pp.849-855

Murray, R.K., Grannee, D.K., Mayer, P.A., and Rodwell, V.W. (1993). Harper's Biochemistry. Appleton and Lange Co., Connecticut:pp.78-83

Nelson, N. (1944)."A Photometric Adaptation of the Somogyi Methods for the Determination of Glucose". *J. Biol. Chem.*, pp.153, pp. 375-377

Pharmacia Inc. (1987). Electrophoresis Calibration Kits Instruction Manual. Uppsla, pp62-68

Sharp, P. (2008). *Sugarcane: Past and Present* http:// www siu. edu/~ebl/leaf lets/ sugar. html (Accessed 15 November,2015)

Slater, R.J. (1986). *Experiment in Molecular Biology*; Clifton, New Jersey: Humana Press, pp. 269-270

Wiseman, A. (1985). *Handbook of Enzyme Biotechnology*. New York: Second Edition, John Wiley and Sons Inc., pp.112-117

Yamamoto, T. (1988). Handbook of Amylases and Related Enzymes. Oxford: Pergamon Press, pp. 1-203