J. Natn. Sci. Coun. Sri Lanka 1977 5(1) : 59—74

Cultivation, Isolation, Purification and some Properties of the Enzyme Glucoamylase from *Aspergillus Niger*

E. R. Jansz, Nirmala Pieris, E. E. JeyaRaj and Nimali de Silva
Industrial Microbiology Section, Ceylon Institute of Scientific and Industrial Research, Colombo 7, Sri Lanka.

(*Paper accepted :* 1 *April* 1977).

Abstract: Glucoamylase from *Aspergillus niger* was purified eleven-fold and its properties studied. The preparation was apparently free of α amylase and transglucosidase. Studies on the effect of temperature revealed that although the enzyme begins to be deactivated at 60°C, this temperature would be most effective in the commercial conversion of starch to glucose. An easy method of assay of α amylase levels in culture filtrates is also described. Studies with DEAE-cellulose also revealed the presence of minor glucohydrolases.

1. Introduction

Glucoamylase also known as amyloglucosidase but more correctly named α 1-4 glucanglucohydrolase (3.2.1.3) is a common starch hydrolysing enzyme present in many species of fungi (notably in *Aspergillus*[3,15] and *Rhizopus*[2,4,8] species) and also in certain yeast and bacteria.[5] The enzyme (an exoenzyme) hydrolyses starch in a stepwise manner from the non-reducing termini to produce glucose.[5] In addition to hydrolysing the α 1-4 bond, the enzyme can also hydrolyse the α 1-6 bond[5] (present in amylopectin).

The enzymic reaction is of considerable importance as the conversion is used in the commercial production of glucose.[10] Considerable work has been done on the purification of glucoamylase from many species of *Aspergillii*;[5,9,13] the enzyme has also been crystallised.[16] Studies on the enzyme have shown that its pH and temperature optima vary from strain to strain.[9,10] The enzyme is also reported to exist in the form of isoenzymes which differ in their pH optimum.[13]

Our studies in this paper have been undertaken with a strain (selected as previously described[6]) which produces larger quantities of the enzyme than three type strains of *Aspergillus* (which are noted for producing high yields of glucoamylase) when the strains are compared by growing them on locally available media.[7]

Our studies have been directed towards :

(1) The preparation of a reasonably pure preparation of glucoamylase (from this strain) which is free of transglucosidase (amylo 1-4, 1-6 transglucosidase) and α amylase.

(2) The study of its properties mainly with respect to pH and temperature in order to evaluate the best conditions of hydrolysis of starch to glucose using glucoamylase from this strain of *A. niger*.

15799—10

2. Experimental

2.1 Production of enzyme

The medium[5] consisted of manioc starch, 20g ; soya bean meal, 5g ; soya bean meal digest, 5g ; $(NH_4)_2HPO_4$, 1g ; NH_4Cl, 2.5g ; $MgSO_4.7H_2O$, 0.5g ; $FeSO_4$, 10mg ; and KCl, 0.5g ; per litre. The soya meal digest was prepared as follows :—

Fresh soya beans were dried to approximately 5% moisture and ground in an edgerunner mill. The flour thus produced was extracted with petroleum ether (60 to 80) in a soxlet for 24 h, dried and reground into a fine powder. The powder was sieved (100 mesh) and suspended in water (1g for 20ml water). To this suspension was added standard papain (0.2g) and the mixture shaken (on a reciprocal shaker) for 4 h at room temperature (28°C to 30°C). The fungus was grown by inoculating approximately 10^7 spores (of strain CISIR—N_4) suspended in 'Tween 80' (0.1ml 'Tween 80' per l) into 250 ml Erlenmeyer flasks containing 50 ml of medium. The flasks were then swirled on an eccentric shaker for 5 days at room temperature (28°C to 30°C), after which the fungal pellets were filtered to give a clear filtrate containing the exo-enzyme.

2.2 Purification of enzyme

Glucoamylase was purified using the standard methods of protein purification, namely, solvent precipitation, salt precipitation, Sephadex gel chromatography and DEAE-cellulose chromatography. Preliminary studies showed that the bulk of the activity was precipitated at concentrations between 70% and 80% isopropyl alcohol. Further, most of the glucoamylase was precipitated at concentrations of $(NH_4)_2SO_4$ between 70% and 90% saturation. This data was used in the purification of glucoamylase. The procedure adopted was as follows : to the crude filtrate was added isopropyl alcohol to 80% by volume and the mixture left to stand at 0°C for 4 h to precipitate the enzyme. The protein was separated by centrifugation and dissolved in a few ml 0.04M acetate buffer pH 5.5 and introduced into a column of Sephadex G-200 (bed volume 92 ml). The mixture of proteins was eluted at 20 ml/h with 0.04M acetate buffer pH 5.5 collecting 2 ml fractions. The fractions containing saccharogenic activity was pooled and concentrated by $(NH_4)_2SO_4$ precipitation (overnight at 0°C) retaining the protein precipitated between 70% and 90% saturation. The precipitate was dissolved in 0.02M acetate buffer, pH 5.5 and was dialysed against the same buffer (three times with 800 ml buffer). The dialysate was introduced into a column of DEAE-cellulose (40 ml bed volume equilibrated in 0.02M acetate buffer pH 5.4) and eluted using the same buffer containing the following concentration of NaCl, (*a*) No NaCl (50 ml) ; (*b*) 0.1M NaCl (15 ml) ; 0.15M NaCl (15 ml) ; 0.20M NaCl (15 ml) ; 0.25M NaCl (15 ml) ; 0.3M NaCl (30 ml) ; 0.4M NaCl (50 ml) and 0.5M NaCl (80 ml) at an elution rate of 20 ml/h while collecting fractions of 3 ml and 2 ml.

2.3 Measurement of activity

Total saccharogenic activity was measured by quantitating the glucose released by the method of Nelson.[12] The reaction mixture contained 7.5 ml starch (2%), 1 ml of 0.2M acetate buffer (pH 4.3) and sufficient quantity of enzyme (in 0.1 ml). The mixture was incubated at the specified temperature (generally 55°) for 1h, aliquots (0.1 ml to 0.3 ml) being removed at zero time and at 15 min intervals. The aliquots were introduced into the Nelson reaction mixture which was immediately placed in a

boiling water-bath. The colour developed was measured using a Klett Summerson Colorimeter (green filter). The amount of glucose liberated at various times was calculated using a standard curve. Enzyme activity, expressed in μ moles/ml/min, was calculated using the progress curve of the reaction (which was generally linear). Corrections were made for protein by using blank and zero time readings which are subtracted from reducing sugar volumes. It was found that these values are very low (< 5 Klett) due to the small amount of protein present in comparison to the experimental readings which were in the order 50 to 225 Klett.

2.4 Properties of the enzyme

The effect of pH and temperature on enzyme activity were studied using the above conditions using a thermostated water bath and a buffer (acetate) of the appropriate pH (0.4M). Reaction time was varied according to the purpose of the experiment (30 min or 20 h).

2.5 Starch-iodine reaction

The reaction was performed by the standard method[14] using 1 ml of 1/10 diluted reaction mixture and reading the absorption at 600 nm on a UNICAM SP3 spectrophotometer.

2.6 Estimation of protein

This was done by the method of Lowry.[11]

2.7 Chromatography

Paper chromatography of reaction products was performed using Whatman No. 1 chromatography paper with butanol : pyridene : water (4 : 6 : 3) as solvent.[16]

3. Results and Discussion

3.1 Some properties of the crude extract

The supernatant resulting from 5 days fungal growth was a dark pink easily filterable liquid of pH 6 to 7. It generally contains a total saccharifying activity equivalent to about 40 μ moles glucose/ml/min which is mainly the result of glucoamylase activity. However, varying amounts of α amylase were also present. This was especially evident about a day before the optimum period of growth for glucoamylase production.

Evidence for α amylase activity was the rapid clearing of starch without liberation of sufficient quantities of reducing sugar. The variation of α amylase levels of cultures is shown in Figure 1. The decrease in absorption (at 600 nm) of the starch-iodine reaction is proportional to α amylase content. The percentage decrease in absorption when 20% of the starch is converted to glucose gives a good estimate of the α amylase activity of the extract.[6] This may be given a numerical value by plotting a standard curve with known ratios of α amylase and glucoamylase (Figure 2). The α amylase used in this experiment was BDH α amylase and the glucoamylase used was a crude extract incubated at pH 2.5 for 4 days at 0°C to destroy α amylase. Although this gives a good estimate of α amylase levels it is not strictly valid because: (1) BDH α amylase (bacterial) and not the α amylase from this *A.niger* strain was used and (2) experiments carried out later showed that the glucoamylase used probably had low levels of α amylase (compare with Figure 5).

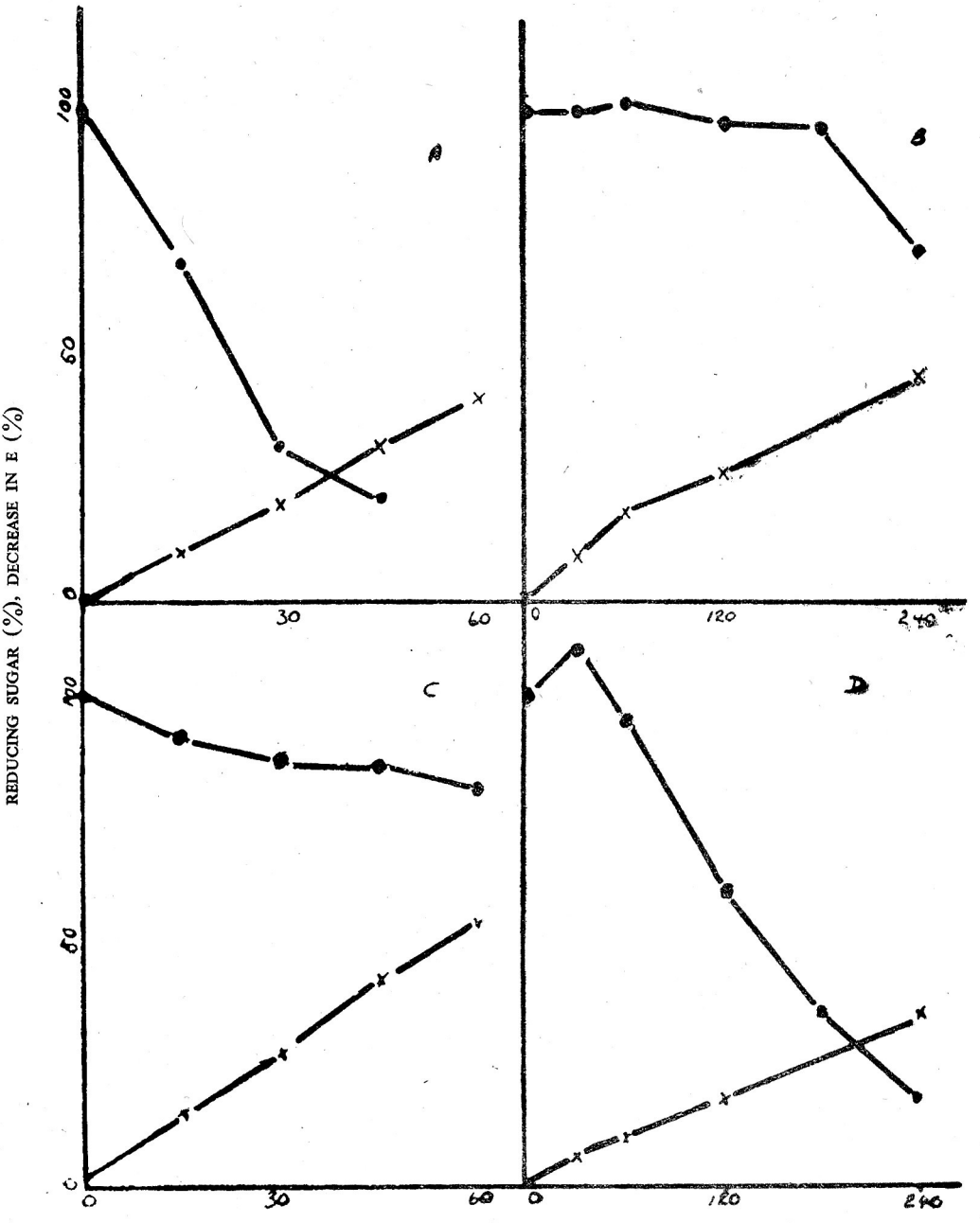

Figure 1. α Amylase levels of culture filtrates

Insets, A, B, C and D show the % release of reducing sugar and % decrease in absorption (E) at 600 nm of the starch iodine colouration by saccharifying enzymes of different culture filtrates. For details see section 3.1.

According to the curve on Figure 2, the α amylase index of A, B, C, and D would be > 30, 0, < 5 and 30 respectively.

X—X, starch iodine colouration.

● — ●, glucose released.

Zero time absorption of the starch-iodine reaction was 0.60 (the assay was carried out as in 2.5). This represents 100% absorption. Total reducing sugar releasable was 400 μmoles.

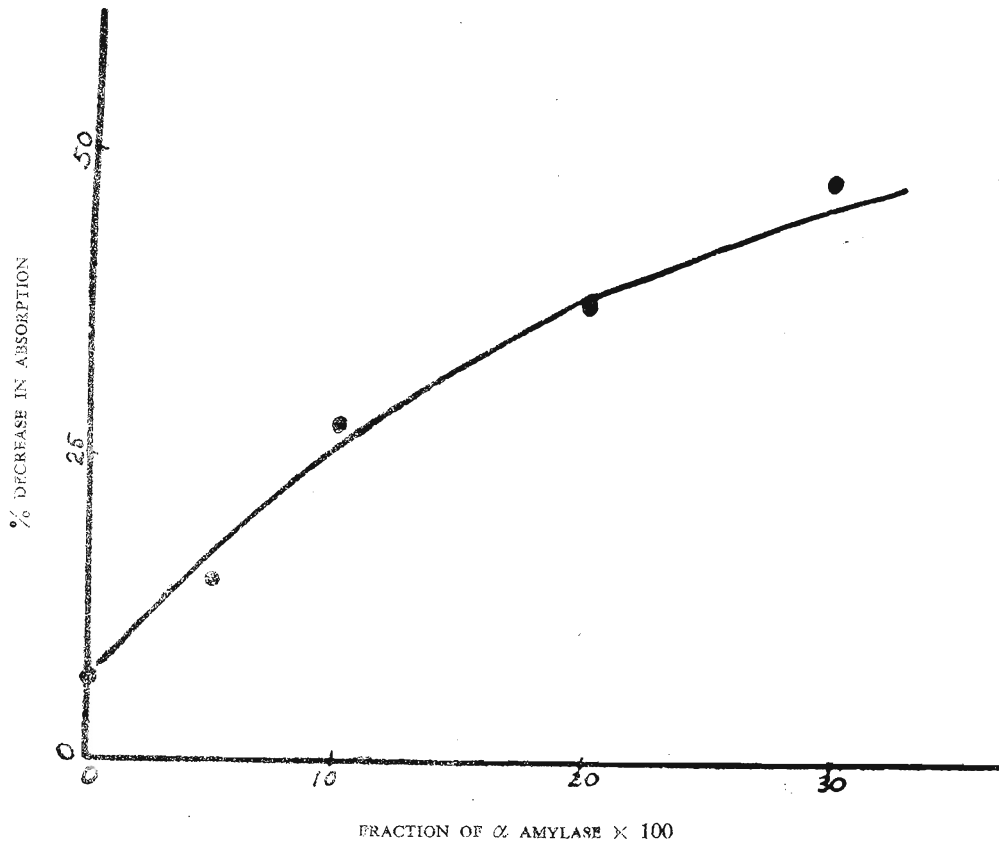

Figure 2. Standard curve for α amylase index.

Percentage decrease of absorption at 600 nm at 20% recovery of reducing sugar (20 D.E.) is plotted vs fraction of α amylase. The activity of α amylase used for the plot being calculated on the release of reducing sugar from starch.

Zero time absorption of the starch-iodine reaction was 0.60 (the assay was carried out as in 2.5). This represents 100% absorption. Total reducing sugar releasable was 400 μmoles.

The saccharogenic activity of crude extracts had a pH optimum that was broad and varied between pH 4.0 and 5.0. The temperature optimum lay between 52°C and 56°C. The extract converted starch to reducing sugar at an efficiency of 85% to 90%. This reflected some transglucosidase activity, which was confirmed by observing the products of hydrolysis by paper chromatography where, in addition to glucose (95% of reducing sugar as determined by the Nelson method after elution of spot) small quantities of other sugars were also present, their R_f values corresponding to that of isomaltose, isomaltotriose and a pentasaccharide.

3.2 Purification of Glucoamylase

During the preparation of glucoamylase for commercial use[7] the procedure used was to concentrate the enzyme at temperatures less than 55° C (under vacuum) followed by dialysis to remove fungal by-products. The process (Table 1) resulted in a sizable loss of activity. Results in section 3.3.3 indicate that the loss of activity might be reduced by evaporation in the presence of starch. Results in Table 2 show that $(NH_4)_2SO_4$ precipitation and precipitation with isopropyl alcohol were more efficient methods of concentration. However, the commercial application of these methods will largely depend on recovery and re-use of the precipitants. The methods used resulted in an eleven-fold purification of the enzyme (Table 2). Although this appears rather low in comparison with the degree of purification achieved for other enzymes, it must be remembered that glucoamylase is an exoenzyme and is present in a relatively high specific activity even in the crude filtrate.

TABLE 1. Concentration of saccharifying activity.

Step	Total Activity (μmoles/min)	Volume (ml)	Total protein (mg)	Sp Activity (μmoles/mg protein/min)	Fold purification	Recovery (%)
1. Crude Extract	2520	100	102.5	24.5	1	100
2. Concentrate (Vacuum evaporation at 55°C)	1375	27.5	68.8	19.1	0.8	55
3. Isopropyl Alcohol precipitation (80%)	1306	10.5	46.2	28.0	1.14	51

Steps 1, 2 and 3 were carried out successively on the same sample.

TABLE 2. Purification of glucoamylase

Stage of Purification	Total Activity (μmoles/min)	Total protein (mg)	Volume (ml)	Sp Activity (μmoles/mg protein/min)	Fold purification	Recovery (%)
1. Crude extract	1314	168	71.0	7.7	1	100
2. Isopropyl alcohol precipitation	1095	35.2	8.7	31.4	4.1	87
3. Sephadex G-200 Gel chromatography	500	8.0	20.0	61.1	8.0	39
4. Salt precipitation $(NH_4)_2SO_4$	393	5.1	5.7	78.4	10.2	31
5. DEAE-cellulose chromatography	139	1.64	8.0	85.0	11.0	10

For experimental details see section 2.2.

Another point of note is that DEAE-cellulose chromatography (Figure 4) results in two minor peaks of saccharogenic activity. Paper chromatographic analysis of the products showed that glucose was the only reducing sugar formed when starch was hydrolysed. However the specific activity of the peaks at eluent volumes of 119 ml and 111 ml were only 9 μmoles and 7 μmoles glucose/mg protein/min. respectively, and the significance of this existence is not known, but it could indicate the presence of isoenzymes[13] as postulated previously.

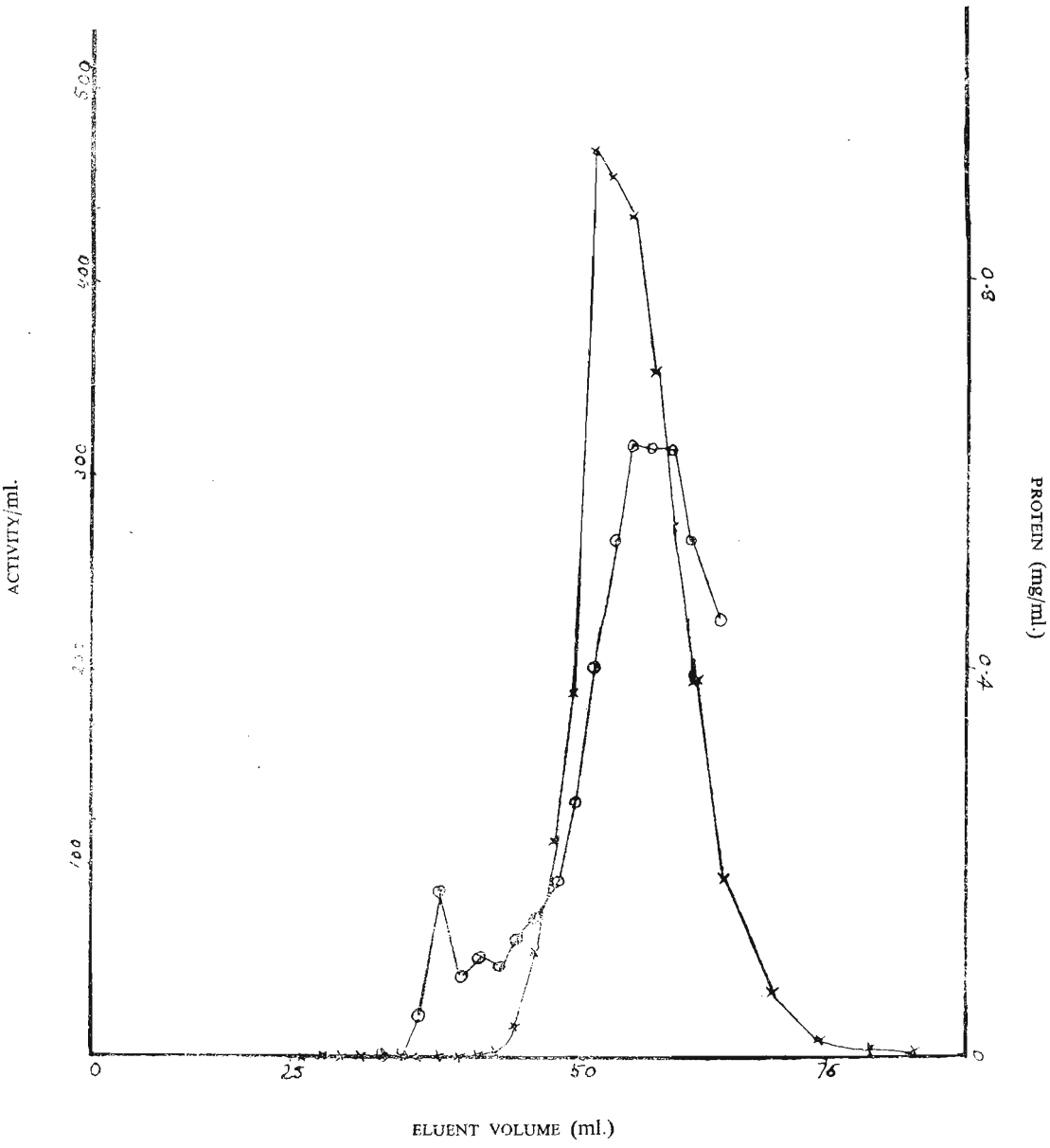

Figure 3. Sephadex-gel chromatography (G-200) of saccharifying activity of *A. niger* filtrates.

For details see section 2.2.

O—O, protein
X—X, activity

Activity expressed in mg glucose/ml/h = μmoles/ml/min x 1/10.8.

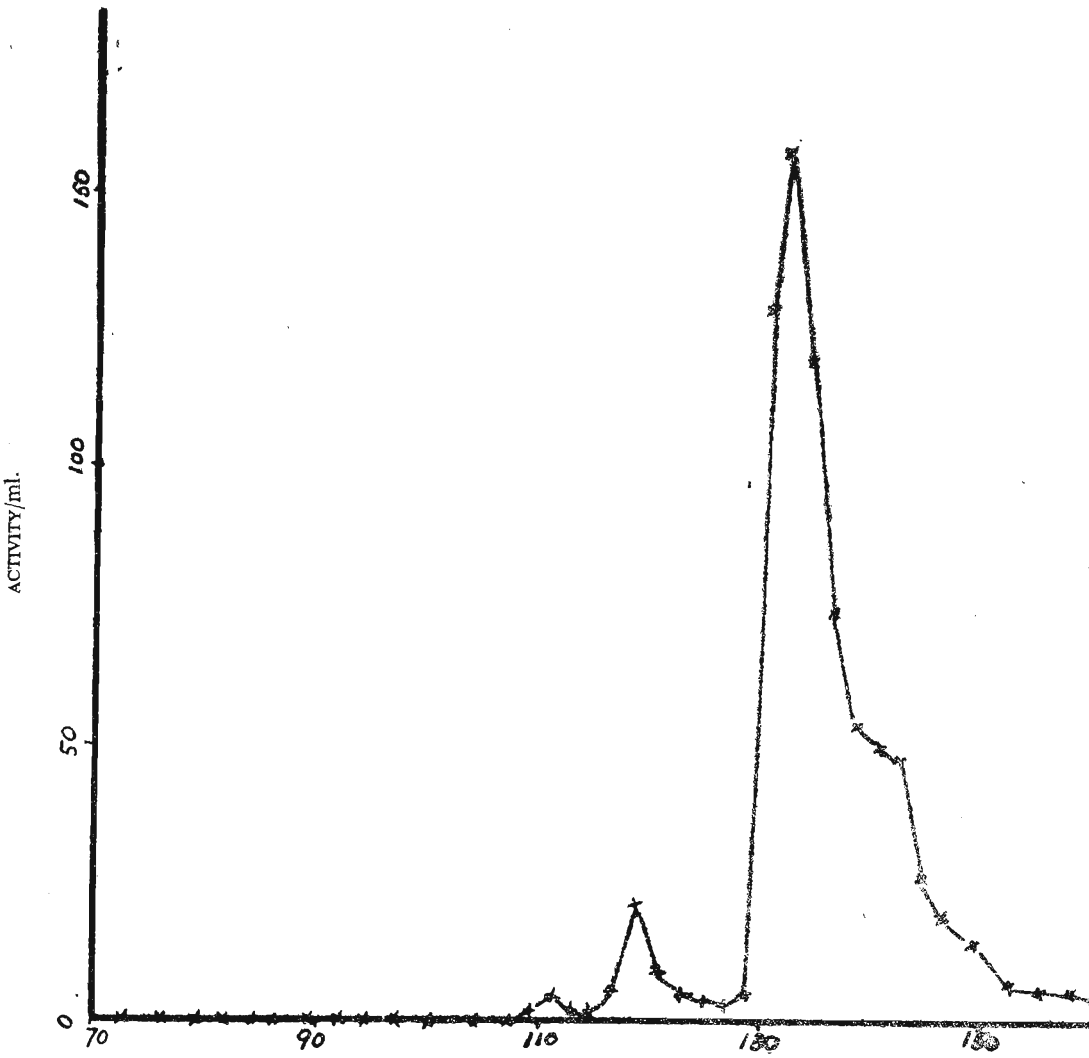

Figure 4. DEAE-cellulose chromatography of glucoamylase containing extracts. For details see section 2.2.

Activity expressed in mg glucose/ml/h = μmoles/ml/min x 1/10.8.

Thinning of starch by extracts (reflecting α amylase activity) was observed up to the DEAE - cellulose stage of purification with glucoamylase activity but was lost after this stage probably due to deactivation of α amylase or its irreversible binding on the column.

3.3 Properties of Glucoamylase (purified)

3.3.1 *Purity*

The enzyme preparation did not produce a significant thinning of starch (as evidenced by the starch-iodine reaction) even after more than 40% of the total theoretical amount of glucose was produced showing that it was free of α amylase (Figure 5). A study of a time course of the reaction (Figure 6) showed that a nearly theoretical amount of glucose (> 97%) was produced from starch. This indicates the complete (or nearly complete) absence of the transglucosidase in the presence of which yields of this magnitude cannot be achieved.[9] Further evidence for the absence of transglucosidase was provided by paper chromatography experiments which showed that glucose was the only product of starch hydrolysis.

3.3.2 *Basic Kinetics*

The effect of concentration of enzyme (Figure 7) did not have any deviations from linearity showing the absence of complicating factors such as the presence of activators or association-dissociation phenomena. Figure 6 also shows the linear nature of the reaction up to nearly 75% conversion of starch to glucose.

3.3.3 *Effect of temperature*

An Arrhenius plot of the enzyme activity with temperature is shown in Figure 8 ; the rates being calculated over 30 min reaction time. The plot clearly illustrates the interplay between two opposing factors, (1) the increase in the rate of reaction with temperature and (2) the increase in extent of denaturation with temperature. The figure shows that even over reaction time of 30 min that some deactivation occurred at 60°C (although the absolute reaction rate became progressively faster even up to 70°C). However, a reaction time of the order of 30 min is of little significance for the commercial process which generally needs 20 h to 100 h enzymic conversion. Results on Table 3 give the extent of reaction at varying temperatures over a reaction time of 20 h. Substrate is not limiting and therefore any deviation from the theoretical rate (calculated from extrapolation of linear slope of the Arrhenius plot) is due to deactivation of the enzyme. The results clearly showed that : (1) over this reaction time the optimum temperature is 60°C and (2) at temperature of 55°C and over there is considerable denaturation which progressively increases with temperature. If a calculation of percent denaturation over 20 h is computed, then it is clear that during successive 20 h periods that lower reaction temperatures will

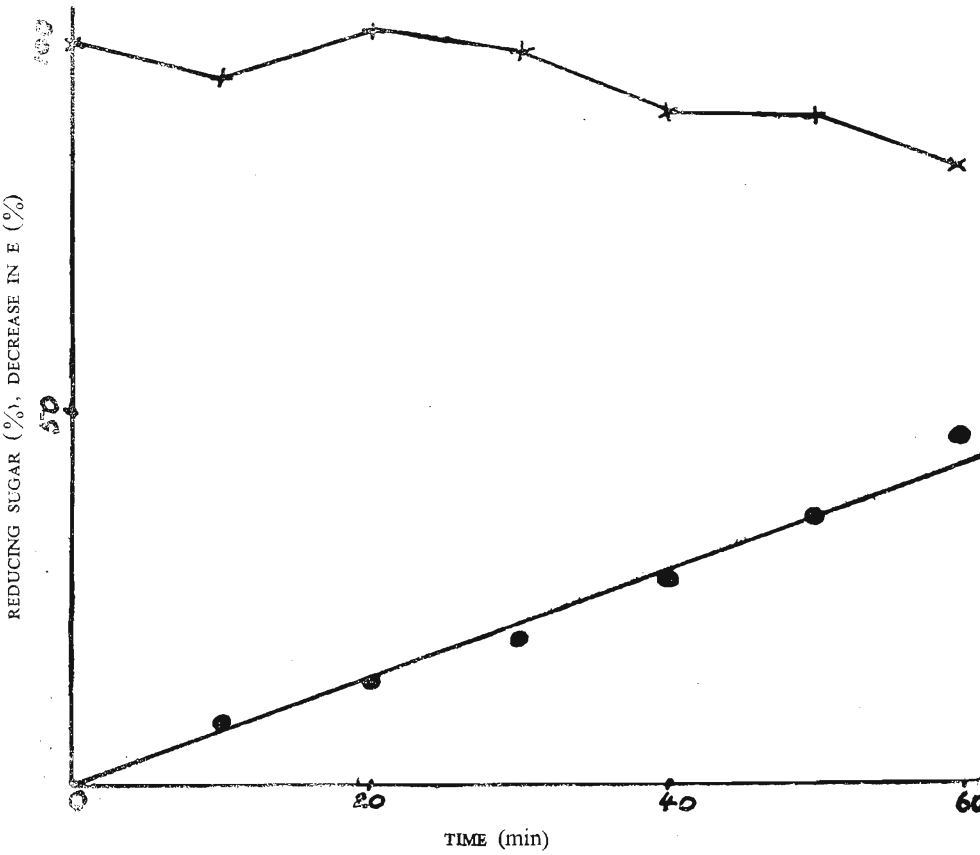

Figure 5. Effect of glucoamylase on the starch-iodine reaction.

Details as in Figure 1 and section 3.1.$\frac{5}{2}$The reaction mixture was the same as in Figure 6 but the glucoamylase added was ~ 3 μmoles/min contained in a volume of 1.5 ml.

X—X, starch iodine absorption (% of zero time).

● — ●, glucose released (% theoretical of starch used).

Zero time absorption of the starch-iodine reaction was 0.60 (the assay was carried out as in 2.5). This represents 100% absorption. Total reducing sugar releasable was 400 μmoles.

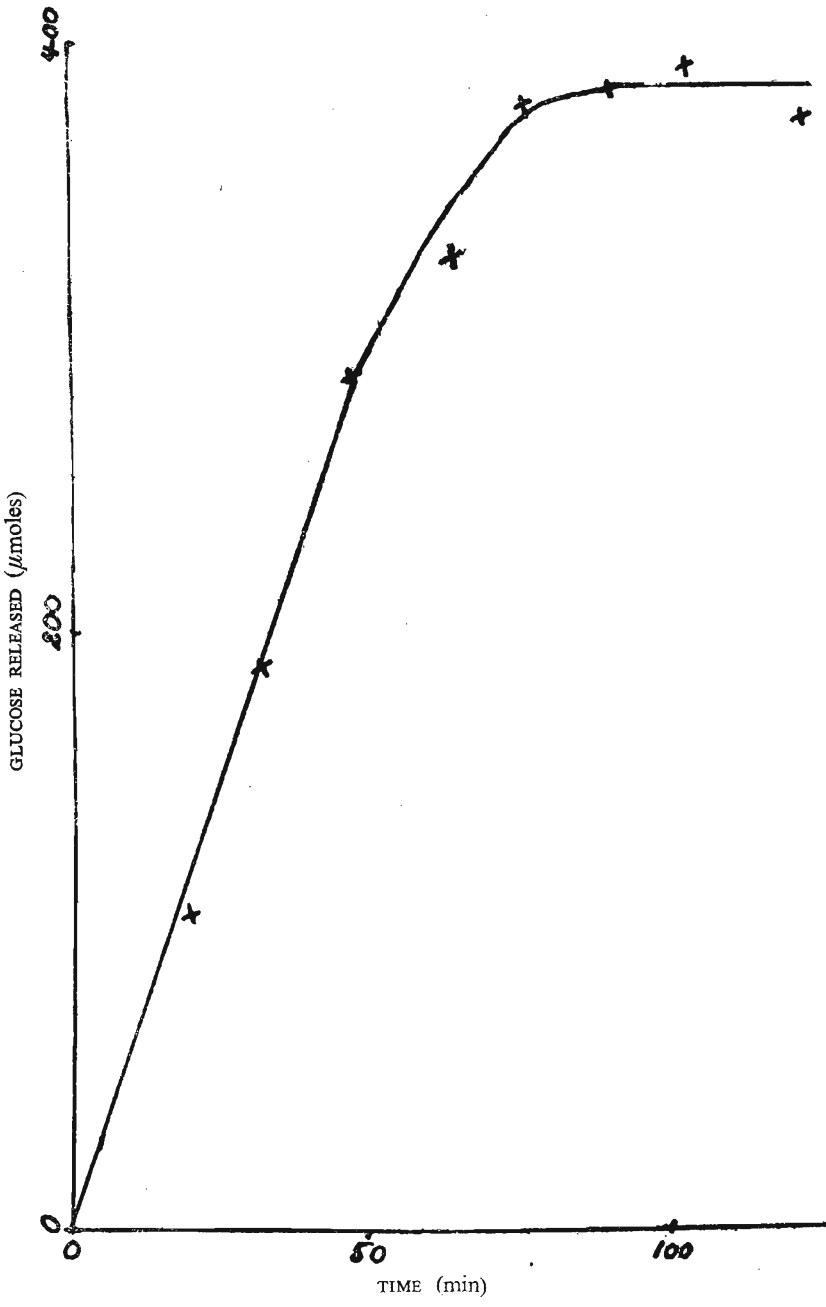

Figure 6. Glucose liberation by glucoamylase.

The reaction mixture contained: 1% starch (7.5 ml) 0.4M acetate buffer, pH 4.3 (1 ml) and glucoamylase (~6 μmoles glucose/min in 0.25 ml). Total theoretical yield of glucose was 407 μmoles

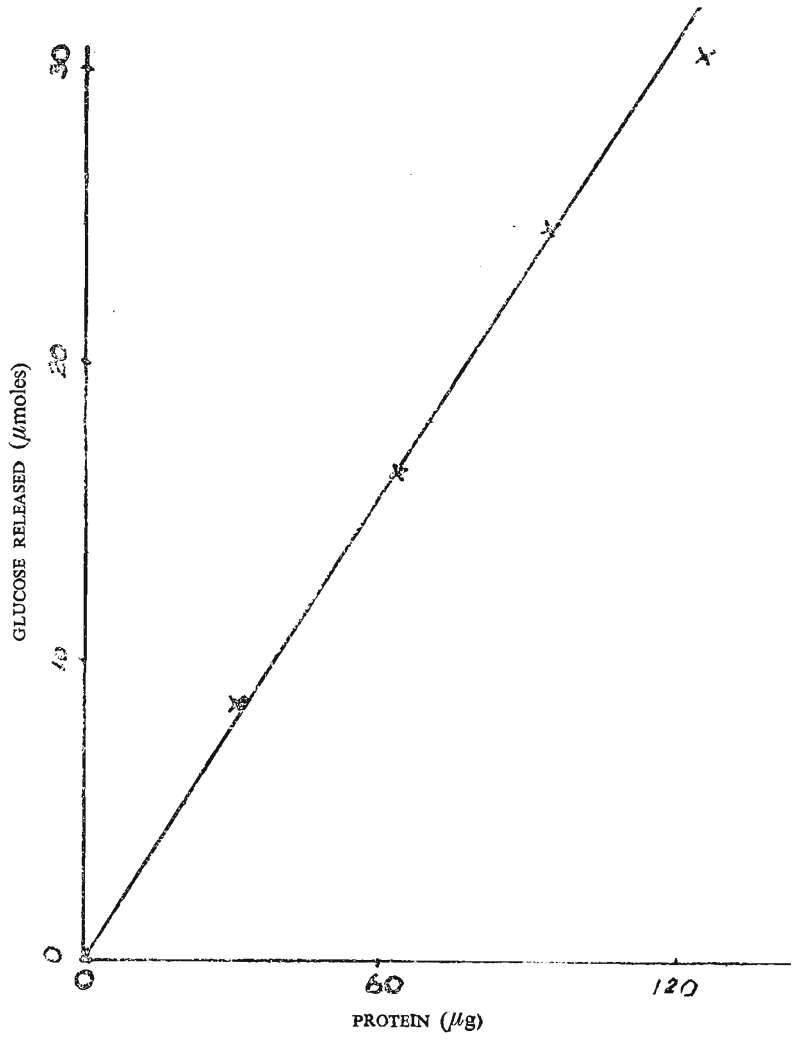

Figure 7. Effect of concentration on the release of glucose from starch by glucoamylase.

become progressively more favourable. Therefore it would be possible to calculate the most favourable temperature of reaction for different reaction times, keeping in mind that there is a limit to lowering temperature as reduced temperature would favour microbial contamination and deterioration. From these results, it appears that a reaction temperature of 60°C would be most favourable if reaction time is of the order of 20 h to 60 h. However, beyond this reaction time a lower temperature (closer to 50°C) could give a better overall rate of reaction.

TABLE 3. Effect of temperature on hydrolysis of starch

Temperature (°C)	Glucose released (mg)	Theoretical glucose (mg)
28	11	20
38	34	34
45	56	64
50	101	98
55	110	152
60	146	202
65	21	304

Experimental glucose release was estimated by using a reaction mixture containing 2% starch (35ml) 0.4M, pH 4.3 acetate buffer (2 ml) and approximately 0.8 μmoles/min glucoamylase.

Theoretical glucose has been calculated using an extrapolation of the Arrhenius plot of Figure 8.

3.3.4 *Effect of pH*

Unlike the effect of temperature the effect of pH does not appear to be critical. Over both reaction times (30 min and 20 h) the activity is relatively high over a wide range of pH. The optimum pH appears to be 4.0 to 4.3 very little difference occurring between pH 3.6 and 4.6. These studies indicate that the enzyme studied probably corresponds to one of the two isoenzymes isolated by Pazur and Okada[13] (which is reported to have a pH optimum of 4.0 ; the other having an optimum pH of 6.5 according to that study).

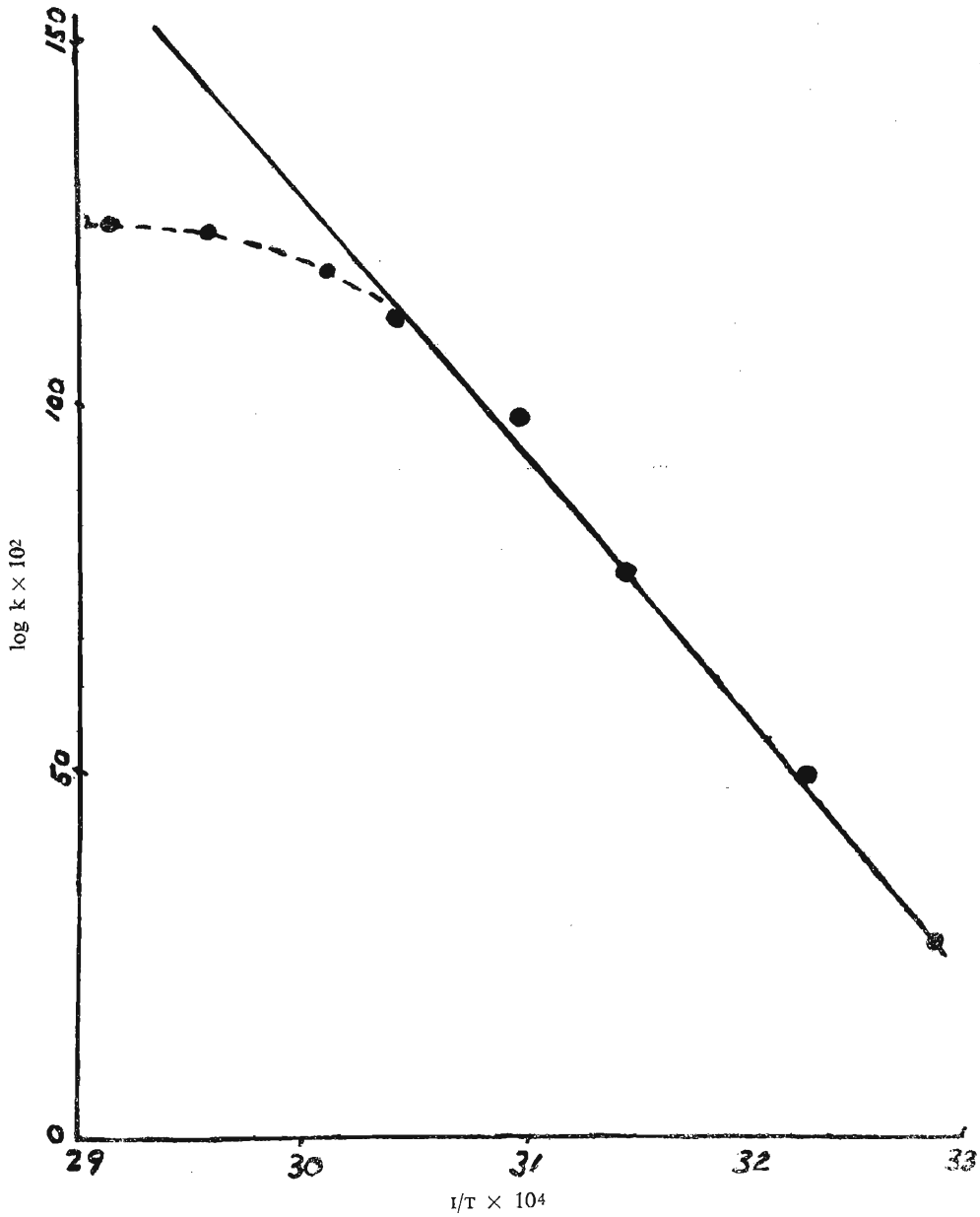

Figure 8. Arrhenius plot for Glucoamylase.

Reaction time was 30 min. K is expressed in terms of μmoles glucose released/ml/min. The extract contains 205 μg protein/ml.

For further experimental details see section 2.3.

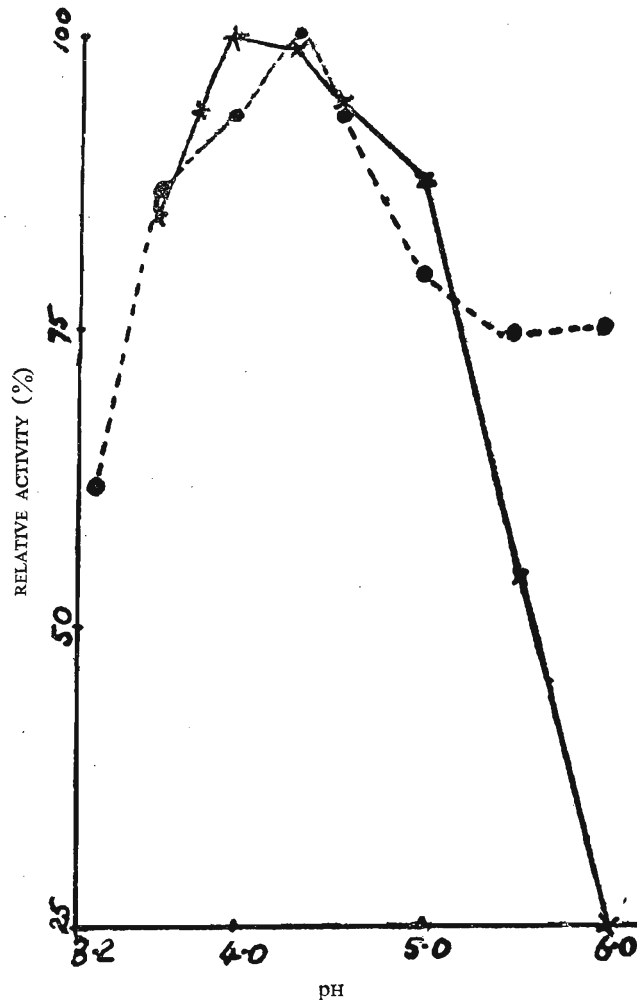

Figure 9. Effect of pH on Glucoamylase activity.

O—O, Reaction time 30 min. Experimental procedure as in section 2.3.

X—X, Reaction time 20h. Experimental procedure as in table 3, but pH varied and temperature constant at 60°C.

100 percent activity represented 17.4 and 18.2 μmoles/mg protein/min. respectively for the experiments.

4. Conclusions

The glucoamylase produced by a strain of *Aspergillus niger* (producing the enzyme in large quantities on locally available substrate) would be most effective, in the commercial conversion of starch to glucose, at pH 4.3 and 60°C even though there is significant enzyme deactivation at this temperature.

Acknowledgements

The authors thank the Director, CISIR, for facilities provided, Mr. D. J. Abeyratne for technical assistance and Miss K. Jayaratne for secretarial assistance.

References

1. Amarakone, S. P., Jansz, E. R., & JeyaRaj, E. E. (1973). *Proc. of the 2nd Annual Session of the Institute of Chemistry*, June 26th 1973 : 5-8.
2. Bendelskii, K. M., Yarovenko, V. L., Ermakova, L. N., & Lukyanova, L. N (1970) *Biochimiya* **35** : 1099-1105.
3. Fukui, T. & Nikuni, Z. (1969). *Agr. Biol. Chem.*. (Tokyo) **6** : 884-891.
4. Fukumoto, J. & Tsujisaka, Y. (1954). *Sci. and Ind.* (Japan) **28** : 92.
5. Greenwood, C. T. & Milne, E. A. (1968). *Advances in Carbohydrate Chemistry* **23**: 281-366.
6. Jansz, E. R., Amarakone, S. P. & JeyaRaj, E. E. (1973). *Proc. of the 2nd Annual Session of the Institute of Chemistry*, June 26th 1973, 1-4.
7. Jansz, E. R. & JeyaRaj, E. E. (1976). Unpublished results—*CISIR reports* 1974-76.
8. Johanides, V., Korculanin, A. & Hrsak, D. (1969). *Microbiologija* **6** : 71-76.
9. Lachmann, A. (1970). In, *Starches and Corn Syrups*. Noyes Data Corporation, New Jersey, U.S.A. p. 161-222.
10. Lachmann, A. (1970). In, "Starches and Corn Syrups". Noyes Data Corporation, New Jersey—U.S.A. p. 225-244.
11. Lowry, O. H. ; Rosebrough, E. J. ; Farr, A. L. & Randell, P. J. (1951). *J. Biol. Chem.* **193** : 265.
12. Nelson, N. (1944). *J. Biol. Chem.* **153** : 375-380.
13. Pazur, J. H. & Okadas (1967). *Carbohydrate Res.* **4** : 371.
14. Radley, J. A. (1968). In *Starch and its Derivatives*, **1**, 4th Edition, Chapmen and Hall.
15. Sternberg, M. (1970). (Miles Labs Inc.) U.S.P. 3, 483, 084, C.A. **72** : 51290.
16. Tsujisaka, Y., Fukumoto, J. & Yamoto, T. (1958). *Nature* **181** : 770.