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Biomass conversion : Synergistic use of immobilized α-amylase and amyloglucosidase for rapid and maximum conversion of starch into glucose

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Enzyme technology is developed for the conversion of starch into glucose syrup, containing 99% (*w/w*) glucose using synergistic action of α-amylase and amyloglucosidase. For repeated and efficient use of these enzymes, they were covalently immobilized on agaropectin, which proved to be an ideal support for immobilization of these enzymes. Parameters for immobilized enzymes were determined and compared with free enzymes. Optimum enzyme starch ratio was determined for rapid and maximum conversion of starch into glucose. Attempts were made to improve quality of glucose by minimizing the reversion product formation.

Starch is a photosynthetic product recovered from edible goods and has industrial importance due to its use as a raw material for glucose production. The role of glucose in health care products is obvious[1].

Starch is a homopolymer of glucose consisting of amylose, a linear fraction containing α (1→4) glycosidic linkage and amylopectin, a branched fraction containing both α (1→4) and α (1→6) glycosidic linkages. To overcome disadvantages of acid hydrolysis of starch, recently α-amylase and amyloglucosidase are most widely used. Enzymic hydrolysis guarantees purity of the product. It is free from bitter taste and toxicity of hydroxymethyl furfuraldehyde produced during acid hydrolysis.

α-amylase (EC 3.2.1.1) is an endoenzyme which randomly hydrolyses α (1→4) glycosidic linkages in amylose, amylopectin and related polysaccharides and yields lower molecular weight products limited by α (1→6) glycosidic bonds, which form branch points[2]. Amyloglucosidase (EC 3.2.1.3) is exoenzyme, catalyses stepwise hydrolysis of α (1→4) links from the non reducing ends, releases glucose. The α (1→6) branch links are also hydrolysed at relatively slow rate and glucose syrups with glucose content of 97% (*w/w*) being formed[2]. About 3% (*w/w*) higher saccharides are formed due to transglycosylation action of amyloglucosidase[3-6].

In recent years, immbolization techniques are used for preparation of insoluble but catalytically active enzymes which permit their recovery and reuse after completion of the reaction[7,8].

Experimental Procedure

Materials

α-amylase—Bacillus licheniformis α-amylase (Sigma Chemicals, USA) was purified using starch adsorption procedure[9]. Raw potato starch was thoroughly washed using 1*M* NaCl and then soaked in potassium phosphate buffer (10m*M*, *p*H 7.0 containing 10m*M* KCl). Enzyme solution, 1 mL (2500 units) was dialysed against the same buffer. Starch suspended in buffer was thermostated at 60°C for two hours with occasional stirring, supernatant discarded and enzyme solution was added to it with continuous stirring at 27°C for 30 min. Then the enzyme adsorbed starch was repeatedly washed using ice cold potassium phosphate buffer (10 m*M*, *p*H 7.0 containing 10 m*M* KCl) till the supernatant was free from α-amylase activity. The adsorbed enzyme was eluted in 10 mL of 10% (*w/v*) maltose prepared in potassium phosphate buffer (10 m*M*, *p*H 7.0 containing 10 m*M* KCl) and dialysed against ice cold potassium phosphate buffer (10 m*M*, *p*H 7.0 containing 10 m*M* KCl) till it was free from maltose, then lyophilized to concentrate.

Amyloglucosidase—Aspergilles niger amyloglucosidase (sigma chemicals, U.S.A.) 1 mL (2500 units) was chromatographed on Bio Gel P-100 into two peaks[10]. The column dimensions were 1.5×72 cm, flow rate 10 mL/h and the eluent buffer was

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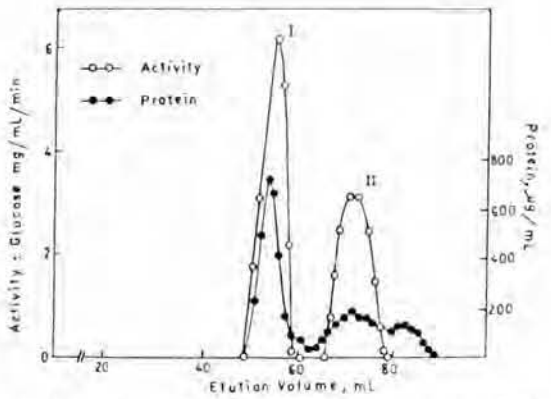

Fig. 1—Elution profile of amyloglucosidase on Bio-gel *P*-100.

potassium acetate (25 m*M*, *p*H 4.5 containing 50 m*M* KCl). The first major peak having maximum amyloglucosidase activity was used for the study.

Methods

Enzyme assay—α-amylase was assayed by measuring the amount of the reducing sugars liberated from 1% (*w/v*) starch at *p*H 6.0, 50°C. Amyloglucosidase was assayed by measuring the amount of glucose liberated from 1% (*w/v*) starch at *p*H 4.5 and 50°C.

Reducing sugars were measured using 3, 5-dinitrosalicilic acid method[11] and glucose was specifically measured using glucose oxidase-peroxidase coupled enzymatic assay method[12].

One unit of α-amylase was defined as the amount responsible for the production of one mg of reducing sugar per minute, and one unit of amyloglucosidase was defined as the amount responsible for the production of one mg of glucose per minute under above mentioned assay conditions.

Preparation of agaropectin—Agaropectin was prepared from agar-agar using ethanol fractional precipitation method[13]. Agar-agar 5.0g was dissolved by boiling in 100 mL of 8M urea solution. Then it was cooled to 40°C and in a thin stream, distilled ethanol (100 mL) was added with continuous stirring to achieve 50% (*v/v*) ethanol concentration. Precipitated agarose was discarded after centrifugation (5000 rpm/15 min) and ethanol concentration of supernatant was raised upto 55% (*v/v*), with the addition of 22.2 ml of distilled ethanol. Again the precipitate was discarded after centrifugation (5000 rpm/15 min) and to the supernatant, equal volume of distilled ethanol added slowly with continuous stirring. The precipitate was agaropectin; it was

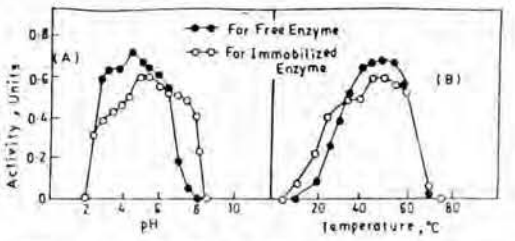

Fig. 2—Effect of (A) *p*H and (B) temperature on activity of amylogucosidase

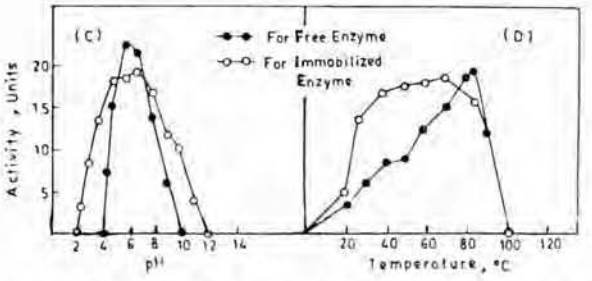

Fig. 3—Effect of (C) *p*H and (D) temperature on activity of α-amylase

repeatedly washed with distilled ethanol till complete removal of urea from it, and then air dried.

Immobilization of enzymes—Agaropectin 2.0g was dissolved in 100 mL distilled water and cooled to 4°C. The gel formed was soaked in ice cold carbonate bicarbonate buffer (200 mM, *p*H 11.5), maintained below 4°C and cynogen bromide (CNBr) crystals 50mg approximately were added and well stirred[14,15]. After overnight storage at 4°C, slurry was dialysed in distilled water at 4°C till it was free from the traces of unreacted cyanogen bromide. It was then soaked in potassium phosphate buffer (50mM, *p*H 7.5) and equally distributed in two aliquots at 4°C. About 2500 units of α-amylase and 2500 units of amyloglucosidase prepared in the same buffer were added in respective aliquots, stirred well and after overnight storage unbound enzymes were removed by repeated washings with ice cold potassium phosphate buffer (50 mM, *p*H 7.0).

The activity of respective bound enzyme was measured using above mentioned assay system. The parameters like optimum *p*H, optimum temperature and Km (Michaelis constant) were determined.

Conversion of 30% starch into glucose—Conversion of 30% starch prepared in potassium acetate buffer (25 mM, *p*H 6.0 containing 50 mM KCl) was studied at 50°C for α-amylase and amyloglucosidase separately as well as synergistically.

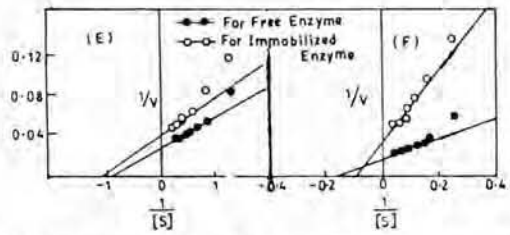

Fig. 4—Lineweaver-Burk plot for (E) α-amylase and (F) amylogucosidase.

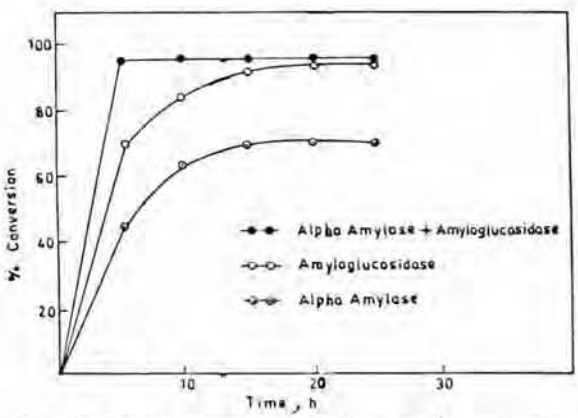

Fig. 5—Enzymic conversion of 30% starch into glucose.

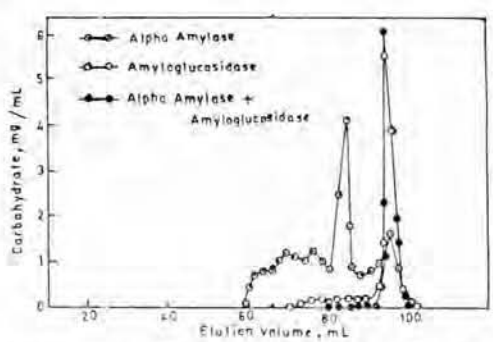

Fig. 6—Elution profile of reaction product on Bio-gel P-2.

Aliquots of reaction mixture were analysed at different time intervals for glucose using glucose oxidase peroxidase method[12]. Starch conversion was studied at different enzyme ratio (1:0, 1:1, 1:2, 1:5, 1:10) of α-amylase and amyloglucosidase, free and immobilized.

Reactor operations—In laboratory reactor operated at 45°C, 30% (*w/v*) starch slurry in potassium acitate buffer (25 mM, *p*H 6.0 containing 50 mM KCl) was used to study its conversion into glucose at 1:1 α-amylase and amyloglucosidase ratio. The reactor was operated separately for studying synergistic use of free forms of α-amylase and amyloglucosidase as well as their bound (immobilized) forms. After

Table 1—Stability study of immobilized α-amylase and amyloglucosidase.

Days	Bound enzyme activity (units)		Activity loss (%)	
	α-Am*	Amgs*	α-Am*	Amgs*
0	2140	2250	0	0
5	2140	2250	0	0
10	2140	2250	0	0
20	2140	2250	0	0
30	2140	2250	0	0
50	2140	2250	0	0
100	2080	2250	2.8	0
150	1700	2050	20.5	8.9

* α-amylase ** Amyloglucosidase

hydrolysis for 16 h, the hydrolysate was boiled for 5 min, cooled and centrifuged at 5000rpm for 15 min. Clear supernatant was collected and analysed for glucose as well as oligosaccharides using the following chromatographic procedures.

Product Analysis—Ascending paper chromatography[16] in butanol:ethanol:water (3:2:1) solvent system was used to characterize the hydrolysate and separated sugars were visualized using benzidine TCA[17] colour spray reagent. The Bio Gel P-2 gel filtration chromatography was also done for quantitative analysis. The column dimensions were 1.5×70 cm, flow rate 12mL/h and elution was done in distilled water.

Results and Discussion

Amylase-free-amyloglucosidase is unable to bring about a complete conversion of starch and glycogen into glucose. Synergistic use of α-amylase and amyloglucosidase restores the conversion. The rate of hydrolysis by amyloglucosidase depends upon the number of non-reducing ends which prompted us to develop experimental conditions which accelerated and completed the hydrolysis within short period.

Agaropectin is a nontoxic, nonbiodegradable component of agar-agar and proved to be an ideal support for immobilization of enzymes. It does not deteriorate under the experimental conditions used during immobilization. It is soluble in water and sets in the form of gel below 4°C. This property of agaropectin was explioted for the immobilization of α-amylase and amyloglucosidase, which were used for rapid saccharification of starch.

Optimum enzyme ratio (α-amylase:amylo-

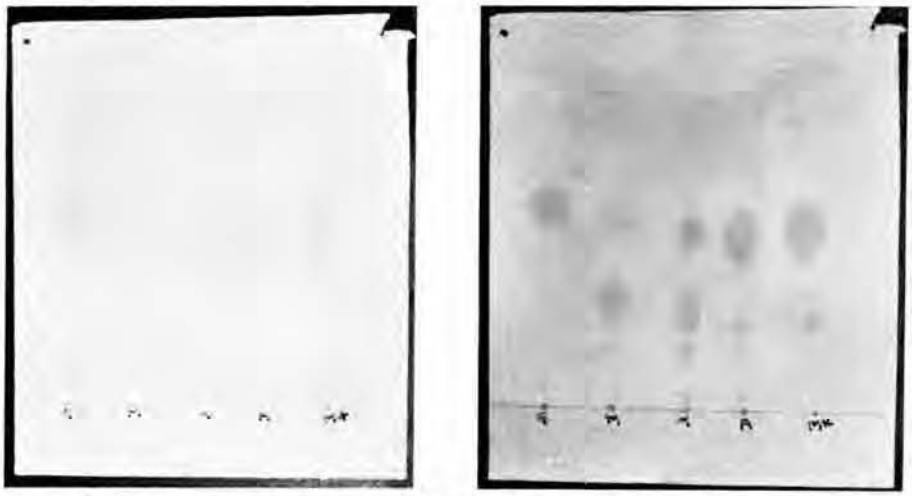

Fig. 7—Paper Chromatographic analysis of reaction product of starch conversion.

glucosidase) was found 1:1 for both free and immobilized enzymes. It gave 97% hydrolysis of 30% starch (Fig. 5). The immobilized enzymes were found stable (Table 1) and used repeatedly for 16 batches without activity loss. The narrow range of *p*H and temperature optima broadened after immobilization for both α-amylase and amyloglucosidase, which were at the favourable side (Figs 2 & 3). The Km (Miachaelis constant) did not change for α-amylase, but changed 6.7 mg/mL of starch to 12.4 mg/mL of starch (Fig. 4).

Ascending paper chromatographic qualitative analysis (Fig. 7) using butanol:ethanol:water (3:2:1) showed that the product of only α-amylase even for extensive duration was little glucose, major maltose, tri- and tetra-saccharides. The product of only amyloglucosidase hydrolysis was 97% glucose along with 3% di- and tetra-saccharides whereas the product of synergistic use of both enzymes was only glucose and traces of di-saccharides. Quantitative analysis using Bio Gel P-2 gel filtration chromatography showed that glucose content of the final product was 99% and only 0.97% di- and trisaccharides were present in it (Fig. 6). Thus, synergistic use of α-amylase amyloglucosidase (1:1) increased the glucose content of hydrolysate within a short period of 16 hr, and simultaneously decreased the reversion product formation. Synergistic action of α-amylase and amyloglucosidase in 30% starch hydrolysis, thus resulted into 4 fold decrease in time (16 h versus 60 h) and improvement in product quality as the reversion product formation was minimized. Parallel results were obtained with the immobilized enzymes system. The immobilized forms of enzymes were preserved at 4^0C and repeatedly used for 16 batches with 2.5% activity loss, indicating that they were stable.

At a stroke, these experimental conditions have reduced energy inputs time and cost of hydrolysis. Its viability needs to be explored for the industrial conversion of starch into glucose.

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