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Partial Purification and Some Properties of α-amylase and Glucoamylase Obtained as By-product From Direct Fermentation of Sago Starch to Solvent by *Clostridium acetobutylicum*

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Abstract: The starch degrading enzymes, α-amylase and glucoamylase, obtained from direct fermentation of sago starch to solvent by *C. acetobuylicum* P262 were partially purified and some of the biochemical characteristics were identified. The α-amylase was found to have maximum activity at pH 5.3 and was stable in a pH range from 3 to 9. The optimum temperature for α-amylase activity was 40°C and only 50 % of its original activity was retained after incubation for 1 h at 60°C. The α-amylase K_m and V_{max} values for soluble starch was 0.31 g/L and 10.03 U/mL, respectively. Hydrolysis rate of partially purified α-amylase was illustrated by the relative values of starch (100%), amylopectin (68%) and amylose (42%). The optimum pH and temperature for glucoamylase was 4.4 and 40°C, respectively. Glucoamylase was stable at a wide pH range (4-8). The enzyme was also stable at high temperatures where 80% of its activity was retained after 1 h incubation at 60°C. Glucoamylase has high affinity towards soluble starch (K_m = 0.039 g/L), followed by maltose (K_m = 4.39 g/L) and maltotriose (K_m = 10.43 g/L).

Keywords: *Clostridium acetobutylicum*, solvent fermentation, sago starch, α-amylase, glucoamylase

Introduction

The production of solvents by *Clostridium acetobutylicum* is of increasing interest worldwide because of its potential commercial significance. One of the research interests aim at reducing cost of raw materials for solvent production is the use of starch material due to its abundance and relatively inexpensive. The utilisation of starches for solvent production has been examined for many years and the occurrence of α-amylase and glucoamylase activities during the fermentation has been reported (Soni *et al.*, 1992). In typical batch fermentation, starch was converted to solvent with a final concentration of approximately 12-20 g/L (Maddox, 1989; Guteirrez *et al.*, 1998). Our research group has interest on direct fermentation of sago starch to solvents by *C. acetobutylicum*. During the fermentation, starch was first hydrolysed to maltose and glucose by α-amylase and glucoamylase secreted by the bacterium, respectively. Although starch was the first industrial substrate for solvent production, very few reports concerning the physiology, purification and the characterization are available on the amylases of *C. acetobutylicum* (Hyun and Zeikus, 1985; Paquet *et al.*, 1991). The α-amylase (1,4-α-D-glucanglucanohydrolase: EC 3.2.1.1) is an endo-acting enzyme that catalyst the random hydrolysis of starch at interior glucosidic lingkages of oligo and polysaccharides which results in a rapid reduction of starch viscocity depending on the specific number of the bonds broken. This enzyme is not inhibited by the α-1,6-glucosidic bond and it hydrolyses amylose to produce maltose and maltotriose (Madi *et al.*, 1987). A remarkable feature of *C. acetobutylicum* α-amylase is its stability at acidic pH that is particularly important for the efficient conversion of starch to solvent (Soni *et al.*, 1987; Paquet *et al.*, 1991).

Glucoamylase (α-D-(1-4-glucan glucohydrolase, EC 3.2.1.3) is an exo-acting enzyme which hydrolysis α-1,6-glucosidic lingkages from the non-reducing chain ends of amylose and amylopectin of starch by successive removal of low molecular weight products from the non-reducing chain ends to produce D-glucose and maltose (Hyun and Zeikus, 1985). The rate of substrate hydrolysis increases with the size of the substrate to a maximum of five and more monosaccharides units (Vandersall *et al.*, 1995). Extracellular glucoamylase has been isolated from bacteria, yeast and molds (Ensley *et al.*, 1975; Fogarty and Benson, 1983; De Mot and Verachtert, 1985; De Mot and Verachtert, 1987). The glucoamylase produced by two Clostridium species has special features for particular industrial interest because they are active at temperature of above 60°C (Hyun and Zeikus, 1985; Madi *et al.*, 1987). The current investigation describes the purification and the properties of α-amylase and glucoamylase produced as by-product during solvent fermentation by *C. acetobuyilicum* using sago starch as a carbon source.

Materials and Methods

Microorganism: The bacterium, *Clostridium acetobutylicum* P262, was used throughout this study. The culture was stored in the form of spore suspension at 4°C in the Reinforced Clostridium Medium (RCM-Oxoid) that was prepared under anaerobic condition using Hungate Technique (Miller and Wolin, 1974).

Direct Fermentation of Sago Starch to Solvent: Batch and continuous fermentations were carried out using medium consisted of gelatinised sago starch (50 g/L), KH_2PO_4 0.75 (g/L), K_2HPO_4 0.75 (g/L), $MgSO_4.7H_2O$ 0.4 (g/L), Mn_5O_4 H_2O 0.01 (g/L), $FeSO_4$ H_2O 0.01(g/L), yeast extract 5.0 (g/L), NH_4NO_3 2.0 (g/L), cystein 0.5 (g/L), resazurin 1.0 (mg/L) and vitamin solution (1mL/L). Vitamin solution used for the preparation of medium consisted of 1 mg/L P-amino benzoic acid and 0.8 mg/L biotin.

Batch fermentation was carried out using 1.5 L stirred tank fermentor (Chemap, Alfa-Laval) equipped with temperature and pH control systems. The medium in fermentor was prepared under strict anaerobic conditions according to Hungate technique by sparging the medium under stream balance of free oxygen N_2 gas. The fermentor and medium was autoclaved at 121°C for 20 min. Prior to inoculation, the vitamin solution prepared under anaerobic conditions was added aseptically into the medium. Batch fermentation was initiated by inoculating 10% (v/v) vegetative cells which was previously grown for 12 h in the RCM medium. A mild mixing was performed by agitation at 100 rpm using 6-bladed Rusthon turbine impeller. The culture was initially sparged with N_2 free oxygen gas to maintain the aerobic conditions until sufficient fermentation activity was reached. Continuous fermentation was

performed using 300 mL jar fermentor (B. Braun Biotech. International, Germany) with the same control system as for batch fermentation. Continuous fermentation was initiated after 16 h of initial batch fermentation when growth reached a stationary phase. During continuous fermentation, a fresh sterile medium containing 30 g/L sago starch was fed continuously at a constant flow rate to get a required value of dilution rate. The culture was agitated using a bar driven by magnetic stirrer at 100 rpm. During continuous fermentation, anaerobic condition was maintained in the culture by sparging a sterile N_2 gas continuously into the fermentor. For both fermentation modes, the temperature within the fermentor was controlled at 35°C. The culture pH was measured continuously using a sterilizable pH electrode (Ingold-Switzerland) and maintained at the desired value by automatic addition of 2 N NaOH. During the fermentation, samples were taken at time intervals for analysis.

Partial Purification of the Amylolytic Enzymes: The α-amylase was partially purified using ethanol precipitation method. Ethanol that was previously chilled to -20°C was added dropwise to the culture filtrate at 4°C with continuous stirring to the final concentration of 75% and the solution was kept at -10°C for 24 h. The protein precipitate formed was dissolved in 0.05 M acetate buffer pH 5.3. Glucoamylase was partially purified using two steps purification procedure. The culture filtrate was first subjected to ammonium sulphate fractionation (30-100% saturation). The precipitated protein was then dissolved in acetate buffer (0.1 M, pH 4.4) and dialysed overnight against distilled water. A volume of chilled acetone (-16°C) was added slowly to the redissolved protein precipitate fraction with a constant stirring. After standing overnight at 4°C, the solution was centrifuged at 10,000 g for 30 min. The resultant precipitate was then dissolved in a small amount of acetate buffer (0.1 M, pH 4.4) and dialysed overnight against distilled water.

Hydrolysis Rate of α-amylase and Glucoamylase: The comparative study of hydrolysis rate of α-amylase was conducted using 1.0 g/L (w/v) substrate. The various substrates were prepared in 0.05 M acetate buffer pH 5.3 and the hydrolysis was carried out at 37°C for 50 min. To study the hydrolysis rate of various substrates by glucoamylase of *C. acetobutylicum*, the substrates (1 g/L) were dissolved in 0.1 M acetate buffer pH 4.4 and the hydrolysis was carried out at 40°C for 50 min. During the hydrolysis, samples were removed from the reaction mixture at time intervals for glucose and other reducing sugars analysis.

Enzyme Kinetics: The kinetic of α-amylase were determined using different concentrations of sago starch (1.0-15.0 g/L), maltose and maltotriose (1-10 g/L). Different types and concentrations of substrate [maltose (2-10 g/L), maltotriose (1-10 g/L) and starch (0.3-5 g/L)] was used for the determination of glucoamylase kinetic. Initial activity of α-amylase and glucoamylase used in the experiment was 0.14 U/mL and 0.17 U/mL, respectively. The value of Michaelis-Menten constant (Km) and the reaction rate at an infinite substrate concentration (V_{max}) for both enzymes were determined using Lineweaver-Burk plot.

Analytical Procedure: Samples were eluted and centrifuged at 5,000 g for 20 min. The supernatant was used for the determination of solvent concentration, amylolytic enzymes activities, sugars and organic acid concentrations. The cell concentration was determined using membrane filtration and oven dry method (Soni *et al.*, 1987).

Solvents (acetone-butanol-ethanol) were determined using gas chromatography (Shimadzu 17-A) equipped with flame ionisation detector (FID). Separation of acetone-butanol-ethanol (ABE) was achieved by using capillary column packed with polar BP 20 and H_2 as a carrier gas. The column temperature was held at 115°C for 8 min and then it was programmed at 5°C min-1 to 170°C with a 10 minutes final hold. The temperature of a detector and injector was set at 270°C and 220°C, respectively. Peak area of the respective compound was integrated against external standard with a Shimadzu C-RCA data processor. Organic acids (acetic and butyric acids) were determined by using UV detector at 210 nm. Separation of organic acids was obtained by using Aminex HPX-87H ion exclusion column (Biorad) with 7 mM H_2SO_4 as a mobile phase. Column was operated at ambient temperature with a flowrate of 0.6 L/min. Reducing sugars were determined using high performance liquid chromatography (HPLC) (LDC Analytical Constametric 3000 RI). Separation of sugars resulted from starch hydrolysis was obtained by using NH_2 Column (MERCK) and 80% (v/v) acetonitrile as a stationary and mobile phases, respectively. The column was operated at ambient temperature with a flowrate of 1.0 mL/min.

Glucoamylase was assayed using the method of Ariff and Webb (1996) and α-amylase was assayed according to the method of Bhella and Altosaar (1984). Glucoamylase activity was expressed as μmole of glucose liberated pe mL broth supernatant (μmole/min.mL) or U/mL. One unit of α-amylase was defined as the quality of protein producing a difference of optical density of 0.1. Protein was determined according to method described by Lowry *et al.* (1951) using serum albumin as a standard.

Results

Batch and Continuous Solvent Fermentations: The time course of batch solvent fermentation by *C. acetobutylicum* using 50 g/L gelatinized sago starch as a carbon source is shown in Fig. 1. During the early stages of growth, sago starch was hydrolysed into maltose and glucose by α-amylase and glucoamylase enzymes. High maltose concentration was detected during the early stages of the fermentation and decreased towards the end (data not shown). Substantially high α-amylase and glucoamylse was produced during the fermentation. The pattern of α-amylase followed growth pattern suggesting that it is growth-associated process. The activity of α-amylase reached a maximum value of 1.4 U/mL after about 4 h and remained constant toward the end of the fermentation. On the other hand, rapid increase in glucoamylase activity was observed during the stationary growth phase, suggesting that the process is either non-growth associated or mixed system. Maximum activity of glucoamylase (4.3 U/mL) was obtained at 24 h of fermentation.

Direct fermentation of sago starch to solvent by *C. acetobutylicum* can be divided into two phases, acidogenic phase and solventogenic phase. An acidogenic phase was observed during the first 12 h of fermentation where *C. acetobutylicum* grew rapidly with organic acid accumulation. The fermentation entered solventogenic phase when growth reached a deceleration phase after 12 h. During this phase, the metabolism of cells undergoes a shift to produce solvent by reassimilation of organic acid. Total solvent reached a maximum concentration (16.2 g/L) after about 16 h of fermentation.

A typical continuous fermentation course, including the initial batch periods and transition to continuous operation is shown in Fig. 2. Cell concentration, total solvent concentration, α-amylase and glucoamylase activities reached their steady-state values after about 3 residence times. Total solvent production and α-amylase activity in continuous fermentation were depended on dilution rate (D) ranging from 0.03 h^{-1} to 0.283 h h^{-1}, though glucoamylase production was not significantly different (data not shown). Total solvent production at steady-state was drastically decreased with increasing dilution rate. On the other hand, α-amylase activity at steady-state was slightly decreased with increasing dilution rate.

The performance of batch and continuous solvent fermentations by *C. acetobutylicum* using gelatinised sago starch as a carbon source, including α-amylase and glucoamylase production is given in Table 1. In comparison to batch fermentation, total solvent productivity (0.332 g/L.h) for continuous fermentation operated at dilution rate of 0.03 h h^{-1} was about 3 times lower. For this fermentation run, α-amylase and glucoamylase productivity was significantly lower than batch fermentation. However, α-amylase and glucoamylase productivity in continuous fermentation operated at high dilution rate (D = 0.123 h h^{-1}) was significantly higher than those obtained in batch fermentation, though total solvent productivity was about 6 times lower.

Partial Purification of α-amylase and Glucoamylase: The crude fluid for partial purification of enzymes was obtained after centrifuging the 28-h-old batch cultures at 5,000 g for 10 min. The clear supernatant contained α-amylase and glucoamylase activity of 1.41 U/mL and 3.24 U/mL, respectively. Result of the partial purification of α-amylase is summarized in Table 2. Ethanol precipitation step yielded α-amylase with a specific activity of 0.82 U/mg protein. This gave a purification factor of 1.69 and 96% yield.

Partial purification of glucoamylase was conducted by using ammonium sulphate fractionation and acetone precipitation. The typical partial purification profile for glucoamylase is given in Table 3. During the initial purification by fractionation using 0-30% and 30-50% ammonium sulphate, precipitation of glucoamylase was not occurred. The precipitation of glucoamylase was obatained at 50-70% ammonium sulphate, though substantial activity of α-amylase was also detected. However, only a trace amount of α-amylase was detected in the precipitation using 70-100% ammonium sulphate. Further purification of this fraction using acetone showed that α-amylase has been totally removed from the fraction. Up to this stage of purification, glucoamylase specific activity was increased to 8.42 U/mg and this was about four times higher than the crude fluid. This gave a purification factor of 3.68 and 52.99% yield.

Temperature Optimum and Stability: The influence of temperature on the activity and stability of partially purified α-amylase and glucoamylase is presented in Fig. 3. Partially purified α-amylase of *C. acetobutylicum* exhibited optimum activity at 40°C. More than 80% of the relative activity was still observed at temperature up to 80°C. However, the enzyme was not very stable at high temperatures. It losses 55% of its original activity when heated at 70°C and the acitivity was completely lost when heated at >80°C for 1 h. The highest glucoamylase activity was also observed at 40°C and was still stable at temperature up to 70°C. The acitivity was greatly reduced to relative activity of about 15% at temperature of above 80°C. The pattern of glucaomylase stability at different temperatures of incubation is more or less similar to the pattern for α-amylase.

pH Optimum and Stability: The effect of pH on activity and stability of partially purified α-amylase and glucoamylase of *C. acetobutylicum* is shown in Fig. 4. The α-amylase displayed optimal pH for activity at 5.3 and was stable at a wide pH range (3-8). The residual activity of α-amylase retained after incubation at pH ranging from 9-11 was between 58-81%. A remarkable feature of α-amylase from *C. acetobutylicum* P262 was its stability at an acidic pH (3- 6). Experiment to study the effect of pH on glucoamylase activity and stability was conducted at pH 3-11. The optimal pH for glucoamylase was 4.4. This enzyme retained less than 50% of its relative activity at pH 10 to 11. Glucoamylase was stable at pH ranging from 4 to 5 by retaining its original activity above 90% after 24 h incubation at this pH range. However, at pH above 10 glucoamylase activity was greatly reduced and less than 30% of its original activity was retained.

Comparative Hydrolysis Rate: Relative rate of hydrolysis of various compounds (1 g/L w/v in 0.05 M acetate buffer, pH 5.3) by partially purified α-amylase is shown in Table 4. From HPLC analysis, it was found that maltose and glucose were the end products of polysaccharides hydrolysis by *C. acetobutylicum* α-amylase (data not shown). *C. acetobutylicum* α-amylase possesses a great ability to hydrolyse large molecular weight compounds as illustrated by the relative values for soluble starch (100%), amylopectin (67.6%) and amylose (41.9%). The rate of hydrolysis by α-amylase increased with degree of polymerization of the homologous maltooligosaccarides series from maltotriose up to amylose. For the different types of starch tested, potato starch showed the higher rate of hydrolysis (100%), followed by corn starch (76.5%) and sago starch (68.6%). The ability of *C. acetobutylicum* α-amylase to hydrolyse maltose was very low (18.9%). This means that the relative rate of hydrolysis for maltose (small molecular weight substrate) was about 80% lower than for potato starch (large molecular weight substrate).

Relative rate of hydrolysis of starch, maltotriose and maltose (1 g/L w/v in 0.1 M acetate buffer pH 4.4) by partially purified glucoamylase is shown in Table 5. In contrary to α-amylase, glucoamylase of *C. acetobutylicum* showed a great ability to hydrolyse small molecular weight substrate. For example, the relative rate of hydrolysis for maltose was only about 15% lower than for sago starch.

Kinetic Parameters: The kinetic parameters of partially purified α-amylase and glucoamylase towards certain substrates are presented in Tables 4 and 5. Both enzymes displayed standard Michaelis-Menten kinetics and the apparent V_{max} and $K_m$values were determined from Lineweaver-Burk plot. The α-amylase $K_m$value (0.31 g/L) for soluble starch was comparatively 100 and 300 times lower than $K_m$value for maltose and maltotriose (Table 4). Low $K_m$value for starch as compared to maltotriose and maltose indicated that α-amylase has higher affinity towards substrates with longer chain length. The α-amylase V_{max} value (10.03 U/mL) was comparatively 10 to 50 times higher than maltotriose and maltose.

The glucoamylase $K_m$value for soluble starch (0.03 g/L) was very low as compared to the value for maltotriose (10.43 g/L) and maltose (4.39 g/L) (Table 5). On the other hand, the V_{max} value for soluble starch (19.27 g/L) was only about two times lower than the values for maltose and maltotriose. This result indicates that glucoamylase of *C. acetobutylicum* has higher affinity towards starch than towards maltose.

Discussion

Since sago starch is an abundant carbon source, it may be used as cheap raw materials for solvent fermentation. In one-step direct fermentation of starch to solvent, starch is first hydrolysed to fermentable sugars by amylolytic enzymes before the sugars are consumed for growth and metabolite production (Soni *et al.*, 1992). The process is depended strongly on the activity of α-amylase and glucoamylase secreted by the solvent-producing-strain at the prevailing fermentation conditions (Hyun and Zeikus, 1985; Annous and Blanschek, 1990). Knowledge on the properties and biochemical characteristics of α-amylase and glucoamylase of solvent-producing-strain is important for the improvement of the process. Starch-degrading enzymes of microbial origin also have a number of

Table 1: Glucoamylase and α-amylase production during direct fermentation of gelatinised sago starch to solevent.

Fermentation mode	Total Solvent (g/L)	α-amylase (U/mL)	Glucoamylase (U/mL)	Total solvent productivity (g/L.h)	α-amylase productivity (g/L.h)	Gluco-amylase productivity (g/L.h)
Batch	16.18	1.2	4.3	1.050	0.050	0.179
Continuous, D = 0.03 h-1	11.07	0.59	3.43	0.332	0.018	0.103
Continuous, D = 0.123 h-1	1.24	1.31	3.82	0.153	0.161	0.470

Table 2: A typical purification profile of the extracellular α-amylase of *C. acetobutylicum* P262.

Purification step	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude fluid	2.98	1.45	0.49	100.0	1.0
Ethanol precipitation	1.70	1.39	0.82	96.1	1.69

Table 3: A typical purification profile of glucoamylase of *C. acetobutylicum* P262.

Purification step	Total protein (mg/mL)	Total activity (U/mL)	Yield (%)	Specific activity (u/mg)	Purification fold
Crude fluid	1.42	3.24	100.00	2.29	1.00
(NH4)2SO4 fraction (70-100% saturation)	0.78	2.65	81.93	3.40	1.49
Acetone prescipitation	0.20	1.72	52.99	8.42	3.68

Table 4: Comparative hydrolysis of various substrates by α-amylase of *C. acetobutylicum* P262.

Substrate (10 g/L)	Lingkage	K_m(g/L)	V_{max} (U/mL)	Relative rate of hydrolysis (%)
Potato starch	α-1,4 and α-1,6	0.3	10.03	100
Corn starch	α-1,4 and α-1,6	ND	ND	76.5
Sago starch	α-1,4 and α-1,6	ND	ND	68.6
Amylopectin	α-1,4 and α-1,6	ND	ND	67.6
Amylose	α-1,4	ND	ND	41.9
Maltotriose	α-1,4	97.7	1.22	21.3
Maltose	α-1,4	40.56	0.24	18.9

ND not determined

Table 5: Comparative hydrolysis of various substrates by glucoamylase of *C. acetobutylicum* P262.

Substrate (10 g/L)	K_m(g/L)	V_{max} (U/mL)	Relative rate of hydrolysis (%)
Sago starch	0.039	19.27	100.00
Maltotriose	10.43	45	100.00
Maltose	4.39	55	86.40
Amylopectin	ND	ND	84.60
Amylose	ND	ND	86.50

ND not determined

industrial applications (Fogarty and Benson, 1983). The amylolytic enzymes produced, as by-product, from solvent fermentation may also be purified and concentrated for commercial applications in starch industry. Partially purified α-amylase of *C. acetobutylicum* P262 exhibited optimum activity at 5.3. This is in the range of optimal pH for the activity of most bacterial α-amylase (Chojecki and Blanschek, 1986; Madi *et al.*, 1987; Tanaka *et al.*, 1987) and yeast α-amylase (De Mot and Verachtert, 1985).

A remarkable feature of *C. acetobutylicum* α-amylase was its stability at an acidic pH (3-6). Similar pH stability profile has also been observed for α-amylase of B. acidocaldarious (Buonocore *et al.*, 1976). Stability of this enzyme at low pH was particularly important in direct fermentation of starch to solvent because the low culture pH (4.4 to 5) during the process was essential for enhancement of solvent production (Soni *et al.*, 1987; Paquet *et al.*, 1991). The optimal temperature (40°C) for the activity of *C. acetobutylicum* α-amylase was low as compared to other bacterial α-amylase. Most α-amylase reported in the literature is thermostable and the optimum temperature for activity is ranging from 50 to 70°C (Buonocore *et al.*, 1976; Morgan *et al.*, 198; Madi *et al.*, 1987). The optimum temperature for activity of many industrial α-amylases is normally at above 100°C (Fogarty and Kelly, 1979).

The optimal pH for the activity (4-4.4) and stability (4-5) of glucoamylase produced by *C. acetobutylicum* P262 are within the range for other sources as reported in the literature (Spencer-Martin and van Uden, 1979; Takahashi *et al.*, 1981). High activity and stability of this enzyme at acidic pH was the most important feature for maltose hydrolysis during solventogenic phase where low pH was required for solvent production (Soni *et al.*, 1992). The optimum temperature for *C. acetobutylicum* glucoamylase (30-50°C) is within the range for other fungal glucoamylase (Takashahi *et al.*, 1981; Vandersall *et al.*, 1995). High activity and stability of glucoamylase at high temperature (60°C) is similar to glucoamylase of two Clostridium species as reported by Hyun and Zeikus (1987).

In this study, it was found that maltose and glucose are the end products of oligosaccharides hydrolysis by α-amylase of *C. acetobutylicum* P262. On the other hand, Paquet *et al.* (1991) reported that maltotriose, maltose and glucose were end products of maltohexose hydrolysis by α-amylase of *C. acetobutylicum* ATCC 824. Similar characteristics have also been shown by

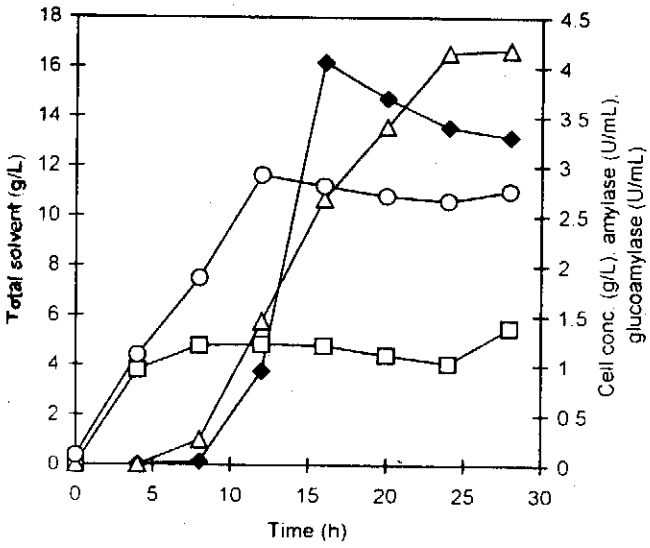

Fig. 1: Time course of batch solvent fermentation by *C. acetobutylicum* P262 using 50 g/L gelatinised sago starch. Symbols represent; total solvent concentration (◆), glucoamylase activity (▲), α-amylase activity (■), cell concentration (○).

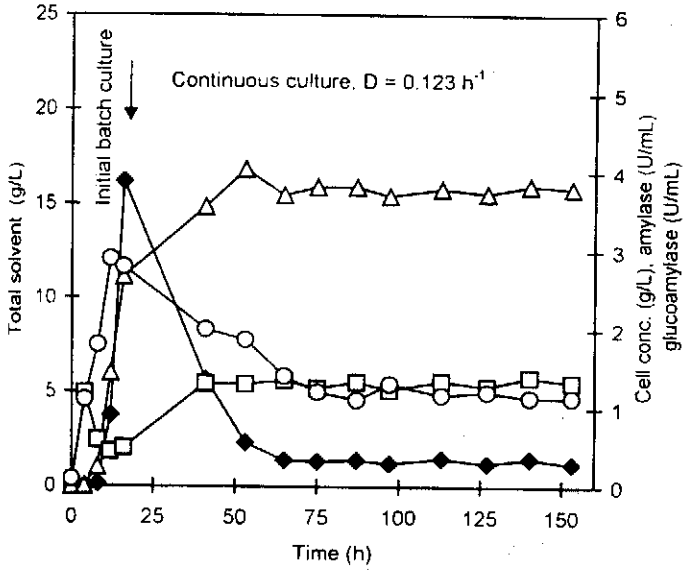

Fig. 2: Time course of continuous solvent fermentation by *C. acetobutylicum* P262 operated at a dilution rate (D) 0.123 h-1. Feeding medium consists of 30 g/L sago starch and the culture pH was controlled at 4.5. Symbols represent; total solvent concentration (◆), glucoamylase activity (▲), α-amylase activity (■), cell concentration (○).

α-amylase of C. butyricum (Tanaka *et al.*, 1987). The rate of α-amylase hydrolysis was increased with the degree of polymerisation of the homologous maltooligosaccharide series for the maltotriose up to amylose. A low activity detected on maltose probably due to the hydrolysis of α-1-4 glucan bonds rather than a debranching activity.

The IK_m value (0.31 g/L) of α-amylase for soluble starch is comparatively 10 times lower than the value (3.6 g/L) for

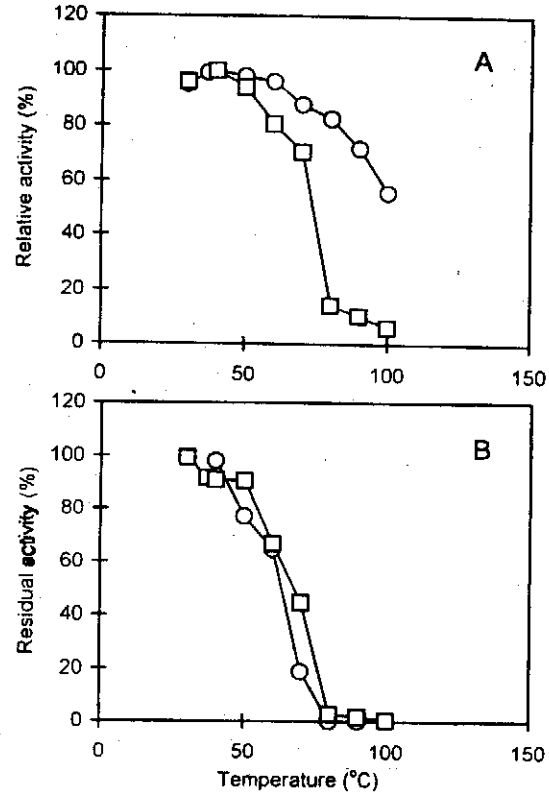

Fig. 3: Activity (A) and stability (B) of α-amylase (○) and glucoamylase (□) of *C. acetobutylicum* P262 at various temperatures. Initial activity of α-amylase and glucoamylase used in the experiment was 0.14 U/mL and 0.17 U/mL, respectively.

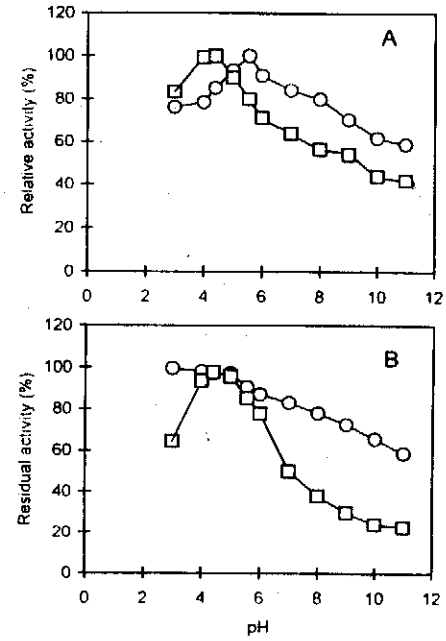

Fig. 4: Activity (A) and stability (B) of α-amylase (○) and glucoamylase (□) of *C. acetobutylicum* P262 at various pHs. Initial activity of α-amylase and glucoamylase used in the experiment was 0.14 U/mL and 0.17 U/mL, respectively.

glucoamylase of other solvent-producing-strains as reported by Paquet *et al.* (1991). Low *C. acetobutylicum* glucoamylase K_m value (0.03 g/L) for soluble starch indicates that the enzyme has high affinity towards this substrate. Similar characteristics have also been reported for other fungal glucoamylase (Vandersall *et al.*, 1995).

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