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Anaerobic Fermentation of Gelatinized Sago Starch-Derived Sugars to Acetone–1-Butanol–Ethanol Solvent by *Clostridium acetobutylicum*

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ABSTRACT. A study of the kinetics and performance of solvent-yielding batch fermentation of individual sugars and their mixture derived from enzymic hydrolysis of sago starch by *Clostridium acetobutylicum* showed that the use of 30 g/L gelatinized sago starch as the sole carbon source produced 11.2 g/L total solvent, *i.e.* 1.5–2 times more than with pure maltose or glucose used as carbon sources. Enzymic pretreatment of gelatinized sago starch yielding maltose and glucose hydrolyzates prior to the fermentation did not improve solvent production as compared to direct fermentation of gelatinized sago starch. The solvent yield of direct gelatinized sago starch fermentation depended on the activity and stability of amylolytic enzymes produced during the fermentation. The pH optima for α-amylase and glucoamylase were found to be at 5.3 and 4.0–4.4, respectively. α-Amylase showed a broad pH stability profile, retaining more than 80 % of its maximum activity at pH 3.0–8.0 after a 1-d incubation at 37 °C. Since *C. acetobutylicum* α-amylase has a high activity and stability at low pH, this strain can potentially be employed in a one-step direct solvent-yielding fermentation of sago starch. However, the *C. acetobutylicum* glucoamylase was only stable at pH 4–5, maintaining more than 90 % of its maximum activity after a 1-d incubation at 37 °C.

The production of solvents by *Clostridium acetobutylicum* has promoted research into physiology of this commercially important bacterium. *C. acetobutylicum* is known to utilize a wide range of sugar substrates. However, our knowledge of the mechanism and regulation of its utilization of sugar is still limited. Research interests aim at reducing the cost of raw materials for solvent production by using starch materials which are abundant and relatively inexpensive.

The main characteristic of sugar fermentation by *C. acetobutylicum* is the metabolic transition from an acidogenic growth phase to the solvent producing phase (Jones *et al.* 1986). A few studies have been carried out to examine the fermentation of hemicellulose-derived sugars such as xylose and arabinose but there has been no detailed report on solvent fermentation of starch-derived sugars such as maltose and glucose (Linden *et al.* 1985; Fond *et al.* 1986; Ounine *et al.* 1985). Although glucose serves as a suitable substrate, the inability of *Clostridium* sp. to ferment maltose is not well known (Ounine *et al.* 1985; Barton *et al.* 1972; Fond *et al.* 1985). Starch has been used as substrate for industrial solvent production, but reports on the purification and characterization of amylolytic enzymes of *C. acetobutylicum* are scarce (Hyun *et al.* 1985; Paquet *et al.* 1991) and so are reports on direct fermentation of sago starch, which is an abundant carbon source, to solvents and other products (Rosfarizan *et al.* 1998). The direct fermentation of starch to value-added products should not be limited by the activity of amylolytic enzymes produced by the microorganisms employed, *i.e.* the enzymes should be excreted in sufficient amounts, and possess sufficient activity and stability under the fermentation conditions.

During the direct fermentation of sago starch to solvents by *C. acetobutylicum*, the starch is first hydrolyzed to maltose and glucose by α-amylase and glucoamylase secreted by the bacterium. These two enzymes can be partially adsorbed on cells and their synthesis is generally induced by starch or starch degradation products, and repressed by glucose (Ensley *et al.* 1975; Chojecki *et al.* 1986).

Our study of *C. acetobutylicum* concerned the regulation of uptake of sugars produced by sago starch hydrolysis and serving as substrates for solvent production, the effect of different carbon sources on the secretion of α-amylase and glucoamylase and properties of these amylolytic enzymes.

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MATERIALS AND METHODS

Microorganism and medium. *C. acetobutylicum* strain P262 was maintained as spores in sterile RCM (Reinforced Clostridia Medium, *Oxoid*) medium and stored under anaerobic condition at 4 °C.

The basal medium consisted of (in g/L) NH_4NO_3 2.0, KH_2PO_4 0.75, K_2HPO_4 0.75, $MgSO_4 \cdot 7H_2O$ 0.4, $MnSO_4 \cdot 4H_2O$ 0.01, $FeSO_4 \cdot 5H_2O$ 0.01, yeast extract 5.0, cysteine 0.5 and resazurin 0.001. Different concentrations of carbon sources (glucose, maltose, gelatinized starch and their combinations) were added according to the need of each experiment. One mL of a solution containing 1 mg/L 4-aminobenzoic acid; 80 μg/L biotin was added to 1 L of the medium. Gelatinized starch was prepared by heating starch slurry to slightly above 70 °C for 30 min. Sago starch was hydrolyzed enzymically to maltose and glucose according to Arbakariya *et al.* (1990). Gelatinized sago starch was liquefied to a maltose hydrolyzate by using α-amylase (Termamyl 120L). To prepare a glucose hydrolyzate, gelatinized starch was liquefied and saccharified by α-amylase and glucoamylase (Dextrozyme 225/70L). Both enzymes were obtained from *Novo*, Kuala Lumpur (Malaysia).

Fermentations. The anaerobic batch fermentation was conducted in 250-mL Schott (Duran) bottles. These bottles have flat bottom (internal diameter 50 mm) and a mouth of 35 mm diameter with screw cap. The initial pH of the medium was adjusted to 6.0 by 1 mol/L HCl. Anaerobic culture medium was prepared by sparging free oxygen with nitrogen and distributed into 250-mL tightly screw-capped Schott bottles using the Hungate technique (Miller *et al.* 1974). The medium in Schott bottles was sterilized at 121 °C for 20 min. Vitamin solution was filter sterilized and added aseptically into the sterilized medium. Batch culture was initiated by inoculating 1 mL spore suspension that was previously grown on RCM medium. The Schott bottles were incubated at 35 °C without shaking in *Memert* incubator under anaerobic conditions. All fermentations were carried out in triplicate and the results are average values.

Partial purification of amylolytic enzymes and their properties. α-Amylase was partially purified by ethanol precipitation. Ethanol pre-chilled to –20 °C was added dropwise to the culture filtrate at 4 °C with continuous stirring to a final concentration of 75 % and the solution was kept at –10 °C for 1 d. The precipitated protein was dissolved in 50 mmol/L acetate buffer of pH 5.3.

Glucoamylase was partially purified using a two-step purification procedure. The culture filtrate was subjected to ammonium sulfate fractionation (30–100 % saturation) and the precipitated protein was dissolved in acetate buffer (100 mmol/L, pH 4.4) and dialyzed overnight against distilled water. A volume of chilled acetone (–16 °C) was added slowly to the redissolved protein precipitate fraction under constant stirring. After standing overnight at 4 °C, the solution was centrifuged at 10 000 ***g*** for 30 min. The resulting precipitate was then dissolved in a small amount of acetate buffer (100 mmol/L, pH 4.4) and dialyzed overnight against distilled water.

The *rate of hydrolysis* by α-amylase of different substrates was measured using 1.0 g/L (*W/V*) substrate. The substrates were prepared in 50 mmol/L acetate buffer (pH 5.3) and the hydrolysis was carried out at 37 °C for 50 min. Samples were removed at time intervals for reducing sugar assay. To study the rate of hydrolysis of various substrates by glucoamylase, the substrates (1 g/L) in 100 mmol/L acetate buffer (pH 4.4) were hydrolyzed at 40 °C for 50 min. Samples were removed from the reaction mixture at time intervals for glucose assay.

Analytical procedures. The samples were eluted at appropriate time intervals and centrifuged at 50 Hz for 20 min. The supernatant was used for determining solvent concentration, enzyme activities, concentrations of sugars and organic acids. Cell concentration was determined according to Soni *et al.* (1987). Acetone–1-butanol–ethanol was determined by gas chromatography on a *Shimadzu* 17-A instrument equipped with flame ionisation detector. Acetone, 1-butanol and ethanol were separated on a capillary column packed with polar BP 20 phase and H_2 as a carrier gas. The column temperature was held at 115 °C for 8 min, programmed at 5 K/min to 170 °C with a 10-min binal hold. Detector and injector temperatures were set at 270 °C and 220 °C, respectively. The peak area of the respective compound was integrated against an external standard with a *Shimadzu* C-RCA data processor. Acetic and butyric acid were determined by HPLC with a UV detector at 210 nm. The acids were separated on an Aminex HPX-87H ion exclusion column (*Biorad*) with 7 mmol/L H_2SO_4 as a mobile phase. The column was operated at room temperature with a flow rate of 0.6 L/min. Reducing sugars were determined by HPLC on LDC analytical constametric 3000 RI instrument. Sugars produced by starch hydrolysis were separated on a NH_2 column (*Merck*) and 80 % acetonitrile as stationary and mobile phases, respectively. The column was operated at room temperature with a flow rate of 1.0 mL/min. Glucoamylase was assayed by the method of Ariff and Webb (1996) and α-amylase was assayed according to Bhella and Altosaar (1984). Protein was determined according to Lowry.

RESULTS AND DISCUSSION

Solvent production from individual carbon sources. The time course of solvent fermentation with different carbon sources (starch, maltose and glucose) is shown in Fig. 1. Table I compares the fermentation characteristics with these substrates. In all cases, the time course of the fermentation can be divided into two distinctive phases, acidogenic and solventogenic. During the acidogenic phase, organic acids and biomass were rapidly produced. The drop in culture pH during this phase was due to the rapid secretion of butyric and acetic acids into the medium. In the second, solventogenic, phase these organic acids were reassimilated, concomitantly with sugar utilization, for solvent production. The consumption of the acids caused an increase in culture pH.

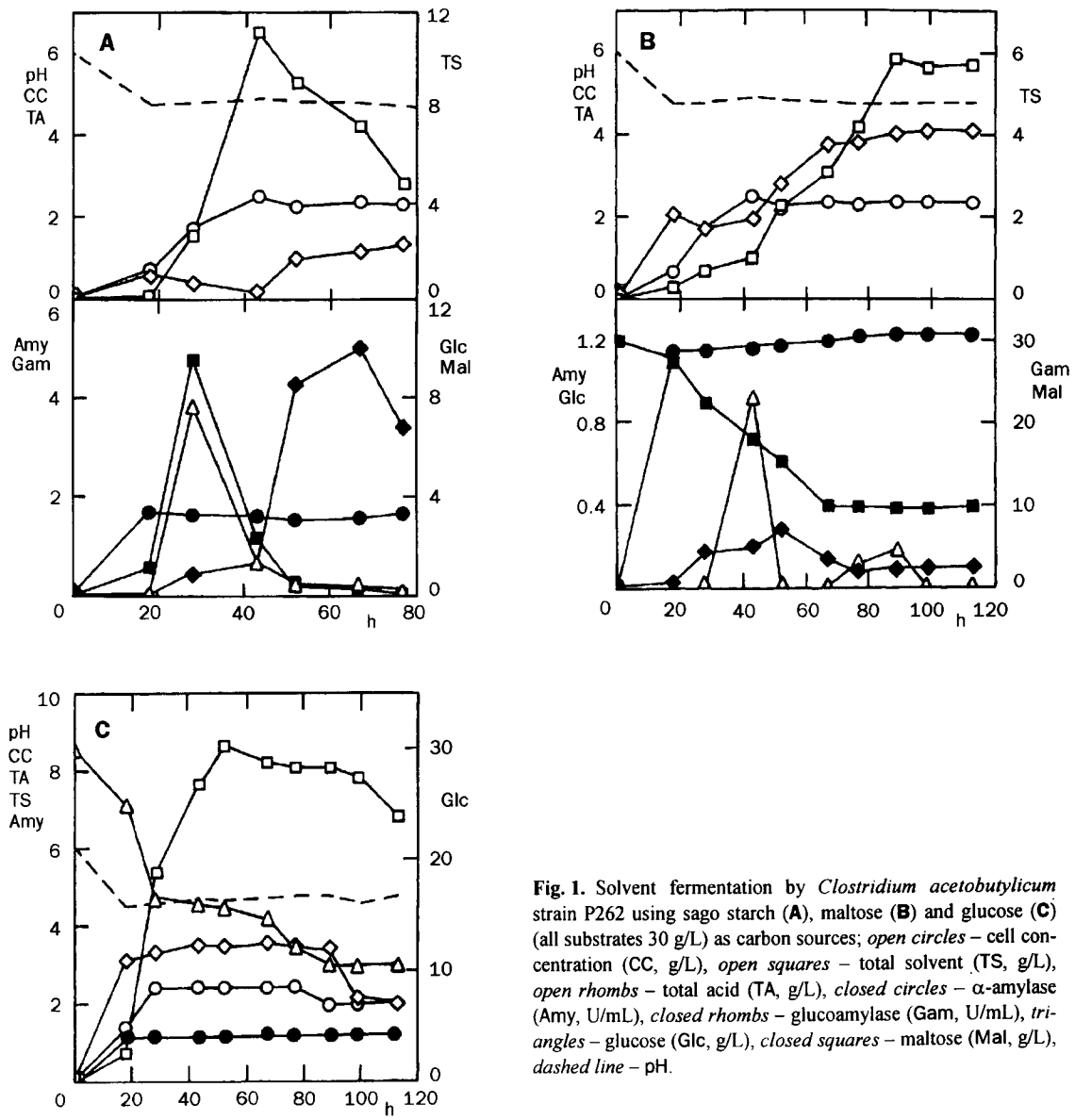

Fig. 1. Solvent fermentation by *Clostridium acetobutylicum* strain P262 using sago starch (**A**), maltose (**B**) and glucose (**C**) (all substrates 30 g/L) as carbon sources; *open circles* – cell concentration (CC, g/L), *open squares* – total solvent (TS, g/L), *open rhombs* – total acid (TA, g/L), *closed circles* – α-amylase (Amy, U/mL), *closed rhombs* – glucoamylase (Gam, U/mL), *triangles* – glucose (Glc, g/L), *closed squares* – maltose (Mal, g/L), *dashed line* – pH.

When the direct fermentation of sago starch to solvents proceeded, maltose and glucose were rapidly produced during 28 h of fermentation owing to the induction of α-amylase and glucoamylase (Fig. 1A). The pattern of α-amylase activity followed the growth pattern, suggesting that it is growth-associated. On the other hand, a rapid increase in glucoamylase activity was observed when growth reached the stationary phase, suggesting that it is either not growth-associated, or a mixed event. Similar patterns of amylolytic enzyme production by *Clostridium* spp. has been reported by Annous *et al.* (1990) and Soni *et al.* (1992). During the acidogenic or active growth phase, glucose concentration decreased owing to its consumption for intermediate organic acid production. Maltose concentration also dropped due to its hydrolysis

to glucose. At the end of the fermentation, almost all maltose was hydrolyzed to glucose. This result indicates that the glucoamylase secreted during the fermentation was sufficient for the hydrolysis of maltose to glucose. In other words, starch hydrolysis to fermentable sugars was not the main problem in direct fermentation of 30 g/L sago starch to solvent by *C. acetobutylicum*. During the solventogenic phase, the organic acids were consumed concomitantly with glucose to produce the solvents. Total solvent production reached a maximum (11.2 g/L) after about 42 h, at the time when glucose became depleted. This gave an overall solvent production rate of 260 mg/L per h.

Table I. Performance and kinetic parameters of solvent fermentation† by *C. acetobutylicum* on different sugars††

Kinetic parameters and/or performance‡	Sago 30	Maltose 30	Glucose 30	Sago starch 15 + glucose 15	Sago starch 15 + maltose 15	Maltose 15 + glucose 15
Maximum cell concentration, g/L	2.5	1.90	2.4	1.79	1.76	1.40
Specific growth rate, 1/h	0.28	0.28	0.2	0.29	0.20	0.27
Maximum α-amylase activity, U/mL	1.67	1.23	1.08	1.64	1.72	1.34
Maximum glucoamylase activity, U/mL	5.0	7.0	–	6.0	5.8	2.8
Maltose not hydrolyzed, g/L	0.02	9.9	–	5.4	10.3	4.3
Glucose unconsumed, g/L	0.13	–	10.5	5.7	0.47	4.1
Glucose consumed, g/L	32.5	22.1	19.5	19.5	19.7	22.7
Maltose hydrolysis rate, g/L	0.38	0.30	–	0	0.13	0.16
Specific maltose hydrolysis rate, mg/U per h	0.60	0.06	–	0	0.04	0.08
Maximum butyric acid concentration, g/L	1.14 ± 0.08^{b}	1.70 ± 0.02^{c}	2.7 ± 0.06^{d}	0.66 ± 0.05^{a}	0.76 ± 0.07^{a}	0.74 ± 0.07^{a}
Maximum acetic acid concentration, g/L	0.38 ± 0.06^{a}	3.0 ± 0.18^{c}	1.16 ± 0.02^{b}	0.25 ± 0.08^{a}	1.09 ± 0.06^{b}	0.89 ± 0.07^{b}
Total organic acid concentration, g/L	1.52	4.7	3.8	0.91	1.85	1.63
Final culture pH	4.8	4.8	4.8	4.9	4.6	4.6
Maximum acetone concentration, g/L	2.4 ± 0.9^{d}	1.75 ± 0.45^{c}	1.09 ± 0.16^{a}	1.87 ± 0.57^{c}	1.48 ± 0.28^{b}	2.7 ± 0.45^{d}
Maximum butanol concentration, g/L	8.4 ± 0.5^{c}	4.0 ± 0.01^{a}	7.4 ± 0.24^{c}	4.7 ± 0.16^{b}	4.6 ± 0.1^{b}	3.9 ± 0.13^{a}
Maximum ethanol concentration, g/L	0.38 ± 0.2c	0.09 ± 0.02^{a}	0.19 ± 0.02^{b}	0.18 ± 0.08^{b}	0.20 ± 0.03^{b}	0.20 ± 0.08^{b}
Total solvent concentration, g/L	11.2 ± 0.21^{d}	5.6 ± 0.26^{a}	8.2 ± 0.77^{c}	6.7 ± 0.22^{b}	6.3 ± 0.95^{b}	6.7 ± 0.19^{b}
Fermentation time, h	42	89	89	99	67	89
Cell yield, g cell per g carbon	0.19	0.22	0.31	0.23	0.22	0.16
Solvent yield, g/g carbon	0.86	0.66	1.05	0.85	0.80	0.74
Overall total solvent production rate, g/L solvent per h	0.26	0.09	0.17	0.09	0.09	0.08

†Data were obtained from the time course of each fermentation run; fermentation time was the time needed to reach a maximum total solvent concentration.

††Concentration 15 and 30 g/L.

‡Results are means of three replicates (±SD, if given).

[a–d]Values in the same row with different superscripts differ significantly ($p < 0.05$).

Growth on maltose was slightly lower than on starch (Fig. 1B). Glucoamylase and α-amylase production was enhanced in fermentation on maltose and the maximum activities attained were comparable to the fermentation on gelatinized starch. This is in contrast with several reports which showed that the synthesis of amylolytic enzymes was induced when starch was used as a carbon source but severely repressed during growth on glucose, xylose and maltose (Srivastiva *et al.* 1984; Hyun *et al.* 1985). Although substantial glucoamylase activity (7 U/mL) was detected during the fermentation on maltose as the sole carbon source, a significant amount of maltose (9.9 g/L) was not hydrolyzed at the end of the fermentation, when a slight deactivation of glucoamylase was also observed. It is well known that glucoamylase possesses

a considerable ability to hydrolyze large molar mass compounds (Fogarty and Benson 1983). Starch, which has a higher molar mass than maltose, was hydrolyzed to glucose at a higher rate. In addition, α-amylase activity in fermentations on maltose was slightly lower than that detected in fermentations on starch. The presence of transglucosidase lowers the efficiency of the saccharification of maltose due to the formation of unfermentable sugars (Maher 1968). In fermentations on maltose, the solvent production reached a maximum concentration (5.6 g/L) after about 90 h when glucose in the culture became exhausted. It is important to stress that a high total acid level (4.7 g/L), about 3 times higher than fermentation on starch, was accumulated during the maltose fermentations. This suggests that organic acids were not efficiently utilized for solvent production in maltose fermentations. A lower rate and degree of maltose hydrolysis may be another cause of the reduced solvent production in maltose fermentations. The total solvent yield based on glucose consumed for maltose fermentation was slightly lower than in starch fermentation and the overall solvent production rate (85 mg/L per h) was more than 2 times lower than in starch fermentation.

The time course of solvent fermentation using glucose as the sole carbon source is shown in Fig. 1C. The maximum cell concentration was about the same as with starch. However, the cell yield based on carbon consumed in fermentation on glucose (0.31 g/g) was higher than with starch (0.19) and maltose (0.22). It has been reported that monosaccharides were a more effective growth substrates for *Clostridium* spp. than disaccharides (Ennis *et al.* 1985; Schoutens *et al.* 1985). *C. acetobutylicum* P262 was found to produce similar levels of α-amylase for growth on either glucose or starch, what is in agreement with the data of Ensley *et al.* (1975). α-Amylase synthesis is known to be subject to catabolite repression caused by the presence of free glucose in the culture (Scott *et al.* 1958; Chojecki *et al.* 1986). Glucoamylase was not produced in our fermentations on glucose. The maximum total solvent production (8.2 g/L), which was about 27 % lower than with starch, was obtained after about 50 h. Overall solvent production rate (0.17 g/L per h) was about 35 % lower than in fermentations on starch while solvent yield based on carbon consumed (1.05 g/g) was about 16 % higher than with starch (0.86 g/g). Glucose was not utilized after 90 h and about 10.5 g/L of glucose was not consumed at the end of the fermentation. In addition, total acids accumulated during the fermentation (3.8 g/L) were more than two times higher than with starch. This result indicates that the fermentation was subject to product (solvent and organic acid) inhibition.

Fermentation on a mixture of carbon sources. The effect of addition of glucose to starch and maltose on growth, amylolytic enzyme secretion and solvent production is given in Table I. On adding glucose to starch, the maximum cell concentration attained was somewhat lower than with starch alone. Although the production of α-amylase and glucoamylase was not affected by the addition of glucose, a substantially higher amount of maltose was not hydrolyzed at the end of the fermentation. This means that although the addition of glucose did not repress the secretion of the two amylolytic enzymes, the degree of maltose hydrolysis was lower. We cannot explain this. However, the amount of glucose consumed was the same as in fermentations on glucose alone. A very low total acid level was detected during the fermentation. The total solvent accumulated (6.7 g/L) was almost double that produced on starch alone.

When maltose was added to starch, both carbon sources were hydrolyzed concomitantly during the early stages of the fermentation (*data not shown*). The maximum activities of α-amylase and glucoamylase were the same as with starch alone, indicating that the presence of a high maltose concentration during the initial stages of the fermentation did not inhibit α-amylase production. Although very little glucose remained unconsumed at the end of the fermentation, the amount of unhydrolyzed maltose was very high (10.3 g/L). However, the total amount of glucose consumed during the fermentation was the same as with glucose alone. The maximum total solvent concentration (6.3 g/L) was slightly lower than that obtained in a fermentation with a mixture of glucose and starch.

Growth was significantly reduced in fermentations on a mixture of maltose and glucose. Like with maltose alone, up to 1.34 U/mL α-amylase was detected during the fermentation. Glucoamylase production (2.8 U/mL) was reduced to half as compared with fermentations on maltose alone and was significantly lower than with starch alone. The amount of maltose not hydrolyzed (4.28 g/L) was lower than that obtained in fermentations on a mixture of starch and maltose (10.3 g/L). The presence of α-amylase and glucoamylase enhanced the hydrolysis of maltose to glucose. The total solvent production was comparable to fermentations with a mixture of starch and glucose or maltose whereas cell yield, solvent yield and overall solvent production rate were slightly lower.

Fermentation on sago starch enzymic hydrolysate. The fermentations on high concentrations of gelatinized sago starch, glucose hydrolyzate and maltose hydrolyzate obtained by enzymic hydrolysis of sago starch are characterized in Table II. The maximum cell concentration attained in fermentation with 70 g/L starch was slightly higher than with 30 g/L starch (*see* Table I). The α-amylase activity in fermentations on 30 and 70 g/L gelatinized starch was comparable whereas glucoamylase activity at 70 g/L starch was half that at 30 g/L starch. This suggests that high starch concentration inhibited glucoamylase product-

ion but not α-amylase. Although a very high amount of glucose (59.2 g/L) was consumed in fermentations on 70 g/L starch, the maximum concentration of the produced solvent was the same as with 30 g/L starch. A very low amount of total acids (0.24 g/L) was accumulated during the fermentation, indicating that glucose was not efficiently used for acid and solvent production in fermentations on high starch concentration.

Table II. Performance and kinetic parameters of solvent fermentation† by *C. acetobutylicum* using high concentration (70 g/L) of gelatinized sago starch, glucose hydrolyzate and maltose hydrolyzate

Kinetic parameters and/or performance	Gelatinized sago starch	Maltose hydrolyzate	Glucose hydrolyzate
Maximum cell concentration, g/L	2.9	3.0	2.4
Maximum α-amylase activity, U/mL	1.55	1.40	1.57
Maximum glucoamylase activity, U/mL	2.7	0	0
Maltose not hydrolyzed, g/L	5.8	15.9	0
Glucose unconsumed, g/L	11.4	14.4	50.4
Glucose consumed, g/L	59.2	56.0	19.6
Maximum butyric acid concentration, g/L	0.10 ± 0.02^a	0.31 ± 0.03^b	4.5 ± 0.08^c
Maximum acetic acid concentration, g/L	0.14 ± 0.07^a	0.76 ± 0.04^b	1.74 ± 0.05^c
Total organic acid concentration, g/L	0.24	1.08	6.2
Final culture pH	4.8	4.9	4.6
Maximum acetone concentration, g/L	3.4 ± 0.05^c	2.7 ± 0.06^b	1.91 ± 0.08^a
Maximum 1-butanol concentration, g/L	8.1 ± 1.69^b	8.4 ± 1.20^b	6.1 ± 1.65^a
Maximum ethanol concentration, g/L	0.22 ± 0.02^b	$0.24b \pm 0.01^b$	0.15 ± 0.08^a
Total solvent concentration, g/L	11.7 ± 0.50^b	11.3 ± 0.21^b	7.9 ± 1.58^a
Fermentation time, h	42	75	75
Cell yield, g cell per g carbon	0.12	0.08	0.31
Solvent yield, g/g carbon	0.49	0.20	1.01
Overall total solvent production rate, g/L solvent per h	0.28	0.22	0.11

†See footnotes to Table I.

The total solvent production (11.3 g/L) in a fermentation on 70 g/L maltose hydrolyzate was comparable to that in a fermentation on 70 g/L gelatinized starch. In comparison to fermentations of 30 g/L maltose, the total solvent production was about two times higher, suggesting that high maltose concentration did not inhibit growth of *C. acetobutylicum* and solvent production. Low acid production (1.07 g/L) may be one of the factors that enhance the solvent production in fermentations with maltose hydrolyzate. The amount of unconsumed glucose (14.4 g/L) was similar to the fermentation with gelatinized starch. However, the amount of maltose not hydrolyzed (15.9 g/L) was higher than that observed in fermentation with gelatinized starch, suggesting that the hydrolysis of maltose by the secreted glucoamylase was not effective.

The amount of glucose consumed in a fermentation on 70 g/L glucose hydrolyzate (19.6 g/L) was similar to that consumed in fermentations with 30 g/L of pure glucose (*see* Table I). In addition, the solvent production (7.9 g/L) and maximum cell concentration (2.4 g/L) were comparable to those attained at 30 g/L glucose, suggesting that high glucose concentration did not inhibit either growth or solvent production. However, the solvent production in a fermentation on glucose hydrolyzate was about 32 % lower than with gelatinized starch. In contrast to the fermentations using gelatinized starch, a very high amount of total acid (6.2 g/L) was accumulated with glucose hydrolyzate. The lower solvent production in fermentations using glucose hydrolyzate may be due to the high amount of acid, especially butyric acid, accumulated during the fermentation. Dissociated form of butyric acid is inhibitory to cell growth and solvent production at a concentration as low as 2.5 g/L (Ennis *et al.* 1985). Although the solvent yield referred to glucose consumed in fermentation with glucose hydrolyzate (1.01 g/g) was twice that obtained in fermentation using gelatinized starch, the overall production rate (0.11 g/L per h) was about two times lower. The α-amylase production in fermentations on glucose hydrolyzate was similar as with starch, though glucoamylase was not detected during the fermentation. In terms of maximum solvent concentration and overall productivity, the use of glucose hydrolyzate prepared from enzymic hydrolysis of sago starch did not show any improvements as compared to fermentations using gelatinized sago starch.

Some properties of C. acetobutylicum *amylolytic enzymes.* Knowledge on the properties of amylolytic enzymes produced by *C. acetobutylicum* might be useful for the improvement of direct fermentation of sago starch to solvent. The pH optima for α-amylase and glucoamylase were found to be at 5.3 and 4.0–4.4, respectively (Fig. 2). α-Amylase showed a broad pH stability profile, retaining more than 80 % of its maximum activity at pH 3.0–8.0 after a 1-d incubation at 37 °C. A similar pH stability profile has been reported for α-amylase of *B. acidocaldarious* (Buonocore *et al.* 1976). The optimal culture pH for solvent fermentation was in the range of 4.4 to 5.3 (Soni *et al.* 1987; Paquet *et al.* 1991). The high activity and stability of *C. acetobutylicum* α-amylase at low pH indicated that this strain may function well in a one-step direct fermentation of sago starch to solvent. On the other hand, glucoamylase was only stable at pH between 4 to 5, retaining more than 90 % of its maximum activity after a 1-d incubation at 37 °C. High activity and stability of glucoamylase at acidic pH was the most important feature for maltose hydrolysis during the solventogenic phase which required low pH for high solvent production (Soni *et al.* 1987).

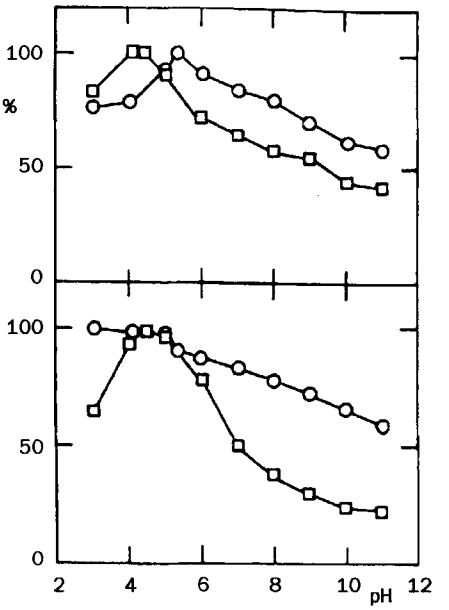

Fig. 2. Relative activity (%) (*above*) and stability (*below*) of α-amylase (*circles*) and glucoamylase (*squares*) of *C. acetobutylicum* at various pH; initial activity of α-amylase and glucoamylase was 0.14 and 0.17 U/mL, respectively.

Hydrolysis of various substrates by partially purified α-amylase and glucoamylase of *C. acetobutylicum* is shown in Table III. The α-amylase possesses a great ability to hydrolyze high molar-mass compounds as illustrated by the relative values for potato starch (100 %), amylopectin (67.6) and amylose (41.9). The rate of α-amylase hydrolysis increased with the degree of polymerization of the homologous maltooligosaccharides from maltotriose up to amylose. Maltose and glucose were the end products of oligosaccharide hydrolysis by α-amylase. By contrast, glucoamylase has higher affinity towards starch (100 %) than to maltose (86.4 %). Table III also shows the substrate specificity of partially purified α-amylase from *C. acetobutylicum.* The highest reducing sugar was detected from potato starch, followed by corn starch, sago starch, amylopectin, amylose, maltotriose and maltose. This indicates that α-amylase had a higher affinity for longer-chain substrates.

Table III. Comparative hydrolysis of various substrates (0.1 %) by α-amylase and glucoamylase of *C. acetobutylicum* strain P262

Substrate	Linkage	Relative rate of hydrolysis, %		Substrate specificity of α-amylase[a]
		α-amylase	glucoamylase	
Potato starch	α-1,4 and α-1,6	100	–	1.71
Corn starch	α-1,4 and α-1,6	76.5	–	1.30
Sago starch	α-1,4 and α-1,6	68.6	100	1.17
Amylopectin	α-1,4 and α-1,6	67.6	84.6	1.16
Amylose	α-1,4	41.9	86.5	0.80
Maltotriose	α-1,4	21.3	100	0.71
Maltose	α-1,4	18.9	86.4	0.32

[a]Measured by total reducing sugar produced from the hydrolysis (g/L).

A lower solvent production in fermentation of hydrolyzed whey permeate and potato starch has been reported, *e.g.*, by Gutierrez *et al.* (1998) and Ennis *et al.* (1986). Obviously, the efficiency of fermentation of high concentrations of gelatinized starch was limited by the ability of amylolytic enzymes to hydrolyze it to fermentable sugars. In addition, fermentation of high starch concentrations tended to reduce the production of glucoamylase. Furthermore, the main amylolytic enzymes in direct fermentation of starch by *C. acetobutylicum* (α-amylase and glucoamylase) have different pH optima. Inefficiency of the amylolytic enzymes

secreted during the fermentation in hydrolyzing starch and maltose to glucose is the main problem in direct fermentation of sago starch to solvent. Although *C. acetobutylicum* appears to be a potential strain for the direct fermentation of sago starch to solvent, it is clear that incomplete utilization of maltose or glucose is still one of the problems. Since this strain is capable of producing total solvent concentrations up to 15 g/L in batch fermentation (Long *et al.* 1984), it can be suggested that product inhibition was not the sole problem in the cessation of direct fermentation of starch to solvent. The formulation of a medium for optimal amylolytic enzyme and solvent production is one of the approaches that can be used to improve direct fermentation of sago starch to solvent. The culture pH for optimal rate of starch hydrolysis may be different from that required for the enhancement of solvent production. Development of a pH control strategy is another approach to be used in improving the direct fermentation of sago starch to solvent.

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