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Production of Solvent (acetone-butanol-ethanol) in Continuous Fermentation by *Clostridium saccharobutylicum* DSM 13864 Using Gelatinised Sago Starch as a Carbon Source

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ABSTRACT

Solvent production by *Clostridium saccharobutylicum* DSM 13864 was carried out in a single stage continuous culture using 2 L stirred tank fermenter with gelatinised sago starch as a carbon source. From the study it was found that the condition could be adjusted to suit for acids production (high dilution rate and high pH) or solvent production (low dilution rate and low pH) by manipulating the dilution rate and culture pH of single stage continuous fermentation. The highest solvent concentration in outflow (9.10 g/L) was obtained at pH 4.5 and dilution rate of 0.05 h^{1}, which corresponds to overall productivity of 0.46 g/L.h. However, the highest total solvent productivity (0.85 g/L.h) was obtained at dilution rate of 0.11 h^{-1} and pH 4.5, which gave a total solvent yield of 0.29 g solvent/g sago starch. Although the total solvent productivity was greatly increased in continuous culture, the final solvent concentration attained in outflow was decreased by about 53% as compared to batch culture.

Keywords: Acetone-butanol-ethanol fermentation; *Clostridium saccharobutylicum*; chemostat culture; sago starch

INTRODUCTION

The most important economic factor in solvent fermentation is the cost of substrate, which made up about 60% of the overall cost of production (Ennis *et al*., 1986). Thus, the availability of an inexpensive raw material, sago starch for example, is essential if solvent fermentation is to become economically viable. In Malaysia, sago starch represents an alternative cheap carbon source for fermentation processes that is attractive in both economic and geographical considerations (Suraini, 2002). Furthermore, the occurrence of amylolytic enzymes such as α-amylase and glucoamylase during growth of *Clostridium* spesies on starch has been reported (Paquet *et al*., 1991; Mitchell, 1998). Substantial activities of amylolytic enzymes during active growth phase are needed to hydrolyze the starch to fermentable sugars that are subsequently used for production of organic acids and solvent. Thus, the use of an amylolytic enzyme-producing microorganism in solvent production by direct fermentation of sago starch is very important in order to eliminate the enzymatic hydrolysis step for the conversion of starch to fermentable sugars and hence, reduce the production cost greatly.

The use of a single-stage and/or two-stage continuous fermentation for the improvement of solvent production as compared to the classical batch fermentation have been reported (Fick *et al*., 1985, Frick and Schiigerl, 1986; Soni *et al*., 1987). Substantial improvement was achieved when high concentration of glucose and nitrogen in the feed medium was used. In addition, high solvent production can also be achieved in either phosphate or nitrogen limited continuous fermentation using synthetic medium (Monot *et al*., 1983b). Increased productivity could be achieved in a single-stage continuous fermentation when operated at slow dilution rate and pH was maintained between 4.3 to 4.8 (Monot *et al*., 1983b; Godin and Engasser, 1988). However, stable steady-state conditions in a single-stage continuous fermentation was relatively difficult to be achieved due to either the toxic effect of butanol at high solvent concentration or to the flocculation of the bacteria employed (Fick *et al*., 1985). Currently, there was no report available in the literature regarding solvent production in a single stage continuous fermentation using sago starch as a carbon source. Most of the research works on the production of solvent in continuous fermentation were focussed on the use of glucose as a carbon source (Monot and Engasser, 1983b; Fick *et al*., 1985; Soni *et al*., 1987; Mollah and Stuckey, 1992).

The present study was undertaken to investigate the feasibility of using a single stage continuous fermentation for the improvement of direct fermentation of sago starch to solvent. The effect of dilution rate and culture pH for solvent production by *C. saccharobutylicum* DSM 13864 using gelatinised sago starch as a carbon source was investigated.

MATERIALS AND METHODS

Microorganism and Strain Maintenance

The solvent-producing-bacterium, *C. saccharobutylicum* DSM 13864 was used throughout the study. This strain

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was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. The culture was maintained on Reinforced Clostridia Medium (RCM) at 35°C for 5 days to induce sporulation, followed by storage at 4°C.

Inoculum Preparation

The RCM was used for the preparation of inoculum. Stock culture was initially heat shocked at 80°C for 1.5 min followed by cooling in ice-cold water for 1 min. A 0.1 mL stock culture was then transferred into serum bottle (sealed with rubber stopper) containing 10 mL RCM and incubated at 35°C. After 24 h, the culture was transferred into fresh RCM, incubated at 35°C for 16 h and used as an inoculum. Optical density of the 16 h culture (measured using spectrophotometer at 680 nm) was approximately 0.8. An inoculum size of 10% (v/v) was used in all fermentations carried out in this study.

Batch Fermentation

Batch fermentation was performed in 2 L stirred tank fermenter (Biostat B, B. Braun Biotech International, Germany) with a working volume of 1.5 L and the basal medium composition (in g/L) was: sago starch 50.0, glycerol 6.0, KH_2PO_4 0.75, K_2HPO_4 0.75, $MgSO_4.7H_2O$ 0.4, $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, yeast extract 5.0, NH_4NO_3 2.0, NaCl 0.5, resazurin 0.001, cysteine 0.5, P-amino benzoic acid 0.001 and biotin 0.0008. Gelatinized starch was prepared by heating slurry to slightly above 70°C. The pH of the medium was adjusted to 6 with 5 M HCl before autoclaving. The medium was then autoclaved at 121°C for 20 min. Cysteine and vitamin solution (P-aminobenzoic acid and biotin) were filter sterilised through 0.20 μm filter and added to the medium when cooled to room temperature. After autoclaving, the fermentation broth was sparged with oxygen-free nitrogen until the temperature of medium dropped to around 35°C to achieve anaerobiosis. Flushing by the N_2 gas was limited to the headspace of the fermenter before and after inoculation and was terminated as soon as the cells started to produce the fermentation gases. The batch fermentation was started by inoculating a 10% (v/v) of inoculum, which was 16 h vegetative culture grown in a 250 mL Schott Duran bottle. In all experiments, the temperature was maintained at 35°C and the agitation was fixed at 100 rpm.

Continuous Fermentation

Continuous fermentations were also performed in 2 L stirred tank fermenter and were initially started as batch fermentations with a working volume of 700 mL. The initial batch fermentation was started by inoculating a 10% (v/v) of inoculum, which was 16 h vegetative culture grown in a 100 mL Schott Duran bottle. The continuous fermentation was started, by feeding a feed medium at a required constant flow rate when solvent concentration in the culture was increased to around 4 to 5 g/L (i.e., after about 16 h of initial batch fermentation). The preparation and the composition of feed medium was similar to the basal medium except that the concentration of sago starch, yeast extract and NH_4NO_3 used was 30 g/L, 2.5 g/L and 1.0 g/L, respectively. After autoclaving at 121°C for 20 min, the medium was maintained in anaerobic condition by sparging nitrogen free oxygen gas continuously into the medium reservoir. The medium was stirred using a hot plate to ensure that well mix condition was achieved.

The volume of the culture in the fermenter was kept constant at 700 mL by using an overflow tube set to a constant height within the fermenter vessel so that as fresh medium was pumped into the fermenter an equal volume of cultures enters the overflow tube and passes to the effluent tank. The culture outlet was connected with a peristaltic pump, which was operated continuously at a slightly higher rate than the feed rate.

The continuous fermentation was operated at dilution rate ranging from 0.03 h^{-1} to 0.22 h^{-1}, which was lower than the maximum specific growth rate of *C. saccharobutylicum* DSM 13864 (0.24 h^{-1}) as calculated from batch fermentation data (data not shown). In experiment to investigate the effect of dilution rate on the fermentation performance, the culture pH was controlled at pH 4.5. In experiment to investigate the effect of culture pH on fermentation performance, continuous fermentation was conducted at a dilution rate of 0.11 h^{-1}. The pH was controlled at desired value using 2 M NaOH or 2 M HCl. The temperature was maintained at 35°C and the agitation was fixed at 100 rpm throughout the fermentations.

The fermenter was assumed to be operating at steady-state when a constant concentration of solvent and cell were achieved in the reactor. The samples were taken from the fermenter and effluent tank at time interval for analysis. In all cases, the cell concentration in the fermenter and outflow was almost the same, indicating that the fermenter was well mixed system. The occurrence of contamination was regularly checked by microscopic observation.

Analytical Procedure

All fermentations were carried out at least in triplicate and the results presented were the average values. During the fermentation samples were withdrawn at appropriate time intervals for analysis. Cell concentration was determined using filtration and oven dry method as reported elsewhere. The concentrations of solvent and organic acids were determined by using gas chromatography (Agilent 6890 series, USA) equipped with the flame ionisation detector (FID). Separation was achieved by using a 2 m capillary column packed with polar BP 20 (SGE) and nitrogen as the carrier gas. The column temperature was held at 65°C for 8 min and then programmed with the increment of 20°C to 140°C with 10 min final hold. The temperature of the injector and detector was set at 220°C and 270°C, respectively. Samples (1 mL) were acidified with a drop of HCl (5%

v/v). The acidification was necessary to ensure that butyrate and acetate were in the acid forms. The injection volume was 1 µl. Data acquisition used to integrate the data was Chemstation, Agilent Techonologies, Hewlett Packard, Wilmington, USA. Sugars (maltose and glucose) and glycerol in the samples were determined using a high performance liquid chromatography (HPLC), Waters 2690 Alliance separation module with a refractive index (RI) detector (Waters 2410). Separation of compounds achieved by using Shodex KC-811 packed column (8 mm x 300 mm) and 3 mM H_2SO_4 as a stationary phase and mobile phase, respectively. The flow rate and the column temperature were conducted at 0.8 mL/min and 80°C, respectively. The injection volume was 10 µl. Data acquisition used to integrate the data was Millennium 32v.3.2. Concentration of starch was determined colorimetrically based on the study of Smith and Roe (1948). Determination of α-amylase activity was conducted according to the method as described by Soni *et al*. (1992). Glucoamylase activity was assayed using method as described by Ariff and Webb, (1996) using maltose as a substrate.

Statistical Procedure

Data were statistically analysed using The Statistical Analysis System (SAS) program (New Jersey, USA). Duncan multiple range tests were used to compare the difference between treatment means. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Batch Fermentation of *C. saccharobutylicum* DSM 13864

The time course of solvent fermentation using 50 g/L sago starch in 2 L stirred tank fermenter by *C. saccharobutylicum* DSM 13864 in batch culture is shown in Figure 1. Typical solvent fermentation using glucose as a carbon source can be divided into two distinctive phases, acidogenic phase and solventogenic phase. However, the direct fermentation of sago starch to solvent as reported in this study can be divided into three phases; i) sago starch hydrolysis phase, (ii) acidogenic phase and (iii) solventogenic phase (Figure 2).

During the early stages of fermentation sago starch was hydrolyzed into maltose and glucose by the action of α-amylase and glucoamylase secreted out into the medium by *C. saccaharobutylicum*. Both extracellular enzymes increased gradually and reached their maximum level after around 12-16 h. The maximum α-amylase and glucoamylase obtained was 10.89 U/mL and 13.83 U/mL, respectively. A gradual decreased in amylolytic enzyme occurred after the maximum activity was reached. This can be explained by a proteolytic denaturation of the enzymes by acidolysin, a protease produced by many bacteria (Soni *et al*., 1992). Sago starch concentration was not detected after 20 h. During the fermentation, the highest maltose and glucose accumulated were 21.33 g/L and 10.84 g/L, respectively.

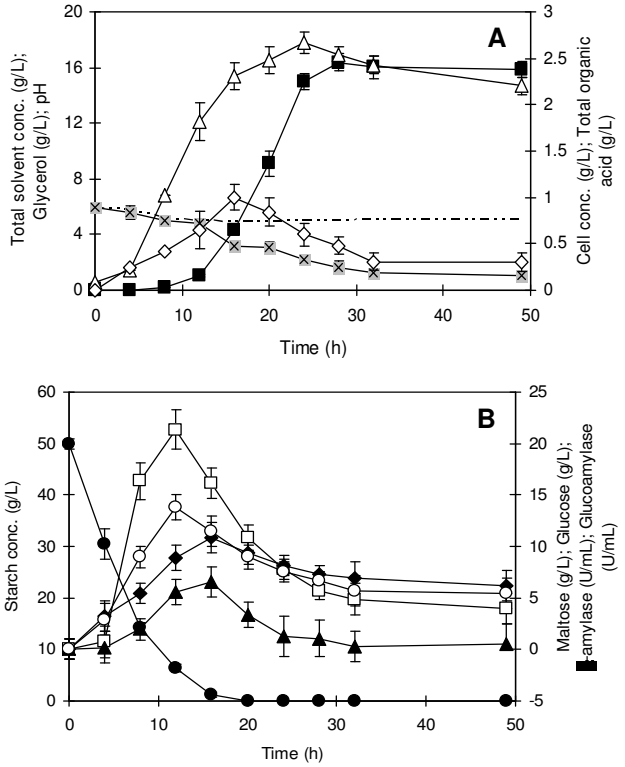

Figure 1: The time course of batch solvent production using 50 g/L sago starch in 2 L stirred tank fermenter.
Note: Symbols represent for figure A, (■) total solvent, (-) pH, (Δ) cell concentration, (◊) total organic acid, (×) glycerol concentration; Symbols represent for figure B, (●) starch concentration, (□) maltose, (▲) glucose, (♦) α- amylase, (o) glucoamylase. Bar indicates standard error for three replicates.

The acidogenic phase was observed during the first 16 h where cell biomass and organic acids (acetic acid and butyric acid) were rapidly produced. The maximum total organic acid accumulated was 1.00 g/L. Rapid secretion of organic acids from *C. saccharobutylicum* caused a reduction of culture pH from 6 to 4.86 and only small amount of solvent was produced.

When growth reached a stationary phase, solventogenic phase was started where intermediate acids were reassimilated for solvent production. During this phase, organic acids were utilized concomitantly with reducing sugars for the production of solvent. Active uptakes of organic acids caused the increment of pH. Increased in solvent concentration was observed during this phase where total solvent concentration reached their maximum value of 16.38 g/L (acetone 6.11 g/L; butanol 10.11 g/L; ethanol 0.16 g/L) at 28 h. The maximum cell concentration (2.67 g/L) was obtained at 24 h.

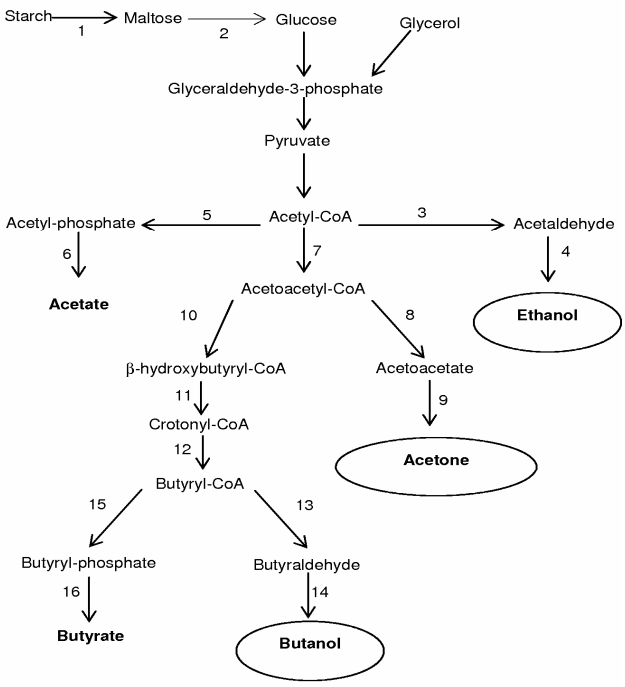

Figure 2: Metabolic pathways and enzymes involved in direct fermentation of sago starch to solvent.
Note: Enzymes related to solvent fermentation are: 1. α-amylase; 2. Glucoamylase; 3. Acetaldehyde dehydrogenase; 4. Ethanol dehydrogenase; 5. Phosphotransacetylase; 6. Acetate kinase; 7. Thiolase; 8. CoA transferase; 9. Acetoacetate decarboxylase; 10. β-hydroxybutyryl-CoA dehydrogenase; 11. Crotonase; 12.Butyryl-CoA dehydrogenase; 13. Butyraldehyde dehydrogenase; 14. Butanol dehydrogenase; 15. Phosphotransbutyrylase; 16. Butyrate kinase.

Continuous Fermentation

Effect of Different Dilution Rates

The steady state concentration of solvent, organic acids, cell, amylolytic enzymes and reducing sugars at different dilution rates during continuous culture are shown in Figure 3 and the data are summarized in Table 1. The secretion of amylolytic enzymes, α-amylase and glucoamylase were not significantly affected by the dilution rate. Residual starch was not detected at all dilution rates investigated, indicating that it was fully hydrolysed by α-amylase and glucoamylase to maltose and glucose.

Cell concentration at steady state was significantly reduced with increasing dilution rate. Increasing dilution rate from 0.03 h^{-1} to 0.22 h^{-1} decreased the cell concentration from 3.45 to 1.36 g/L. On the other hand, accumulation of maltose and glucose at steady state was increased drastically with increasing dilution rate. The highest maltose (8.70 g/L) and glucose (1.56 g/L) concentrations at steady-state was obtained at a dilution rate of 0.22 h^{-1}. Reduction in cell concentration and high accumulation of reducing sugars at higher dilution rates were related to a less efficient utilization of sugars by the bacterium (Mollah and Stuckey, 1992; Mollah and Stuckey, 1993; Badr *et al.*, 2001).

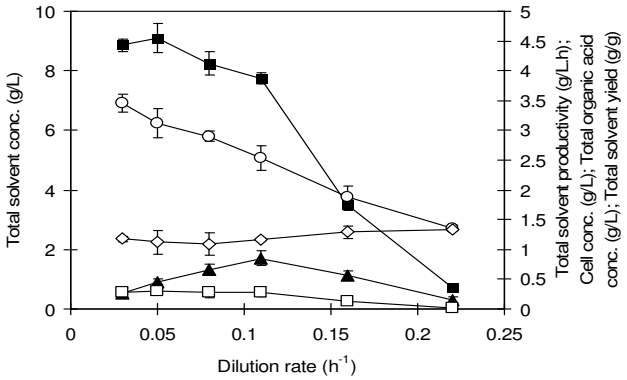

Figure 3: Effect of dilution rate on the performance of a single stage continuous solvent fermentation by *C. saccharobutylicum* DSM 13864 using sago starch as carbon source.
Note: (■) total solvent, (o) cell concentration; (◊) total organic acid; (▲) total solvent productivity; (□) total solvent yield. Bar indicates standard error for three replicates.

Total solvent concentration was decreased substantially with increasing dilution rate. Total solvent concentration obtained was significantly different with different dilution rates studied. The highest total solvent concentration (9.10 g/L) was obtained at dilution rate of 0.05 h^{-1} in which the concentration of acetone, butanol and ethanol was 3.28 g/L, 5.09 g/L and 0.73 g/L, respectively. Low accumulation of acetic (0.50 g/L) and butyric (0.62 g/L) acids at dilution rate of 0.05 h^{-1} enhanced solvent production significantly. At dilution rate higher than 0.16 h^{-1}, solvent production was decreased by about 92.3% as compared to that obtained at D = 0.05 h^{-1}. At D = 0.22 h^{-1}, total solvent concentration at steady state was only 0.7 g/L. It is important to note that ethanol was not detected at dilution rate of above 0.16 h^{-1}. In contrast to solvent production, there was an increase in total organic acid production as the dilution rate increased.

With an increase in dilution rate there was a substantial increase in solvent productivity, and the highest solvent productivity of 0.85 g/L.h was obtained at a dilution rate of 0.11 h^{-1}. At dilution rate higher than 0.11 h^{-1}, solvent productivity decreased, and at 0.22 h^{-1}, dropped to the very low value of 0.15 g/L.h. A maximum total solvent yield of 0.30 g/g was obtained at a dilution rate of 0.05 h^{-1}, and decreased with increasing dilution rate. Nevertheless, up to a dilution rate of 0.11 h^{-1}, the variation in solvent yield was small. At a dilution rate of 0.16 h^{-1} the solvent yield decreased to a value of 0.14 g/g,

Table 1: Performance and kinetic parameter values of solvents fermentation by *C. saccharobutylicum* DSM 13864 using gelatinised sago starch at different dilution rates in a single stage continuous culture at pH 4.5

Kinetic Parameters	Dilution rate (h^{-1})					
	0.03	0.05	0.08	0.11	0.16	0.22
Cell concentration (g/L)	3.45±0.14[a]	3.12±0.37[b]	2.90±0.43[c]	2.54±0.53[d]	1.88±0.29[e]	1.36±0.31[f]
α - amylase activity (U/mL)	5.13±0.39[a]	5.46±0.93[a]	4.78±0.89[a]	5.78±0.84[a]	6.18±1.12[a]	6.50±1.03[a]
Glucoamylase activity(U/mL)	8.23±0.88[a]	8.88±1.03[a]	8.78±0.97[a]	9.01±0.74[a]	9.17±0.67[a]	9.34±0.85[a]
Acetic acid concentration (g/L)	0.63	0.50	0.41	0.56	0.49	0.56
Butyric acid concentration (g/L)	0.55	0.62	0.68	0.61	0.80	0.78
Total organic acid (g/L)	1.18±0.43[c]	1.12±0.25[c]	1.09±0.14[d]	1.17±0.23[c]	1.29±0.14[b]	1.34±0.21[a]
Acetone concentration, A (g/L)	3.50	3.28	3.48	3.38	1.57	0.31
Butanol concentration, B (g/L)	4.68	5.09	4.29	3.97	1.93	0.39
Ethanol concentration, E (g/L)	0.68	0.73	0.48	0.39	0	0
Total solvent concentration, P_m (g/L)	8.86±0.82[b]	9.10±0.74[a]	8.25±0.63[c]	7.74±0.55[d]	3.50±0.42[e]	0.70±0.08[f]
Ratio of A:B:E	0.40: 0.53: 0.08	0.36: 0.56: 0.08	0.42: 0.52: 0.06	0.44: 0.51: 0.05	0.45: 0.55: 0	0.44: 0.56: 0
Residual maltose (g/L)	0.68	2.50	2.63	5.04	6.25	8.70
Residual glucose (g/L)	0.09	0.34	0.55	0.97	1.14	1.56
Total reducing sugar consumed (g/L)	32.19	30.03	29.68	26.72	25.28	22.28
Cell yield, $Y_{x/s}$ (g/g)	0.11	0.10	0.10	0.10	0.07	0.06
Total solvent yield,$Y_{p/s}$ (g/g)	0.28	0.30	0.28	0.29	0.14	0.03
Total solvent productivity, P_r (g/L.h)	0.27[e]	0.46[d]	0.66[b]	0.85[a]	0.56[c]	0.15[f]

Note: Total reducing sugar was calculated as glucose concentration where 1 g/L maltose was assumed to produce 1.053 g/L glucose. 1 g/L starch was assumed to produce 1.1 g/L glucose. Values are means of three replicates ± errors. [a-f] Mean values in same row with different superscripts are significantly different ($P<0.05$).

and as the dilution rate increased further up to 0.22 h^{-1}, the solvent yield was reduced to only 0.03 g/g.

It was found that in continuous solvent fermentation, the condition could be adjusted to suit for acid production (high dilution rate) or solvent production (low dilution rate). The result obtained was in agreement those reported in the literature (Andersch *et al*., 1982; Bahl *et al*. 1982a; Godin and Engasser 1988). In continuous solvent fermentation, the specific growth rate (μ) of *C. saccharobutylicum* was controlled by the dilution rate (D). When low dilution rate was applied, the production of total solvent was higher and this is in general agreement with the concept that obtained in batch culture where solventogenic cells are always associated with a lowering of the specific growth rate (Monot and Engasser, 1983a). On the other hand, high growth rates are associated with acidogenic phase as indicated by increased in total organic acids when high dilution rate was applied. This might due to cells was mainly in a logarithmic growth phase (Frick and Schügerl, 1986) and acids production occurs to provide the cells with excess ATP required for growth (Meyer and Papoutsakis, 1989; Mollah and Stuckey, 1992).

Effect of Different Culture pHs

The effect of different culture pHs on growth of *C. saccharobutylicum* and solvent production is shown in Table 2. Cell concentration was significantly decreased with increasing culture pH. The activities of amylolytic enzymes (α-amylase and glucoamylase) were not significantly influenced by the culture pH. However, the amylolytic enzymes secreted by *C. saccharobutylicum* were enough to hydrolyse all starches in a medium to reducing sugars. Residual maltose (5.04 g/L) and glucose (0.97 g/L) at pH 4.5 was lower as compared to pH 5.0 and 5.5, indicating that low pH was more efficient for conversion of reducing sugars to solvent.

Accumulation of acetic and butyric acids was increased substantially with increasing culture pH where butyric acid production was dominant as compared to acetic acid at pH of above 4.5. Inversely, concentration of acetone, butanol and ethanol at steady state was decreased with increasing culture pH. The highest total solvent concentration (7.74 g/L) was obtained at pH 4.5, in which the concentration of acetone, butanol and ethanol was 3.38 g/L, 3.97 g/L and 0.39 g/L, respectively. The concentration of total organic acid achieved at pH 4.5 was only 1.17 g/L (acetic acid (0.56 g/L) and butyric acid (0.61 g/L) whereas at pH 5.5 the total organic acid accumulated was 6.78 g/L in which the concentration of butyric acid and acetic acid was 4.74 g/L and 2.04 g/L, respectively.

At pH 5.0 and 5.5, total solvent production was only 2.17 g/L and 0.54 g/L, respectively. Ethanol concentration was not detected at pH of above 4.5. This result indicates that low pH (4.5) was required to repress acids accumulation, which in turn, enhance the solvent production. As the culture pH increased, total solvent productivity and total solvent yield decreased. The maximum value of total solvent productivity and total solvent yield that obtained at pH 4.5 was 0.85 g/L.h and 0.29 g/g, respectively.

Solvent production was not observed *when C. acetobutylicum* was grown in continuous culture at pH values of 6.5 or 5.7 (Bahl *et al*.,1982a; Bahl *et al*.,1982b). Bahl *et al*. (1982a) showed that the product spectrum of *C. acetobutylicum* DSM 1731 was governed by physiological parameters. It was found that continuous cultures operated at neutral pH and varying dilution rates, the only fermentation products formed were acetic acid, butyric acid, CO_2 and H_2 gas. However, this acid production phase over weeks could be shifted to produce solvent by reducing the culture pH to 4.3 and by adding appropriate amount of butyric acid to the medium.

The regulation of pH played an important role in the expression of products and enzymes during solvent fermentation. The specific activity of enzymes relevant to solvent production such as phosphate butyryltransferase, thiolase, crotonase, butyryl-CoA dehydrogenase and β-hydroxylbutyryl-CoA dehydrogenase during solvent fermentation using glucose as a carbon source was greatly influenced by the culture pH (Hartmanis and Standtman, 1982; Hartmanis and Gatenbeck, 1984). During continuous culture, the production of solvent was dominant as compared to organic acids production when the culture pH was low (pH 4.5), suggesting that secretion of enzymes relevant to solvent formation may be greatly enhanced at low pH.

Comparison of Performance Between Batch and Continuous Culture

The kinetic performance of batch and continuous fermentation are given in Table 3. It is worth to note that the total solvent productivity obtained in continuous culture was greatly increased by about 44% as compared to batch culture. However, total solvent concentration was decreased by about 53% as compared to that obtained in batch fermentation. The total solvent concentration of 7.74 g/L was significantly lower than that obtained in batch fermentation, which was 16.38 g/L. The total solvent yield was also decreased from 0.35 g/g for batch culture to 0.29 g/g for continuous culture. As mentioned by Fick *et al*. (1985), a continuous process must first yield the high final concentration and conversion yields of solvents which can be obtained in a batch process in order to be industrially attractive. Therefore, it can be concluded that a single stage continuous fermentation was not economically superior to conventional batch fermentation as demonstrated in this study. In term of total solvent concentration, production of solvent by *C. saccharobutylicum* DSM 13864 in single stage continuous culture using sago starch as substrate was lower than those reported in the literature for different strains of *Clostridium* (Table 4). However, the fermentation performance was comparable in term of total solvent productivity.

The production of acids and acetone along with butanol during continuous culture indicates that the production and reutilization of acids occurs concurrently

Table 2: Performance and kinetic parameter values of solvent fermentation by *C. saccharobutylicum* DSM 13864 using gelatinised sago starch at different pHs in a single stage continuous culture at dilution rate 0.11 h^{-1}

Kinetic parameter	pH		
	4.5	5.0	5.5
Cell concentration, X_m (g/L)	2.54±0.25[a]	2.14± 0.21[b]	1.88± 0.09[c]
α - amylase activity (U/mL)	5.78[a]	5.92[a]	6.14[a]
Glucoamylase activity (U/mL)	9.01[a]	9.22[a]	9.87[a]
Acetic acid concentration (g/L)	0.56	0.79	2.04
Butyric acid concentration (g/L)	0.61	1.26	4.74
Total organic acid (g/L)	1.17± 0.07[c]	2.05± 0.12[b]	6.78± 0.32[a]
Acetone concentration, A (g/L)	3.38	0.97	0.15
Butanol concentration, B (g/L)	3.97	1.20	0.39
Ethanol concentration, E (g/L)	0.39	0	0
Total solvent concentration, P_m (g/L)	7.74± 0.41[a]	2.17± 0.27[b]	0.54± 0.05[c]
Ratio of A:B:E	0.44: 0.51: 0.05	0.45: 0.55: 0	0.28: 0.72: 0
Residual maltose (g/L)	5.04	6.58	7.77
Residual glucose (g/L)	0.97	1.22	1.68
Total reducing sugar consumed (g/L)	26.72	24.85	23.14
Total solvent yield, $Y_{p/s}$ (g/g)	0.29	0.09	0.02
Total solvent productivity, P_r (g/L.h)	0.85[a]	0.24[b]	0.06[c]

Note: Total reducing sugar was calculated as glucose concentration where 1 g/L maltose was assumed to produce 1.053 g/L glucose. 1 g/L starch was assumed to produce 1.1 g/L glucose. Values are means of three replicates ± errors. [a-c] Mean values in same row with different superscripts are significantly different (P<0.05).

Table 3: Comparison of performance between batch and continuous fermentations for production of solvent using sago starch as substrate.

	Batch culture	Single stage continuous culture
Acetone, A (g/L)	6.11 ± 0.32[a]	3.38 ± 0.52[b]
Butanol, B (g/L)	10.11 ± 0.54[a]	3.97 ± 0.12[b]
Ethanol, E (g/L)	0.16 ± 0.02[a]	0.39 ± 0.08[b]
Total solvent concentration, P_m (g/L)	16.38 ± 0.72[a]	7.74 ± 0.41[b]
Total solvent yield, $Y_{p/s}$ (g/g)	0.35 ± 0.03[a]	0.29 ± 0.07[b]
Total solvent productivity, P_r (g/L.h)	0.59 ± 0.07[b]	0.85 ± 0.07[a]

Note: The value in batch culture was obtained from the growth of *C. saccharobutylicum* DSM 13864 on the growth of 50 g/L sago starch. Values are means of three replicates ± errors. [a-b] Mean values in same row with different superscripts are significantly different (P<0.05).

Table 4: Comparison of performance of solvent production in a single stage continuous culture using different strains of *Clostridium.*

Strain	Substrate	D (h^{-1})	pH	Total solvent concentration (g/L)	Total solvent productivity (g/L.h)	Reference
C. acetobutylicum DSM 1731	glucose	0.22	5.2	4-5	0.95	Andersch *et al*. (1982)
C. acetobutylicum DSM 1731	glucose	0.03	4.3	13.35	0.33	Bahl *et al*. (1982b)
C. acetobutylicum ATCC 824	glucose	0.03	4.8	12.00	0.40	Monot and Engasser (1983b)
C. acetobutylicum ATCC 824	glucose	0.06	4.8	13.00	0.75	Fick *et al*. (1985)
C. acetobutylicum ATCC 824	glucose	0.29	4.4	7.25	2.08	Soni *et al*. (1987)
C. beijerinkii NCIB 8052	glucose	0.07	4.7	8.29	0.58	Mollah and Stuckey (1992)
C. saccharobutylicum P262 [a]	DSPS	0.13	4.6	7.32	1.00	Badr *et al*. (2001)
C. saccharobutylicum DSM 13864	Sago starch	0.05	4.5	9.10	0.46	This work
C. saccharobutylicum DSM 13864	Sago starch	0.11	4.5	7.74	0.85	This work

Note: [a] Immobilized cell. DSPS is defibered sweet potato slurry

with butanol formation, demonstrating that both acid producing and solvent producing cells coexist in this system. The generation of different populations of cells exhibiting metabolism and growth rates that resulted in the periodic oscillation in cell populations and also decreased the production of end products (Mollah and Stuckey, 1992; Jones, 2001). This phenomenon becomes a major limitation associated with the use of a single stage continuous culture.

In order to overcome the mix population problem, the use of two-stage continuous cultures have been suggested by a number of researchers (Bahl *et al*., 1982b; Godin and Engasser, 1989; Lai and Traxler, 1994). This method of fermentation can be used to separate acidogenic and solventogenic cells into two consecutive fermenters. The first stage fermenter should be operated at conditions that favour for optimal acid production and significant amount of solvent should be induced in the first fermenter, so that the cells were ready for solvent production when they entered the second stage fermenter (Godin and Engasser, 1989; Lai and Traxler, 1994).

CONCLUSION

In a single stage continuous culture with *Clostridium saccharobutylicum* DSM 13864 using gelatinised sago starch as a carbon source, the condition could be adjusted to suit for acids production or solvent production. To trigger solvent production, the fermentation should be carried out at low dilution rate and low pH. On the other hand, high dilution rate and high pH will enhance acid production. The highest total solvent productivity (0.85 g/L.h) was obtained at dilution rate of 0.11 h^{-1} and pH 4.5, which gave a total solvent concentration and a total solvent yield of 7.74 g/L and 0.29 g solvent/g sago starch, respectively. The total solvent productivity obtained in continuous culture was significantly higher than that obtained in batch culture (0.59 g/L.h). However, the final solvent concentration attained in outflow of a single continuous culture was about 53% lower as compared to batch culture (16.38 g/L).

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