Process Development for Lactic Acid Production from Cassava Starch

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Abstract

The continuous single-stage fermentation of *Lactococcus lactis* IO-1, using cassava starch hydrolysate (S_0 = 8.85 g glucose l^{-1}) as carbon source was performed. The maximum volumetric productivity of lactic acid (Q_P = 4.42 g l^{-1} h^{-1}) and the lactic acid concentration of 5.46 g l^{-1} were obtained at 0.81 h^{-1} dilution rate. This volumetric productivity was 2.59 times higher than that of batch fermentation (Q_P = 1.71 g l^{-1} h^{-1}). In continuous single-stage fermentation with cell recycle, the dilution rate (D), the bleed ratio (*B*) and the feed glucose concentration (S_0) were found to affect the growth and the lactic acid production. The volumetric productivity of lactic acid (Q_P = 13.39 g l^{-1} h^{-1}) was maximized at D = 0.96 h^{-1}, *B* = 0.25 and S_0 = 23.03 g l^{-1}, which was 4.13 times higher than that of the batch fermentation (Q_P = 3.24 g l^{-1} h^{-1}). The lactic acid concentration of 13.94 g l^{-1} was also obtained. In continuous two-stage fermentation without cell recycle, the optimal condition obtained for lactic acid production were dilution rates of the first (D_1) and the second (D_2) of 0.60 h^{-1} and S_0 of 44.75 g l^{-1}. As a result, the cells (X_2), lactic acid (P_2) concentrations and total lactic acid productivity ($Q_{P,Total}$) were 2.17, 21.29 g l^{-1} and 6.39 g l^{-1} h^{-1}, respectively. In continuous two-stage fermentation with cell recycle, the optimal condition was obtained by controlling the dilution rates and bleed ratio (B_1) at 0.80, 0.30 h^{-1} and 0.50, respectively, with an initial glucose concentration in fresh medium (S_0) of 39.51 g l^{-1}. As a result, the concentrations of cells (X_2) and lactic acid (P_2) and total lactic acid productivity ($Q_{P,Total}$) were maximized at 4.02, 30.78 g l^{-1} and 7.33 g l^{-1} h^{-1}, respectively.

Key words : *Lactococcus lactis* IO-1, L-lactic acid, cassava starch, continuous fermentation, cell recycle.

Introduction

Cassava starch is agricultural material produced abundantly in Thailand. The starch is used directly as foodstuff or indirectly as raw material for the manufacturing of food additives, sugars and flavoring agents as well as for the production of ethanol and lactic acid. The use of cassava for bioplastics (polylactate) production needs primarily enzymatic hydrolysis of starch to glucose by α-amylase and glucoamylase, followed by L-lactic acid fermentation from starch hydrolysate containing glucose as a main carbon source (Sirisansaneeyakul *et al*., 2000). The lactic acid fermentation by *Lactococcus lactis* IO-1 has been previously reported as high potential process for the production of L-lactic acid

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(Ishizaki and Vonktaweesuk, 1996; Sirisansaneeyakul *et al*., 1998). Therefore, in this work, cassava starch hydrolysate prepared by enzymatic hydrolysis was used as substrate and the continuous production of L-lactic acid was then optimized in both single-stage and two-stage fermentations either with or without cell recycle using *Lactococcus lactis* IO-1, for enhancing the volumetric lactic acid productivity.

Materials and Methods

Industrial grade cassava starch was obtained from Bang-na cassava starch factory in Chaiyaphum province, Thailand. Novo Nordisk supplied the enzymes Termamyl 120 L Type LS (heat stable α-amylase from *Bacillus licheniformis*, 120 KNU/g LOT ATNG 0638; EC 3.2.1.1) and Dextrozyme (a mixture of glucoamylase from *Aspergillus niger* and pullulanase from *Bacillus acidopullulyticus*; 225 AGU/ml, 75 PUN/ml)

Enzymatic hydrolysis of cassava starch

Starch slurries contained 250 g starch (25%), 50 mg $CaCO_3$ (50 ppm) and 1 g benzoic acid (0.1%) in tap water with total volume of 1 l. The slurries were adjusted to pH 6.5 with 1.0 M NaOH and pregelatinized in boiling water for 10 min. Liquefaction was carried out by adding 1.5 ml Termamyl 120L Type LS (0.15% v/v) and incubated at 95 ^{o}C for 2 h. For saccharification, the slurries were adjusted to pH 4.3 with 1.0 M H_3PO_4 and 1.0 ml Dextrozyme (0.10% v/v) was added to the liquefied slurries and incubated at 60 ^{o}C for 24 h (Sirisansaneeyakul *et al*., 2000).

Continuous production of L-lactic acid

Microorganism Lactococcus lactis IO-1 (JCM 7638) (Ishizaki *et al*., 1990) was used throughout the experiments. The stock culture was maintained in thioglycolate (TGC) medium (Difco) and subcultured every two weeks.

Medium The basal medium in 1 l consists of 5.0 g NaCl, 5.0 g yeast extract and 5.0 g polypeptone (Ishizaki and Ohta, 1989). Glucose was obtained from an enzymatic hydrolysis of cassava starch prepared as mentioned above. With an initial glucose concentration of 10 to 45 g glucose l^{-1}, cassava starch hydrolysate was appropriately added into the basal complex medium.

A single-stage fermentation with and without cell recycle The continuous fermentation without cell recycle was carried out with a working volume of 750 ml in a 2-l fermenter (Biostat B, B.Braun Biotech International, Germany) at controlled pH 6.0, temperature 37 ^{o}C and agitation rate of 400 rpm without air feeding. The inoculum was prepared from a 100 μl of TGC stock culture in 5 ml basal medium (10 g glucose l^{-1}) for 18 h at 37 ^{o}C in a static incubator. The 2.5 ml of this preculture was then inoculated into 35 ml of basal medium (10 g glucose l^{-1}) at initial pH 6.0 and incubated for 18 h with gentle shaking at 37 ^{o}C. The preculture was then used as 5% v/v inoculum for starting the 750-ml batch culture. After 10 h of batch cultivation, the continuous culture was further performed at various dilution rates (0.22, 0.47, 0.60, 0.81 and 1.04 h^{-1}). The steady state was obtained approximately after four volumes replacement. In continuous fermentation with cell recycle (Fig. 1), the batch cultivation was initially performed under the above mentioned conditions for 10 h. It was then switched to continuous mode with cell recycle applying a hollow fibre module (Kuraray UF Module KL-U-M 6302, Japan). The feed glucose concentration were 10 and 30 g glucose l^{-1} at dilution rate D = 0.40, 0.80 and 0.96 h^{-1} and recycle ratio of R = 0.10, 0.25 and 0.50.

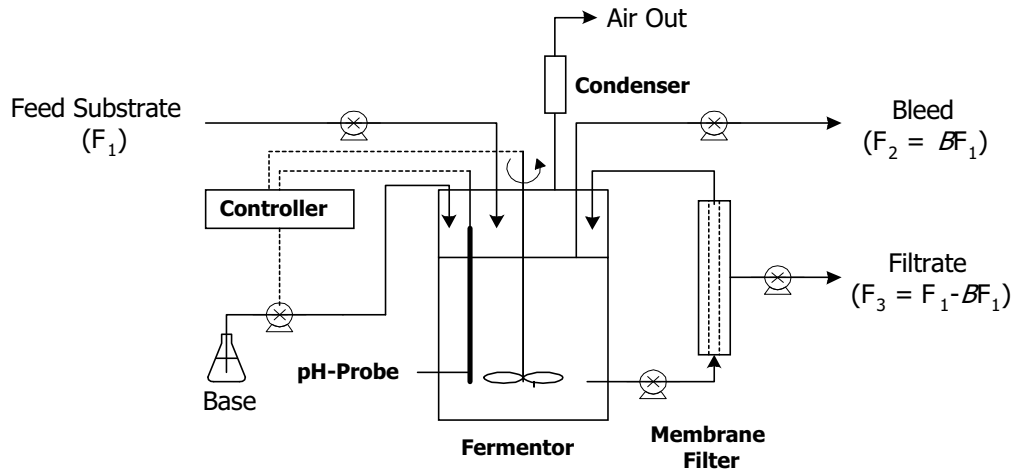


Figure 1 A Scheme of the single-stage fermentation with cell recycle.

A two-stage fermentation with and without cell recycle The fermentation was carried out at controlled conditions (37°C, pH 6.0, 400 rpm agitation) for 10 h in the first (Biostat B, B. Braun Biotech International, Germany) and the second (Eyela M-100, Tokyo Rikakikai Co., Ltd, Japan) fermenters with 0.75 and 1.0 l of working volume, respectively. The system was then switched to continuous two-stage fermentation with a constant feed rate varying C_S^0 = 10, 30, 40, 45 g/l, in which D_1 = 0.60-0.80 h^{-1} and D_2 = 0.46-0.80 h^{-1} were obtained. The steady state of a two-stage fermentation without cell recycle was arrived in 70 h of culture time. In continuous fermentation with cell recycle (Fig. 2), the batch cultivation was performed under the above mentioned conditions for 10 h. It was then switched to continuous mode with cell recycle applying a hollow fibre module (Kuraray UF Module KL-U-M 6302, Japan) at the first fermenter. The feed glucose concentration were 30 and 40 g glucose l^{-1} at dilution rate D = 0.83 h^{-1} and recycle ratio of R = 0.50.

Analytical methods

Cell concentration was monitored by optical density readings at 562 nm (OD_{562}) (Spectrophotometer, Photomech 301-D, Optima, Japan) and converted to dry cell weight (DCW) from calibration curve. The viable count during fermentation were carried out by pour plate counts on thioglycolate (TGC) medium with 1.5% agar and were reported as colony forming unit (CFU/ml). Plates were incubated at 37 °C for 48 h before counting (Roy *et al*., 1986). Glucose and L-lactic acid were determined by HPLC column for organic acids (MetaCarb 87H, 300x7.8 mm), controlled at 35 °C. H_2SO_4 (0.008 N) was used as mobile phase at controlled flow rate of 0.6 ml/min. Refractometer (Waters) was used as a refractive detector in the HPLC system. L(+) lactic acid (2-hydroxypropionic acid), litium salt (L-2250, Sigma) was used as a standard for lactate determination.

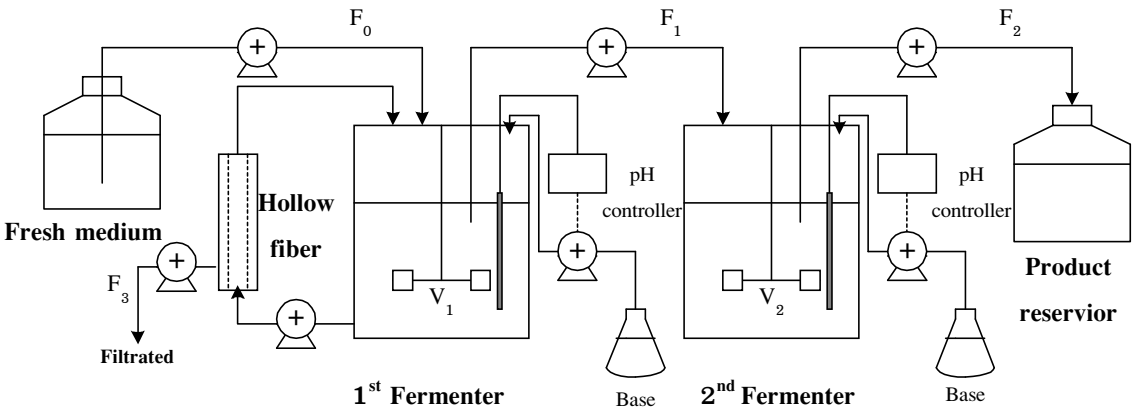


Figure 2 A Scheme of the two-stage fermentation with cell recycle.

Mathematical Model

For lactic acid fermentation with *Lactococcus lactis* IO-1 using starch hydrolysates as substrate, the substrate and product inhibition play a very important role of cell growth. Based on our previous work, the cell growth rate is expressed with Monod kinetics including substrate and product inhibition (Sirisansaneeyakul *et al*., 1998).

$$dX/dt = [(\mu_m S)/(K_S + S + (S^2/K_i))][1-(P/P^*)]^n X \quad (1)$$

Where X, S and P represent the cell, substrate and product concentrations (g l^{-1}), respectively. While μ_m, K_S, K_i, P* and n are the specific growth rate (h^{-1}), the saturation constant (g l^{-1}), the substrate inhibition constant (g l^{-1}), the critical concentration of lactic acid at which cell growth is equal to zero (g l^{-1}) and the constant used to describe product inhibition, respectively.

The lactic acid formation rate is explained by the Luedeking-Piret equation, considering growth and non-growth associated constants (Luedeking and Piret, 1959).

$$dP/dt = \alpha(dX/dt) + \beta X \quad (2)$$

Where α and β are growth associated constant (g g^{-1}) and non-growth associated constant (g g^{-1} h^{-1}) for lactic acid formation, respectively.

The glucose consumption rate is described by balancing the substrate utilized for cell growth and maintenance and lactic acid formation.

$$dS/dt = -[(1/Y_G)(dX/dt) + m_S X + (1/Y_P)(dP/dt)] \quad (3)$$

Y_G and Y_P are the stoichiometric constants describing the theoretical yields of lactic acid (g g^{-1}) and cell mass (g g^{-1}) respectively, whereas m_S is the maintenance coefficient (g g^{-1} h^{-1}) of glucose used for cell maintenance process.

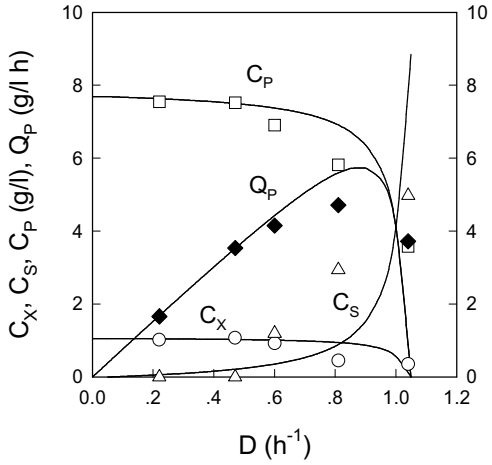
In case of continuous production of lactic acid, the balance equations for cell growth, lactic acid formation and substrate consumption are considered with the flow rate of feed medium in term of dilution rate (D = F/V). With cell recycling system, the dilution

rate of either single- or two-stage fermentation can be properly increased depending on the adjustment of a recycle stream (R) or bleed stream (*B*) (Sirisansaneeyakul *et al*., 1998).

Results and Discussion

(1) A single-stage fermentation with and without cell recycle

The continuous fermentation without cell recycle was carried out using cassava starch hydrolysate (8.85 g glucose l^{-1}) as feed substrate. After varying dilution rates of 0.22, 0.47, 0.60, 0.81 and 1.04 h^{-1}, the volumetric lactic acid productivity of 1.66, 3.52, 4.13, 4.42 and 3.52 g lactic acid l^{-1} h^{-1} were obtained, respectively (Fig. 3). Without both inhibitions of substrate and product, a balance equation for cell growth (Eq. 1) can be simplified to the Monod type kinetics. Therefore, μ_m = 1.08 h^{-1} and K_S = 0.58 g/l were estimated using Hanes-Woolf plot. As a result, theoretical-maximal dilution rate, D_m = 0.81 h^{-1} was absolutely closed to the observed dilution rate, in which the maximal volumetric productivity of 4.42 g lactic acid l^{-1} h^{-1} was achieved in the experiment.



μ_m = 1.08 h^{-1}, K_S = 0.58 g/l, $Y_{X/S}$ = 0.12 g/g, $Y_{P/S}$ = 0.88 g/g

Fugure 3 Glucose, lactic acid, cell concentrations and volumetric lactic acid productivity plotted as a function of dilution rate at steady state.

Symbols :○ Cells (C_X) △ Glucose (C_S) □ Lactic acid (C_P)
◆ Volumetric lactic acid productivity (Q_P)
—— Calculated from ous previously proposed model (Sirisansaneeyakul *et al*., 2000)

From our previous work, we found that the formation of lactic acid depended on non-growth associated constant (β) rather than growth associated constant (α) under no inhibition of lactic acid. Therefore, the specific rates of glucose consumption (q_S) and lactic acid formation (q_P) were increased with an increase of the specific growth rate at higher dilution rates of continuous fermentation. As a result, the volumetric productivity of lactic acid (Q_P) was improved at optimal dilution rate (D_m). The volumetric productivity was 2.58 times higher than that of batch fermentation which used 8.41g l^{-1} glucose of

cassava starch hydrolysate (Table 1). The specific rate of lactic acid production was also 4.4 times higher than that of batch fermentation. However, the residual glucose of 2.67 g l^{-1} and the lower concentration of 5.46 g l^{-1} lactic acid were moderately obtained at this optimal productivity.

In a single-stage fermentation with cell recycle, the volumetric lactic acid productivity was increased to 6.64 g/l h with 8.47 g glucose l^{-1} at D = 0.80 h^{-1} and a bleed ratio (*B*) of 0.5 (Table 1). The higher concentrations of 1.33 g cell l^{-1} and 8.30 g lactic acid l^{-1} were also obtained, and glucose was completely utilized. When using 26.59 g glucose l^{-1} at D = 0.80 h^{-1} and $\dot{B}$= 0.5, the volumetric lactic acid productivity of 8.58 g lactic acid l^{-1} h^{-1} was further improved, resulting in the concentrations of lactic acid and residual glucose of 10.73 and 15.19 g l^{-1}, respectively. The experiment was confirmed using cassava starch hydrolysate as a sole substrate (31.99 g glucose l^{-1} at D = 0.87 h^{-1} and $\dot{B}$= 0.5), The volumetric productivity and concentration of lactic acid were 11.09 g l^{-1} h^{-1} and 12.75 g l^{-1}, respectively. The better results are obtained because of a slightly higher dilution rate and glucose concentration. However, the substrate utilization at these higher feeding concentrations of glucose was reduced to 42.88-44.61 %. The glucose utilization was improved to 61.09 %, when decreasing the dilution rate to 0.40 h^{-1}. As a result, the less volumetric productivity and concentration of lactic acid were occurred instead. When decreasing the bleed ratio (*B*) to 0.10 and 0.25, the maximal lactic acid productivity of 13.39 g lactic acid l^{-1} h^{-1} was achieved at D = 0.96 h^{-1} and $\dot{B}$= 0.25. The concentrations of lactic acid and residual glucose were 13.94 and 7.95 g l^{-1}, respectively. This indicated that a high dense culture could enhance the volumetric productivity of lactic acid by monitoring the stream of cell recycle. A higher recycle ratio (R) or a less bleed ratio (*B*) gives a higher density of cells which results an improved productivity of lactic acid. Therefore, the volumetric productivity lactic acid of 11.41 g l^{-1} h^{-1} was obtained at the lower feed concentration of 23.02 g glucose l^{-1} and the bleed ratio (*B*) of 0.10, as compared with that of 31.99 g glucose l^{-1} and *B* = 0.50. The cell concentration of 11.53 g l^{-1} was 4.71 times higher, showing more glucose utilization for cell growth rather than lactic acid formation. The process optimization should therefore emphasize more on efficient consumption of substrate for producing lactic acid under above optimal conditions. Alternatively, the fermentation with two-stage system should be considered for investigating the better performance of lactic acid production.

Table 1 Fermentation kinetics of lactic acid production by *L. lactis* IO-1 with single-stage cell recycling system (SSFR) under steady state condition.

Fermentation	Fermentation conditions			Concentrations ($g\ l^{-1}$)			Yields ($g\ g^{-1}$)		Specific rates ($g\ g^{-1}\ h^{-1}$)		Glucose consumption (%)	Volumetric lactic acid productivity (Q_P) ($g\ l^{-1}\ h^{-1}$)
	Feed glucose, (S_0) ($g\ l^{-1}$)	Dilution rate, (D) (h^{-1})	Bleed ratio, (*B*)	Cells (X)	Glucose (S)	Lactic acid, (P)	$Y_{X/S}$	$Y_{P/S}$	q_S	q_P		
Batch* (2)	8.41	-	-	1.69	0.00	7.87	0.20	0.90	2.12	1.95	100.00	1.71
SSF** (2)	8.85	0.81	1.00	0.52	2.67	5.46	0.08	0.88	9.70	8.58	69.81	4.42
SSFR-1 (1)	8.47	0.80	0.50	1.33	0.00	8.30	0.08	0.98	5.11	5.00	100.00	6.64
SSFR-2 (1)	26.59	0.80	0.50	1.88	15.19	10.73	0.08	0.94	4.84	4.56	42.88	8.58
SSFR-3 (2)	31.99	0.87	0.50	2.45	17.72	12.75	0.09	0.89	5.07	4.53	44.61	11.09
SSFR-4 (1)	25.93	0.40	0.50	1.96	10.09	14.65	0.06	0.92	3.24	3.00	61.09	5.86
SSFR-5 (1)	23.02	0.96	0.25	4.06	7.95	13.94	0.07	0.93	3.56	3.30	65.47	13.39
SSFR-6 (1)	24.27	0.86	0.10	11.53	8.55	13.27	0.07	0.84	1.17	0.99	64.79	11.41

* Fermentation condition controlled at 37 ^{o}C and pH 6.0 using cassava starch hydrolysate (Sirisansaneeyakul *et al*., 2000)

** A single-stage fermentation without cell recycle (SSF) using cassava starch hydrolysate at dilution rate, D = 0.81 h^{-1}

(1) Glucose as carbon source (2) Cassava starch hydrolysate as substrate

(2) A two-stage fermentation with and without cell recycle

To compare with a single-stage fermentation, a process with two-stage fermentation for lactic acid production was performed to utilize the residual glucose from the first fermenter. Both two-stage fermentations with and without cell recycling system were studied to elucidate their lactic acid production. For an example, in a single-stage fermentation without cell recycle, the glucose concentration of 2.67 g l^{-1} was remained at the condition of $D = 0.81\ h^{-1}$ and $S_0 = 8.85$ g glucose l^{-1}. Similarly, the two-stage fermentation was conducted under the condition of $D_1 = 0.83\ h^{-1}$, $D_2 = 0.62\ h^{-1}$ and $S_0 = 8.99$ g glucose l^{-1}. As a result, glucose was completely consumed to produce 6.93 g lactic acid l^{-1}. The overall lactic acid productivity ($Q_{P,Total}$) was decreased to 2.42 g l^{-1} h^{-1}, due to the decreased dilution rate of the whole system ($D_{Total} = 0.35\ h^{-1}$) (Table 2). This volumetric lactic acid productivity was still 1.42 times higher than that from batch fermentation ($Q_p = 1.71$ g l^{-1} h^{-1}) (Sirisansaneeyakul *et al*., 2000), but 1.83 times lower than that from a single-stage fermentation without cell recycle ($Q_p = 4.42$ g l^{-1} h^{-1}) (Table 1).

Either increasing feed concentrations or optimizing dilution rates, the overall volumetric productivity of lactic acid could be further improved to 4.75-6.39 g l^{-1} h^{-1} in a two-stage fermentation without cell recycle, as shown in Table 2. The lowest concentration of residual glucose (10.47 g l^{-1}) was obtained at the condition of $D_1 = D_2 = 0.60\ h^{-1}$ and $S_0 = 29.96$ g glucose l^{-1}. Whereas the highest concentration, P = 21.29 g l^{-1} and overall productivity, $Q_{P,Total} = 6.39$ g l^{-1} h^{-1} of lactic acid were achieved at the condition of $D_1 = 0.80\ h^{-1}$, $D_2 = 0.46\ h^{-1}$ and $S_0 = 44.75$ g glucose l^{-1}. This overall productivity and concentration of lactic acid were increased 3.74 and 1.45 times, as compared with batch fermentation and a single-stage fermentation with cell recycle, respectively. However, the residual glucose concentration of 19.41 g l^{-1} was not entirely minimal. When decreasing the feed concentration to 29.93 g glucose l^{-1}, glucose was completely utilized at $D_1 = 0.80\ h^{-1}$ and $D_2 = 0.30\ h^{-1}$ in a two-stage fermentation with cell recycle ratio (R) of 0.10. Similarly, the overall lactic acid productivity, $Q_{P,Total} = 6.42$ g l^{-1} h^{-1} was obtained with the final lactic acid concentration of 25.97 g l^{-1}. The overall lactic acid productivity of 7.33 g l^{-1} h^{-1} was slightly improved under the same conditions with an increased feed concentration to 39.51 g glucose l^{-1}. The final concentration of lactic acid was maximized to 30.78 g l^{-1}, as well as the residual concentration of glucose was minimized to 4.29 g l^{-1}.

Conclusion

In this work, we compared four types of continuous lactic acid production by *L. lactis* IO-1 (single-stage and two-stage either with or without cell recycle). The results showed that the best single-stage fermentation with cell recycle produced the highest volumetric lactic acid productivity of 13.39 g l^{-1} h^{-1} with the final concentration of lactic acid at 13.94 g l^{-1}. Although the best two-stage fermentation with cell recycling provided the lower productivity of 7.33 g l^{-1} h^{-1}, the higher lactic concentration of 30.78 g l^{-1} and the lower residual glucose of 4.02 g l^{-1} were interesting. This provides the implementation of two-stage with cell recycling system for producing lactic acid from cassava starch hydrolysate, which is much more superior than that of batch fermentation,

Table 2 Lactic acid fermentation by *L. lactis* IO-1 in a two-stage with and without cell recycle under steady state condition.

Fermentation	Concentrations ($g\ l^{-1}$)				Yields** ($g\ g^{-1}$)		Specific rates**		$Q_{P,Total}$** ($g\ l^{-1}h^{-1}$)
	S_0	X*	S*	P*	$Y_{X/S}$	$Y_{P/S}$	q_S	q_P	
TSF-1 (1)	8.99	1.45	0.01	6.93	0.16	0.77	2.17	1.67	2.42
TSF-2 (1)	29.96	2.05	10.47	17.49	0.10	0.90	3.33	2.99	6.12
TSF-3 (2)	44.75	2.17	19.41	21.29	0.08	0.84	3.50	2.94	6.39
TSF-4 (3)	42.54	2.96	15.85	21.01	0.11	0.79	2.61	2.06	6.09
TSF-5 (4)	37.88	2.72	19.04	13.98	0.14	0.74	2.36	1.75	4.75
TSFR-1 (5)	29.93	3.61	0.02	25.97	0.08	0.86	4.32	3.74	6.42
TSFR-2 (5)	39.51	4.02	4.29	30.78	0.08	0.86	4.94	4.32	7.33

A two-stage fermentation without cell recycle (TSF) at steady state of (1) $D_1 = 0.83\ h^{-1}$, $D_2 = 0.62\ h^{-1}$; (2) $D_1 = 0.60\ h^{-1}$, $D_2 = 0.60\ h^{-1}$; (3) $D_1 = 0.80\ h^{-1}$, $D_2 = 0.46\ h^{-1}$; (4) $D_1 = 0.60\ h^{-1}$, $D_2 = 0.80\ h^{-1}$.

A two-stage fermentation with cell recycle (TSFR) at steady state of (5) $D_1 = 0.80\ h^{-1}$, $D_2 = 0.30\ h^{-1}$, R = 0.10.

* Concentrations at steady state of the 2nd-fermenter in a two-stage fermentation.

** Overall yields, specific rates and volumetric lactic acid productivity ($Q_{P,Total}$), when $D_{Total} = F/(V_1+V_2)$, but those for TSFR were average values taken from the 1st- and 2nd fermenters.

Acknowledgement

We acknowledge the previous financial support of Japan NEDO-RITE International Joint Research grant in FY 2000/2001, as well as the present KURDI research grant (05012344) in FY 2004. Moreover, the authors would like to thank Ms. Kasorn Kittikusolthum and Mr. Meechai Luddee for their experimental works due to master course.

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