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https://doi.org/10.5109/24339

出版情報：九州大学大学院農学研究院紀要. 44 (3/4), pp.367-375, 2000-02. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **44** (3·4), 367–375 (2000)

pH–Dependent Continuous Lactic Acid Fermentation by *Lactococcus lactis* IO–1 Using Hydrolyzed Sago Starch.

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(Received October 28, 1999 and accepted November 5, 1999)

High dilution rate and high cell density continuous lactic acid production by *Lactococcus lactis* IO–1 in hydrolyzed sago starch medium was investigated using a pH–dependent feed system coupled with cross flow filtration and turbidity control. Lactate production and productivity were dependent on cell density, dilution rate and feed glucose concentration. At a cell concentration of 15 g/*l* and a feed glucose concentration of 50g/*l*, volumetric lactate productivities of 14.9, 23.5 and 33.0 $g \cdot l^{-1} \cdot h^{-1}$ were obtained at dilution rates of 0.35, 0.5 and 1.1 h^{-1} respectively. The respective residual glucose concentrations in the permeate were 0.8, 1.0 and 3.7 g/*l*. On increasing the feed glucose concentration to 64 g/*l*, volumetric lactate productivities of 12.4 and 32.3 $g \cdot l^{-1} \cdot h^{-1}$ at dilution rates of 0.21 and 0.70 h^{-1} were obtained while the respective residual glucose concentrations in permeate were 2.7 and 12.2 g/*l*.

INTRODUCTION

Over the last decade, lactic acid production has gained considerable attention because, besides its conventional applications in the food and pharmaceutical industries (Yin *et al*, 1987; Marshall, 1987; Atkinson and Mavituna, 1991) it is a raw material for the synthesis of polylactic acid (PLA), an essential biodegradable plastic material, oxygenated chemicals, green chemical/solvents, and plant/growth regulators (Datta *et al*, 1995). However, the high cost of lactic acid production, either petrochemically or fermentatively, has been the bottleneck for practical application. Presently, fermentation accounts for half of the world lactic acid production (Vick Roy, 1985). One advantage of this bio-process is that carbon dioxide is not released, making this process environmentally friendly (Ishizaki, 1997). Therefore, there has been a lot of interest in the development and improvement of the kinetics of fermentative lactic acid production.

To this end, it has been attempted to replace batch fermentation with continuous processes to reduce end–product inhibition and improve lactate productivity. Further improvements in productivity have been achieved using higher cell densities, employing cell immobilization (Senthuran *et al*, 1997; Mehaia and Cheryan, 1987a; Zayed and Winter, 1995), and by using cell–recycling modules (Ohleyer *et al*, 1985a; Bibal *et al*, 1991; Xavier, *et al*, 1995), different fermentation feedstocks and different lactate–producing strains (Linko and Janavainen, 1996; Chatterjee *et al*, 1997; Hofvendahl and Hahn–hägerdal, 1997, Moldes *et al*, 1999). For example, productivities between 56 and 88 $g \cdot l^{-1} \cdot h^{-1}$ have been reported in lactose–based media using different *Lactococci* and *Lactobacilli* (Ohleyer, *et al*, 1985b; Mehaia and Cheryan, 1987b). However they were

obtained with low specific lactate productivities. Additionally, some fermentation systems have incorporated lactate recovery units such as electrodialysis (Boniardi *et al*, 1997; Vonktaveesuk *et al*, 1994), extractive fermentation (Ye and Shimizu, 1996) and ion exchange resins (Evangelista *et al*, 1994) to increase both lactate production and productivity. Nevertheless, an efficient modern process for lactic acid fermentation that produces organic acid for industrial purposes has not yet been established.

Like any industrial organic acid or solvent fermentation, the basic economic factors remain the choice, and cost, of the substrate and the cost of recovery (or downstream processing). Substrates/feedstocks that would reduce the processing cost would greatly enhance the competitiveness of fermentative lactate production. Sago starch obtained from the processing of the sago palm is cheap and its possible harvest have been reported to be 25 t/h year (Ishizaki, 1997). Work in our laboratory has established a method for the efficient enzymatic hydrolysis of sago starch to glucose. In this study, we report on the use of hydrolyzed sago starch to produce lactic acid in batch and continuous cultures. We report on the improvements made in the productivity of lactic acid fermentation employing pH-dependent substrate feeding combined with cross flow filtration and turbidity control of the cell concentration using hydrolyzed sago starch as the substrate.

MATERIAL AND METHODS

Microorganism and growth medium The microorganism was *Lactococcus lactis* IO-1 (Ishizaki *et al*, 1990). Stock cultures were maintained at 4 °C on thioglycolate medium (Difco Laboratories, Detroit, MI). The growth medium, per liter of deionized water, consisted of: glucose, 10 g; polypeptone, 5 g; yeast extract (Difco Laboratories, Detroit, MI), 5 g; and NaCl, 5 g.

Media Sago starch was obtained through the University of Sarawak, Malaysia (UNIMAS), and stored at room temperature until use. The hydrolysis procedure has been described in the section on sago starch hydrolysis. Batch culture experiments were conducted using both glucose obtained from the sago starch and technical grade glucose as carbon source, by increasing the glucose concentration of the growth medium until 75 g/*l*. Continuous fermentation experiments were carried out using sago glucose only. An initial glucose concentration of 30 g/*l* was used and the respective concentrations of polypeptone and yeast extract doubled to 10 g/*l* each in order to increase cell density. The glucose concentration of the feed medium was varied between 50 and 64 g/*l*.

Sago starch hydrolysis Two enzymes, Kleistase T5, a thermostable amylase from *Bacillus subtilis*, purchased from Daiwa Kasei Co., Ltd. (Osaka) and glucozyme, from *Rhizopus delemar*, purchased from Amano Pharmaceutical Co., Ltd. (Nagoya) were used. Their respective enzymatic activities as defined by the manufacturers were 5,000 AU/g and 4,200 AU/g. Starch hydrolysis was carried out in accordance with a method described previously (Tripetchkul *et al*, 1992) as follows. 30 g of starch was suspended in 100 cm^3 of tap water. pH was adjusted to 6.5 by adding 1M NaOH. Liquefaction was carried out by adding 0.2% (v/v) of Kleistase T5 to the slurry at pH 6.0; incubating at 95 °C for 2 h, heating to 130 °C for 10 min, and then adding 0.1% of Kleistase

T5 to the slurry at pH 6.5 and incubating for 1 h at 95 °C. Saccharification was carried out by adding 8 units of glucozyme per gram of starch at pH 5.5 and incubating at 50 °C for 24 h.

Cell recycling In this study, two hollow fiber cartridges (MICROZA PSP–103 from Asahi Kasei Co., Ltd. and H1MP01–43 from Amicon, Inc. Beverly, MA) were used to recycle cells back to the fermentor.

Turbidostat Turbidity control consisted of a laser probe (LA–300 LT, Automatic System Research Co. Ltd., Tokyo), installed in the fermentor, for online measurements of turbidity (Yamane, *et al* 1992). It was preset to control the cell density at specific concentrations as determined by a previous calibration between the cell concentration and culture turbidity.

Culture systems All fermentations, both batch and continuous, were carried out in a 1-*l* jar fermentor with a working volume of 400 ml, maintained at 37 °C and agitated at 400 rpm without gas flow. The culture pH was controlled at 6.0 by addition of 10 M NaOH throughout the experiments. The batch fermentation setup consisted of a simple pH–controlled system. The setup of the pH–dependent continuous fermentation experiment has been shown in Fig. 1. The system was initially operated in batch mode and switched to continuous mode when the residual glucose concentration was about 2 g/*l*, during which biomass recycling and pH–dependent feeding commenced simultaneously (Ishizaki and Vonktaveesuk, 1996). pH–dependent fresh medium feeding was achieved through separate inflows for alkali and fresh medium controlled by changes

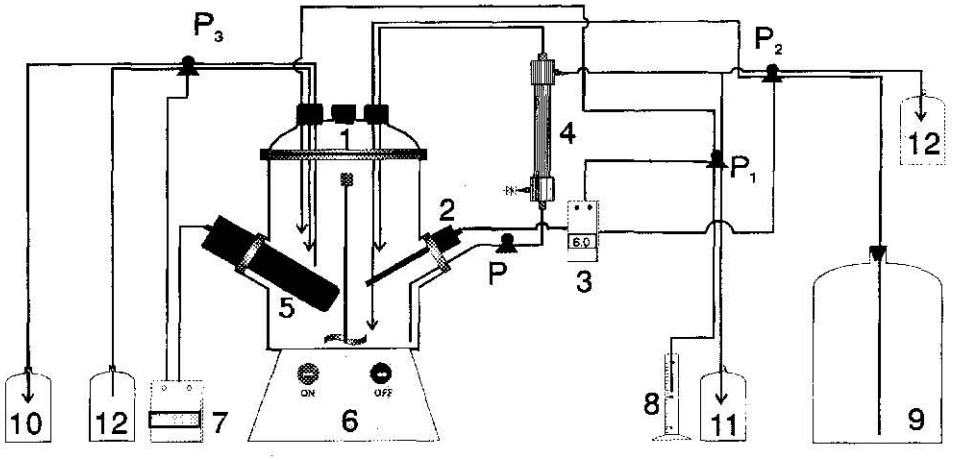

Fig. 1. Schematic Diagram of pH–dependent feed control with turbidity control system.
key: 1. Fermentor; 2. pH electrode; 3. pH Controller; 4. Hollow fiber cartridge; 5. Turbidity probe; 6. Magnetic stirrer; 7. Turbidity controller; 8. NaOH tank; 9. Fresh medium reservoir; 10, 11. Feed–out tank; 12. Glucose free solution reservoir; and P, P_1, P_2, P_3: Pump.

in the pH of the culture broth (Tripetchkul, 1992). This was achieved via a control loop based on the pH of the culture broth and the preset upper and lower pH points. When the microorganism metabolized glucose and produced lactic acid, the pH in the culture fell below 6.0 and alkali was automatically pumped into the fermentor to neutralize the acid to maintain the preset point. As long as glucose was present, the pH drop and alkali feed was repeated until all remaining glucose was spent. When substrate is finally exhausted (glucose concentration below critical level), the pH of the culture is expected to rise. Substrate is fed at the upper preset point. At the preset cell density, the turbidity control system was started, by a feedback loop that consisted of the turbidity controller and the dilution system, by feeding of glucose-free solution. Each inflow route was balanced by an outflow rate as shown in the Fig. 1. Therefore, the dilution rate (D) for this system was calculated based on the equation below:

$$D=(F_1+F_2+F_3)/V \qquad \text{Eq. 1}$$

where F_1 is the inflow rate of alkali into the fermentor (balanced by the outflow of cell-free effluent from the module by the pump P_1), F_2 is the inflow rate of substrate into the fermentor (balanced by the outflow of cell-free effluent from the pump P_2), F_3 is the inflow rate of glucose-free solution into the fermentor for the dilution (balanced by the outflow of culture broth by the pump P_3), and V is the total volume of the culture broth.

Analytical methods Cell density was measured in terms of optical absorbance, at 562 nm, in a spectrophotometer (UVIDEC-320; Japan Spectroscopic Co., Ltd., Tokyo.). Cell growth was determined using a pre-determined standard curve. Glucose and lactate concentrations were determined by HPLC (LC-10AD, RID-6A Refractive Index Detector, Shimadzu, Japan) using an Aminex HPX-87H column (Biorad, USA) at 50 °C and 0.400 ml/min flow rate of 5 mM sulfuric acid as a mobile phase.

RESULTS AND DISCUSSION

Batch culture in glucose from sago starch

In order to exploit sago starch as a feedstock for lactic acid production, we compared the batch culture kinetics of lactic acid production between glucose obtained from sago starch hydrolysis and technical grade glucose. The profiles of cell growth, glucose consumption and lactic acid production observed were similar. As shown in figure 2, the specific growth rate, glucose consumption rate and lactate productivity in the two media were almost identical. It was concluded that glucose obtained from sago starch hydrolysis could be used for fermentative lactic acid production. Following this work, we observed the kinetics of lactate production in 75 g/*l* glucose (Fig. 3). Ohleyer *et al* (1985b) demonstrated that using 35 g/*l* as the initial glucose concentration, 35 g/*l* of lactic acid would be produced. This means that 100% of the glucose is converted into lactic acid. When a concentration of 85 g/*l* was used, however, only a 70% conversion value was obtained. Additionally they found that using a glucose concentration of 60 g/*l*, 100% conversion was again displayed. We obtained similar results when 30 and 75 g/*l* of glucose were used. In general, lactate production was accompanied by a rapid drop in the pH of the culture and alkali was added to neutralize this effect. As shown in Fig. 3, the

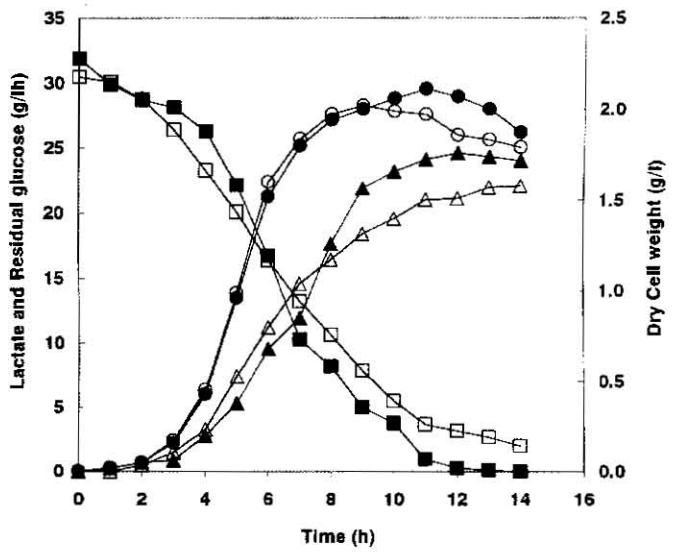

Fig. 2. Time course lactic acid fermentation of *Lactococcus lactis* IO-1 using commercial glucose (close symbols) and glucose from sago starch (open symbols).
Symbols: ■, Residual glucose; ▲, Lactate concentration in broth, ●, Dry Cell Weigh; □, Residual glucose; △, Lactate concentration in broth; ○, Dry Cell Weight

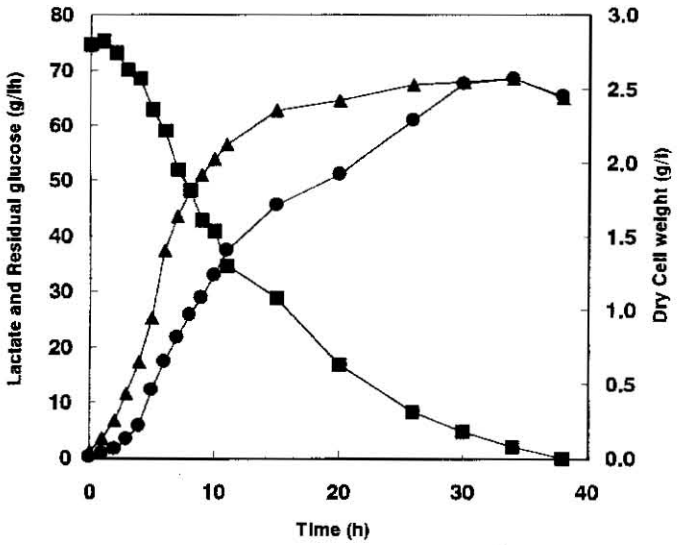

Fig. 3. Batch culture: lactic acid fermentation using an initial glucose concentration of 75 g/l.
Symbols: ■, Residual glucose; ▲, Lactate concentration in broth; ●, Dry Cell Weight.

maximum lactic acid concentration after 34 h was 68 g/l, with residual glucose levels at 2.0 g/l, and at 38 h, glucose had been consumed completely. It was also observed that a lactate concentration between 50–60 g/l resulted in the inhibition of cell growth. From these results we assumed that in continuous culture, using an initial glucose concentration in the range of 50–65 g/l, all the glucose could be converted to lactic acid.

Continuous culture

Effect of cell density and dilution rate

In continuous lactic acid fermentation, the two principal variables influencing volumetric productivity are the dilution rate and the cell concentration. A high cell concentration obtained through cell recycling enhances lactic acid production. Therefore, the complimentary effects of high cell density and high dilution rate on lactate productivity were observed by varying the dilution rate between 0.21 and 1.1 h^{-1}. Table 1 shows the variation of individual kinetic parameters at different cell concentrations and dilution rates. Generally, cell concentration and dilution rate were essential parameters in obtaining high productivities. Volumetric lactate productivity progressively increased from 8.2 $g \cdot l^{-1} \cdot h^{-1}$ at a dilution rate of 0.21 h^{-1} to 33.1 $g \cdot l^{-1} \cdot h^{-1}$ at a dilution rate of 1.1 h^{-1}. However, lactate concentration remained between 37 and 39 g/l until the dilution rate was increased to 1.1 h^{-1}, when it decreased to 30 g/l, while residual glucose concentration was low. Specific productivities in the range 0.31–0.2 $g \cdot g^{-1} \cdot h^{-1}$ have been reported despite the high cell concentration employed (100–178 g/l) (Kamoshita *et al*, 1998). They could not obtain a lactic acid concentration of more than 50 g/l using a stirred ceramic membrane reactor, due to problems encountered in membrane performance when operated at high cell density. From our results, it is clear that by increasing the cell concentration, it is possible to enhance the volumetric productivity and consequently the specific productivity. Using a cell concentration of 15 g/l, we obtained higher specific productivities to those obtained at higher cell concentrations (Bibal, *et al.* 1991, Meihaia and Cheryan, 1987a, Kamoshita, *et al* 1998). This was probably due to our system which operated with high cell viability. We stated that the turbidity control system, which operated at low dilution rates, was responsible for the permission of high cell viability (data not shown).

Effect of maintaining high cell density on lactic acid production and productivity

The effect of using high cell density (15 g/l) while maintaining the feed glucose concentration at 53 g/l has been shown in Table 2. The cell density was maintained at

Table 1. Kinetic parameters in run fermentations using 50 g/l of glucose from Sago starch at different dilution rates and cell concentrations.

Parameter	Dilution rate (h^{-1})				
	0.21	0.44	0.5	0.75	1.1
Dry cell weight (g/l)	4.6	5.0	6.0	10.0	15.3
Residual glucose concentration (g/l)	1.9	0.6	0.24	5.6	3.8
Lactate concentration (g/l)	39.2	36.8	38.6	36.8	30.1
Volumetric lactate productivity ($g \cdot l^{-1} \cdot h^{-1}$)	8.2	14.7	19.3	27.6	33.1
Specific lactate productivity ($g \cdot g^{-1} \cdot h^{-1}$)	1.8	2.6	3.1	2.7	2.2

Table 2. Kinetic parameters in run fermentations using glucose from sago starch at different concentrations and different dilution rates.

Parameter	Dilution rate (h^{-1})			
	0.35	0.5	0.21	0.7
Initial glucose concentration (g/*l*)	53.0	53.0	64.0	64.0
Dry cell weight (g/*l*)	15.0	15.0	15.0	15.0
Residual glucose concentration (g/*l*)	0.8	5.1	2.7	12.2
Lactate concentration (g/*l*)	42.6	38.3	59.1	46.2
Volumetric lactate productivity ($g \cdot l^{-1} \cdot h^{-1}$)	14.9	19.2	12.4	32.3
Specific lactate productivity ($g \cdot g^{-1} \cdot h^{-1}$)	1.0	1.1	0.82	2.1

15 g/*l* to minimize the fouling of the membrane, encountered in these processes. At dilution rates of 0.35 and 0.5 h^{-1} lactate concentration increased to 42.6 and 38.3 g/*l*, which corresponded to volumetric productivities of 14 and 19.1 $g \cdot l^{-1} \cdot h^{-1}$ respectively. At a dilution rate of 0.35 h^{-1}, lactate productivity was comparable to that shown at 0.44 h^{-1}, using a cell concentration of 5 g/*l* (Table 1), but the specific productivity and lactate concentration were higher. Taking into account the results obtained in batch culture with regards to glucose and lactic acid inhibition, we increased the initial glucose concentration to 64 g/*l*. As we have shown in Table 2, when dilution rate was 0.21 h^{-1}, a lactic acid concentration of 59.1 g/*l* was obtained and this corresponds to a volumetric productivity of 12.4 $g \cdot l^{-1} \cdot h^{-1}$. The higher lactic acid concentration obtained was mainly due to the fact that we used a low dilution rate. Therefore we reasoned that, at this dilution rate, *Lactococcus lactis* IO–1 used glucose mainly for producing lactic acid but not for growing while sustaining its lactate production rate and maintaining a high glucose conversion rate of 97%. When dilution rate was increased to 0.7 h^{-1}, a lactate concentration of 46.2 g/*l* was obtained, which corresponds to a productivity of 32.3 $g \cdot l^{-1} \cdot h^{-1}$. This productivity was similar to that obtained when dilution rate was 1.1 h^{-1} (table 1). From these results it is clear that initial glucose concentration, dilution rate and cell concentration all play an important role in enhancing the lactate productivity. Ohleyer *et al* (1985b) reported a volumetric productivity of 151 $g \cdot l^{-1} \cdot h^{-1}$ using *Lactobacillus delbrueckii*, lactose as the substrate, a high dilution rate of 2.55 h^{-1} and a cell concentration of 118 g/*l*. However, the fermentation was sustained for only 18 hours. This is disadvantageous from the point of view of an industrial-scale operation. The specific lactate productivity (1.28 $g \cdot g^{-1} \cdot h^{-1}$) obtained by Ohleyer *et al*, was lower comparing it with our results (Table 1). Both product concentration and productivity are essential parameters that determine the competitiveness of the production process. We are therefore focusing on improving the cell concentration so that fermentation could be operated at high dilution rates.

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