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◇◇◇ Original Paper ◇◇◇

Preferential Substrate Utilization by *Propionibacterium shermanii* in Kitchen Refuse Medium

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To utilize kitchen refuse as a substrate for polylactic acid (PLA), it is essential to eliminate the L-and D-lactate initially contaminated, while preserving the glucose in it. Both the specific growth rate (μ) and the substrate consumption rate (r_S) of *Propionibacterium shermanii* on kitchen refuse medium (KRM) were compared at each pH 5.0, 5.5, 6.0 and 6.5 with artificial kitchen refuse medium (AKRM), lactate medium (LM) and lactate-glucose medium (LGM) which respectively contains glucose, lactate, and a mixture of these substrates. Lactate initially contaminated in KRM was assimilated prior to glucose by *P. shermanii* at each pH. In KRM and LGM, enhancement and reduction of cell growth by lactate were observed at pH 6.5 and 5.0, respectively, when compared with that in AKRM. As a result, a glucose consumption rate in KRM was more than twice, but was significantly lower than that in AKRM at pH 6.5 and 5.0, respectively. Glucose could be preserved by a low glucose consumption rate when pH was changed from 6.5 to 5.0 after lactate exhaustion in KRM. Preferential substrate utilization of *P. shermanii* and a pH change from 6.5 to 5.0 can increase the optical purity of lactic acid while preserving glucose.

Key words: kitchen refuse, *P. shermanii*, lactate, glucose, pH

1. Introduction

Kitchen refuse contains rich nutrients such as carbohydrates, nitrogen sources, vitamins and inorganic materials and does not include toxic compounds for microbial growth since it comes primarily from cooking waste and the remains of meals. To date, it has been used only partially as a stimulant and substrate for microbial fermentation [1, 2]. A recycling system using kitchen refuse as a renewable substrate for the production of biodegradable polylactic acid (PLA) has been proposed by Shirai [3]; kitchen refuse is collected, hydrolyzed by glucoamylase and fermented for lactic acid production. Through a series of processes for chemical purification, pure lactic acid is produced. Finally, PLA can be produced by polymerization of the lactic acid.

Under this system, contamination of L- and D-lactate by predominant lactic acid bacteria (LAB) existing in the natural environment [4] is unavoidable during the collection, transportation and storage of kitchen refuse. The initial L- and D-lactate causes deterioration of the optical purity of lactic acid even when lactic acid fermentation of kitchen refuse is performed by *Lactobacillus rhamnosus*, which is a representative microorganism for the production of L-lactate form. It is well known that the ratio of L- and D-lactate dramatically affects the characteristics of PLA such as its crystallinity, and glass transition and melting temperatures [5]. Prior to lactic acid fermentation, it is essential to completely remove lactate isomers initially contaminated in kitchen refuse, meanwhile preserving the glucose as the main substrate for lactic acid fermentation of the refuse.

It has been recognized that *Propionibacterium shermanii* assimilates lactate more preferentially than glucose in a mixed medium containing both substrates [6].

The aim of the present work was to investigate whether lactate initially contaminated in kitchen refuse is assimilated by *P. shermanii* as a preferential substrate to the glucose which is the main substrate for lactic acid fermentation at pH 5.0, 5.5, 6.0 and 6.5. The specific growth rate (μ) and substrate consumption rate (r_S) of *P. shermanii* in kitchen refuse medium (KRM) were compared with those of various other media under the above pH conditions. A pH change after initial lactate depletion was also conducted to examine whether such a measure can preserve glucose in KRM.

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2. Materials and Methods

2.1 Microorganisms

Propionibacterium freudenreichii subsp. *shermanii* (NBRC12426), a propionic acid bacterium, was obtained from the NITE Biological Resource Center (National Institute of Technology and Evaluation, Chiba, Japan). Stock culture was stored in growth medium with 25% glycerol at −35°C. Two successive subcultures of the stock culture (24 h and 30 h with 10% (v/v) inoculation at 35°C under shaking conditions (100 rpm)) were conducted immediately prior to all experiments.

2.2 Medium

The growth medium for the subcultures of *P. shermanii* contained the following components per liter: 5.0 g (+) glucose, 5.0 g yeast extract, 5.0 g polypeptone, and 1.0 g $MgSO_4 \cdot 7H_2O$.

The kitchen refuse used in the present experiments was collected from the Yahata Royal Hotel (Kitakyushu, Fukuoka, Japan) during the summer. It was divided into three categories: carbohydrates (43% (w/w wet kitchen refuse)), protein (19%), and fruits and vegetables (38%), following Sakai et al. [7], who studies refuse generated from various kinds of commercial kitchens. The 43% consisting of carbohydrates primarily contained cooked rice, rice cakes and a small amount of bread; the 19% designated as protein included fish, beef and fried shrimp; and the remaining 38% identified as fruits and vegetables approximately consisted of cabbage, onion, radishes, carrots, watermelon peel and oranges. 20 kg of wet kitchen refuse was mixed with 10 kg of water (50% (w/w)) and then hydrolyzed by glucoamylase (300 mg/kg wet kitchen refuse) in a water bath at 50°C and at 50 rpm for 6 h using a drum-shaped reactor. After hydrolysis, kitchen refuse broth was sieved by wire mesh (5 × 5 mm) and two consecutive centrifugations (GS-6 Centrifuge, Beckman, USA) were conducted, first at 3,500 rpm for 10 min and then at 4,000 rpm for 5 min. The supernatant was filtered by a 3-μm filter to remove suspended solids and oils in the kitchen refuse broth. After autoclaving at 121°C for 15 min (Labo Autoclave, Sanyo Electric Co., Osaka, Japan), centrifugation was again performed at 4,000 rpm for 5 min to remove the solids which arose during sterilization. The clear supernatant was used as KRM for the fermentations.

Artificial kitchen refuse medium (AKRM) not including initial lactate was prepared for comparison. The same items as those found in KRM were purchased from a local dairy market. The pretreatment of AKRM was essentially identical to that of KRM except that two centrifugations and filtration were conducted at 2°C in order to prevent contamination of lactate. No lactic, propionic or acetic acid was detected in the AKRM. AKRM was diluted (1 : 1.1) with distillated water to adjust it to the same glucose concentration as that of KRM. Lactate medium (LM) and lactate-glucose medium (LGM) were also prepared for comparison with KRM. The composition of the various media used in the present study is shown in Table 1.

Table 1 Composition of the various media used in the present study.

Medium code	Ingredients				
	Glucose (90.0 gL^{-1})	Lactate (9.0 gL^{-1})	Yeast extract (5.0 gL^{-1})	Polypeptone (5.0 gL^{-1})	$MgSO_4 \cdot 7H_2O$ (1.0 gL^{-1})
LM	−	+	+	+	+
AKRM	+	−	−	−	−
LGM	+	+	+	+	+
KRM	+	+	−	−	−

2.3 Fermentation

Figure 1 is a schematic diagram of the bioreactor equipped with a 350-mL glass facultative anaerobic fermentor. All fermentations were carried out at 30°C and 150 rpm agitation by hung magnetic bar under controlled pH conditions. The pH was automatically maintained at a constant value (each pH ± 0.03 unit) by 6N NaOH or 2N HCl using micro-tube pumps (MP−3, Eyela, Tokyo Rikakikai, Japan). The pH probe (PH−6, Eyela) was sterilized separately and was transferred aseptically to the fermentor prior to each fermentation. The concentrated cell which was centrifuged with 90 mL of 30-hr-old culture at 3,500 rpm for 5 min to remove the effects of organic acids in inoculum was inoculated into each 300 mL of medium aseptically. At the appropriate time intervals, 1.5 mL of medium was taken from the sampling port. All fermentations were duplicated.

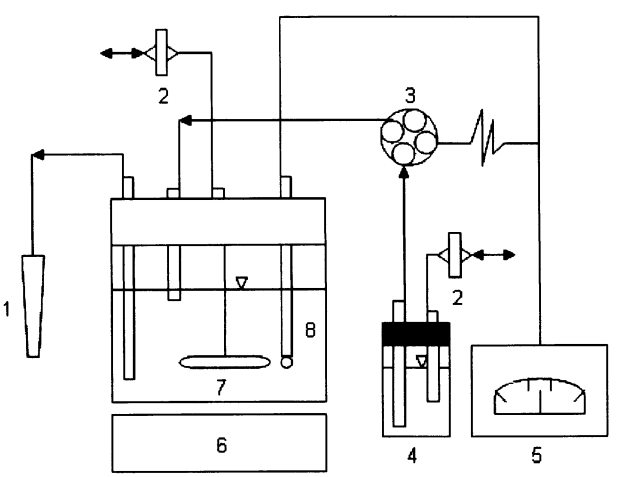

Fig. 1 A schematic diagram of the bioreactor. 1, sampling port; 2, air filters; 3, micro-tube pump; 4, NaOH or HCl bottle; 5, pH controller; 6, stirrer; 7, magnetic bar; 8, pH probe.

2.4 Analysis

Bacterial growth was monitored by measuring the optical density of fermented broth at 0.0~0.5 unit of OD_{550nm} using a spectrophotometer (UV mini 1240, Shimadzu Corporation, Tokyo, Japan). After checking the OD, the remaining volume of the sample was filtered by a 0.45-μm membrane filter and frozen for further analysis. Glucose concentration was determined by glucose oxidase-peroxidase enzyme (glucose test kit, Toyobo, Osaka, Japan). The concentrations of lactic, propionic and acetic acid in the samples were determined by a high-performance liquid chromatography (HPLC) system equipped with a Shimadzu CDD-6A detector. A shim-pack SCR-102H column (Shimadzu) was used with 5 mM *p*-toluenesulfonic acid aqueous solution as mobile phase at an elution speed of 0.8 $mLmin^{-1}$ and the column temperature was maintained at 40°C. A 20-μL cell-free sample was injected into the analysis column, and the buffer phase used was 5 mM *p*-toluenesulfonic acid, 10 μM EDTA and 20 mM Bis-Tris at a flow rate of 0.8 $mLmin^{-1}$.

3. Results

3.1 Propionic acid fermentation of LGM

Fig. 2 presents the profiles of substrate consumption, product accumulation and cell growth obtained from each

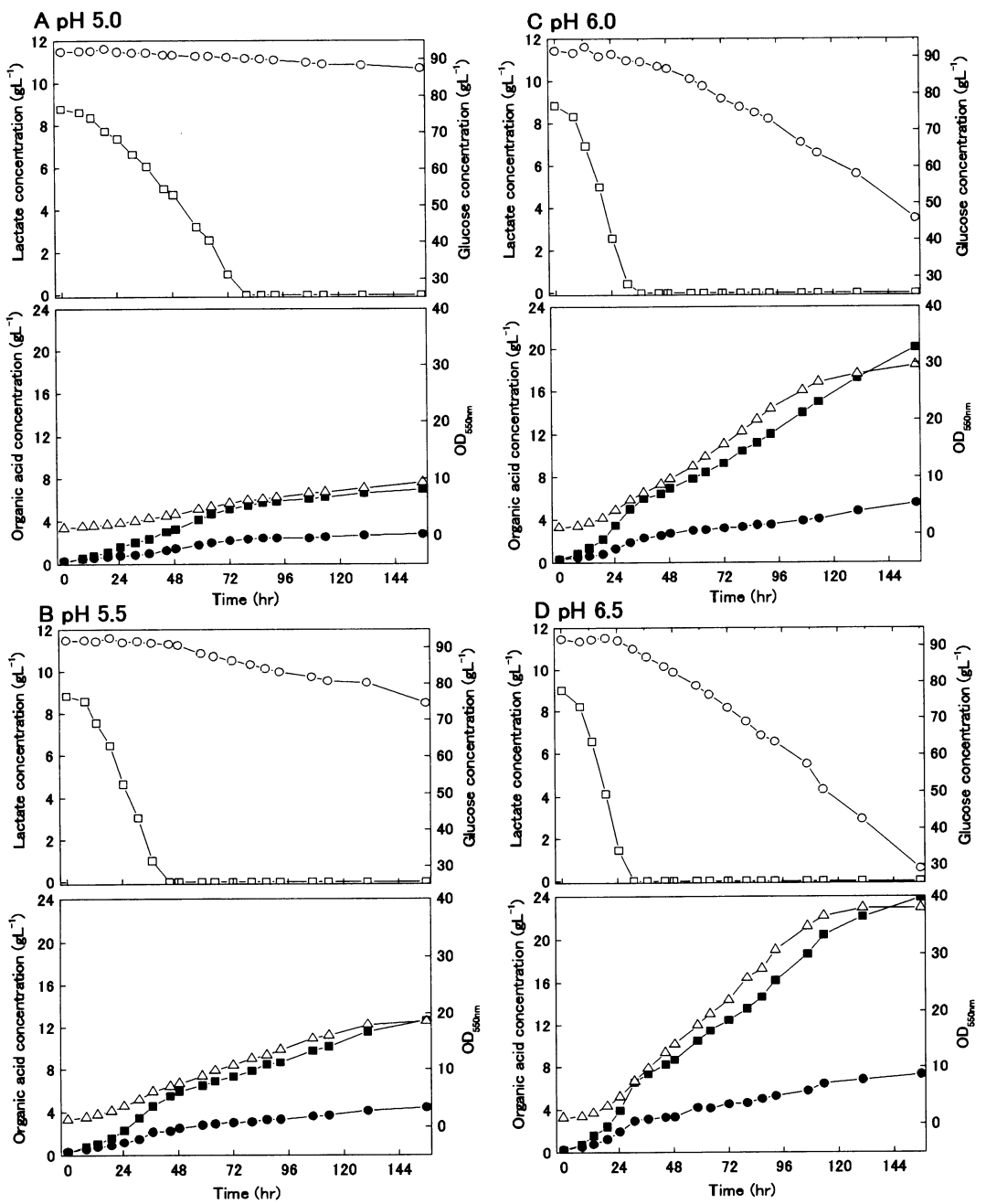

Fig. 2 Propionic acid fermentation of LGM at (A) pH 5.0, (B) pH 5.5, (C) pH 6.0, and (D) pH6.5. Concentrations of ○, glucose; □, lactate; ■, propionate; ●, acetate and △, OD_{550nm} are indicated.

fermentation of *P. shermanii* in LGM at pH levels of 5.0, 5.5, 6.0 and 6.5. The LGM includes amounts of lactate and glucose as a carbon source equivalent to those of KRM. An analogous inclination at each pH level was observed, however, at acidic pH 5.0, cell growth and substrate consumption were markedly slow. We found that *P. shermanii* preferentially assimilates lactate prior to glucose at each pH level. A small amount of glucose was assimilated at each pH level when lactate was completely consumed (upper graphs of Fig. 2). Propionic acid and acetic acid were the two main products of propionic acid fermentation. A significant change in product concentration was observed during sequential substrate utilization at each pH level. Until the lactate was reduced to extremely low levels, specifically at 78 h, 46 h, 37 h and 30 h at pH 5.0, 5.5, 6.0 and 6.5, respectively, product concentration, especially that of propionic acid, increased rapidly. After lactate consumption was completed at each pH, new increasing rates in propionate and acetate concentration were observed with glucose consumption however, the increasing rates in product concentration at that point were lower than those observed during lactate consumption.

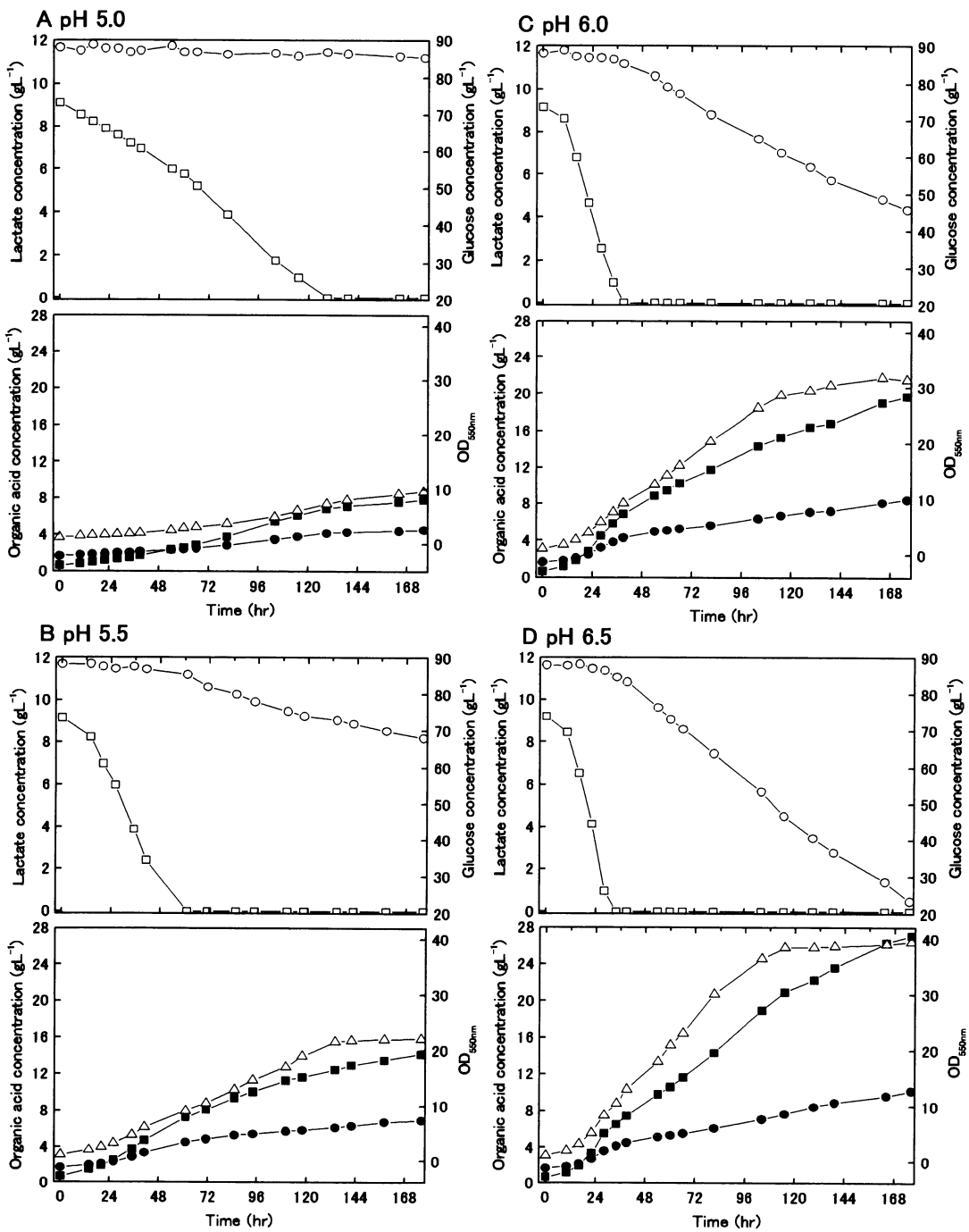

Fig. 3 Propionic acid fermentation of KRM at (A) pH 5.0, (B) pH 5.5, (C) pH 6.0, and (D) pH 6.5. Concentrations of ○, glucose; □, lactate; ■, propionate; ●, acetate and △, OD_{550nm} are indicated.

3.2 Propionic acid fermentation of KRM

Fig. 3 shows the outline of substrates, products and cells during each fermentation of KRM at various pH levels. The concentrations of lactate and glucose as a carbon source in KRM were approximately 9 gL^{-1} and 90 gL^{-1}, respectively. Additionally, trace amounts of acetic and propionic acid (1.7 and 0.6 gL^{-1}, respectively) initially contaminated were also observed. The pattern of fermentation of KRM was quite similar to that observed for LGM at each pH level. Lactate was consumed prior to glucose as a preferential substrate by *P. shermanii*. A small amount of glucose was found to be consumed during lactate utilization at each pH level (upper graphs of Fig. 3). Also, changes in product concentration similar to those observed in the case of LGM were observed after lactate exhaustion at 129 h, 58 h, 38 h and 32 h at pH 5.0, 5.5, 6.0 and 6.5, respectively.

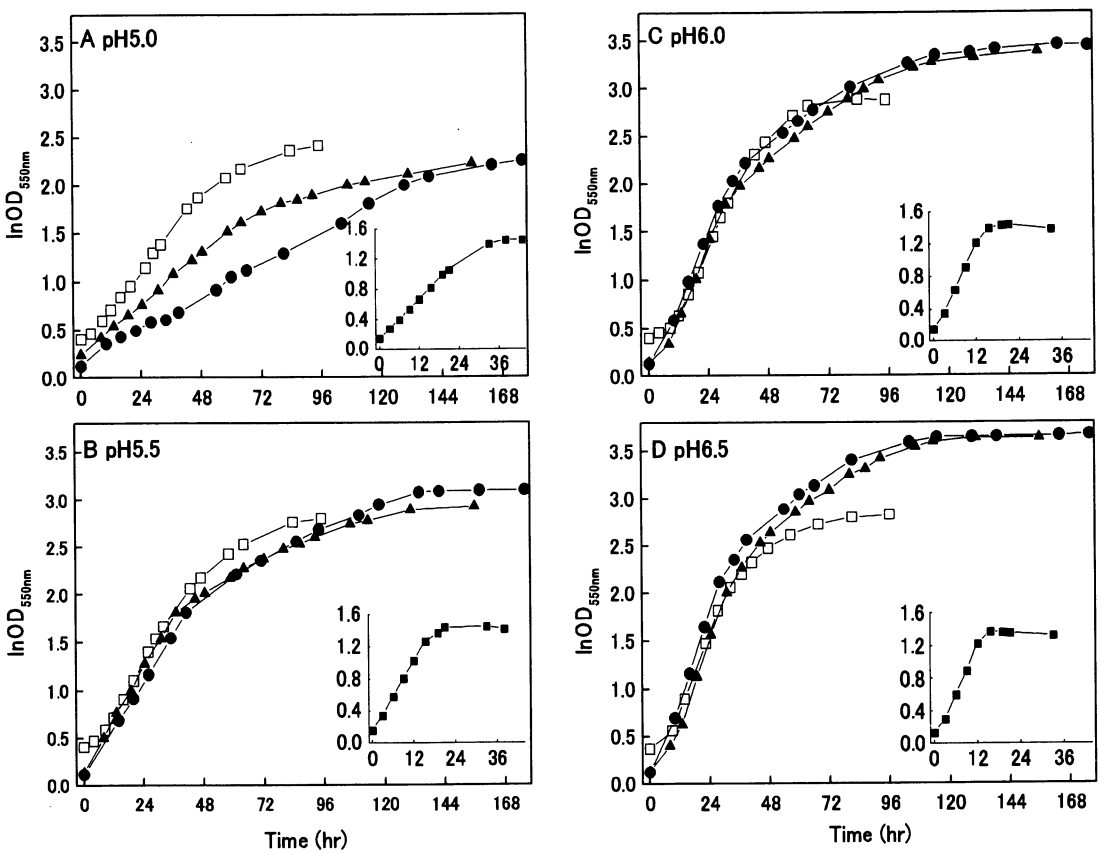

Fig. 4 The effect of pH on cell growth in various media at (A) pH 5.0, (B) pH 5.5, (C) pH 6.0, and (D) pH 6.5. ■, LM; □, AKRM; ●, KRM; ▲, LGM.

Table 2 The specific growth rate (μ) and the volumetric substrate consumption rate (r_S) for each medium at the tested pH levels.

Media code	Substrate	pH 5.0		pH 5.5		pH 6.0		pH 6.5	
		μ (h^{-1})	r_S ($gL^{-1}h^{-1}$)	μ (h^{-1})	r_S ($gL^{-1}h^{-1}$)	μ (h^{-1})	r_S ($gL^{-1}h^{-1}$)	μ (h^{-1})	r_S ($gL^{-1}h^{-1}$)
LM	Lactate	0.050	0.189	0.078	0.523	0.106	0.608	0.113	0.650
AKRM	Glucose	0.032	0.061	0.050	0.151	0.059	0.198	0.067	0.223
LGM	Lactate +	0.021[a]	0.115[a]	0.045[a]	0.260[a]	0.065[a]	0.348[a]	0.081[a]	0.411[a]
	Glucose	0.006[b]	0.037[b]	0.014[b]	0.160[b]	0.019[b]	0.319[b]	0.018[b]	0.456[b]
KRM	Lactate +	0.014[c]	0.074[c]	0.042[c]	0.216[c]	0.066[c]	0.339[c]	0.079[c]	0.416[c]
	Glucose	0.004[d]	0.029[d]	0.013[d]	0.178[d]	0.019[d]	0.330[d]	0.020[d]	0.466[d]

μ and r_S were calculated by liner regression equations derived from each log phase and from the amount consumed during each log phase, respectively. [a]The growth phase on lactate in LGM; [b]the growth phase on glucose in LGM; [c]the growth phase on lactate in KRM; and [d]the growth phase on glucose in KRM.

3.3 Effect of pH on the specific growth rate (μ) and the volumetric substrate consumption rate (r_S)

The cell growth of *P. shermanii* in LM, AKRM, LGM and KRM at each pH level is shown in Fig. 4. Table 2 shows the specific growth rate (μ) calculated from the linear part of the semilogarithmic plot of OD_{550nm} versus time from Fig. 4, and the volumetric consumption rate (r_S) calculated by linear regression equations determined from the amount consumed for each exponential phase of each experiment.

3.4 Preservation of glucose by pH control in KRM

An additional fermentation was conducted to examine the preservation of glucose after lactate consumption by decreasing pH from 6.5 to 5.0 in KRM. During lactate consumption, pH was maintained at 6.5 and it was then reduced to 5.0, as shown in Fig. 5. At this time, cell growth of *P. shermanii* almost ceased and the production of propionic acid and acetic acid also stopped. During lactate consumption, when pH was maintained at 6.5, the specific growth rate on lactate and the lactate consumption rate were 0.074 h^{-1} and 0.391 $gL^{-1}h^{-1}$, respectively, while at the period of pH 5.0, we obtained the dramatically low specific growth rate and the glucose consumption rate (0.002 h^{-1} and 0.021 $gL^{-1}h^{-1}$, respectively).

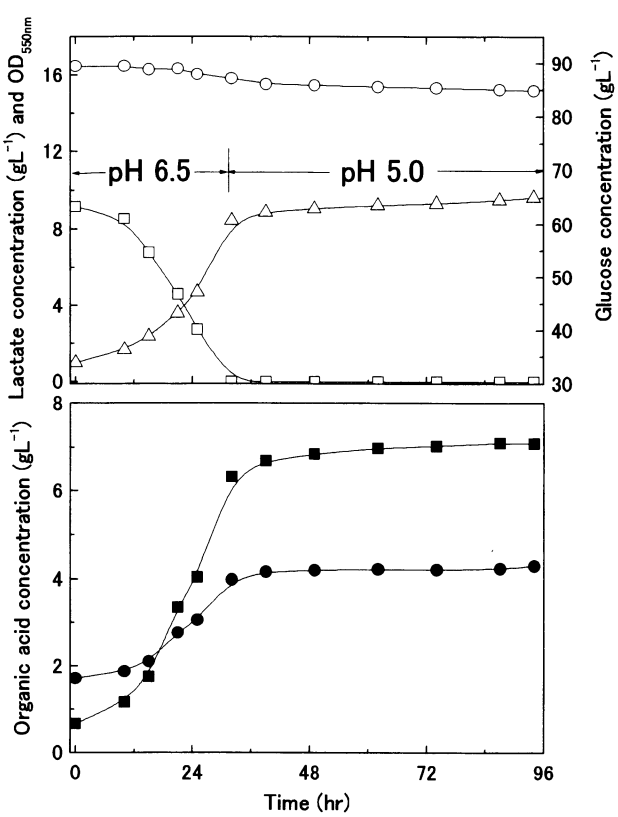

Fig. 5 Preservation of glucose in KRM by pH control. Concentrations of ○, glucose; □, lactate; ■, propionate; ●, acetate and △, OD_{550nm} are indicated.

4. Discussion

P. shermanii assimilates lactate in preference to glucose at all pH levels in KRM and LGM. There was no significant difference in the pattern of substrate utilization under the different pH conditions. A low level of glucose was consumed during the late period of lactate depletion, but not during the early or middle stages (Figs. 2 and 3). Similar results were also observed by Marcoux et al. [8], who found that lactate was utilized preferentially to lactose in their various types of supplemented whey media. Additionally, Lee et al. [9, 10] observed that *P. shermanii* preferentially uses lactate when presented with a medium containing both glucose and lactate. Piveteau et al. [11] also report that lactose was not utilized in whey containing lactate. The reason for this preferential assimilation of lactate by *P. shermanii* in medium containing both lactate and glucose remains unclear, however, it is possible that there might be a shorter metabolic pathway to pyruvate from lactate than from glucose.

The specific growth rates (μ) of AKRM at each pH were approximately 40% lower than those of LM (Table 2). Given the fact that the specific growth rates were very similar to those of *P. shermanii* for lactate and glucose [9, 12], the reduction of the μ in AKRM might result from inhibition by the high (90 gL^{-1}) glucose concentration [13].

During sequential substrate utilization of lactate and glucose in KRM and LGM, the growth of *P. shermanii* was divided into two separate phases according to each carbon source. The dividing point of these growth phases corresponded almost exactly with the time of lactate exhaustion and the change in product concentration at each pH level, as mentioned above (Figs. 2 and 3). As shown in Fig. 4, after lactate exhaustion at each pH level, a new growth phase of *P. shermanii* on glucose was observed before it reached a stationary phase. The growth phase of *P. shermanii* on glucose was dramatically lower than that on lactate at each pH level. Changes in product concentration and two specific growth rates from mixed substrates of lactate and glucose at pH 5.8 were also observed by Liu and Moon [12]. These phenomena were not observed in the single substrate media of AKRM and LM.

The effect of pH on both cell growth and the glucose consumption rate of *P. shermanii* in LGM and KRM was greater than that in AKRM. At pH 6.0 and 6.5, dramatically higher cell growth was obtained in LGM and KRM at the stationary phase than that in AKRM (Figs. 4 (C) and (D)). Enhanced cell growth of *P. shermanii* by supplemented lactate has also been observed by Marcoux et al. [8], who

found that an increase in the fermented ammoniated condensed milk permeate (FACMP) / whey ratio promotes the growth of *P. shermanii*. As a result, we found that glucose consumption rates (r_S) in LGM and KRM (0.456 $gL^{-1}h^{-1}$ and 0.466 $gL^{-1}h^{-1}$, respectively) were found to be greater than twice that in AKRM (0.223 $gL^{-1}h^{-1}$) at pH 6.5. Nevertheless, at pH 5.0, cell growth in LGM and KRM was considerably slower than that in AKRM (Fig. 4 (A)), with the result that the glucose consumption rates in LGM and KRM (0.037 $gL^{-1}h^{-1}$ and 0.029 $gL^{-1}h^{-1}$, respectively) were significantly lower than that in AKRM (0.061 $gL^{-1}h^{-1}$). Based on these comparisons between KRM or LGM and AKRM, we assume that the lactate in KRM and LGM acts as a cell growth stimulant on *P. shermanii* at pH 6.5, but as an inhibitor at pH 5.0.

The parameters of KRM were very similar to those of LGM at pH 5.5, 6.0 and 6.5, while at pH 5.0, the specific growth rate on lactate (0.014 h^{-1}), the lactate consumption rate (0.074 $gL^{-1}h^{-1}$), the specific growth rate on glucose (0.004 h^{-1}), and the glucose consumption rate (0.029 $gL^{-1}h^{-1}$) were all lower in KRM than in LGM (Table 2). The reason for this is not obvious, however, it may be that the growth of *P. shermanii* at pH 5.0 is further inhibited by acetate and propionate initially contaminated in KRM, as mentioned earlier.

During the present study, pH 5.0 was recognized as the optimal pH level from the viewpoint of glucose preservation in KRM. However, the fermentation time for the removal of lactate initially contaminated in KRM is almost 4 times as long as that at pH 6.5 (Figs. 3 (A) and (D)). Nevertheless, this drawback can be overcome by a pH change after lactate consumption (Fig. 5). Each value of the parameters obtained from each period of fermentation at pH 6.5 and 5.0 was found to be almost equivalent to those observed at constant pH conditions of 6.5 and 5.0 in KRM (Table 2).

In conclusion, KRM showed good potential as a substrate for the growth of *P. shermanii* in terms of both μ and r_S when compared with LGM containing rich nutrients such as yeast extract and polypeptone. The lactate initially contaminated in KRM was assimilated before glucose by *P. shermanii* under all pH conditions. During assimilation of sequential substrates in KRM, two specific growth rates and a change in product concentration were observed according to each carbon source. Lactate initially contaminated in KRM functioned as a stimulant at pH 6.5, but as an inhibiter at pH 5.0 on the cell growth of *P. shermanii*. Overall, it can be stated that the removal of lactate initially contaminated can be stimulated at pH 6.5 by enhanced cell growth and that glucose can be preserved at pH 5.0 by means of a radically low glucose consumption rate in KRM. Therefore, our work suggests that preferential substrate utilization of *P. shermanii* and a change in pH level from 6.5 to 5.0 is a promising method of increasing the optical purity of lactic acid while preserving glucose for subsequent lactic acid fermentation.

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◇◇◇ 和文要約 ◇◇◇

プロピオン酸菌 *P. shermanii* による生ゴミ培地中の優先的基質資化

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高品質なポリ乳酸の製造には，高い乳酸の光学純度が要求される．生ゴミの貯蔵・運搬の際に，L (+)/D (−)乳酸が蓄積される．生ゴミを培地として，乳酸発酵によりポリ乳酸の製造を目指す場合，高い光学純度を達成するためには，すでに蓄積したL (+)/D (−)乳酸のみを除去すると同時に，引き続く乳酸発酵の基質となるグルコースの消費を抑える必要がある．

プロピオン酸菌 *P. shermanii* は，グルコースと乳酸を基質として両方含む場合，優先的に乳酸を資化することが報告されている．そこで，プロピオン酸菌 *P. shermanii* のグルコースに対する乳酸の優先的資化性を，生ゴミ培地中の初期混入乳酸の選択的除去へと応用した．本研究では，生ゴミ培地および合成培地を用いて，各種pHにおける培養工学的パラメータを算出し，*P. shermanii* の示す基質資化性について詳細に検討した．

実際に排出された生ゴミを調製して，基質としてグルコースと乳酸を含む生ゴミ（KRM）培地，生ゴミ培地と同じ組成の食品を調製することにより，基質としてグルコースのみ含む模擬生ゴミ培地（AKRM），プロピオン酸菌の増殖培地において，基質として乳酸のみ含む乳酸増殖培地（LM），乳酸とグルコースの両方を含む乳酸グルコース増殖培地（LGM）の4種類の培地を調製し，pH 5.0，5.5，6.0，6.5の条件下で培養を行った．

KRM培地とLGM培地において，いずれのpH条件下でも，グルコースより優先的に乳酸が資化された．乳酸とグルコースを資化する過程において，生成物阻害により，2つの顕著に異なる比増殖速度のモードが観測された．すなわち，pHが高いと増殖が活性化される一方，pHが低いと増殖が阻害される．ここでは，pH6.5において乳酸の資化が活性化されることと，pH5.0において，菌体増殖速度とグルコース消費速度が共に低いことを見出した．生ゴミ培地（KRM）を用いて，pH6.5から5.0にシフトさせた場合にも，上記現象が確認された．

したがって，生ゴミを基質とする場合，まずpH6.5においてプロピオン酸菌 *P. shermanii* で乳酸を資化し，その後pH5.0に下げることによりグルコースの過消費を防げるため，引き続く乳酸発酵で，効率的に高い光学純度が得られる可能性が示唆された．

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