Bioconversion of Dairy Processing Waste into Value-Added Chemicals

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Abstract

More than 145 million tons of liquid whey is produced world-wide as dairy processing waste per year, and half of it is discarded without proper treatment. Due to its high nutrient value, the environmental impact can be significant. Bioconversion of cheese whey can provide an effective way to reduce the waste and, at the same time, generate economically attractive value-added chemicals. In this study, cheese whey was fermented with *P. acidipropionici* to produce propionic acid which has a high market value for chemical and pharmaceutical industries. In order to specifically enhance propionic acid production, acetic acid production was suppressed using *o*-iodosobenzoic acid as an enzyme inhibitor. When grown in the presence of the inhibitor, propionic acid production rate increased by a factor of 2 while acetic acid production rate decreased by a factor of 3. Furthermore, when 0.3 mM of *o*-iodosobenzoic acid was used, the incipient stage(creeping growth period) was considerably reduced. Therefore, the inhibitor helps the cells begin to grow earlier and speed up the production of propionic acid. Although the production rate of propionic acid effectively increased, the final concentration(or production yield) remained unchanged due to product inhibition. Methods that can reduce product inhibition are being tested combined with *o*-iodosobenzoic acid to optimize both the production rate and yield. The results are expected to be informative for controlling the other byproducts for other applications.

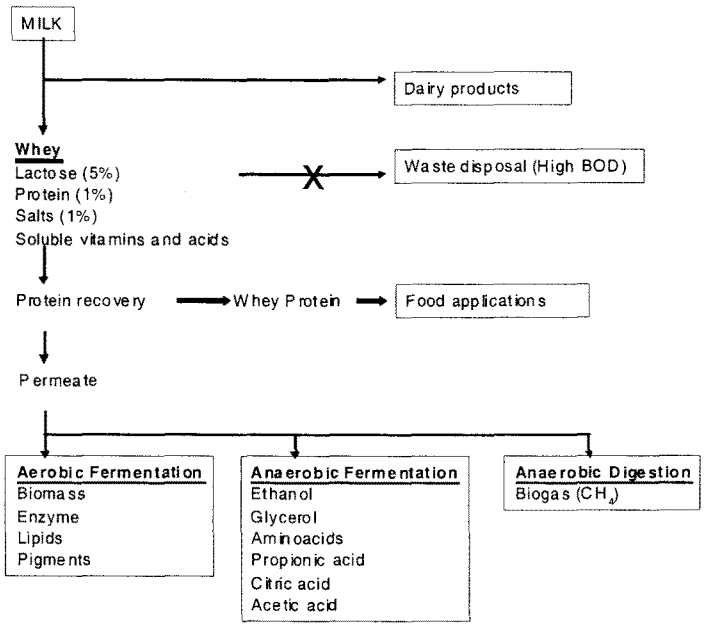
Keywords : Bioconversion, Cheese whey, Dairy processing waste, Propionic acid

Ⅰ. Introduction

According to a 2003 USDA report, the total world cow milk production in 2002 was estimated at 390 million tons (Korea; 2 million tons in 2002), and more than 25 million tons of dairy products (Korea, 100 thousand tons) including cheese (12.6 million; Korea, 20 thousand), butter (6.3 million; Korea, 5 thousand), and dry milk (6.6 million; Korea, 45 thousand) are produced worldwide (USDA, 2003; Korea Dairy Committee, 2002). The remaining after the production of these dairy products is high in organic matters and amounts, and therefore, presents a high potential of environmental pollution. The remaining includes more than 145 million tons of liquid whey per year, with 6 million tons of lactose world-wide (Castillo, 1990). When discarded untreated, cheese whey represents an important environmental problem because of the high volumes produced and the high BOD_5, 30,000-50,000 ppm, and COD, 60,000-80,000 ppm, with lactose being largely responsible for the high BOD and COD (Mawson, 1994). Although several possibilities for cheese-whey exploitation have been investigated over the past 50 years, approximately half of world cheese whey production is not treated, but is discarded as waste. The liquid whey production in Korea is estimated as 0.19 million tons (190 thousand tons) in 2002, and the most of it was disposed into the landfill.

Cheese whey represents about 85-95% of the milk volume and retains 55% of milk nutrients. In order to utilize these nutrients, whey is processed to separate it into protein (1% w/v), lactose (5% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried extract) (Marwaha and Kennedy, 1988). By using these nutrients as a substrate for producing value-added byproducts, both the pollution problem and disposal cost can be abated simultaneously. One of the ways to use these nutrients for producing value-added byproducts is bioconversion of whey lactose to SCP (single cell protein), ethanol, methane, or organic acids as shown in Figure 1.

<Figure 1> Systematic sketch of an ideal biotechnological utilization of whey (von Stockar and Marison, 1990)

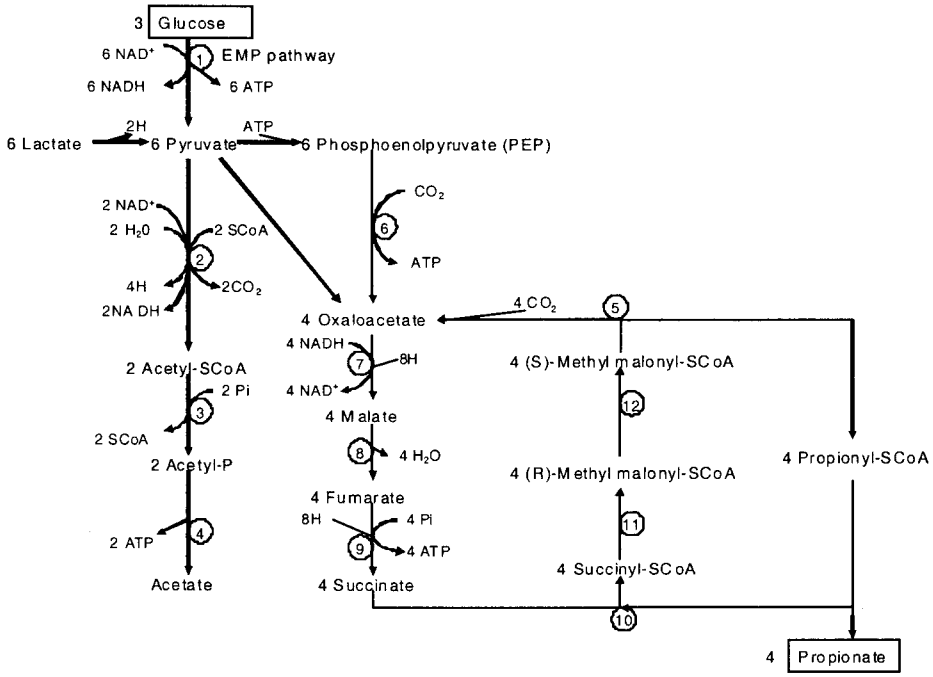


In this study we have focused on optimizing the bioconversion of cheese whey into propionic acid through fermentation. Propionic acid is widely used in food, cosmetic, and pharmaceutical industries (Colomban et al., 1993). It is used chiefly in the form of its salts, propionates, for animal feed preservation, foods, mainly in baked goods and cheeses. Propionic acid is also used as an important intermediate in the synthesis of herbicides, and pharmaceuticals. In 2002 its US market demand was 204 million pounds (93,000 tons) and is expected to grow to 219 million pounds in 2006. Currently, almost all propionic acid is being manufactured by chemical synthesis from ethyl alcohol and carbon monoxide (Jin and Yang, 1998). The current price for one pound of propionic acid is 0.51 - 0.54 US Dollars. Compared to other common intermediate chemicals such as acetic acid ($0.47 - 0.51) or phenol ($0.37), propionic acid has a high commercial market value. The controlling technique for the acid production obtained in this research can be used to enhance the production of organic acids, ethanol, or biogases such as methane or hydrogen. These byproducts can be used as an alternative energy resource for conventional energy production or fuel cell applications.

In fermentation of lactose included in cheese whey, various organic byproducts are

produced through different metabolic pathways as shown in Figure 2. Usually acetic acid is the most abundant byproduct among the other organic acids such as pyruvic acid, propionic acid, and succinic acid. In order to increase the production of propionic acid, the acetic acid metabolic pathway was blocked using an enzyme inhibition method in this study based on an assumption that the metabolic flux for the acetic acid production may be diverted to the other organic acid production while the acetic acid production is suppressed.

<Figure 2> Propionic acid formation via succinate-propionate pathway



Key to enzymes : (1) Enzymes of glycolysis, (2) Pyruvic dehydrogenase, (3) Phosphotrans-acetylase, (4) Acetyl kinase, (5) Trnascarboxylase, (6) Phosphoenolypyruvvic carboxyl transphosphorylase, (7) Malic dehydrogenase, (8) Fumarase, (9) Fumarate redcutase, (10) CoA transferase, (11) Methlymalonyl isomerase, (12) Methylmalonyl racemase (Boyer et al., 1962).

In this study, we used *Propionibacterium acidipropionici* (ATCC 4875) for propionic acid production in lactose fermentation. In order to suppress the acetic acid production, *o*-iodosobenzoic acid was used during fermentation. The inhibitory effects of *o*-iodosobenzoic acid (in short *BA*, hereafter) have been the focus of research efforts because of its selectivity to protein SH (sulfurhydryl) groups near or at their active sites

at neutral pH (Webb, 1963c). Previous studies have shown that *BA* is implicated in glucose utilization, respiration, protein synthesis, phosphorylation and cellular activity (Webb, 1963a). *E. coli* has been found to have a similar propionic acid formation via succinate-propionate pathway to that of *Propionibacterium acidipropionici* indicating inhibitory affects on acetic acid production in propionic acid fermentation. It is thought that BA is effectively and specifically bound to acetyl kinase (④ in Figure 2) blocking the conversion of acetyl-P into acetic acid in the acetic acid metabolic pathway. It was reported that using 2 mM *BA* on *E. coli* exhibited 100% inhibition and 50% inhibition of acetyl kinase using 0.3 mM *BA* during 30 minutes of exposure (Webb, 1963b). In addition, Boyer et al. (1962) have also shown the inhibitory effect of 0.35mM *o*-iodosobenzoic acid on acetyl kinase.

In this study, various *o*-iodosobenzoic acid concentrations were examined to determine the effects of enzyme inhibition on the rates of cell growth and byproduct production in propionic acid fermentation. *P. acidipropionici* was inoculated in an anaerobic reactor with lactose-based nutrients, which was separated from cheese whey, and the number of cells and concentrations of the byproduct acids were measured during the incipient and exponential growth phases at different inhibitor concentrations.

Like most organic acid fermentation, cell metabolism is inhibited by the end products, i.e., propionic and acetic acids. Nanba et al. (1983) studied the inhibitory effects of propionic and acetic acids on the growth of *P. shermanii*. Propionic acid was found, much more inhibitory than acetic acid, particularly in acidic conditions and mode of inhibition was non-competitive type. In our preliminary experiments in a batch reactor, product inhibition was also observed. As the concentration of acid increased, the cell growth rate slowed down due to the product inhibition. Because the effect of *BA* on propionic acid production was the main concern in this study, only the early stage of exponential growth phase, where the product inhibition was not significant, was used for data analysis.

II. RESEARCH METHODS

Preparation of Agar Slant for Culture Maintenance

A 100 mL tomato juice agar culture medium was obtained by mixing 1 g of yeast

extract, 1 g of tryptone, 2 g of agar, 20 mL of tomato juice and 80 mL of distilled water into an Erlenmeyer flask (Vandana, 2000). The tomato juice was stirred at a medium speed and the rest of the components were slowly added to the solution until completely dissolved. The cultivation medium was autoclaved, and 10 mL of solution was poured in a sterilized test tube with cap, and cooled at room temperature to form a agar slant. The slants were inoculated with the seed culture obtained from ATCC (American Type Culture Collection) and incubated in anaerobic gas jar at 30°C for 48 hours. Finally the slants were stored at 4°C for preservation of the bacteria.

Preparation of Cultivation Medium

The cultivation medium for *P acidipropionici* fermentation was prepared by ultrafiltration of cheese whey obtained from Country Fresh Milk in Toledo, Ohio. The cheese whey permeate (See Figure 1) after ultrafiltration was assayed for lactose concentration and was measured as approximately 50 g/L.

Inoculum Development

Three loops of cells were taken from the agar slant, and transferred to the 50-mL cultivation medium. Once the optical density reached a value of one, a 10 ml of inoculum culture solution was transferred to a 300-ml cultivation medium solution (cheese whey permeate) previously poured in a 500-ml serum flask. The flasks were kept at temperature 30°C and 200 rpm. The flasks were flushed with nitrogen gas to ensure anaerobic conditions. Solution samples were taken every four hours and analyzed for optical density of the cells using a U.V. spectrophotometer and for organic acid concentrations using HPLC.

Inhibitor effects

In order to determine the effect of *o*-iodosobenzoic acid (*BA*) in propionic acid fermentation on the production rates of the byproducts, three molar concentrations of *BA* (0.1 mM, 0.3 mM and 0.6 mM) were used. It turned out that 0.3 mM concentration of o-iodosobenzoic acid buffer solution in cultivation medium resulted in the highest production rate of propionic acid (data not shown).

Acid Concentration Measurement

HPLC was used to analyze the concentration of lactose, propionic, acetic, succinic and pyruvic acids in the cultivation medium solution using a HPX-87H column operated at 58°C with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/minute. Peaks were detected using U.V. spectrophotometer at a 660 nm wave length and peak areas were compared with the calibration data to calculate the respective concentrations. The fermentation samples (2-3 mL) were taken every four hours, and filtered through 0.2 μm Millipore filters to remove impurities before injecting into HPLC.

Ⅲ. EXPERIMENTAL RESULTS

As stated earlier, the incorporation of *o*-iodosobenzoic acid (*BA*) in the cultivation medium was designed to enhance the propionic acid production rate in the anaerobic reactor. The concentrations of propionic, acetic, pyruvic, and succinic acids within cultivation medium were measured. The results were plotted in terms of the concentration (g/L) and a period of time (hours). Also, the optical density was plotted to determine the effects of the inhibitor on the cell growth rate.

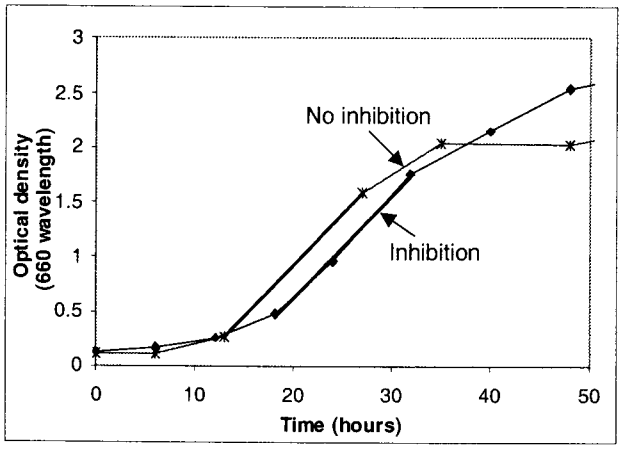
Figure 3 shows the impact of *o*-iodosobenzoic acid on the bacterial cell growth rate during the 1-day fermentation process. The cell growth rate was estimated by taking the slope of growth curve during the early exponential growth period. The darker lines represent the slopes for the exponential growth phase. The cell growth rate with no addition of the inhibitor, 0.3 mM *BA* in the cultivation medium was estimated to be 0.0917 g/L·h. The cell growth rate with the inhibitor was slightly higher, 0.0946 g/L·h. The optical density reached a maximum value of 2.028 g/L in the U.V. spectrophotometer. The final cell density slightly decreased, and the growth rate barely increased with the inhibitor.

The concentrations of propionic and acetic acids are shown in Figure 4. The experiment continued until steady-state was reached as the maximum concentration value was maintained for several readings. For propionic acid, steady-state was reached in approximately 15 hours with a presence of 0.3 mM *BA* in the cultivation medium. On the other hand, it took 35 hours for 0 mM *BA* to reach steady-state in the cultivation medium.

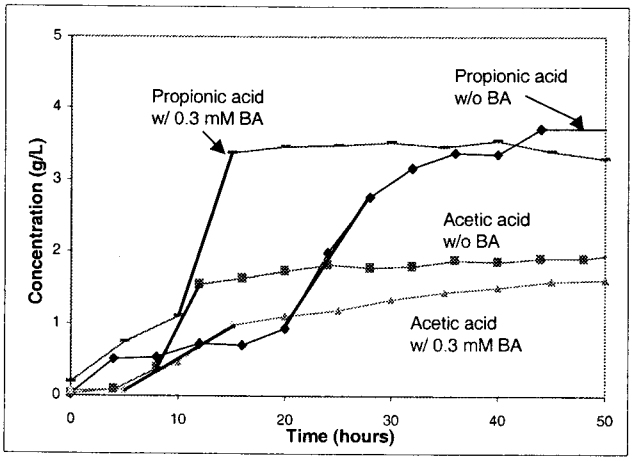
Creeping growth period (or incipient stage) was observed in the cell growth and acid

production after transferring the cells from the cultivation medium to the anaerobic reactor. The lengths of the incipient stage were quite different for propionic acid production with and without the inhibitor. In Figure 4, the long incipient stage (~20 hours) of the propionic acid was observed with no inhibitor while around 10 hours of incipient stage with the inhibitor. The longer incipient stage for propionic acid indicates that the bacteria were producing acetic acid during this period. The rapid increase in acetic acid was observed at a higher rate during this period for no inhibitor case as expected.

<Figure 3> The optical density in the anaerobic cultivation solution with or without the *o*-iodosobenzoic acid inhibitor.



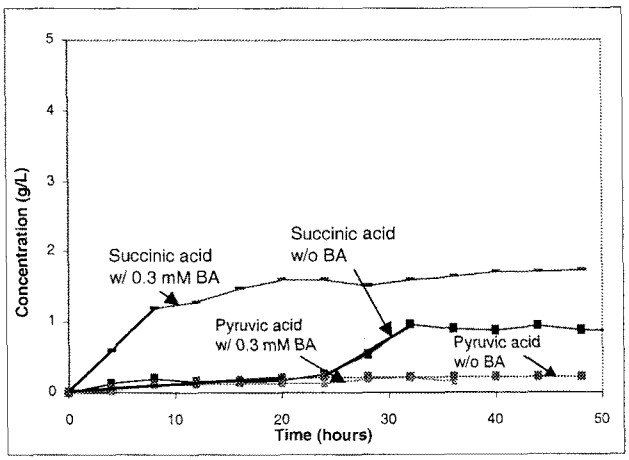
<Figure 4> The concentrations of propionic and acetic acids with 0M and 0.3 mM *o*-iodosobenzoic acid within a 30-hour period.



The maximum concentration recorded for propionic and acetic acids were 3.6 and 1.9 g/L with no *BA* present in the anaerobic reactor. With *BA* added to the cultivation medium, the corresponding maximum concentration of propionic and acetic acids were 3.4 and 1.1 g/L respectively.

In Figure 5, the concentrations of succinic and pyruvic acids were plotted with respect to time. For succinic acid with a presence of 0.3 mM *BA* in cultivation medium, a steady-state of 1.5 g/l was reached in approximately 12 hours. Without *BA*, the final concentration of succinic acid was 0.8 g/L in 32 hours. The concentration of pyruvic acid was 0.192 g/L regardless of the presence of *BA*. Compared to almost zero incipient stage of the succinic acid curve with 0.3 mM BA, with no inhibition it took up to 32 hours for succinic acid to start the exponential growth phase. Again, the longer incipient stage observed in the succinic acid curve with no inhibitor suggested that the bacteria were producing pyruvic acid at a higher rate than the other acids during this incipient stage.

<Figure 5> The concentrations of succinic and pyruvic acids with 0M and 0.3 mM *o*-iodosobenzoic acid.



For *P acidipropionici*, the results in Figures 4 and 5 showed that without inhibition the order in acid production was first pyruvic acid followed by acetic, and lastly succinic and propionic acids. Pyruvic acid production started at the beginning of the culture, whereas the succinic and propionic acid production was delayed for approximately 20 hours.

The patterns in the incipient stage differed when 0.3 mM *BA* was present in the anaerobic reactor. In the presence of *BA*, the incipient stage of propionic acid was the

largest (10 hours), followed by succinic acid (11 hours) and acetic (5 hours) and pyruvic acids. The results on the incipient stage are summarized in Table 1.

The maximum concentrations for succinic and pyruvic acids were 0.937 and 0.022 g/L with no inhibitor present in the anaerobic reactor. On the other hand, when *BA* is present, the corresponding maximum concentrations of both succinic and pyruvic acids increased to 1.709 and 0.092 g/L respectively.

<Table 1> Comparison of incipient stages

	Incipient period (hours)	
	No inhibitor	0.3 mM BA
Pyruvic acid	~ 2 hrs	~ 2 hrs
Acetic acid	~ 5 hrs	~ 3 hrs
Succinic acid	~ 23 hrs	~ 0 hrs
Propionic acid	~ 20 hrs	~ 10 hrs
Cell growth	~ 16 hrs	~ 10 hrs

Ⅳ. Discussion

The acid production rates are compared in Table 2 to determine the impact of the inhibitor. Table 2 shows that with the presence of 0.3 mM *BA* in the cultivation medium, the production rates of propionic, pyruvic and succinic acids were greater than the production rates without the inhibitor in the medium. The production rates of propionic acid, succinic acid and pyruvic acid increased by a factor of 2 when the inhibitor was added into the cultivation medium. On the other hand, the acetic acid production rate decreased by a factor of 3 with addition of the inhibitor.

<Table 2> Acid production rates in *Propionibacterium* fermentation

	rate constant (h^{-1})		Steady-state concentration (g/L)	
	No inhibitor	0.3 mM BA	No inhibitor	0.3 mM BA
Propionic acid	0.2307	0.4582	5.548	3.286
Acetic acid	0.2903	0.0884	2.245	1.056
Succinic acid	0.0884	0.1475	0.937	1.709
Pyruvic acid	0.0094	0.0187	0.022	0.092
Cell growth	0.0917	0.0946	2.229	2.068

The ratios of propionic and acetic acid production rates ($\mu_{propionic}/\mu_{acetic}$) were compared for the 0 mM and 0.3 mM *BA* cases in Table 3. The ratio of $\mu_{propionic}/\mu_{acetic}$ with no inhibition in the cultivation medium was of approximately 0.79 which indicates that the production of acetic acid is about 1.3 times faster than that of propionic acid. The ratio of $\mu_{propionic}/\mu_{acetic}$ increased to 5.18 with the addition of the inhibitor. In other words, *o*-iodosobenzoic acid sped up the production of propionic acid while slowing down the acetic production. In addition, the value of $\mu_{succinic}/\mu_{pyruvic}$ without addition of *o*-iodosobenzoic acid was 9.40 and with addition of 0.3 mM *o*-iodosobenzoic acid it was approximately 7.89, which indicates that the pyruvic acid production rate was enhanced with the inhibitor. Furthermore, the $\mu_{propionic}/\mu_{succinic}$ value with no inhibitor was 2.61; and with an inhibitor, the ratio value was increased to 3.11. This result indicates that with addition of the inhibitor into the system, some of the metabolic fluxes for succinic acid production were diverted toward propionic acid metabolic pathway.

<Table 3> Production rate ratios for *Propionibacteria* fermentation with no pH control

Ratio of the rates	Ratio	
	(no inhibition)	(inhibitor, 0.3 mM)
$\mu_{propionic}/\mu_{acetic}$	0.79	5.18
$\mu_{succinic}/\mu_{pyruvic}$	9.40	7.89
$\mu_{propionic}/\mu_{succinic}$	2.61	3.11

It is interesting to notice that the cell growth rates for both the inhibition and no inhibition cases were very similar while the production rates of the organic acids and creeping cell growth period (incipient stage) significantly changed depending on the presence of the inhibitor. It appears that the reduction of the acetic acid production rate was compensated by the increase of the propionic acid production rate. In other words, the enhanced propionic production rate with the inhibitor is thought to occur because of the diversion of metabolic flux from acetic acid. In the presence of the inhibitor, the metabolic fluxes were routed to 3 organic acids (pyruvic, succinic, and propionic acids) instead of 4, which resulted in greater production rates of those 3 acids (Figure 2). However, again, this production rate increase did not necessarily result in an increase in the cell growth rate in this case. As shown in Figure 3, the cell growth rates were similar

for both cases, although the rate for the inhibitor case was slightly higher. It is thought that the cell growth rate was also affected by the abort of 2 moles of ATP in the step (in Figure 2 due to the inhibition of acetyl kinase. Despite the lack of 2 moles of ATP, the creeping growth period (or, incipient stage) was shortened in the presence of the inhibitor. The cells started the exponential growth earlier and produced the acids earlier with a shorter creeping growth period as shown in Figure 3. Although the reason for the early start of the exponential production of acids needs more in-depth study, it is thought that the slower production of acetic acid during the creeping growth period with the inhibitor helped cells start the exponential growth earlier.

It was observed that, regardless of the presence of the inhibitor, the final steady state concentrations of propionic acid stayed the same. Although propionic acid began to be produced earlier (shorter incipient stage) and the production rate was higher in the presence of inhibitor, the final concentration of propionic acid was observed to be unchanged. However, the production rate and final concentration of acetic acid considerably decreased and those of succinic acids increased in the presence of inhibitor. By blocking the last step (() in acetic acid pathway in Figure 2, the diverted metabolic flux seems to enhance the succinic acid production. Because propionic acid is produced from the succinate and oxaloacetate cycle, the increased metabolic flux led to the increase in the propionic acid production rate. The unchanged final concentration of propionic acid is thought to be due to the product inhibition.

There have been many studies to reduce product inhibition in propionic acid production (Boyaval et al., 1994; Ozadali et al., 1996; Park and Glatz, 1991; Paik and Glatz, 1994; Yang et al., 1995), and these methods combined with the BA enzyme inhibitor are being tested for optimum propionic production.

V. Conclusions

Cheese whey is nutrient-rich waste that may cause severe environmental impacts because of its high BOD and COD. Utilizing the nutrients in waste to produce value-added chemicals not only helps reduce the potential risk of environmental pollution, but also generates the economic benefits. Bioconversion of the nutrient-rich waste is a promising

way to utilize food processing waste such as cheese whey and agricultural wastes.

In order to selectively increase the production of a desired byproduct, propionic acid in this case, enzyme inhibition technique was used. By blocking the enzyme that produces acetic acid using *o*-iodosobenzoic acid, acetic acid production was suppressed and the propionic acid production rate was increased successfully. Furthermore, the incipient stages of the cell growth and acid production were significantly reduced, which helps speed up the entire bioconversion process.

However, the increased production rate of propionic acid did not lead to the higher production yield due to the product inhibition. Further research is necessary to enhance the production yields of propionic acid.

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