Dairy technology3 (Fermented Milks and By-Products) (Code FS 0705)

- Lecture 5, for Food safety program, level 3, 2019-2020

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Propionic acid fermentation

Introduction

- Propionic acid (PA) is an important building block chemical and finds a variety of applications in organic synthesis, food, feeding stuffs, perfume, paint and pharmaceutical industries.
- Presently, PA is mainly produced by petrochemical route. With the continuous increase in oil prices, public concern about environmental pollution, and the consumers' desire for bio-based natural and green ingredients in foods and pharmaceuticals.
- Propionic acid (PA), a colorless liquid with a pungent odor and is an important C3–based building block chemical with a formula of CH_3CH_2COOH.

Table 1. Applications of propionic acid in various fields

Applications	Instructions
Feed and grain preservation	PA is inhibitory to *Aspergillus flavus*, aerobic *Bacillus*, *Salmonella* and yeast, and has been used as a mold inhibitor for animal feed, wet corn, silage and grain (Balamurugan *et al.*, 1999).
Food preservatives	*Propionibacterium* have been granted a GRAS (generally recognized as safe) status by the United States Food and Drug Administration (FDA) (Salminen *et al.*, 1998). PA can be used as preservatives in food industries to prevent the foods such as bread and cake from molding.
Herbicide synthesis	PA can be used for the synthesis of sodium 2, 2-dichloropropionate used as herbicide.
Perfume intermediates	PA is a precursor for the chemical synthesis of propionic ether and benzyl propionate, which can be used as additives in food and cosmetics (Kumar and Babu, 2006).
Pharmaceuticals intermediates	PA can be used for the synthesis of propionic andydride and chloropropionic acid as pharmaceutical intermediates (Kumar and Babu, 2006).
Synthesis of cellulose acetate propionate	PA can be used as the precursor for the synthesis of cellulose acetate propionate.
Other applications	PA can be used as an intermediate in the production of plastics, plasticizers, textile, and rubber auxiliaries, as well as dye intermediates.

Culture methods developed for microbial PA production

1-batch fermentation.

2- fed-batch fermentation.

3- cell-immobilized fermentation.

Microbial PA production

- **1-Strains used for PA production**

Typical strains for PA production are *Propionibacterium* spp., which are Gram-positive, non-motile, non-sporulating, rod-shaped, facultative anaerobes.

These strains include *P. thoenii*, *P. freuden- reichii*, *P. shermanii*, *P. acidipropionici*, and *P. beijingense*.

Table 2. Strains and corresponding details of microbial PA production

Strain	Culture mode	Substrates	PA production (g/L)	Productivity ($g\ L^{-1}h^{-1}$)	References
Propionibacterium acidipropionici	fed-batch	glycerol	44.62	0.20	Zhu *et al.*, 2010
	fibrous bed bioreactor	glycerol/ glucose/ lactate	~100	–	Zhang and Yang, 2009a, 2009b
	extractive fermentation	lactose	75	~1	Jin and Yang, 1998
	batch	lactate/sugarcane /molasses	15.06	0.26	Coral *et al.*, 2008
	fibrous bed bioreactor	glucose	71.8	–	Suwannakham *et al.*, 2006;
	batch	cheese whey	3.30	–	Morales *et al.*, 2006
	fed-batch	glucose/lactate	~30	–	Martinez-Campos. 2002
	immobilized cell fermentation	lactose	18.61	0.31	Coronado *et al.*, 2001
	cell recycle fermentation	xylose	–	2.7	Carrondo *et al.*, 1988
	batch/fed-batch/ extractive fermentation	lactose/glucose/ lactate	~15	–	Hsu and Yang, 1991; Lewis and Yang, 1992b, 1992c
	batch	glucose/ glycerol	~42	0.167	Barbirato *et al.*, 1997
Propionibacterium freudenreichii	multi-point fibrous-bed bioreactor (fed-batch)	glucose	67.05	0.14	Feng *et al.*, 2010a
	batch	wheat flour	20	–	Border *et al.*, 1987
Propionibacterium shermanii	batch	glucose	12.5	–	Quesada-Chanto *et al.*, 1998a
	batch	glucose/glycerol	~9	–	Himmi *et al.*, 2000
Propionibacterium microaerophilum	batch	glucose	~	–	Koussemon *et al.*, 2003
Propionibacterium beijingense	batch	glucose	11.32	–	He Y and Jin, 1990

2- Biosynthetic pathway of PA in Propionibacterium

PA synthesis includes two steps:

1) the formation of succinic acid by the condensation of two molecules of acetic acid.
2) 2) the formation of PA and CO2 via the intermediate dissimilation of succinic acid. In

Propionibacterium PA is synthesized according to Eq. (1):

- $3CH_3CHOHCOOH \rightarrow 2CH_3CH_2COOH+CH_3COOH+CO_2+H_2O$ (1)

- The biosynthesis of PA in *Propionibacterium* is related to the EMP pathway and dicarboxylic acid pathway. Theoretically, 2 moles of glucose can yield 3 moles of PA, 1 mole of acetic acid (AA), 1 mole of CO2, and 1 mole of H2O. Three moles of lactate can be converted to 2 moles of PA, 1 mole of AA, 1 mole of CO2, and 1 mole of H2O; and 1 mole of glycerol can generate 1 mole of PA and 1 mole of H2O. The reactions for three different carbon sources (glucose, lactate, and glycerol) are as follows :

- $1.5C_6H_{12}O_6 \rightarrow 2CH_3CH_2COOH+CH_3COOH+CO_2+H_2O$ (2)
- $3CH_3CHOHCOOH \rightarrow CH_3CH_2COOH+CH_3COOH+CO_2+H_2O$ (3)
- $CH_2OHCHOHCH_2OH \rightarrow CH_3CH_2COOH+H_2O$ (4)

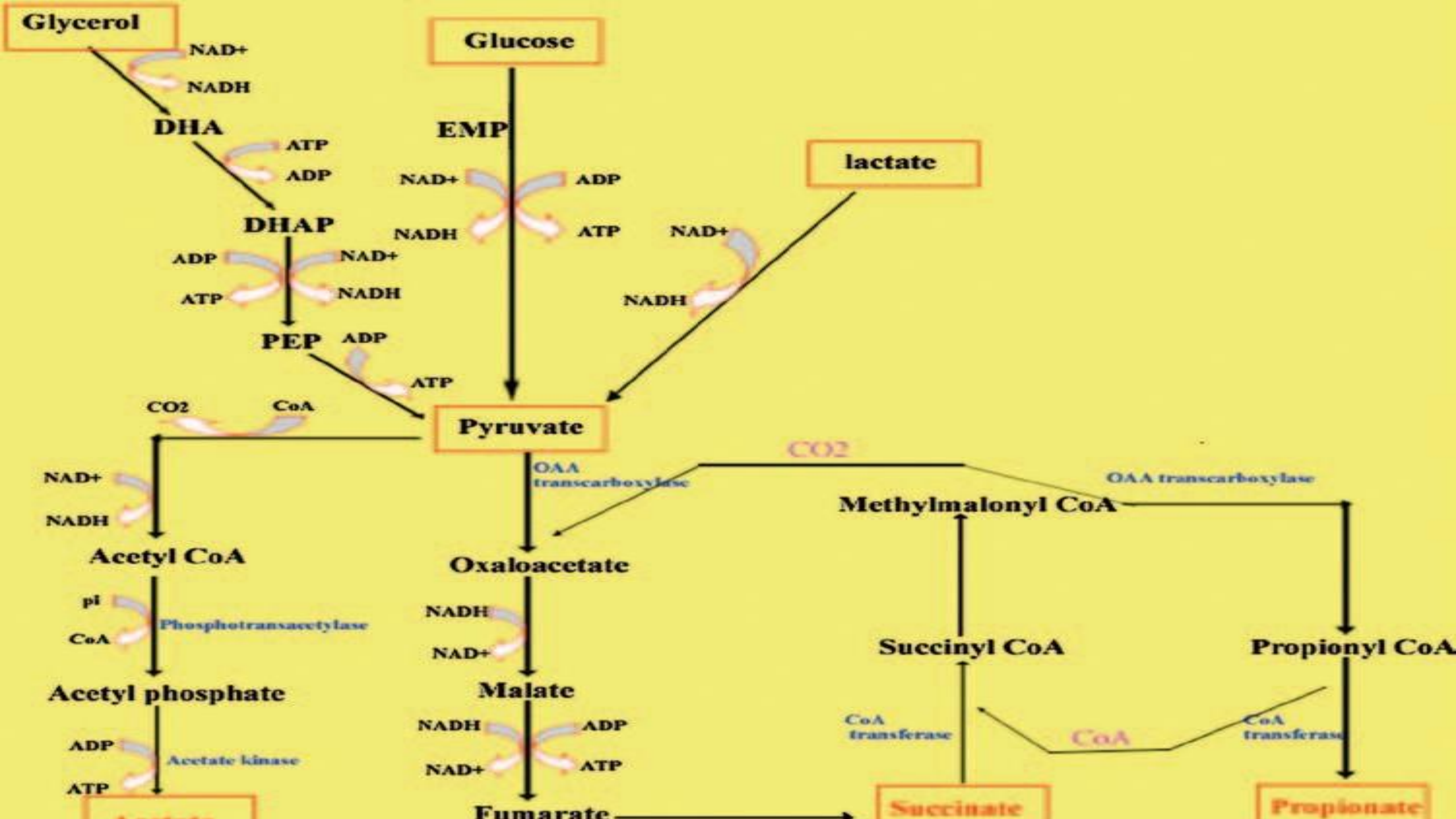
- The carbon sources (glycerol, glucose, and lactate) are metabolized into the same intermediate, pyruvate, which is a key metabolic node in the metabolic network of PA synthesis.
- A portion of the pyruvate is converted into acetate, and the rest is metabolized into malate and fumarate, which are then converted into succinate as a precursor of PA synthesis.
- There are three important cofactors involved in the regulation of PA synthesis, namely, ATP/ADP, NADH/NAD^+, and CoA/AcCoA. The regeneration rate of these cofactors determines the consumption rate of carbon sources and the synthetic rate of PA.
- Therefore, the regulation of the regeneration rate of these cofactors is a necessary part of metabolic engineering of *Propionibacterium* for enhanced PA production.

3- Carbon/nitrogen sources

- Several carbon sources such as glucose, fructose, maltose, sucrose, molasses, xylose, lactate, whey lactose hemicellulose and glycerol have been used for PA production.
- The oxidation state of the carbon source has a significant impact on the production of PA; the lower the oxidation state, the more favorable for PA synthesis due to the accelerated regeneration rate of NAD+, which is necessary for PA synthesis in *P. acidipropionici* .

4-Culture conditions

- The culture conditions, such as temperature and pH, also impact PA production.
- A temperature of 30°C is usually adopted for microbial PA production.
- A two-stage pH control strategy, involving a controlled pH of 6.5 for 48 h and then a pH of 6.0, was shown to enhance PA production.
- With this pH control strategy, the maximal PA concentration and glucose conversion efficiency achieved 19.21 g/L and 48.03%, respectively, and these parameters achieved 14.58 g/L and 36.45%, respectively, with a constant pH operation.


Glycerol
NAD+
NADH
DHA
ATP
ADP
DHAP
ADP
NAD+
ATP
NADH
PEP
ADP
ATP
Glucose
EMP
NAD+
ADP
NADH
ATP
lactate
NAD+
NADH
CO2
CoA
Pyruvate
NAD+
NADH
Acetyl CoA
pi
CoA
Phosphotransacetylase
Acetyl phosphate
ADP
ATP
Acetate kinase
OAA transcarboxylase
CO2
OAA transcarboxylase
Oxaloacetate
NADH
NAD+
Malate
NADH
ADP
NAD+
ATP
Fumarate
Methylmalonyl CoA
Succinyl CoA
Propionyl CoA
CoA transferase
CoA
CoA transferase
Succinate
Propionate

- **5- Fermentation modes**
- ***Batch and fed-batch fermentation***
- Batch culture is commonly used for microbial PA pro- duction (Barbirato *et al*., 1997; Coral *et al*., 2008; Feng *et al*., 2010b). Though significant improvement of PA production in batch culture has been achieved in the last decades, drawbacks exist. For example, the substrate (lactate, glycerol, or glucose) in high concentration is unfavorable for cell growth (Barbirato *et al*., 1997; Lewis and Yang, 1992c; Zhu *et al*., 2010) and the distribution of metabolic flux is adversely affected (Gu *et al*., 1998; Koussemon *et al*., 2003), resulting in a low conversion yield of substrate. For example, as the PA concentration

- increased from 2.77 to 30.41 g/L, cell growth declined by two-thirds, and specific PA productivity and glucose consumption rate decreased from 0.059 to 0.015 g PA /g cell/h and 0.11 to 0.04 g glucose/g cell/h, respectively (Gu et al., 1998). The excess PA also altered bacterial metabolism to produce more by-products such as acetic, lactic, and succinic acid resulting in a decreased yield of PA from 0.52 to 0.41g PA/g glucose (Gu et al., 1998).
- To alleviate the inhibition caused by the substrate, fed-batch fermentation was performed (Coronado et al., 2001; Eaton and Gabelman, 1995; Goswami and Srivastava, 2000). For example, glycerol can be efficiently utilized by P. acidipropionici for PA production (Barbirato et al., 1997; Himmi et al., 2000; Zhang and Yang, 2009a), and feeding glycerol at a constant rate is effective for the enhancement of PA yield and productivity. The maximum PA production and productivity reached 44.62 g/L and
- 0.20 g / (L· h) at 220 h, respectively, when concentrated glycerol (400 g/L, 500 mL) was fed at a rate of 0.01 L/h from 72 h to 120 h with an initial glycerol concentration of 30 g/L (Zhu et al., 2010).

Extractive fermentation

- The accumulation of PA, even at low concentration in the culture medium, can cause severe inhibition of cell growth and results in low PA yield and productivity (Gu *et al*., 1999; Woskow and Glatz, 1991). To resolve this challenge, extractive fermentation was performed during microbial PA production (Gu *et al*., 1999; Jin and Yang, 1998; Keshav *et al*., 2008; Lewis and Yang, 1992b; Ozadali *et al*., 1996; Solichien *et al*., 1995). This process removes the inhibitory PA product from the bioreactor resulting in better pH control and higher PA yield and productiv- ity. In addition, the PA product is present in a relatively pure and concentrated form resulting in savings in downstream recovery and purification costs (Kumar and Babu, 2006). In extractive fermentation, the fermentation products, mainly PA and acetic acid, are continuously removed by solvent extraction in an extractor. The sol- vent containing the extracted PA and acetic acid are then back-extracted in a second extractor with a base solution to simultaneously regenerate the solvent and to produce concentrated PA (Jin and Yang, 1998). The most impor- tant parameter of extractive fermentation is the selection of an extractant with high extraction coefficient and low toxicity to the cells. The mixture of Alamine 336/2-octa- nol (Lewis and Yang, 1992b) and the liquid extractant consisting of trilaurylamine, oleyl alcohol, and activated charcoal (Nakano *et al*., 1996) are ideal extractants for PA production. A membrane-based extractive process with continuous substrates feeding and continuous cell-free PA removal increased PA productivity by 300% (Jin and Yang, 1998).
- However, extractive fermentation has some disadvan- tages. First, the selection of extractant is difficult; an ideal candidate should have a high extraction coefficient and low cell toxicity. Nearly all extractants are chemicals and are more or less harmful to the growth of strain (Gu *et al*., 1999). Second, extractive fermentation is highly depen- dent on pH (Lewis and Yang, 1992b). The distribution coefficient, Kd, is nearly zero at pH 7.0 and increases with decreasing pH, reaching the maximum value at pH 4.0 (Yang *et al*., 1991). On the other hand, cells grow better at pH values higher than 5.0, with an optimum around pH
- 7.0. Thus, a higher pH favors cell growth and a lower pH favors the extraction, making it difficult to facilitate both. Third, the cost of extractive fermentation is relatively high and its application on an industrial scale is restricted (Cho and Shuler, 1986).
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Cell-immobilized fermentation

- Cells are immobilized on a matrix, resulting in a rapid increase in cell density and significant improvement of PA production (Czaczyk *et al*., 1997; Feng *et al*., 2010a; Lewis and Yang, 1992a; Paik and Glatz, 1994; Wodzki *et al*., 2000; Yang *et al*., 1995; Yang *et al*., 2004). Calcium alginate (Rickert *et al*., 1998) and cotton fiber (Feng *et al*., 2010a) are the commonly used materials for immobiliza- tion. Goswami and Srivastava developed an *in situ* cell retention bioreactor for continual PA fermentation with spin filters (pore sizes 5 μm and 10 μm). PA productivity (0.9 g/(L·h)) was enhanced by approximately four-fold compared to conventional batch fermentation (0.25 g/ (L·h)). The *in situ* cell retention (5-μm pore size spin fil- ter) bioreactor was operated continuously for 8 days at a dilution rate of 0.05 h−1 (Goswami and Srivastava, 2001). Paik and Glatz produced PA with a propionate-tolerant strain *P. acidipropionici* immobilized in calcium alginate beads, obtaining 57 g/L PA and 0.96 g/(L·h) volumetric productivity (Paik and Glatz, 1994).
- The packed-bed bioreactor (Lewis and Yang, 1992a), recycle batch immobilized cell bioreactor (Yang *et al*., 1995), *in situ* cell retention bioreactor with spin filters (Goswami and Srivastava, 2001), and multi-point fibrous- bed bioreactor (Feng *et al*., 2010a) have been developed for the continuous production of PA. The maximum PA concentration reached 67.05 g/L after 496 h, and the pro- portion of PA to total organic acids was approximately 78.28% (w/w) (Feng *et al*., 2010a). In the cell- immobi- lized bioreactor, cells are protected from the inhibitor, and the growth rate, substrate consumption rate, and PA production rate were improved significantly compared to conventional fermentations.
- However, problems still exist for cell-immobilized fermentation, such as the significant decrease in mass transfer rate. Also, productivity must be increased to improve commercial competition with the petrochem- ical process. The integration of extractive fermentation with cell immobilization may be an effective approach for the microbial production of PA, and whether this novel culture method can be applied on an industrial scale needs further investigation. In addition, more efficient and less expensive immobilization materials should be explored.

Prospects and opportunities: Application of metabolic engineering to improve acid tolerance and reduce by-product formation

- The emergence of metabolic engineering provides an opportunity to strengthen the commercial competence of microbial PA production. Metabolic engineering is defined as the manipulation of the cellular metabolism to achieve a desired goal (Bailey, 1991; Desai *et al*., 1999; Suwannakham, 2004). Maximal production or produc- tivity can be achieved via the deletion or overexpression of key genes. Few studies have been conducted on the genetic modification of propionibacteria and much work still needs to be conducted to improve the acid tolerance and reduce the formation of by-products via metabolic engineering.
- Currently, the available tools to improve acid toler- ance include adaptive evolution and genome shuffling. During adaptive evolution, cells obtained from culture media with the highest PA concentration that are able to grow are repeatedly transferred into fresh broth containing that concentration of PA. Once the growth rate of the tolerant strain reaches approximately 80% of that of the unchallenged parental strain, the toler- ant strain is transferred into broth containing a slightly higher amount of PA, and the process is repeated until the acid tolerance of the strain is satisfactory (Woskow and Glatz, 1991; Zhu *et al*., 2010). This evolutionary approach has been proven to be a powerful tool for strain improvement (Rosenberg 2001; Woskow and Glatz, 1991; Zhu *et al*., 2010). Another tool for the improvement of acid tolerance is genome shuffling, which involves the generation of mutant strains with improved phenotypes, followed by multiple rounds of protoplast fusion (recursive fusion) to allow recombi- nation between genomes (Wang *et al*., 2007). Genome shuffling is useful for engineering multitrait pheno- types because it is unlikely that all of the mutations are needed to improve a complex trait and maintain robust growth (Zhang *et al*., 2002). This approach has recently been used to improve acid tolerance in *Lactobacillus* (Patnaik *et al*., 2002; Wang *et al*., 2007).
- However, neither adaptive evolution nor genome
- shuffling can identify the key genes or proteins respon- sible for improved acid tolerance; reverse metabolic engineering is an effective tool to identify specific genes or proteins (Cakir *et al*., 2009; Lum *et al*., 2004; Soranzo *et al*., 2007). Therefore, to further improve acid tolerance of a strain at the genetic level, as shown in Figure 2, reverse metabolic engineering may be an effective alternative. After acid tolerance is improved via adaptive evolution or genome shuffling, the key factors (genes, proteins and metabolites) responsible for the acid tolerance can be identified by comparing the transcriptomes, proteomes, and metabolomes of the wild-type and evolved strains. Finally, the targeted genes can be manipulated for further improvement of acid tolerance at the molecular level.

- Eliminating the formation of by-products, such as acetic acid and succinic acid, is another potential approach for obtaining industrial production of PA. According to the traditional approach, genes respon- sible for the synthesis of by-products can be deleted to achieve this goal. However, this approach may have limitations because the consequences of gene dele- tion must be considered in the context of the entire metabolic network. For example, the metabolically engineered mutant ACK-Tet, which has the acetate kinase gene knocked out, can produce more PA and less acetic acid in comparison with its parent strain (Suwannakham *et al*., 2006). However, the mutant ACK-Tet strain grew more slowly than the parent due to deletion of the acetate kinase gene, resulting in a longer fermentation time and lower PA productivity (Suwannakham *et al*., 2006). The emergence of systems metabolic engineering allows us to overcome this limi- tation through the use of genome-wide high-through- put omics data and genome-scale computational analysis (Park and Lee, 2008). In systems metabolic engineering, targets are determined by considering
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- entire metabolic and regulatory networks together with midstream (fermentation) and downstream (recovery and purification) processes. During the actual meta- bolic engineering, the impact of altering these targets on the entire metabolism is examined to provide feed- back. Systems metabolic engineering has been used for strain improvement for the efficient overproduction of various bioproducts (Becker *et al*., 2011; Lee *et al*., 2007). For example, the overproduction of L-threonine by genetically engineered *Escherichia coli* using sys- tems metabolic engineering is a successful case (Lee *et al*., 2007). The feedback inhibition due to aspartoki- nase I and III (encoded by thrA and lysC, respectively) and transcriptional attenuation regulation (located in thrL) were removed from this strain. Pathways for Thr degradation were removed by deleting tdh and mutat- ing ilvA, and the metA and lysA genes were deleted to make more precursors available for Thr biosynthesis. Further target genes to be engineered were identified by transcriptome profiling combined with *in silico* flux response analysis, and their expression levels were manipulated accordingly. The final engineered *E. coli*

- strain was able to produce 82.4 g/l Thr by fed-batch cul- ture (Lee *et al*., 2007).
- For the systematic engineering of propionibacteria to increase PA production, the key factors including enzymes, metabolic pathways, and cofactors should be manipu- lated to increase the carbon flux towards PA synthesis. Fox example, glycerol dehydrogenase could be over-expressed in *P. acidipropionici* to accelerate the consumption rate of substrate glycerol, and oxaloacetate transcarboxylase could be over-expressed to increase the carbon flux from pyruvate to malate and fumarate. CoA is directly involved in the synthesis of PA and thus the over-expression of CoA transferase would be expected to accelerate the regenera- tion rate of CoA and to improve PA productivity. The genes encoding phosphotransacetylase could be deleted to block the carbon flux from pyruvate to acetate.
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References

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