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REVIEW ARTICLE

Microbial production of propionic acid from propionibacteria: Current state, challenges and perspectives

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Abstract

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, PA production from propionibacteria has attracted considerable attention, and substantial progresses have been made on microbial PA production. However, production of PA by propionibacteria is facing challenges such as severe inhibition of end-products during cell growth and the formation of by-products (acetic acid and succinic acid). The integration of reverse metabolic engineering and systematic metabolic engineering provides an opportunity to significantly improve the acid tolerance of propionibacteria and reduce the formation of by-products, and makes it feasible to strengthen the commercial competition of biotechnological PA production from propionibacteria to be comparable to the petrochemical route.

Keywords: Propionic acid, microbial production, propionibacteria, reverse metabolic engineering, systematic metabolic engineering, acid tolerance, petrochemical synthesis

Introduction

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. PA has received much attention in recent years due to its wide range of applications in organic synthesis, in the food, perfume, paint, and pharmaceutical industries (Balamurugan *et al.*, 1999; Suwannakham and Yang, 2005; Yang *et al.*, 1995; Zhang and Yang, 2009a). The various applications of PA are listed in Table 1. The application of PA in feed and food preservatives accounts for about 66% of its use, whereas the synthesis of herbicides accounts for about 19%, the synthesis of cellulose acetate propionate accounts for about 11%, and other applications as intermediates account for about 4% (Wang *et al.*, 2007). BASF is the largest PA producer in the world, with 176 million pounds per year of dedicated capacity. The other major PA manufacturers include Chemische Werke Hüls (Germany), Distillers Company (Britain), USSR (Russia), Celanese Chemical Company (US), and Eastman Chemical (US).

Presently, PA is mainly produced by the petrochemical route. The annual PA production in 2008 reached 130, 000 tons worldwide (Sauer *et al.*, 2008). The commercial price for PA from the petrochemical route is about 1.0 USD/kg, while the commercial price for the PA from the biotechnological route is about 1.5–2.0 USD/kg. However, the recent rise in oil prices and consumers' desire for bio-based chemical products makes producing PA from bio-renewable feedstock an attractive alternative. Microbial PA production possesses several advantages over

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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.

petrochemical synthesis, such as the ability to label the product as a "natural preservative" and the opportunity to use food processing wastes as fermentation substrates, thus lowering disposal costs (Woskow and Glatz, 1991).

Various culture methods have been developed for microbial PA production. Techniques such as batch fermentation (Zhu *et al.*, 2010), fed-batch fermentation (Woskow and Glatz, 1991; Zhu *et al.*, 2010), extractive fermentation (Solichien *et al.*, 1995), and cell-immobilized fermentation (Yang *et al.*, 1995) have been developed for the production of PA. The highest PA production reaches 100 g/L in a fibrous-bed bioreactor with an engineered *Propionibacterium acidipropionici* (Zhang and Yang, 2009a).

In this study, we comprehensively analyze and discuss the current advances in microbial PA production from propionibacteria, and propose the emphasis for future research.

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 (Kumar and Babu, 2006). These strains include *P. thoenii*, *P. freudenreichii*, *P. shermanii*, *P. acidipropionici*, and *P. beijingense*. Table 2 presents the main strains and the corresponding culture details for microbial PA production described in the literature.

2) Biosynthetic pathway of PA in Propionibacterium

Figure 1 shows the biosynthetic pathway for PA in *Propionibacterium*; there is a metabolic cycle in the synthetic network called the Wood-Werkman cycle (Wood and Werkman, 1936). PA synthesis includes two steps: 1) the formation of succinic acid by the condensation of two molecules of acetic acid, and 2) the formation of PA and CO_2 via the intermediate dissimilation of succinic acid. In *Propionibacterium* PA is synthesized according to Eq. (1):

$$3CH_3CHOHCOOH \rightarrow 2CH_3CH_2COOH+CH3COOH+CO_2+H_2O \quad (1)$$

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 CO_2, and 1 mole of H_2O. Three moles of lactate can be converted to 2 moles of PA, 1 mole of AA, 1 mole of CO_2, and 1 mole of H_2O; and 1 mole of glycerol can generate 1 mole of PA and 1 mole of H_2O. The reactions for three different carbon sources (glucose, lactate, and glycerol) are as follows (Wood, 1981).

$$1.5C_6H_{12}O_6 \rightarrow 2CH_3CH_2COOH+CH_3COOH+CO_2+H_2O \quad (2)$$

$$3CH_3CHOHCOOH \rightarrow CH_3CH_2COOH+CH_3COOH+CO_2+H_2O \quad (3)$$

$$CH_2OHCHOHCH_2OH \rightarrow CH_3CH_2COOH+H_2O \quad (4)$$

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.

The actual conversion yield of substrates to PA is about 0.5 g/g, which is much lower than the theoretical conversion yield of substrates to PA, and the productivity is lower than 0.3 g/(L·h) (Barbirato *et al.*, 1997; Coral *et al.*, 2008; Feng *et al.*, 2010a; Himmi *et al.*, 2000; Lewis and Yang, 1992a, 1992b, 1992c; Zhang and Yang, 2009a). Consequently, microbial PA production is inefficient and cannot compete effectively with the petrochemical route.

3) Carbon/nitrogen sources

Several carbon sources such as glucose (Feng *et al.*, 2010a; Himmi *et al.*, 2000), fructose (Rehberger and

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

Glatz, 1998), maltose, sucrose, molasses (Coral *et al.*, 2008), xylose (Carrondo *et al.*, 1988), lactate (Eaton and Gabelman, 1995; Lewis and Yang, 1992c; Zhang and Yang, 2009b), whey lactose (Lewis and Yang, 1992b), hemicellulose (Ramsay and Hassan, 1998), and glycerol (Barbirato *et al.*, 1997; Zhang and Yang, 2009a; Zhu *et al.*, 2010) 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* (Barbirato *et al.*, 1997; Suwannakham and Yang, 2005). Glycerol is an ideal carbon source for *Propionibacterium* fermentation, giving a higher PA yield compared to glucose or lactic acid, thus the cost of PA purification can be reduced due to the less formation of by-products such as acetic acid and succinic acid (Barbirato *et al.*, 1997). In addition, glycerol is an abundant and inexpensive carbon source due to its generation as a by-product during biofuel production (Yazdani and Gonzalez, 2007), and has become an ideal feedstock for the microbial production of bio-based chemicals (Blankschien *et al.*, 2010; Zhang and Yang, 2009a). It is feasible to produce microbially-derived PA from crude glycerol present in a biodiesel wastewater (Zhang and Yang, 2009a). Besides crude glycerol, cellulose is another potential carbon source for microbially produced PA. To achieve this goal, the efficient treatment of cellulose and engineering of cells for efficient utilization of cellulose-derived sugars need further research. In addition to the carbon source, the nitrogen source also has a significant effect on microbial production of PA. Corn steep liquor, peptone, and yeast extract can be used effectively by *Propionibacterium* spp (Quesada-Chanto *et al.*, 1998a).

4) Culture conditions

The culture conditions, such as temperature and pH, also impact PA production (Czaczyk *et al.*, 1997; Koussemon *et al.*, 2003; Quesada-Chanto *et al.*, 1998a; Rehberger and Glatz, 1998). 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 (Feng *et al.* 2010b). 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.

5) Fermentation modes

Batch and fed-batch fermentation

Batch culture is commonly used for microbial PA production (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

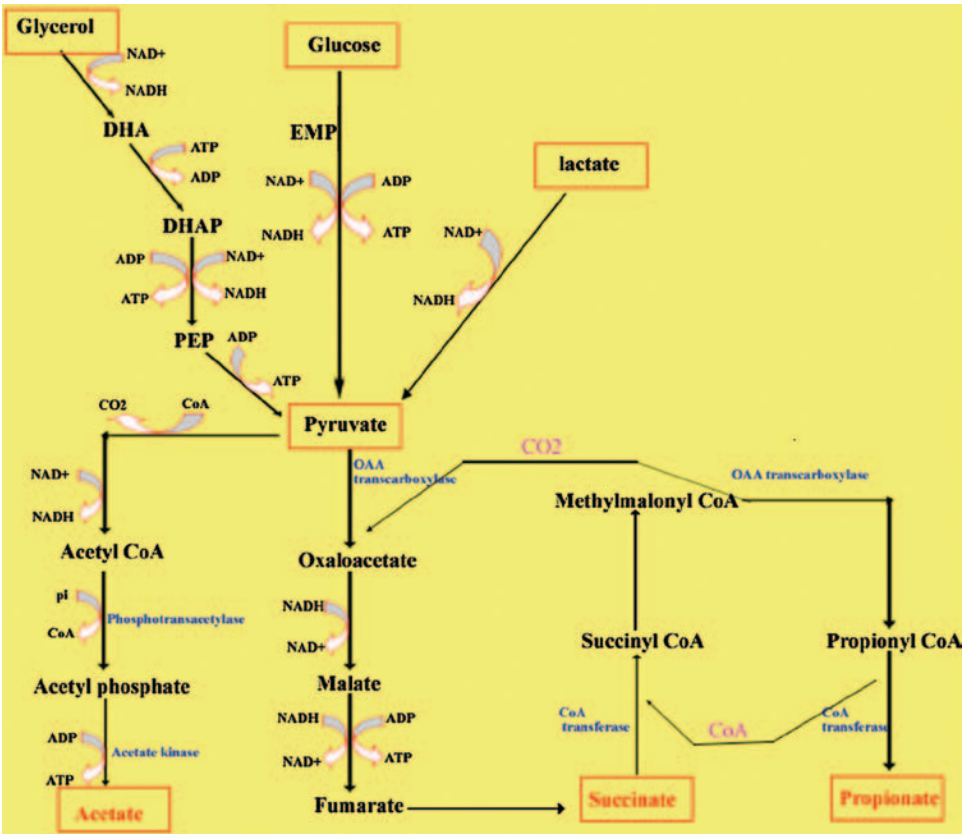

Figure 1. The biosynthetic pathway of PA production in Propionibacterium.

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 productivity. 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 solvent 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 important 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-octanol (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 disadvantages. 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 dependent on pH (Lewis and Yang, 1992b). The distribution coefficient, K_d, 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).

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 immobilization. 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 filter) 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 proportion of PA to total organic acids was approximately 78.28% (w/w) (Feng *et al.*, 2010a). In the cell- immobilized 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 petrochemical 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 productivity 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 tolerance 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 tolerant 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 recombination between genomes (Wang *et al.*, 2007). Genome shuffling is useful for engineering multitrait phenotypes 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 responsible 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.

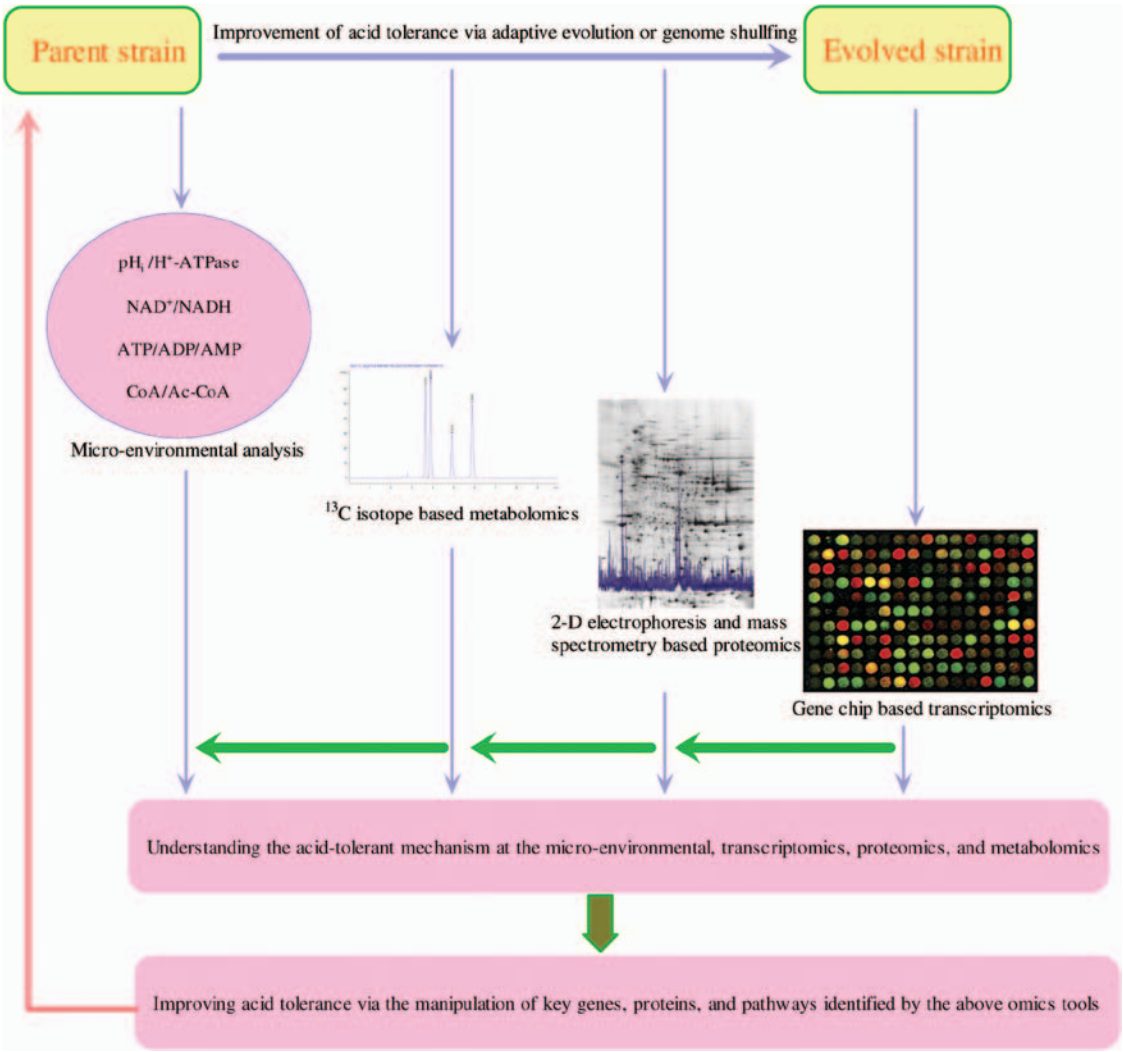

Figure 2. Omics-based systematic approaches for the increase of acid tolerance of propionic acid bacteria.

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 responsible for the synthesis of by-products can be deleted to achieve this goal. However, this approach may have limitations because the consequences of gene deletion 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 limitation through the use of genome-wide high-throughput omics data and genome-scale computational analysis (Park and Lee, 2008). In systems metabolic engineering, targets are determined by considering entire metabolic and regulatory networks together with midstream (fermentation) and downstream (recovery and purification) processes. During the actual metabolic engineering, the impact of altering these targets on the entire metabolism is examined to provide feedback. 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 systems metabolic engineering is a successful case (Lee *et al.*, 2007). The feedback inhibition due to aspartokinase 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 mutating 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 culture (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 manipulated 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 regeneration rate of CoA and to improve PA productivity. The genes encoding phosphotransacetylase could be deleted to block the carbon flux from pyruvate to acetate.

Concluding remarks

Recently, microbial production of PA has drawn much attention due to an increasing desire for bio-based natural and green additives to foods and pharmaceuticals. The inhibition of PA on cell growth and the formation of by-products, such as succinic acid and acetic acid, are the two major factors limiting PA yield and productivity. The optimization of culture conditions and the development of novel culture methods, such as extractive fermentation and cell-immobilized fermentation, have been used for the improvement of PA production. The emergence of systems metabolic engineering will allow us to improve PA production more efficiently via increased acid tolerance and reduction of by-products at the molecular level.

Declaration of interest

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