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**Enhanced propionic acid production from whey lactose with  
immobilized *Propionibacterium acidipropionici* and role of trehalose  
synthesis in acid tolerance**

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## ABSTRACT

Whey lactose is a major by-product of the cheese and casein manufacturing industry. Utilization of this cheap and renewable resource is an area of interest among biotechnologists and bulk chemists. The objective of the present work was to determine the suitability of whey lactose for propionic acid production by a food microbe, *Propionibacterium acidipropionici*. Batch and fed-batch fermentations of whey lactose with both free cells and immobilized cells in a fibrous bed bioreactor (FBB) were examined and compared. It was observed that cells immobilized on polyethylenimine-treated Poraver (PEI-Poraver) in FBB favored higher productivity and yield of propionic acid as compared to the free-cell fermentation. Interestingly, *P. acidipropionici* accumulated high levels of trehalose, especially in response to acid stress. An analysis of the *P. acidipropionici* genome sequence revealed the presence of two putative trehalose biosynthesis pathways (OtsA-OtsB and TreY-TreZ), and their functions in response to acid stress were followed studied. To further improve propionic acid production, enhanced trehalose synthesis mutant by over-expression of *otsA* gene encoding enzyme belonging to OtsA-OtsB pathway was conducted. In this mutant, the maximum concentration of propionic acid reached  $135 \pm 6.5$  g/L in a fed-batch fermentation approach, which to our knowledge is among the highest propionic acid concentration ever produced in the traditional fermentation process. In view of these results it was concluded that the FBB fed-batch fermentation with enhanced trehalose synthesis mutant from whey lactose is therefore considered as a good candidate for the large-scale production of propionic acid in the near future.

KEYWORDS: *Propionibacterium acidipropionici*; propionic acid; whey lactose; trehalose synthesis; immobilization; acid tolerance

## 1. Introduction

Propionic acid is a 3-carbon short chain fatty acid with wide applications in food and dairy, pharmaceutical, cosmetic, agro-industries. It is chiefly used as a mold inhibitor for animal feeds and as a preservative for foods such as cheeses and baked goods.<sup>1</sup> In other fields, propionic acid can be used for animal therapy, and for the manufacture of perfume, herbicides, cellulose plastics, and flavoring agents.<sup>2</sup> Currently, almost all propionic acid is synthesized commercially via petrochemical routes, with an annual market demand of more than 400,000 tons in the world.<sup>3</sup> However, as the crude oil prices had surpassed US\$100 per barrel, and the demand by consumers and manufacturers for using bio-based natural ingredients in foods, cosmetics, and pharmaceuticals, there have been increasing interests in propionic acid production from microbial fermentation. *Propionibacteria* have been granted “Generally Recognized As Safe (GRAS)” status by the United States Food and Drug Administration (FDA).<sup>4</sup> Therefore, fermentation by *Propionibacteria* has a good potential for the production of natural propionic acid to satisfy the market demand, and also opens the door with a more sustainable approach for propionic acid manufacturing in the case with limited petroleum supply.

Several fermentation processes have been studied for bio-production of propionic acid by genus *Propionibacteria* from renewable and low-cost substrates, such as corn meal,<sup>5</sup> cane molasses,<sup>6</sup> Jerusalem artichoke hydrolysate,<sup>7</sup> flour hydrolysate,<sup>8</sup> and crude glycerol.<sup>9</sup> In general, carbon source is usually used in relatively higher concentration compared to other medium components and thus has high share in the raw material cost. Therefore, the continuous exploitation of cheap, renewable carbon sources has been strongly stimulated. Whey is a by-product of cheese and casein production and an inexpensive raw material which composes mainly of 4.5-5% (w/v) lactose, 0.6-0.8% (w/v) soluble proteins, 0.4-0.5% (w/v) lipids and mineral salts (8-10% of dried extract).<sup>10</sup> Much of whey is currently used as animal feed, fertilizer and as a food ingredient or additive.<sup>11</sup> Nevertheless, as much as 180 million tons of the annual world whey production remains underutilized.<sup>12</sup> Because whey is considered an environmentally high-strength wastewater pollutant due to its high biological oxygen

demand (~50,000 mg/L) and chemical oxygen demand (~80,000 mg/L) contents, disposal of whey can be costly.<sup>13</sup> Several other problems will also be encountered in whey disposal, including uneconomical transport due to its high water content and the difficulty of prolonged storage because of whey susceptibility to spoilage by microorganisms. Therefore, both environmental and economic concerns all demand that whey should be converted into value-added products. To our knowledge, whey lactose has only been used in a few cases to produce propionic acid by *Propionibacteria* so far.<sup>14</sup> However, the yield and productivity of propionic acid were not satisfying for the industrial application, resulting in difficulty in product recovery and decreased cost-effectiveness. It is thus of important to develop an effective fermentation technology for economical production of propionic acid.

Numerous attempts have been made to improve propionic acid fermentation, and resulted in the development of innovative culture technologies and mutant strains.<sup>15</sup> To date, the most promising bioprocess employs a fibrous-bed bioreactor (FBB) in a fed-batch cultivation mode with cells immobilized in cotton towel packed in the reactor, which has been demonstrated to successfully produce several organic acids (acetic acid, propionic acid, lactic acid, and butyric acid) with significantly improved reactor productivity, final product yield and concentration.<sup>5,16-18</sup> Despite the high final concentration obtained, the propionic acid fermentation was usually time-consuming (97.0 g/L in FBB at 2,000 h and 67.1 g/L in MFB at 496 h of fermentation),<sup>17,19</sup> which makes cotton fiber inefficient for the industrial production of propionic acid. In addition, cotton fiber has a small specific surface area and a low porosity, which not only reduces the number of immobilized cells on the fiber but also decreases its update rate. In the present work, production of propionic acid from whey lactose was investigated using cells of *Propionibacterium acidipropionici* immobilized on Poraver, one kind of porous recycled glass beads, treated with a cationic polymer, polyethylenimine.<sup>20</sup> Immobilized-cell bioreactors were operated in batch and fed-batch fermentation modes, respectively, to determine process efficiency and economics.

Trehalose is a non-reducing disaccharide (1-*O*- $\alpha$ -dglucopyranosyl- $\alpha$ -d-

glucopyranoside) naturally accumulated in microbes, insects, and plants. Trehalose has many advantages due to its inertness property and ability to stabilize biomolecules and production of this disaccharide is of commercial importance.<sup>21</sup> Initially, trehalose was thought to act solely as a reserve energy and carbon source in a manner similar to that of starch and glycogen, but a growing number of studies indicate that this sugar instead has important biological function of playing a major role in cell protection against various physical and chemical stresses, such as heat, cold, dehydration, desiccation, oxygen radicals, and acidic/alkali environmental conditions.<sup>22-24</sup> Moreover, the ability of the microorganisms to survive in these environments correlates with their trehalose content.<sup>25,26</sup> In recent years, trehalose was reported in cell extracts obtained from different species of the genus *Propionibacteria*, and the intracellular trehalose content increased considerably in response to osmotic, oxidative and acid stress.<sup>27-29</sup> Inspired by this observation, the second goal was to elucidate the trehalose metabolism and regulation in *P. acidipropionici*, as well as its contribution to the enhancement of propionic acid resistance during the immobilization process in the FBB. We anticipated that the accumulation of trehalose engineered by strain manipulation could be a good strategy to improve the stress resistance and allow extended fermentation.

## 2. Materials and methods

### Chemicals

Propionic acid, acetic acid, and succinic acid were all of analytical grade, and were purchased from Guoyao Reagent Co., Ltd. (Shanghai, China). Whey powder was obtained from Anhui Huayang Farming Co., Ltd. (Anhui province, China). Yeast extract and trypticase were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). Poraver® beads (6-8 mm diameter) were obtained from Germany marcom Co., Ltd (Beijing Office, China). Polyethylenimine (PEI, average Mw=25,000) and antibiotics were procured from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals of analytical grade were obtained from standard sources.

### Preparation of whey lactose

Two hundred grams of whey power (Spray dried powder containing lactose >65%

(w/v) and protein >11% (w/v)) was added to 100 mL of sodium phosphate buffer (pH 7.5) and mixed vigorously. The mixture was centrifuged at 12,000g for 20 min, and the supernatant was adjusted to pH 7.5. The mixture was centrifuged again at 12,000g for 20 min. The lactose concentration of the supernatant was approximately 20% (w/v) and the prepared lactose solution was stored at 4 °C for further use.<sup>30</sup>

### Strains and media

*Propionibacterium acidipropionici* CGMCC 1.2232 (equal to *Propionibacterium acidipropionici* ATCC 4875) was purchased from the China General Microbiological Culture Collection Center (CGMCC) and designated as the wild-type. *Escherichia coli* strains DH5 $\alpha$  and JM109 ( $\lambda$  *pir*) were used for the cloning, maintenance and propagation of plasmids. *E. coli* S17-1 ( $\lambda$  *pir*) was used to transfer plasmid DNA via conjugation to *P. acidipropionici*. The primers used in this work are displayed in Table S1 in the supplemental material.

*E. coli* strains were grown at 37 °C aerobically in Luria-Bertani (LB) medium. When necessary, antibiotics were added at the following final concentrations: 50  $\mu$ g/mL each for ampicillin, kanamycin, streptomycin and erythromycin, and 25  $\mu$ g/mL for spectinomycin. The stock culture of *P. acidipropionici* was kept in serum bottles under anaerobic conditions at 4 °C.<sup>31</sup> For *P. acidipropionici*, a detailed description of the culture medium has been given elsewhere.<sup>7</sup> Acid stress was imposed by the addition of 100 g/L propionic acid to the culture medium. The basal culture medium (without the carbon source) and the concentrated carbon source solution were sterilized separately by autoclaving at 121 °C and 15 psig for 20 min to avoid undesirable reactions.<sup>32</sup> They were mixed aseptically before use in the fermentation study.

### Construction of $\Delta$ *otsA*, $\Delta$ *treY* and $\Delta$ *otsA* $\Delta$ *treY* mutants of *P. acidipropionici*

Plasmid isolation, restriction enzyme digestion, DNA ligation, and the transformation of *E. coli* were performed as described by Sambrook and Russell.<sup>33</sup> The PCR primers used in this work are displayed in Table 1. An 1.0-kb DNA fragment with the 5' region of the *otsA* gene, which was generated using the *otsA*\_f1 primer and *otsA*\_r1 primer with *P. acidipropionici* DNA by the PCR reaction. This DNA fragment was

digested and ligated with XbaI-EcoRI digested pBluescriptII SK+ (Stratagene), resulting in pBOTSAL. An 1.1-kb DNA fragment with the 3' region of the *otsA* gene was generated using *otsA\_f2* primer and *otsA\_r2* primer. This DNA fragment was digested, ligated with EcoRI-HindIII digested pBOTSAL, resulting in pBOTSAIL. The 2.0-kb  $Sm^r$ / $Spc^r$   $\Omega$  cassette was inserted into the EcoRI site of pBOTSAIL to create pBOTSALW. It is flanked by short inverted repeats with the T4 transcription-termination signal. The 4.1-kb XbaI-KpnI restriction fragment from pBOTSALW was inserted into the  $Km^r$ -suicide vector pJP5603, which predigested with XbaI and KpnI, resulting in pJPOTSA. An 1.0 kb DNA fragment with the 5' region of the *treY* gene was generated using *treY\_f1* primer and *treY\_r1* primer by PCR reaction. Then it was digested, ligated with XbaI-EcoRI digested pBluescriptII SK+, resulting in pBTREYI. An 1.2-kb DNA fragment with the 3' region of the *treY* gene was generated using *treY\_f2* primer and *treY\_r2* primer. This fragment was digested and then ligated with digested pBTREYI by the EcoRI-HindIII, resulting in pBTREYII. The  $Sm^r$  / $Spc^r$   $\Omega$  cassette was inserted into the *EcoRI* site of the pBTREYII to create pBTREYW. The 4.2-kb XbaI-it KpnI restriction fragment from pBTREYW was inserted into the XbaI and KpnI sites of pJP5603 to create pJPTREY. Then the plasmid was transferred into *P. acidipropionici* cells by conjugation with mobilizing strain *E. coli* S17-1 ( $\lambda$  *pir*).  $Spc^r$   $Km^s$  cells were selected as double-crossover candidates, and the chromosomal insertion was confirmed by PCR amplification and DNA Sequencing. To generate strains carrying double gene deletion, single deletion strain was used instead of the wild-type. A two-step recombination system was used for inactivating the trehalose biosynthesis genes of *P. acidipropionici* to make double mutant strain  $\Delta$ *otsA* $\Delta$ *treY* described elsewhere.<sup>34</sup>

#### **Construction of over-expression mutant of *P. acidipropionici***

To overexpress *otsA* gene in OtsA-OtsB pathway, the 1.82-kb full-length *otsA* gene from *P. acidipropionici* CGMCC 1.2232 was amplified by PCR using genomic DNA as the template and the oligonucleotide primers *otsA\_f* (5'-ATATGATCAATGTCCGAACCCGTTTCAGG-3') and *otsA\_r* (5'-TATCCGCGGTCACCTCGCCCTCCAGCTGGT-3'). The PCR product was ligated



into the pGEM-T Easy vector to create pGEM-*otsA*EX. The pGEM-*otsA*EX was subsequently digested with BclI and SacII, and the 1.82-kb fragment containing *otsA* was inserted into the BclI and SstII sites of the shuttle vector pBRESP36A, yielding plasmid pBRESP36A-*otsA*. Expression of genes inserted into this vector was constitutively with the Ery<sup>r</sup> and Amp<sup>r</sup> genes.<sup>35</sup>

#### **Fermentation kinetic studies with fibrous bed bioreactor**

The fermentation system consisted of a 5 L stirred-tank fermentor (B. Braun, B. Braun Biotech International, Melsungen, Germany) connected to a 0.5 L FBB through a recirculation loop and operated at 32 °C, agitated at 150 rpm, and pH controlled at 6.0 by adding NH<sub>4</sub>·OH facilitated by an on-line sensing and dosing system.<sup>36</sup> The immobilization matrices of Poraver coated with PEI were prepared as described elsewhere.<sup>20</sup> The batch and fed-batch mode was started with 2 L of fresh medium, and the fed-batch mode was followed with pulse feeding concentrated whey lactose whenever its concentration in the fermentation broth was close to zero. The results shown are the mean of analyses performed in duplicates for all the fermentations.

#### **Trehalose determination**

To determine the intracellular trehalose level in *P. acidipropionici*, 10 mL of broth from the culture medium was collected at regular of time and centrifuged at 16,000g for 10 min at 4 °C. A few grams of pellets obtained were then extracted with 80% ethanol (5 ml per 1 g wet weight), suspended and homogenized well. The suspension was boiled at 100 °C for 5 min and centrifuged at 16,000g for 10 min. The supernatant was filtered with a Minisart-RC 4 filter (Sartorius) and added with phosphate buffer (100 mM, pH 5.7) to make the final volume to 1 mL. Trehalose was established by assaying the release of free glucose following incubation of samples with trehalase (EC 3.2.1.28; Sigma-Aldrich).<sup>37</sup> Samples for analysis (100 μL) were added to 100 μL phosphate buffer (100 mM, pH 5.7), 800 μL water and 10 μL trehalase, then incubated at 37 °C for 1 h and stopped by incubation in boiling water for 5 min. Glucose was then measured by using a HPLC system as described below.

#### **Analysis Methods**

Cell growth was analyzed by measuring the optical density of the cell suspension at a

wavelength of 600 nm ( $OD_{600}$ ) with a spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience) after an appropriate dilution. Dry cell weight (DCW) was calculated from the OD value by using a correlated equation ( $DCW \text{ (g/L)} = 0.64 \times OD_{600}$ ).<sup>36</sup> Quantitative analysis of lactose, glucose and organic acid products (propionic, acetic, and succinic acids) in the fermentation broth was performed by using a HPLC system equipped with an organic acid column (Bio-Rad, HPX-87H). The HPLC analysis was operated at 45 °C using 5 mM  $H_2SO_4$  as the eluant, with a flow rate of 0.6 mL/min. All results were obtained from the means of triplicate determinations.

### 3. Results and discussion

*P. acidipropionici* was chosen as a candidate for propionic acid fermentation based on earlier reports which showed the bacterium to provide the higher conversion yield and productivity among the *Propionibacteria* spp. investigated,<sup>7,19,38</sup> including fermentation on a semi-industrial scale (10 m<sup>3</sup>),<sup>39</sup> as well as the available genome sequence.<sup>31</sup> Lactose from cheese whey is superior to other carbon sources because of its low market price with comparable level of propionic acid yield. Theoretically, its metabolism yields 0.79 gram propionic acid per gram of lactose; however, under experimental conditions, the yield ranges from 0.40 to 0.70 g/g lactose with succinic acid and acetic acid as the major by-product.<sup>40</sup>

#### Batch fermentation of whey lactose with free cells

The kinetics of propionic acid batch fermentation of whey lactose using free *P. acidipropionici* cells was illustrated in Fig. 1. Lactose (60 g/L) was entirely consumed in  $132 \pm 10$  h yielding  $27 \pm 1.6$  g/L propionic acid at a productivity of  $\sim 0.20$  g/(L h). Total organic acids yield from lactose was 0.63 g/g, 70.7% (g/g) of which was propionic acid. As shown in Fig. 1, the free-cell fermentation was characterized by a very long lag phase of about 25 h, and production of organic acids were minimal in this period but increased afterward and reached the peak values. However, when the propionic acid concentration reached up to 20 g/L, the growth rate approximated to zero, cells were still metabolically active and continued to produce propionic acid at a lower rate.<sup>7</sup> As a weak organic acid, the non-dissociated propionic acid can pass

through cell membrane into cytoplasm and release protons due to the intracellular alkaline environment that results in the disturbance of the pH gradient across the cell membrane and affects the nutrients transfer and inhibits cell growth.<sup>17</sup>

#### **Batch fermentation with cell immobilized on PEI-Poraver**

Poraver is a trade name for foamed highly porous recycled glass beads, which has been studied previously in biofilm reactor.<sup>41</sup> Its surface is covered by numerous macropores which increase the surface area and support cell attachment and settlement. However, native Poraver beads did not exhibit significant adsorption of microorganisms even after a few days incubation with the cells (data not shown). Based on adsorption studies, cell adsorption using the charged cationic polymer polyethylenimine (PEI) is an extremely simple and straightforward procedure resulting in the negatively charged cells becoming firmly attached to the matrix. Cell immobilization was performed using the procedure described earlier by us for adsorbing *Clostridium tyrobutyricum* on PEI coated cotton fiber,<sup>42</sup> with the exception that the cells were grown to high density in the reactor vessel before circulation through the column packed with the matrix. This helped to overcome the inhibitory effect of PEI exerted on cell growth and shorten the immobilization period.

As a result, the batch fermentation of whey lactose with immobilized cells on PEI-Poraver in FBB was illustrated in Fig. 2. Compared with those under free-cell fermentation conditions (Fig. 1), bacteria immobilized on PEI-Poraver grew faster and exhibited more lactose consumption, a shorter fermentation period, and a faster rate of propionic acid formation in the FBB. With an initial lactose concentration of 60 g/L, the maximum propionic acid concentration reached  $32.4 \pm 1.4$  g/L at  $58 \pm 4$  h in the FBB (productivity  $\sim 0.56$  g/(L h)), which showed that whey lactose could be efficiently metabolized by immobilized *P. acidipropionici*, in accordance with the previous reports.<sup>43,44</sup> A comparable observation was also recorded by Coral et al.<sup>45</sup> in processes with both glycerol and lactose as culture media carbon sources. At  $\sim 130$  h of batch fermentation lactose introduced with medium was exhausted while half of glycerol initial amount remained. Moreover, the amount of organic acid by-products of acetic acid and succinic acid were also reduced in the FBB, which could be benefic

to the separation and purification of propionic acid in the industrial production.

#### **Fed-batch fermentation with immobilized cells**

The use of concentrated lactose solutions (up to 200 g/L) is important in an actual industrial production process since it will permit high propionic acid titres (up to 10-15% v/v) at the end of fermentation, therefore further reducing considerably the propionic acid distillation costs. However, natural lactose-fermenting *Propionibacteria* cannot ferment efficiently (i.e., rapidly and with high conversion yields) such high concentrations of lactose. Since fed-batch fermentation can alleviate the inhibition of high sugar concentration and minimize the loss of remaining substrate, but maximize the final concentration and yield of a target product, it is often favored over batch fermentation. Based on the previous findings, fed-batch fermentation approach with immobilized cells on PEI-Poraver was performed hereby to improve the final concentration of propionic acid from whey lactose (Fig. 3). As can be seen in Fig. 3, the *Propionibacteria* grew exponentially in the first fed-batch and then entered the stationary phase in the middle of the second batch. It continued to produce propionic acid until the final concentration reached  $125 \pm 9.6$  g/L within the whole period of ~250 h, which was much higher than that obtained in the batch fermentation ( $32.4 \pm 1.4$  g/L). Another further positive effect of fed-batch supply was the higher selectivity of propionic acid production discussed above, which is, the ratio of propionic acid to total organic acids (g/g) was increased from batch culture to fed-batch culture (82.5% vs. 78.1%), a clear indication that the metabolic pathway in the bacterial had been shifted. Chen et al has proved through metabolic flux analysis that the fluxes of propionic acid synthesis and the pentose phosphate pathway in immobilized-cell fermentation increased by 84.7% and 228% compared with free cell fermentation, as well as the a decrease in succinic and acetic acid fluxes.<sup>46</sup> However, the productivity decreased dramatically at the propionic acid concentration higher than 100 g/L, and at the end of fermentation productivity reached 0.50 g/(L h), which decreased by 10.7% compared with the batch fermentation, indicating a strong product inhibition at this level (Table 2).

The common defence strategy of microbes against environmental stresses includes

accumulation of several organic compatible solutes.<sup>47</sup> Realizing the importance of trehalose as a well know compatible solute accumulated in microbes, it is not surprising to find that the cells accumulated abundant trehalose in response to increased propionic acid concentration in the culture medium (Fig. 1 and 2), especially upto 100 g/L (Fig. 3). As can be seen in Fig. 3, the maximum trehalose yield with respect to biomass was  $423 \pm 23$  mg/g, which was more than 3 times higher than that obtained with pure glycerol ( $128 \pm 3$  mg/g) by genus *Propionibacteria*.<sup>9</sup> Thus it can be predicted that the presence of other certain impurities in whey except for lactose are probably responsible for the enhancement of trehalose production.

Previously, enhanced trehalose yield under environmental stress (e.g., osmotic stress) was reported in microbes, including *P. freudenreichii*, *E. coli*, *C. glutamicum* and yeast.<sup>27,48-50</sup> Further experiments were thus conducted to search the genome sequence of *P. acidipropionici* with homology to genes involved in trehalose biosynthesis in other bacteria, and constructed mutants by using gene inactivation and over-expression of various enzymes in trehalose biosynthesis metabolic pathway to compare their phenotypes and regulatory properties under acid stress with those of the wild-type strain.

#### **Trehalose synthesis pathway in *P. acidipropionici***

The availability of complete genome sequence data of *P. acidipropionici* CGMCC 1.2232 ([http://www.ncbi.nlm.nih.gov/genome/13350?project\\_id=179069](http://www.ncbi.nlm.nih.gov/genome/13350?project_id=179069)) makes it possible to screened for the presence of ORFs sharing homology with genes known to be involved in trehalose biosynthesis in other bacteria. The ORFs with high similarity to four genes involved in two different pathways were found in *P. acidipropionici* and the results are summarized in Table 3. A BLAST search based on the known *E. coli* OtsA-OtsB pathway genes identified ORFs PACID18560 and PACID18570 as a homologue of trehalose 6-phosphate synthase (OtsA) and trehalose 6-phosphate phosphatase (OtsB) as having 36% and 31% overall identity at amino acid level respectively. The ORFs PACID05880 and PACID05850 were identified as treY and treZ, respectively, because they have 39% and 46% identity with the *Rhizobium* sp. TreY-TreZ pathway genes.

In order to characterize the role of two distinct trehalose-biosynthesis pathways proposed from genome sequence data analysis in *P. acidipropionici*, two mutants were constructed by chromosomal inactivation of one gene of each pathway, and named  $\Delta otsA$  and  $\Delta treY$  respectively (Fig. 4). Based on the single mutants, the combination of double mutant named  $\Delta otsA\Delta treY$  was constructed by the two-step recombination system. Also, over-expression was carried out via replicative plasmid pGEM-T containing *otsA* gene in OtsA-OtsB pathway from *P. acidipropionici*. First, we measured the intracellular trehalose level among *P. acidipropionici* wild-type and trehalose biosynthesis gene mutants under acid stress with culture media containing 100 g/L propionic acid. As can be seen in Table 4, the  $\Delta otsA$  mutant showed a significant decrease in the intracellular trehalose level in comparison with the wild-type strain ( $95 \pm 10$  vs.  $311 \pm 17$  mg/g biomass), while the level in the  $\Delta treY$  mutant also decreased to some extent ( $230 \pm 12$  vs.  $311 \pm 17$  mg/g biomass). The results led to the proposal that trehalose synthesis in *P. acidipropionici* cells responded to acid stress predominantly depend on the OtsA-OtsB pathway and only marginally on the TreY-TreZ pathway, which were in accordance with those in *R. sphaeroides*, but inconsistent with those in *C. glutamicum*.<sup>34,48</sup> For example, in the later organism, it has been shown that the mutant with a deletion of *otsA* gene accumulates levels of trehalose comparable with those of the wild-type, with the synthesis proceeding via the TreY-TreZ pathway, which revealed an enormous disparity in the function of trehalose synthesis pathways. However, there was no detectable intracellular trehalose in the  $\Delta otsA\Delta treY$  mutant strain, and experiment with *otsA* over-expression mutant strain found a significant increase in the intracellular trehalose level (upto  $566 \pm 29$  mg/g biomass), which was 1.8 times higher than the value in wide-type strain.

We next examined the growth characteristics under acid-stressed conditions (100 g/L propionic acid), and the significant difference in growth curve of strains was observed (Fig. 5). As can be seen in Fig. 5, the growth was slightly compromised in both  $\Delta otsA$  and *otsA* over-expression mutant strain and strongly compromised in  $\Delta otsA\Delta treY$  mutant strain compared to the wild-type strain. A wild-type strain reached

the maximum DCW about 8.06 g/L, while  $\Delta otsA$  and *otsA* over-expression mutant strain reached the maximum DCW about 6.78 g/L and 8.11 g/L, respectively. Additionally,  $\Delta otsA\Delta treY$  mutant strain reached the maximum DCW about 4.85 g/L. The results showed that the acid-stressed condition showed a significant growth suppression in  $\Delta otsA\Delta treY$  mutant strain compared to the wild-type strain. However, there was a recovery of acid tolerance in  $\Delta otsA\Delta treY$  mutant strain when adding 10 mM trehalose to the acid-containing-medium (Fig. 5), and no remarkable growth difference was observed between wild-type and  $\Delta otsA\Delta treY$  mutant strain under this condition, which indicates that the growth suppression was caused by inability of trehalose synthesis. Previous researches have revealed that accumulation of constitutive trehalose in *E. coli* under stress conditions is thought to contribute to maintenance of cell integrity and protein stability.<sup>50</sup> Consistent with such a role, the presence of free trehalose in apparently acid-stressed *Propionibacteria* may likewise have the same protective function.

#### **Production of propionic acid with enhanced trehalose synthesis**

Concerning the growth profiles between wild-type and mutants, as well as the finding that the OtsA-OtsB pathway is the major route for trehalose synthesis under acid stress, *otsA* over-expression mutant strain with enhanced trehalose synthesis was further used to improve propionic acid production. Fig. 6 and 7 showed the free-cell batch and immobilized-cell fed-batch fermentation kinetics with the *otsA* over-expression mutant, respectively. The results were further compared with the wide-type strain listed in Table 2. As can be seen in Fig. 6, by-products acetic acid and succinic acid yield in *otsA* over-expression mutant declined very slightly in contrast to the wild-type in free-cell fermentation process. In contrast, the propionic acid yield with the mutant was 11% higher ( $0.50 \pm 0.041$  vs.  $0.45 \pm 0.028$ ) than that with wide-type strain. In the immobilized-cell fermentation mode shown in Fig. 7, the *otsA* over-expression mutant strain produced 21% less acetic acid ( $0.058 \pm 0.010$  vs.  $0.074 \pm 0.007$  g/g) and 22% less succinic acid ( $0.046 \pm 0.010$  vs.  $0.059 \pm 0.007$  g/g) than the wide-type strain. In contrast, the propionic acid yield with the *otsA* over-expression mutant strain was 11% higher ( $0.50 \pm 0.041$  vs.  $0.45 \pm 0.028$ ) than



that with wide-type strain. However, over-expression mutant had a more higher propionic acid production performance in FBB fed-batch fermentation mode even in the first batch. The maximum concentration of propionic acid reached  $135 \pm 6.5$  g/L with a corresponding productivity and yield of  $0.61 \pm 0.10$  g/(L h) and  $0.67 \pm 0.067$  g/g lactose, respectively, which to our knowledge is among the highest propionic acid concentration ever produced in the traditional fermentation process. So far, the maximum propionic acid concentration previously produced in fermentation was  $136.23 \pm 6.77$  g/L by *P. freudenreichii* CCTCC M207015 with glucose as the substrate.<sup>46</sup> Zhang and Yang (2009) also reported that 97 g/L of propionic acid was produced from glucose by immobilized cells of *P. acidipropionici* in fed-batch fermentation.<sup>17</sup> The higher propionic acid concentration obtained in these fed-batch immobilized fermentations indicated that the bacteria in the FBB had adapted and acquired a higher tolerance to propionic acid.

Herein we showed that the enhancement of the OtsA-OtsB pathway in *P. acidipropionici* was a response common to high acid concentration stress imposed. As expected, *P. acidipropionici* accumulated intracellular trehalose to remarkable levels 20 hour in advance as compared to the wide-type strain. As shown in Fig. 7, its content increased considerably in response to acid accumulation, and the highest yield based on biomass consumed was improved 1.8 times than that in wide-type strain ( $764 \pm 47$  mg/g vs.  $423 \pm 14$  mg/g). Recent studies have demonstrated the ability of trehalose in cell protection against diverse stresses, such as osmotic stress, oxidative stress, heat stress as well as acid stress.<sup>51</sup> It is conceivable that trehalose plays a role in the stabilization of cell membranes, which must withstand a strong pH gradient in such acidic environments. In addition, it also can be seen in Fig. 7 that there was a slow decrease in trehalose concentration with growth in late stationary phase, which maybe ascribed to the degradation of trehalose by hydrolytic enzymes. However, trehalase, trehalose phosphorylase, trehalose-6-phosphate phosphorylase and trehalose-6-phosphate hydrolase identified in other organisms involved in trehalose degradation are all absent in the genome sequence of *P. acidipropionici*, indicating a new catabolism pathway of trehalose in this food microbe.



#### 4. Conclusions

Herein, we demonstrated the advantages of using immobilized cells for propionic acid fermentation from whey lactose to achieve the desired improvement in fermentation efficiency. In particular, immobilization on PEI-Poraver considerably enhanced propionic acid properties with both final propionic acid productivity ( $0.56 \pm 0.075$  g/(L h) vs.  $0.29 \pm 0.034$  g/(L h) with free-cell fermentation) and yield ( $0.54 \pm 0.022$  g/g vs.  $0.45 \pm 0.028$  g/g with free-cell fermentation). In addition, *P. acidipropionici* accumulated intracellular trehalose to remarkable levels, and its content increased considerably in response to acid accumulation. Two pathways (OtsA-OtsB, TreY-TreZ) to synthesize trehalose were identified in *P. acidipropionici*, and their functions were then studied by characterizing strains defective in individual trehalose biosynthetic routes. A phenotypic comparison revealed that trehalose synthesis in *P. acidipropionici* is mediated mainly by the OtsA-OtsB pathway and, to some extent, by the TreY-TreZ pathway. Strains with the simultaneous inactivation of *otsA* and *treY* gene belonging to these two pathways were completely unable to synthesize trehalose. On the other hand, mutant strain with over-expression of *otsA* gene could significantly increase the synthesis of trehalose up to  $764 \pm 47$  mg/g biomass, which then promoted the production of propionic acid. The highest propionic acid concentration of  $135 \pm 6.5$  g/L was superior to those reported earlier in either batch or fed-batch modes of operation with equivalent productivities. To further improve the final concentration of propionic acid, a deeper and global profile of the trehalose assimilation and metabolism in *P. acidipropionici* should be performed in further trials.

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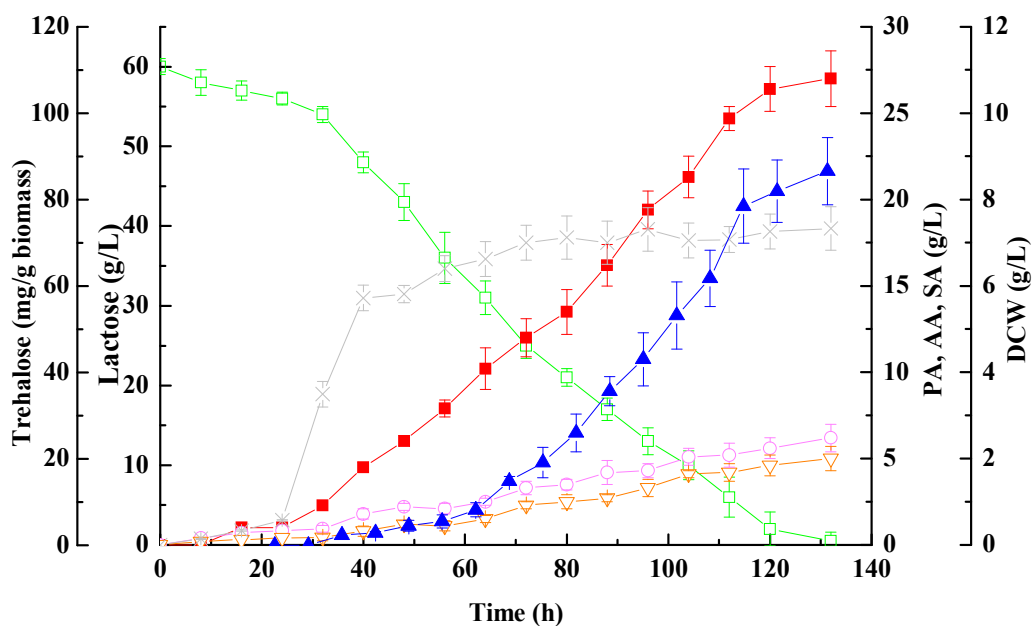
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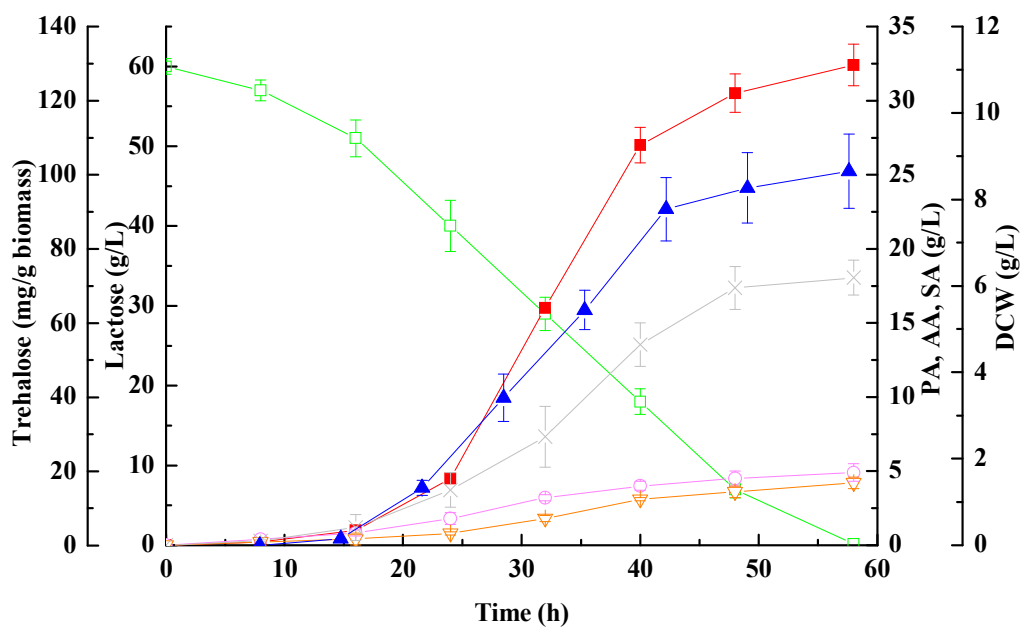
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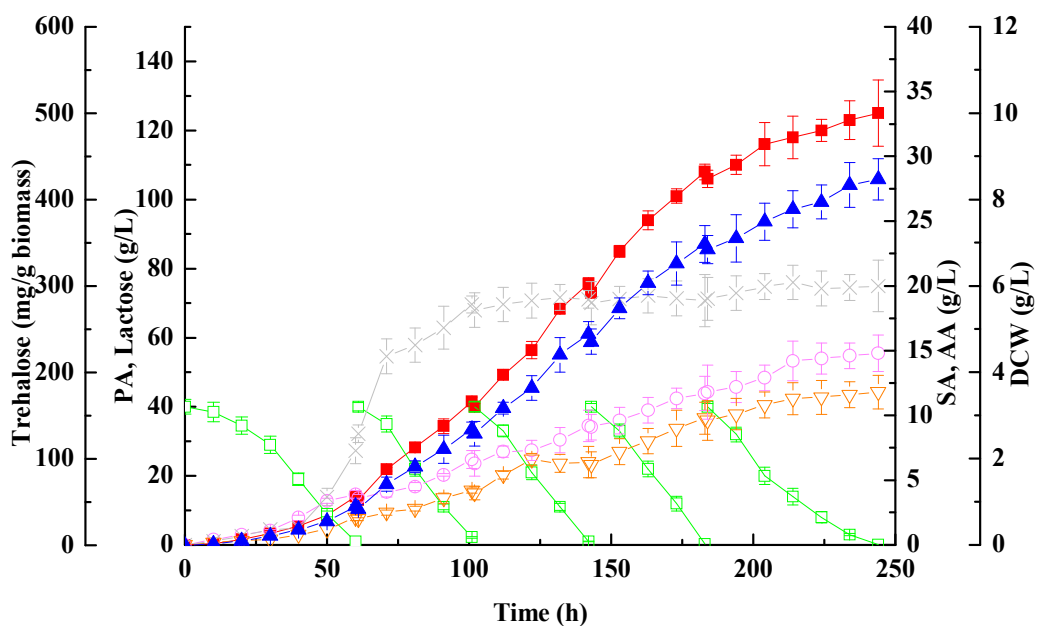
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**Fig. 1.** Kinetics of propionic acid batch fermentation of whey lactose using free *P. acidipropionici* cells of wide-type strain. Propionic acid, ■; Acetic acid, ○; Succinic acid, ▼; Lactose, □; DCW, ×; Trehalose, ▲.

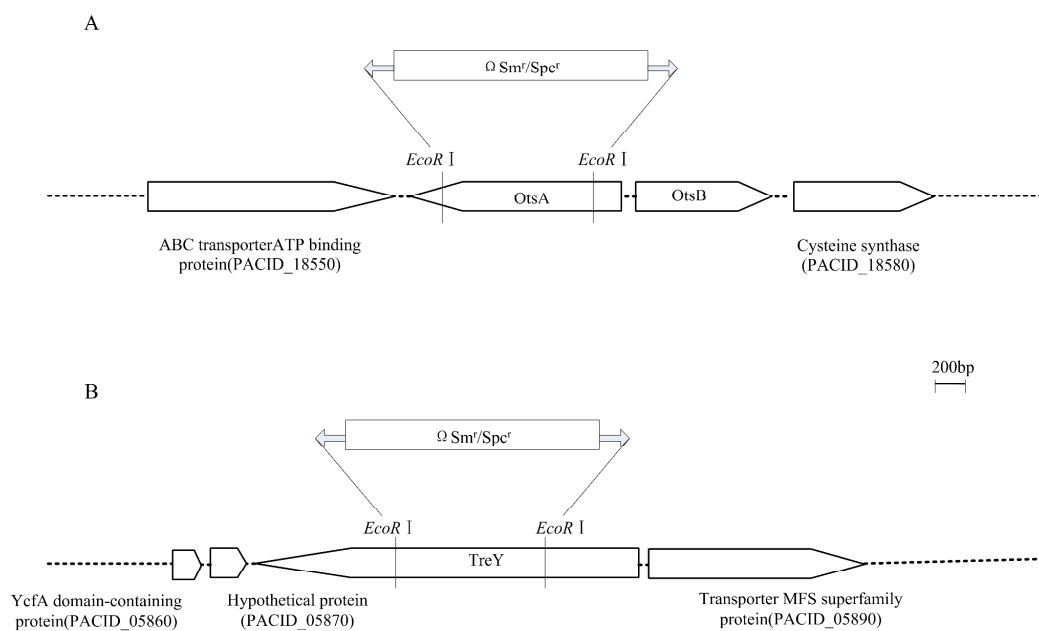


**Fig. 2.** Kinetics of propionic acid batch fermentation of whey lactose with immobilized wide-type strain on PEI-Poraver in FBB. Propionic acid, ■; Acetic acid, ○; Succinic acid, ▼; Lactose, □; DCW, ×; Trehalose, ▲.

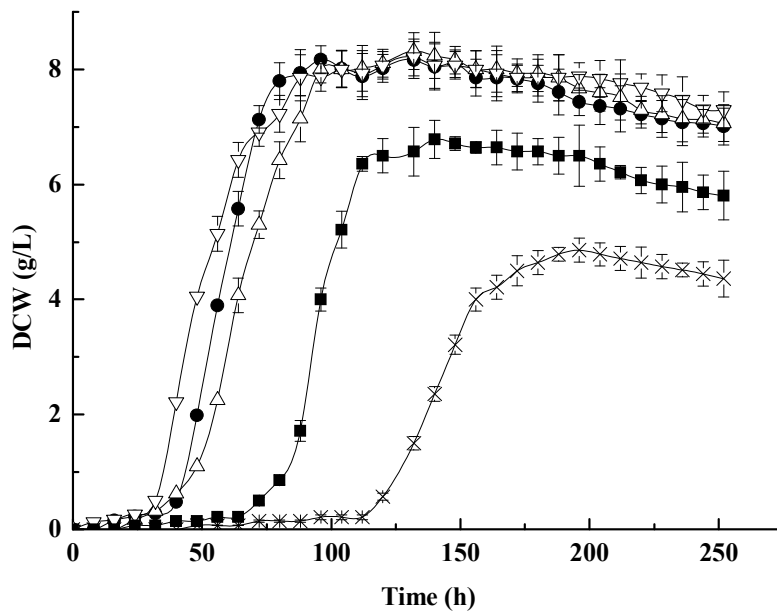


**Fig. 3.** Kinetics of propionic acid fed-batch fermentation of whey lactose with immobilized wide-type strain on PEI-Poraver in FBB. Propionic acid, ■; Acetic acid, ○; Succinic acid, ▼; Lactose, □; DCW, ×; Trehalose, ▲.

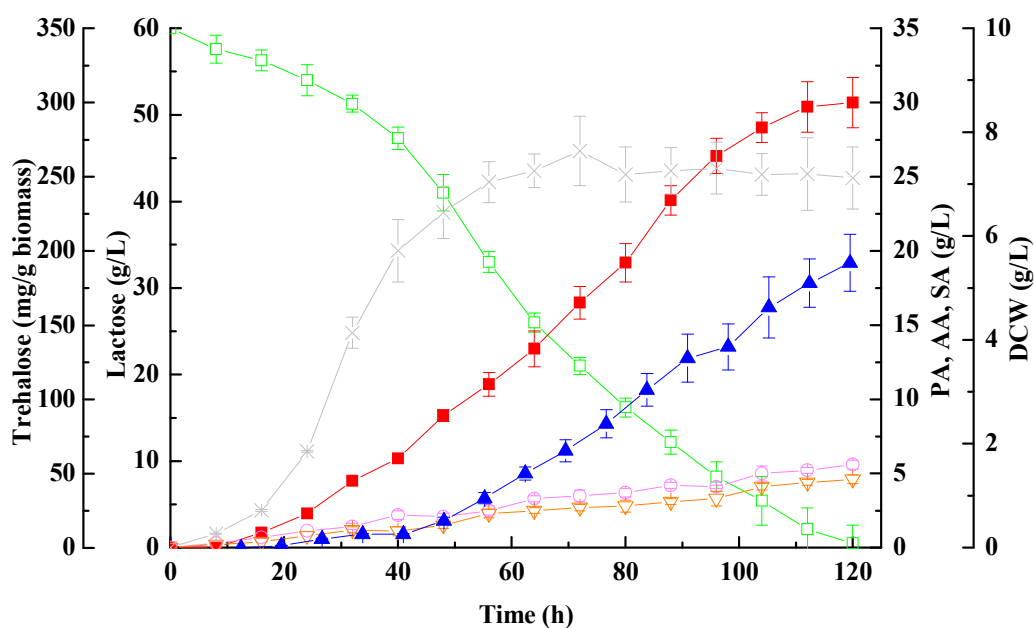




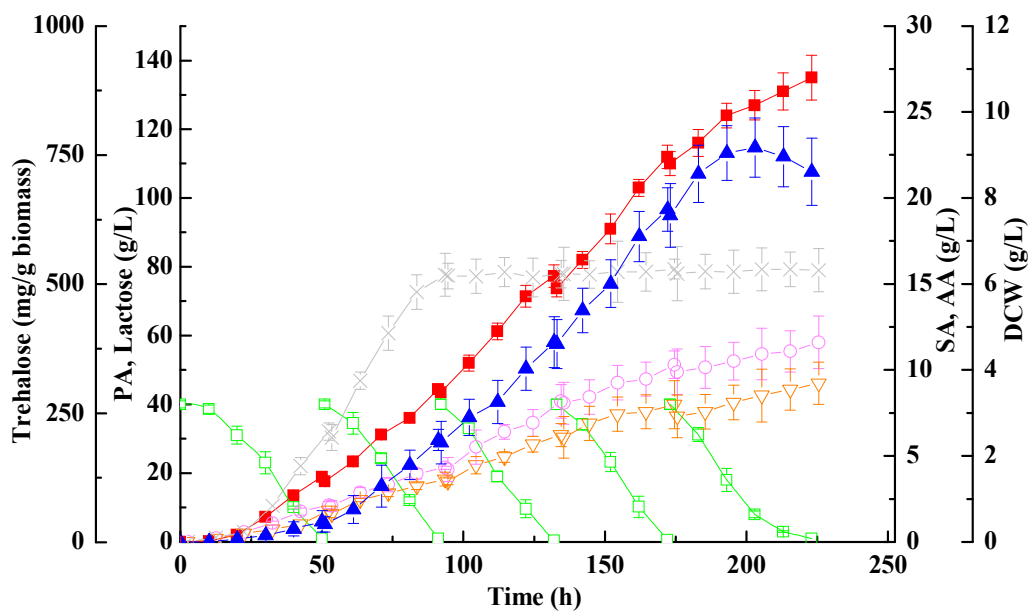
**Fig. 4.** Physical and genetic maps of *P. acidipropionici* *otsA* (A) and *treY* (B) regions. ORFs and their directions of transcription are represented by open arrows. Insertion of the Sm<sup>r</sup>/Sp<sup>c</sup>r<sup>f</sup>Ω is indicated. The constructions of double mutant is described in Materials and methods.



**Fig. 5.** Growth profiles of *P. acidipropionici* wild-type and trehalose biosynthesis gene mutants strains under acid-stressed conditions (100 g/L propionic acid). Wild-type strain, ●;  $\Delta otsA\Delta treY$  mutant strain+10 mM trehalose, △; *otsA* over-expression mutant strain, ▽;  $\Delta otsA$  mutant strain, ■;  $\Delta otsA\Delta treY$  mutant strain, ×.

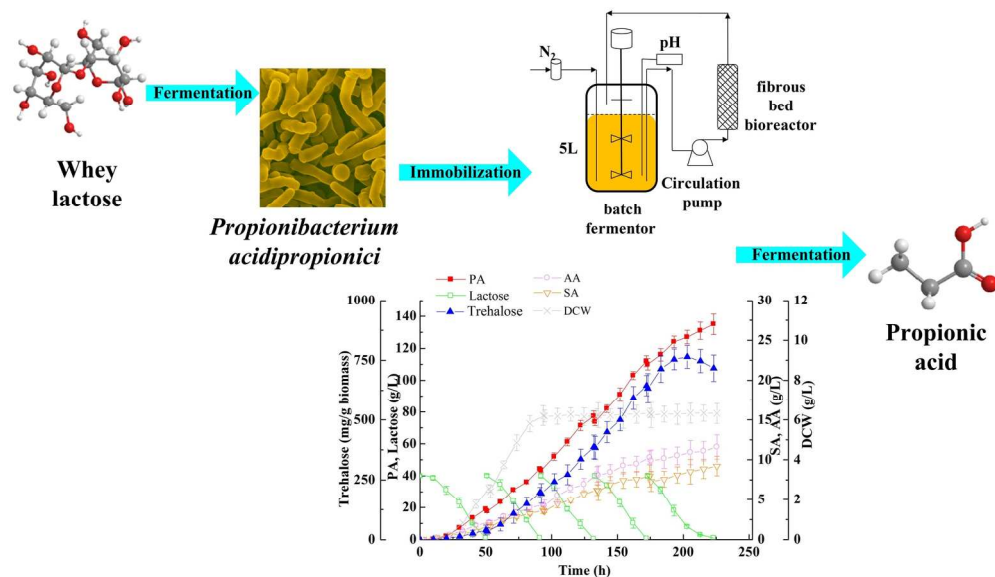


**Fig. 6.** Kinetics of propionic acid batch fermentation of whey lactose using free cells of *otsA*-overexpression mutant strain. Propionic acid, ■; Acetic acid, ○; Succinic acid, ▼; Lactose, □; DCW, ×; Trehalose, ▲.



**Fig. 7.** Kinetics of propionic acid fed-batch fermentation of whey lactose with immobilized *otsA*-overexpression mutant strain on PEI-Poraver in FBB. Propionic acid, ■; Acetic acid, ○; Succinic acid, ▼; Lactose, □; DCW, ×; Trehalose, ▲.

High propionic acid production could be performed with enhanced trehalose synthesis mutant immobilized on PEI-Poraver in FBB fed-batch fermentation.



170x100mm (300 x 300 DPI)

Table S1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Source or reference
<i>Propionibacterium acidipropionici</i> 1.2232	Wild type	China General Microbiological Culture Collection Center
$\Delta otsA$	$\Delta otsA::\Omega$ : Wild type with insertion of $\Omega$ cassette and deletion in the <i>otsA</i> ORF	This work
$\Delta treY$	$\Delta treY::\Omega$ : Wild type with insertion of $\Omega$ cassette and deletion in the <i>treY</i> ORF	This work
$\Delta otsA\Delta treY$	$\Delta otsA::\Omega/\Delta treY$ : Wild type with deletion in the <i>otsA</i> and <i>treY</i> ORFs and insertion of $\Omega$ cassette in the <i>otsA</i> ORF	This work
<i>E. coli</i>		
DH5 $\alpha$	<i>supE44\Delta lacU169</i> ( $\Phi 80$ <i>lacZ\Delta M15</i> ) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> $\lambda^-$	Vazyme Biotech (Nanjing) Co., Ltd.
JM109	<i>endA1 recA1 gyrA96 hsdR17 supE44\lambda^- \Delta (lac-proAB)/F'</i> [ <i>tra\Delta 36 proAB</i> <sup>+</sup> <i>lacZ\Delta M15</i> ]	Vazyme Biotech (Nanjing) Co., Ltd.
JM109 ( $\lambda$ <i>pir</i> )	JM109 lysogenized with $\lambda$ <i>pir</i> bacteriophage	Vazyme Biotech (Nanjing) Co., Ltd.
S17-1	Tp <sup>r</sup> Sm <sup>r</sup> <i>hsdR pro recA</i> RP4-2-Tc::Mu-Km::Tn 7 in chromosome	Vazyme Biotech (Nanjing) Co., Ltd.
S17-1 ( $\lambda$ <i>pir</i> )	S17-1 lysogenized with $\lambda$ <i>pir</i> bacteriophage	Vazyme Biotech (Nanjing) Co., Ltd.
Plasmid		
pBluescript II SK+	Ap <sup>r</sup> ; cloning vector	Stratagene
pTK106	Ap <sup>r</sup> Sm <sup>r</sup> /Spc <sup>r</sup> ; source of the Sm <sup>r</sup> /Spc <sup>r</sup> gene ( $\Omega$ cassette)	Prentki and Krisch (1984)
pJP5603	Km <sup>r</sup> ; R6K-based suicide vector	Kai M. Thormann et al. (2004)
pHSGSKm	Cm <sup>r</sup> Km <sup>r</sup> ; pHSG396 carrying the Km <sup>r</sup> gene and <i>sacRsacB</i>	Masuda et al. (2002)
pJPMobsac	Km <sup>r</sup> ; R6K-based suicide vector carrying <i>sacRsacB</i>	This work
pBOTSA I	pBII with PCR fragment containing upstream of <i>otsA</i>	This work
pBOTSA II	pBII with PCR fragments containing upstream and downstream of <i>otsA</i>	This work

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pBOTSAX	pBOTSAX II with $\Omega$ cassette insertion between PCR fragments	This work
pJPOTSA	pJP5603 carrying PCR fragments of <i>otsA</i> with $\Omega$ cassette insertion	This work
pBTREY I	pBII with PCR fragment containing upstream of <i>treY</i>	This work
pBTREY II	pBII with PCR fragments containing upstream and downstream of <i>treY</i>	This work
pBTREYX	pBOTSAX II with $\Omega$ cassette insertion between PCR fragments	This work
pJPTREY	pJP5603 carrying PCR fragments of <i>treY</i> with $\Omega$ cassette insertion	This work
pJ <b>P</b> <i>mobsac</i> TREY	pJ <b>P</b> <i>mobsac</i> carrying PCR fragments of <i>treY</i>	This work

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