Metabolic flux analysis of simultaneous production of vitamin B_{12} and propionic acid in a coupled fermentation process by *Propionibacterium freudenreichii*

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Abstract

The metabolic processes involved in simultaneous production of vitamin B_{12} and propionic acid by *Propionibacterium freudenreichii* are very complicated. To further investigate the regulatory mechanisms of this metabolism, a simplified metabolic network was established. The effects of glucose feeding, propionic acid removal, and 5,6-dimethylbenzimidazole (DMB) addition on the metabolic flux distribution were investigated. The results showed that synthesis of propionic acid can be increased by increasing the metabolic flux through the oxaloacetate and methylmalonyl-CoA branches in the early and middle stages of the coupled fermentation. After DMB addition, the synthesis of vitamin B_{12} was significantly enhanced via increased metabolic flux through the δ-aminolevulinate branch, which promoted the synthesis of uroporphyrinogen III, a precursor of vitamin B_{12}. Therefore, the analysis of metabolic flux at key nodes can provide theoretical guidance for the optimization of *P. freudenreichii* fermentation processes. In an experimental coupled fermentation process, the concentrations of vitamin B_{12} and propionic acid reached 21.6 and 50.12 g/L respectively, increased by 105.71% and 73.91% compared with batch fermentation, which provides a new strategy for industrial production.

Introduction

Vitamin B_{12} (cobalamin) is a complex cobalt-containing cyclic tetrapyrrole coenzyme that is widely used in the pharmaceutical and food industries. The industrial production of vitamin B_{12} by chemical methods is very difficult, complicated and expensive, with > 70 steps. Therefore, the production of vitamin B_{12} is exclusively preformed using aerobic and anaerobic fermentation by microorganisms; vitamin B_{12} synthesis involves > 30 genes [1]. The main strains used for industrial production of vitamin B_{12} are *Pseudomonas denitrificans*, *Propionibacterium freudenreichii*, and *Propionibacterium shermanii* [2].

Propionic acid is an important chemical intermediate, and among the top 30 candidate platform chemicals according to the US Department of Energy [3]. Propionic acid and its salts can be used as antibacterial agents, anti-inflammatory drugs, herbicides, food preservatives, and artificial foods [4]. Industrially, propionic acid is produced from petroleum-based chemicals via the hydrocarboxylation of ethylene, which causes substantial damage to the environment. As an alternative, renewable route, the preparation of propionic acid or its salts by microbial fermentation has been investigated extensively and is considered a safe and pollution-free process [5].

P. freudenreichii is a “generally recognized as safe” bacterial strain. However, the production of vitamin B_{12} by anaerobic fermentation suffers from formation of byproducts such as propionic acid, acetic acid, and succinic acid. In the traditional batch fermentation process, the concentration of propionic acid can reach 20–30 g/L, which causes feedback inhibition of cell growth [6]. Considerable effort has been made to improve the fermentation efficiency. A highly-efficient vitamin B_{12}-producing strain was constructed by Rhone-Poulenc Rorer, Inc. (France) using random mutagenesis [7]. Some necessary genes were overexpressed, such as the *cobF–cobM* cluster, *cobA*, and *cobE*, while some genes, such as *hemZ*, were

silenced with an antisense RNA strategy [8, 9]. Finally, an engineered strain of *P. freudenreichii* for vitamin B_{12} production was successfully constructed [10–12]. However, the engineered strain was unbalanced at the systems level because of the limited available genetic tools [13].

Traditional strategies are still crucial for industrial production, for example, medium optimization (glucose, glycerol, corn steep liquor, and Co^{2+}); cell immobilization [Ca alginate beads, polyethylenimine-treated Poraver (PEI-Poraver)]; and process regulation [5,6-dimethylbenzimidazole (DMB) addition, *in-situ* product removal (ISPR) processes] [14–15]. ISPR can remove propionic acid in time to effectively overcome its feedback inhibition effect, and help to realize a semi-continuous fermentation process. Various ISPR processes have been developed using *in situ* cell retention reactors [16], plant fibrous-bed bioreactors [17–19], and PEI-Poraver immobilized bioreactors [4]. However, these studies focused on production of only a single product, and few works have reported on simultaneous production of vitamin B_{12} and propionic acid.

To set up a cost-effective semi-continuous fermentation process to realize the simultaneous production of vitamin B_{12} and propionic acid, a new ISPR process characterized by expanded bed adsorption was built [20]. Membrane separation was introduced into the fermentation system to realize fixed-bed removal of propionic acid, based on vitamin B_{12} *ex situ* conversion [21].

In this study, to further investigate the regulatory mechanisms of vitamin B_{12} and propionic acid biosynthesis, the metabolic flux in the coupled fermentation process was analyzed based on the metabolic network of *P. freudenreichii*. In view of the complexity of *P. freudenreichii* metabolism, a simplified metabolic network was established. The metabolic flux distribution at key nodes in different fermentation conditions was analyzed, and the influencing factors were assessed. Our findings provide theoretical guidance for the optimization of simultaneous production of vitamin B_{12} and propionic acid.

Materials And Methods

Microorganism and medium

P. freudenreichii CICC 10019 was obtained from the Chinese Industrial Microorganism Conservation Center. The stock culture was incubated in agar slants at 30 °C, stored at 4 °C, and transferred to fresh agar monthly. For long-term preservation, the stock was stored at −80 °C.

Inoculum medium contained glucose (35 g/L), corn steep liquor (21 g/L), $(NH_4)_2SO_4$ (5 g/L), KH_2PO_4 (4 g/L), $CoCl_2$ (5 mg/L), and distilled water (pH 6.8–7.0). Fermentation medium contained glucose (60 g/L), corn steep liquor (41 g/L), KH_2PO_4 (4.6 g/L), $CoCl_2$ (12.7 mg/L), and distilled water (pH 6.8–7.0). For medium preparation, glucose was autoclaved separately.

Corn steep liquor was purchased from Lingshanhe Plant Protein Manufacturing Co., Ltd. (Xingtai, Hebei, China). Glucose and inorganic salts were of analytical grade and produced by Shanghai Macklin

Biochemical Co., Ltd. (China).

Batch/fed-batch fermentation of *P. freudenreichii*

P. freudenreichii CICC 10019, stored on a agar slant, was activated at 30 °C for 24 h. One loopful of culture from the activated deep agar slant was inoculated aseptically into 50 mL of inoculum medium and cultivated statically at 30 °C for 48 h. This culture was used to inoculate 500 mL of fresh inoculum medium. After 48 h, the culture was used to inoculate fermentation medium in a 7-L fermenter filled to 70% capacity, and cultivated at 30 °C, 50 rpm. During the fermentation, no sterile air was required and the pH was controlled at 7.0 by the automatic addition of $NH_3{\cdot}H_2O$ or H_3PO_4. In the batch fermentation process, 0.90 mg/L DMB was added as a precursor for vitamin B_{12} biosynthesis at 84 h [22].

In fed-batch fermentation of *P. freudenreichii*, 500 g/L glucose were added when the glucose concentration fell below 10 g/L, to maintain cell growth and product synthesis.

During the fermentation process, the concentrations of glucose, propionic acid, acetic acid, succinic acid, and lactic acid were determined every 12 h. After DMB addition, the concentrations of vitamin B_{12} and heme were also analyzed.

Membrane separation-coupled fermentation of *P. freudenreichii*

The membrane separation-coupled fermentation process of *P. freudenreichii* was established by Wang *et al*. [21]. During the fermentation process, a multifunctional membrane separation device equipped with a G13 plunger diaphragm pump (RNF 0460-011) and a 0.22-μm spiral membrane module (Type 1812) was used to separate *P. freudenreichii* cells online, while the penetrant flowed through a chromatography column packed with ZGA302 anion exchange resin and propionic acid in the filtrate was adsorbed. Details are shown in Fig. 1. After optimizing the adsorption conditions, the concentration of propionic acid was reduced to <10 g/L. Then, the deacidified filtrate was returned to the fermenter.

Cell concentration assay

Cell growth was analyzed photometrically at 600 nm. Before measuring the cell concentration, *P. freudenreichii* cells were centrifuged at 10,000 × *g* for 10 min, and then resuspended in isovolumetric phosphate buffer (20 mM, pH 7.0) to eliminate the effect of corn steep liquor and other fermentation components.

Assay of organic acids and glucose

After centrifugation, fermentation supernatant was used to analyze the concentration of organic acids and glucose.

High-performance liquid chromatography (HPLC) analysis of propionic acid, lactic acid, acetic acid, and succinic acid was performed using a Shimadzu LC-20AT HPLC system fitted with a Bio-Rad HPX-87H

column (5 μm, 380 mm × 4.6 mm) and an SPD-20A UV detector operated at 215 nm. The mobile phase was 5 mM H_2SO_4 with a flow-rate of 0.6 mL/min at 55 °C. The injection volume was 10 μL. Commercially available organic acids were used as external standards.

Residual glucose was determined enzymatically using an SBA-40E biological sensor (Shandong, China). The tested supernatant was diluted to a final glucose concentration of 0–1 g/L, and then loaded onto the biological sensor with an injection volume of 25 μL. The glucose concentration was calculated according to a glucose standard solution.

Assay of vitamin B_{12} and heme

After centrifugation, *P. freudenreichii* cells were washed and resuspended in 20 mM phosphate buffer (pH 7.0), then disrupted in a boiling water for 30 min to release intracellular vitamin B_{12} and heme [22]. After removing cell debris by centrifugation at 10,000 × *g* for 10 min, the supernatant was used to analyze the concentration of vitamin B_{12} and heme.

HPLC analysis of vitamin B_{12} was performed with a Hypersil GOLD C18 column (5 μm × 4.6 mm × 250 mm; Dikma) at 254 nm with the same HPLC system as described above [20]. The mobile phase consisted of 0.02 M sodium acetate buffer solution (pH 3.5, adjusted with acetic acid), and acetonitrile (gradient of 5%–24%, 0–30 min). The elution was carried out at 25 °C with a flow-rate of 1.0 mL/min. Commercially available vitamin B_{12} was used as an external standard.

The concentration of heme was measured by colorimetry [23]. Commercially available hemin was used as external standard. First, hemin standard solutions (1–10 μg/mL) were prepared. Pyridine-NaOH (2 mL, 33% pyridine and 0.1 mol/L NaOH) solution was added to 1 mL of hemin standard solution. After mixing, 3 mg sodium sulfite were added and reacted for 30 min to reduce the heme iron. A linear standard calibration curve was obtained using absorbance at 557 nm. When measuring a fermentation sample, there was no need to add sodium sulfite.

Establishment of metabolic flux balance model of vitamin B_{12} and propionic acid

The metabolic network of *P. freudenreichii* is very complicated. According to the literature and preliminary experimental data, the most important metabolic pathways of propionic acid and other related metabolites with glucose as the carbon source were determined, including glycolysis, the Wood-Werkman pathway, succinate synthesis, lactate synthesis, acetate synthesis, and vitamin B_{12} synthesis. Details are shown in Fig. 2 [24-26].

To facilitate analysis and calculation, a simplified ideal metabolic network was established based on the following principles: (1) Metabolic flux analysis was based on the pseudo-steady-state assumption. In the late stage of fermentation, *P. freudenreichii* cells were in the non-growth period and growth rate was ignored to simplify the model; (2) The pentose phosphate pathway and glyoxylate pathway were ignored; (3) A complex interdependent and interactional relationship exists among the tetrapyrrole compounds,

which are derived from δ-aminolevulinate (ALA). However, the synthesis of chlorophyll and coenzyme F430 was ignored; (4) Reactions with fixed stoichiometry and with intermediates without branch points were simplified into a single reaction equation. (5) In the cell growth arrest stage, the total amount of cell maintenance energy and the consumption of ATP were not equal, due to the existence of many invalid cycles. Thus, the balance of total ATP was not considered.

The established metabolic network of simultaneous production of vitamin B_{12} and propionic acid in *P. freudenreichii* included 27 reaction equations, as shown in Table 1. According to the metabolic network and the stoichiometric balance equation of each reaction, the relationship among the reaction rates was obtained. It was assumed that the intermediate metabolites were in a pseudo-steady-state, that is, their concentration change rate was zero. On the basis of the material balance law, the accumulation rate of metabolites can be calculated from the formula:

$$r_i(t) = \sum_{i=1}^{M} a_{ij} r_j(t) - \sum_{i=1}^{N} a_{ik} r_k(t)$$

where $r_i(t)$ is the accumulation rate of intermediate metabolite *i* [mmol/(L·h)]; $r_j(t)$ and $r_k(t)$ are the reaction rates of the j^{th} and k^{th} reactions of substance *i*, [mmol/(L·h)]; and a_{ij} and a_{ik} are the stoichiometric coefficients corresponding to each reaction. An equation based on this principle can be established for each metabolite in the metabolic network.

According to the pseudo-steady-state assumption, ri(t) = 0. The *m* intermediate metabolites and *n* reactions in the metabolic network constitute *m* metabolic flux balance equations with degree of freedom of $F = n - m$. The overall metabolic flux distribution can be determined by measuring *F* reaction rates. The metabolic flux balance model of vitamin B_{12} and propionic acid established in this study included 27 reaction rates (*J*) and 23 equilibrium equations (*K*), with degree of freedom (*F*) = 4. Details are shown in Table 2. Therefore, the metabolic flux distribution can be determined only by measuring just 4-step reaction rates.

During the fermentation of *P freudenreichii*, the concentration of seven substances can be measured, and the corresponding reaction rates were obtained by differentiation: the consumption rate of glucose (r_1), and the production rates of acetic acid (r_{11}), propionic acid (r_{13}), lactic acid (r_{17}), succinic acid (r_{21}), heme (r_{24}), and vitamin B_{12} (r_{27}). Taking these parameters as known variables, substituting them into the metabolic balance equations, listing the matrix, and using the Linprog function in Matlab software, the ideal metabolic flux distribution was obtained. During the calculation process, the glucose consumption rate was assumed to be 100 mmol/(L·h).

Results And Discussion

Fermentation of *P. freudenreichii* in different conditions

In the fermentation process of *P. freudenreichii*, many process variables can affect the production of vitamin B_{12} and propionic acid, including pH, temperature, fermentation time, inoculum size, the carbon and nitrogen sources and their concentrations, as well as fermentation strategies [25]. On the basis of previous studies, the effects of glucose feeding and membrane separation-coupled operation on the simultaneous production of vitamin B_{12} and propionic acid were analyzed. Generally, cell growth was accompanied by the synthesis of propionic acid, acetic acid, and succinic acid in the initial stage of *P. freudenreichii* fermentation, while the synthesis of vitamin B_{12} occurred later. The consumption of glucose and the production of organic acids such as propionic acid, acetic acid, and succinic acid, as well as vitamin B_{12} were analyzed. As shown in Fig. 3, in batch fermentation, the concentrations of vitamin B_{12} and propionic acid were 10.5 mg/L and 28.82 g/L at 132 h, with productivity of 0.08 mg/(L·h) and 0.22 g/(L·h), respectively.

In fed-batch fermentation, when the glucose concentration fell to <10 g/L, a high concentration of glucose was fed into the fermentor to maintain cell growth and product synthesis. After 132 h, the concentration of vitamin B_{12} and propionic acid reached 16.0 mg/L and 32.70 g/L, increased by 52.38% and 13.46% respectively, compared with the batch fermentation process. Therefore, feeding glucose during the fermentation process can improve the fermentation efficiency of *P. freudenreichii*. However, propionic acid fermentation suffers from end-product feedback inhibition, which leads to a decrease in productivity of propionic acid [27]. Our results showed that in the initial stage (0–72 h) and the later stage (72–132 h) of the fermentation process, the productivity of propionic acid was 0.34 g/(L·h) and 0.13 g/(L·h), respectively, with an overall productivity of 0.25 g/(L·h).

To overcome the feedback inhibition of propionic acid synthesis, a membrane separation-coupled fermentation process of *P. freudenreichii* was established. When the concentration of propionic acid reached about 20–25 g/L, *P. freudenreichii* cells were separated online using a multifunctional membrane device, and the penetrant flowed through a ZGA302 anion chromatography column to adsorb propionic acid. When the propionic acid concentration was <10 g/L, the flow-through liquid was returned to the fermenter. After this deacidification, the feedback inhibition effect of propionic acid was relieved, which further promoted cell growth and product synthesis. In this set-up, the concentrations of vitamin B_{12} and propionic acid reached 21.6 mg/L and 50.12 g/L at 132 h, with productivity of 0.16 mg/(L·h) and 0.38 g/(L·h) respectively, increased by 35.00% and 49.12% respectively, compared with the fed-batch fermentation process. The concentration of succinic acid in the coupled fermentation process was 22.09 g/L, increased by 52.03%, implying that the metabolism of propionic acid and succinic acid was inherently related. However, the concentration of acetic acid decreased by 31.86% compared with the fed-batch fermentation process, indicating that more metabolic flux flowed to propionic acid synthesis in the coupled fermentation process. Details are shown in Fig. 3.

The current state of simultaneous production of vitamin B_{12} and propionic acid by *Propionibacterium* is shown in Table 3. Compared with the expanded bed reactor (EBA-reactor)[20,28], the membrane

separation-coupled process does not require complicated ISPR operation and is easy for industrial application. Therefore, this process can improve the fermentation efficiency of *P. freudenreichii* and realize the simultaneous production of vitamin B_{12} and propionic acid. However, the coupled system still needs to be integrated, for example, the removal efficiency of propionic acid around neutral pH (6.5–7.0) was still low, and the adsorption conditions should be further optimized, which is ongoing in our laboratory.

Metabolic flux analysis of key nodes

The cellular metabolic network of *P. freudenreichii* is a complex reaction system with steps catalyzed simultaneously by multiple enzymes, and is affected by interaction of the internal and external environments. To analyze the intracellular metabolic flux distribution in different conditions, a simplified ideal metabolic network of vitamin B_{12} and propionic acid synthesis was established based on the current literature.

During the fermentation process of *P freudenreichii*, the consumption rate of glucose (r_1), and the production rates of acetic acid (r_{11}), propionic acid (r_{13}), lactic acid (r_{17}), succinic acid (r_{21}), heme (r_{24}), and vitamin B_{12} (r_{27}), can be calculated by differentiation, and then the ideal metabolic flux distribution was obtained using the Linprog function in Matlab software. The influence of culture conditions on product synthesis was assessed by analyzing the metabolic flux at key nodes, which provided theoretical guidance for the optimization of simultaneous production of vitamin B_{12} and propionic acid.

Metabolic flux analysis of pyruvate node

In the simplified metabolic network of *P. freudenreichii*, pyruvate was the first key metabolic node. Pyruvate directly or indirectly participates in the synthesis of organic acids, including: (1) direct generation of lactate via catalysis by lactate dehydrogenase; (2) generation of acetyl-CoA, an intermediate in acetate production, via catalysis by pyruvate dehydrogenase; and (3) generation of oxaloacetate via the pyruvate carboxylation pathway, and hence production of succinate. It was found that the flux at the pyruvate node to the above three branches was 84.64:3.91:30.44 in the batch fermentation process, while the flux distribution was 83.47:2.51:33.52 and 75.44:2.32:34.64 respectively in the fed-batch and coupled fermentation processes. Details are shown in Fig. 4. Therefore, the addition of glucose to the culture and the removal of propionic acid changed the metabolic flux distribution at the pyruvate node. That is, the flux to the lactate and acetate branches decreased, while the flux to the oxaloacetate branch increased accordingly, which was consistent with the experimental results. Especially in the later stage of fermentation, the flux to the lactate branch dropped to a negative value.

Metabolic flux analysis of succinyl-CoA node

Succinyl-CoA is another key node in the metabolism of vitamin B_{12} and propionic acid. In the metabolic process, pyruvate can directly generate oxaloacetate via catalysis by pyruvate carboxylase. Then, oxaloacetate can be used to produce succinate via a three-step enzymatic reaction. It was reported that

the last step of propionate synthesis was the transfer of CoA from propionyl-CoA to succinic acid, also generating succinyl-CoA. Succinyl-CoA can be used to synthesize ALA, a vital intermediate of vitamin B_{12} synthesis, by condensation with glycine [29]. In addition, succinyl-CoA can be converted to methylmalonyl-CoA, an important intermediate of propionic acid synthesis.

According to our results (Fig. 5), the flux distribution at the succinyl-CoA node to ALA and methylmalonyl-CoA was 0.02:95.11 in the batch fermentation process. However, the flux to the ALA branch increased significantly (from 0.02 to 4.40) in the fed-batch process, which directly led to a 52.38% increase in the concentration of vitamin B_{12}. The propionic acid concentration only increased by 13.42% because of feedback inhibition. However, when propionic acid was partly removed in the coupled fermentation process and its feedback inhibition effect was relieved, the flux to the ALA and methylmalonyl-CoA branches both increased, with proportion 4.52:100. As a result, the concentrations of vitamin B_{12} and propionic acid increased by 105.71% and 73.91%, respectively, compared with those in the batch fermentation process.

Metabolic flux analysis of uroporphyrinogen III node

Microbial synthesis of vitamin B_{12} can be regulated at both the transcriptional and metabolic levels [29-31]. A cobalamin riboswitch is used to quickly respond to the environment [32]. It was reported that a complex interdependent and interactional relationship exists among the tetrapyrrole compounds [24].

According to the metabolic network, there were two metabolic branches at the uroporphyrinogen III node: one is the synthesis of heme, and the other is the synthesis of sirohydrochlorin, a precursor of vitamin B_{12}. It was found that the metabolic flux to the heme branch significantly increased when glucose was fed or the coupled process was adopted. However, the increase in the metabolic flux to the heme branch did not reduce the metabolic flux to the sirohydrochlorin branch, which implied that the total flux through the uroporphyrinogen III node increased. Details are shown in Fig. 6. In the late stage of vitamin B_{12} synthesis, the metabolic flux was divided into two branches in equal proportion at the sirohydrochlorin node, one of which was the synthesis of siroheme. Relevant enzymes (e.g., HemE, HemZ, HemY, HemH, SirB) can be modified (silenced or knocked-out) to reduce the metabolic flux to the heme and siroheme branches, to increase the concentration of vitamin B_{12}.

Metabolic flux distribution in the coupled fermentation process

According to the metabolic network of *P. freudenreichii*, α-pyrroleribose is activated to form the lower ligand base DMB that is linked to adenosylcobinamide-GDP (Ado-cbi-GDP) to synthesize vitamin B_{12} [33,34]. However, because of its low concentration, α-pyrroleribose was a key rate-limiting factor in vitamin B_{12} synthesis, which led to the accumulation of intracellular Ado-cbi-GDP [35]. During the fermentation process, additional DMB was required to promote the conversion of Ado-cbi-GDP to vitamin B_{12}.

In our experiments, DMB was added to the fermentor at 84 h. To investigate the effect of DMB addition on the metabolic flux distribution, we analyzed the data from the coupled fermentation process for 0–84 and 84–132 h. After DMB addition, the metabolic flux significantly flowed toward vitamin B_{12} synthesis. Details are shown in Fig. 7. The specific effects observed were: (1) Increased synthesis of pyruvate by increased flux of 3-phosphoglycerate and phosphoenolpyruvate. (2) Decreased synthesis of organic acids. The synthesis of organic acids mainly occurred in the early and middle stages of the fermentation process. After DMB addition, the flux to lactic acid, acetic acid, succinic acid, and propionic acid decreased sharply, which was consistent with the experimental results. (3) Increased synthesis of succinyl-CoA and its flux proportion to the ALA branch, which provided the possibility of efficient synthesis of uroporphyrinogen III. (4) The flux increase at the uroporphyrinogen III node further increased the flux to heme and sirohydrochlorin. However, the flux proportion to the sirohydrochlorin branch (the direction of vitamin B_{12} synthesis) was lower than that to heme. In fact, the concentration of heme was slightly higher than that of vitamin B_{12}. Detailed studies should be carried out with the aid of systems biology and synthetic biology to investigate the complex relationship among the tetrapyrrole compounds (e.g., cobalamin, chlorophyll, heme, and coenzyme F430) and its regulatory mechanisms.

Conclusions

The simultaneous production of vitamin B_{12} and propionic acid by *P. freudenreichii* has attracted wide attention. In the membrane separation-coupled fermentation process described here, the concentration of vitamin B_{12} and propionic acid reached 21.6 mg/L and 50.12 g/L after 132 h, increased by 105.71% and 73.91% respectively compared with a batch fermentation process. To investigate the distribution of intracellular materials and energy, a simplified ideal metabolic network of *P. freudenreichii* was established. By analyzing the flux distribution at key metabolic nodes in different conditions, the effects of glucose feeding, propionic acid removal, and DMB addition on the fermentation efficiency were assessed. The results showed that all these methods can improve the metabolic flux through key nodes and increase the product yield, which provides theoretical guidance for the optimization of *P. freudenreichii* fermentation processes for production of vitamin B_{12} and propionic acid.

Declarations

CRediT authorship contribution statement

Yuhan Zhang: Methodology, Validation, Writing-Original Draft; Xiaolian Li: Writing-Review & Editing; Ziqiang Wang: Conceptualization, Writing-Review & Editing, Funding acquisition, Project administration; Yunshan Wang: Resources, Supervision, Project administration; Yuanyuan Ma: Resources, Supervision; Zhiguo Su: Resources, Supervision.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

Not applicable

Consent to Participate

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

Consent to Publish

All the authors listed have seen the manuscript and approved the submission to your journal. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property.

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Tables

Table 1 Metabolic reactions of simultaneous production of vitamin B_{12} and propionic acid by *Propionibacterium freudenreichii*

Flux	Metabolic reactions
r_1	Glucose + ATP → Glucose-6-phosphate + ADP + H^+
r_2	Glucose-6-phosphate → Fructose-6-phosphate
r_3	fructose-6-phosphate + ATP → Fructose-1,6-diphosphate + ADP + H^+
r_4	Fructose-1,6-diphosphate → Dihydroxyacetone phosphate + Glyceraldehyde-3-phosphate
r_5	Dihydroxyacetone phosphate → Glyceraldehyde-3-phosphate
r_6	Glyceraldehyde-3-phosphate + NAD^+ + Pi → 1,3-diphosphoglycerate + NADH + H^+
r_7	1,3-Diphosphoglycerate + ADP → 3-Phosphoglycerate + ATP
r_8	3-Phosphoglycerate → 2-Phosphoglycerate
r_9	2-Phosphoglycerate → Phosphoenolpyruvate + H_2O
r_{10}	Phosphoenolpyruvate + ADP +H^+ → Pyruvate + ATP
r_{11}	Pyruvate + NADH + H^+ → Lactate + NAD^+
r_{12}	Pyruvate + CoA + NAD^+ → Acetyl-CoA + CO_2 + NADH
r_{13}	Acetyl-CoA + ADP → Acetic acid + CoA + ATP
r_{14}	Pyruvate + CO_2 + ATP + H_2O → Oxaloacetate + ADP + 2Pi + $2H^+$
r_{15}	Oxaloacetate + NADH + H^+ → L-malate + NAD^+
r_{16}	L-malic acid → Fumaric acid + H_2O
r_{17}	Fumaric acid + $FADH_2$ → Succinic acid + FAD
r_{18}	Succinic acid + ATP + CoA → Succinyl-CoA + Pi + ADP
r_{19}	Succinyl-CoA → Methylmalonyl-CoA
r_{20}	Methylmalonyl-CoA → propionyl-CoA+CO_2
r_{21}	Propionyl-CoA + ADP → Propionic acid + CoA + ATP
r_{22}	Succinyl-CoA + Glycine → δ-aminolevulinic acid + ADP
r_{23}	δ-aminolevulinic acid → …… → Uroporphyrinogen III
r_{24}	Uroporphyrinogen III → …… → heme

r_{25}	Uroporphyrinogen III → …… → Sirohydrochlorin
r_{26}	Sirohydrochlorin → Siroheme
r_{27}	Sirohydrochlorin →……→ Cob(II)yrinic acid a,c diamide→ ……→ Adenosylcobalamin

Table 2 Equations of metabolic flux of simultaneous production of vitamin B_{12} and propionic acid by *Propionibacterium freudenreichii*

Intermediate	Equations
Glucose-6-phosphate	r_1-r_2=0
Fructose-6-phosphate	r_2-r_3=0
Fructose-1,6-diphosphate	r_3-r_4=0
Glyceraldehyde-3-phosphate	r_4+r_5-r_6=0
Dihydroxyacetone phosphate	r_4-r_5=0
1,3-diphosphoglycerate	r_6-r_7=0
3-Phosphoglycerate	r_7-r_8=0
2-Phosphoglycerate	r_8-r_9=0
Phosphoenolpyruvate	r_9-r_{10}=0
Acetyl-CoA	r_{12}-r_{13}=0
Oxaloacetate	r_{14}-r_{15}=0
L-malic acid	r_{15}-r_{16}=0
Fumaric acid	r_{16}-r_{17}=0
Succinyl-CoA	r_{18}-r_{19}-r_{22}=0
Methylmalonyl-CoA	r_{19}-r_{20}=0
Propionyl-CoA	r_{20}-r_{21}=0
δ-aminolevulinic acid	r_{22}-r_{23}=0
Uroporphyrinogen III	r_{23}-r_{24}-r_{25}=0
Sirohydrochlorin	r_{25}-r_{26}-r_{27}=0
NADH	r_6+r_{12}-r_{11}-r_{15}=0
Pi	2r_{14}+r_{18}-r_6=0
CoA	r_{13}+r_{21}-r_{12}-r_{18}-r_{22}=0
H^+	r_1+r_3+r_6+2r_{14}-r_{10}-r_{11}-r_{15}=0

Table 3 The current state-of vitamin B_{12} and propionic acid production by *Propionibacterium*

Microorganism	Carbon source	Culture system	Vitamin B_{12} (mg/L)	Propionic acid (g/L)	References
P. acidipropionici DSM 8250	beet molasses	Two-stage	34.8	17.0	[36]
P. acidipropionici DSM 8250	reed molasses	Two-stage	28.8	17.7	[36]
P. acidipropionici DSM 8250	sugar (household refined product)	Two-stage	33	30	[36]
P. shermanii PZ-3	glucose	Hollow-fiber module	52	~12	[37]
P. freudenreichii CICC 10019	glucose	EBA bioreactor	42.6	52.5	[20]
P. freudenreichii CICC 10019	corn stalk hydrolysates	EBA bioreactor	47.6	91.4	[28]
P. freudenreichii T82	glucose/fructose	Batch	4-14 ug L^{-1}	23-40	[38]
P. freudenreichii CICC 10019	glucose	Membrane coupled	21.6	50.12	This study

Figures

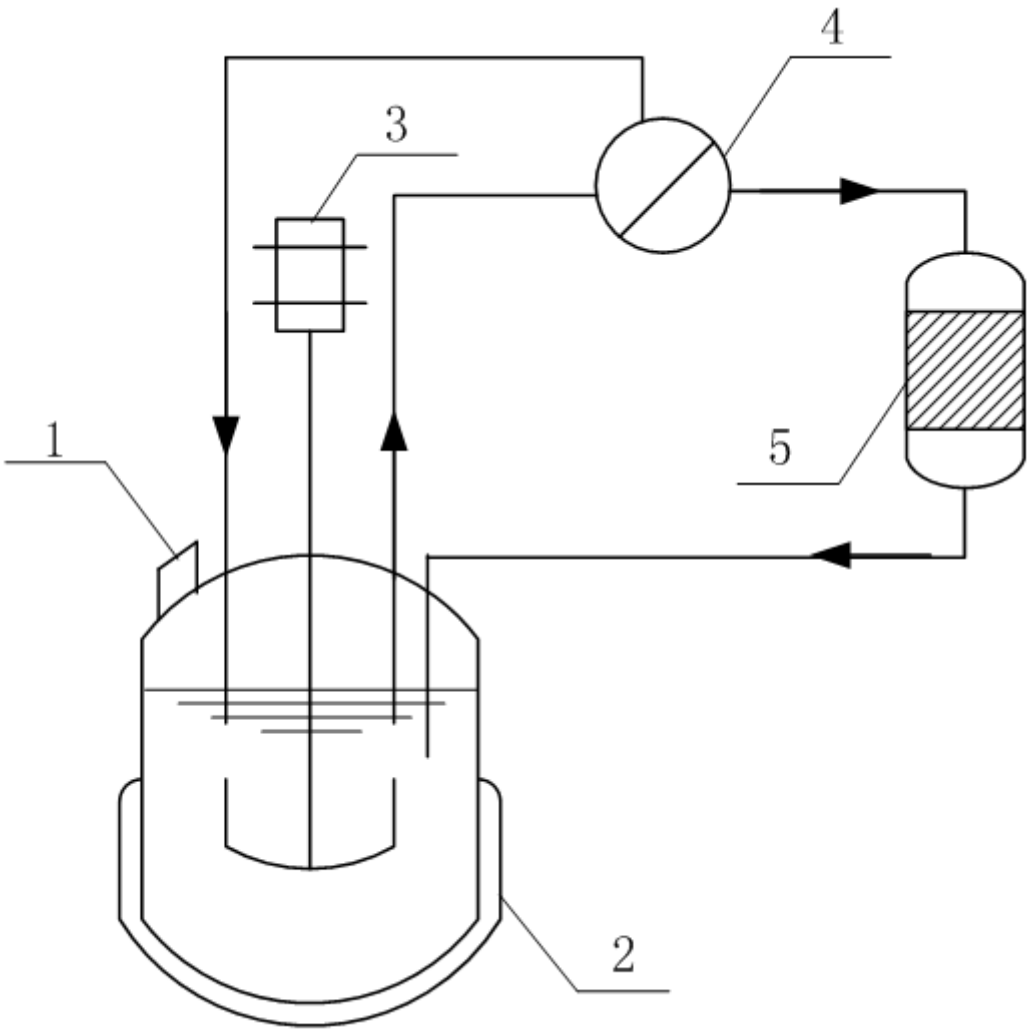

Figure 1

Schematic diagram of membrane separation coupled fermentation of Propionibacterium freudenreichii. 1. Feed port/ Inoculation port; 2. Fermenter; 3. Motor; 4. Membrane device; 5. Chromatographic column. P. freudenreichii cells were separated on line with a membrane device. The penetrant flowed through the chromatography column to adsorb propionic acid, and then returned to the fermenter to continuous fermentation.

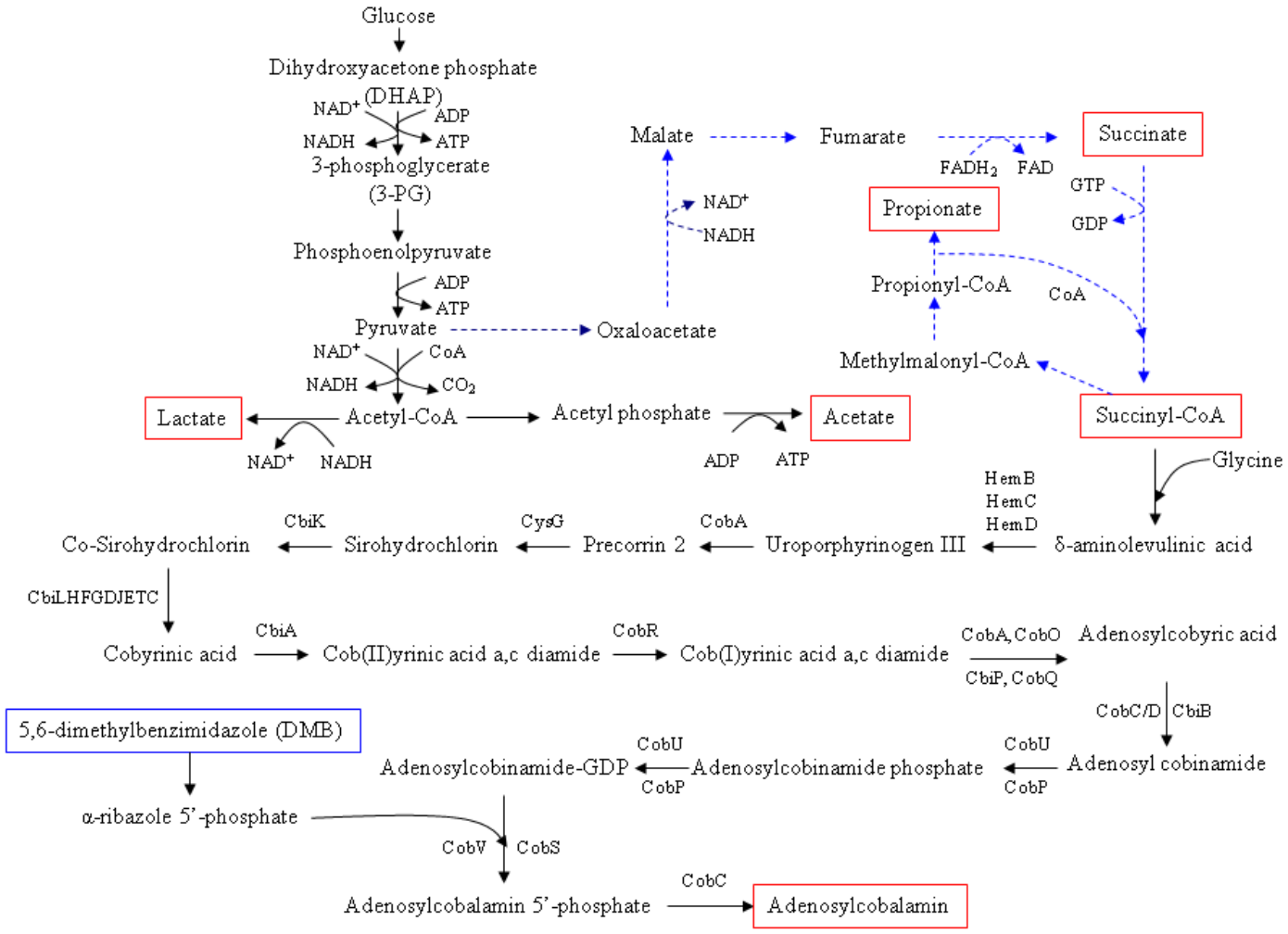

Figure 2

The metabolic network of simultaneous production of vitamin B12 and propionic acid in Propionibacterium freudenreichii.

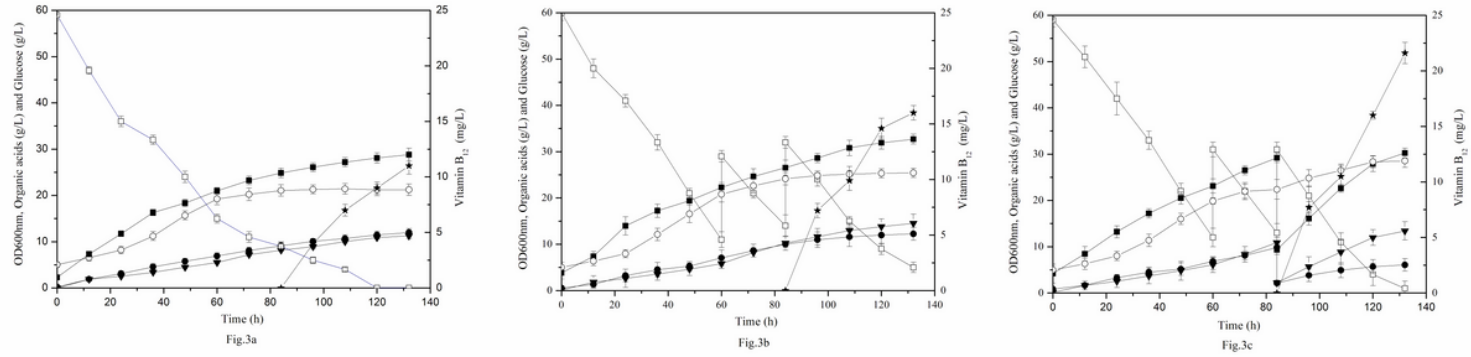

Figure 3

Time courses of glucose (△), propionic acid (●), acetic acid (●), succinic acid (▲), lactic acid (▼), and vitamin B12 (□) in Propionibacterium freudenreichii fermentation process. (a) Batch fermentation; (b) Fed-batch fermentation; (c) Coupled fermentation.

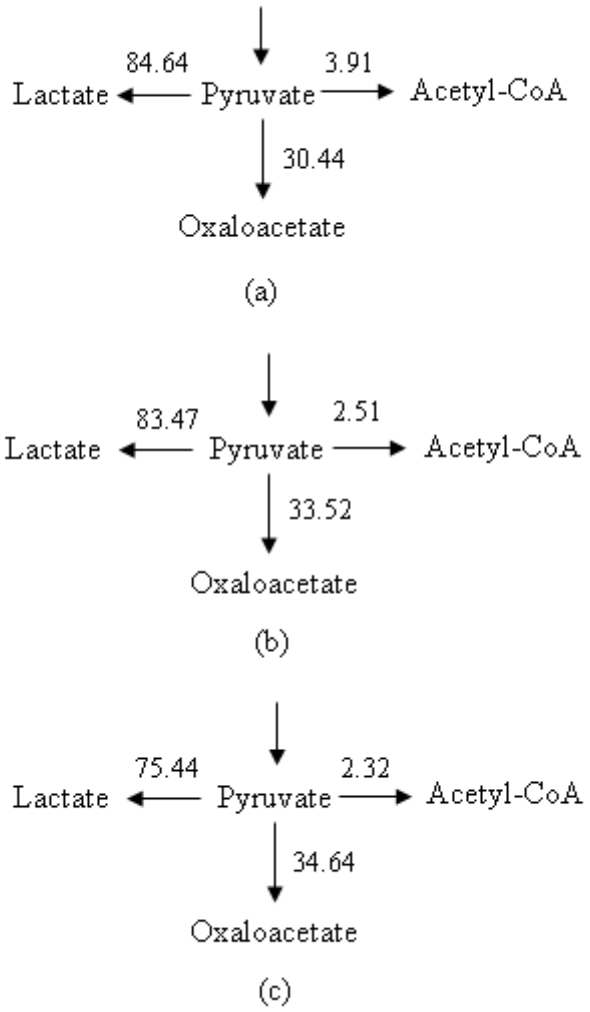

Figure 4

Metabolic flux distribution at pyruvate node. (a) Batch fermentation; (b) Fed-batch fermentation; (c) Coupled fermentation.

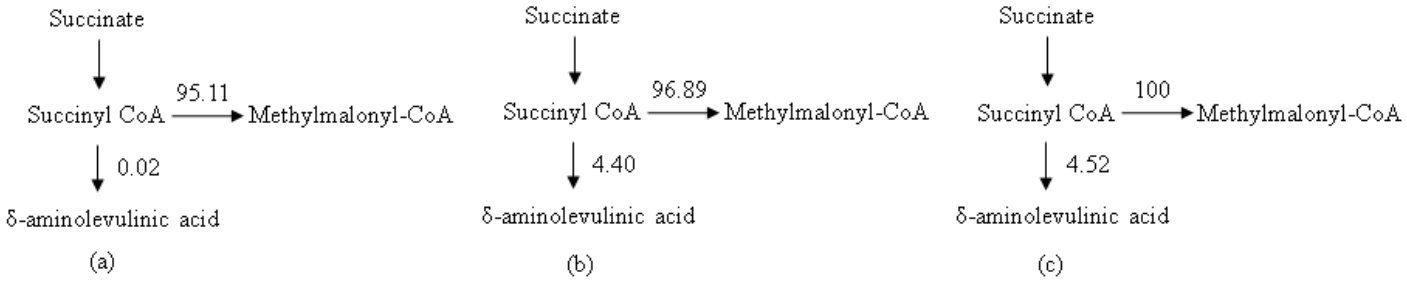

Figure 5

Metabolic flux distribution at succinic acid-CoA node. (a) Batch fermentation; (b) Fed-batch fermentation; (c) Coupled fermentation.

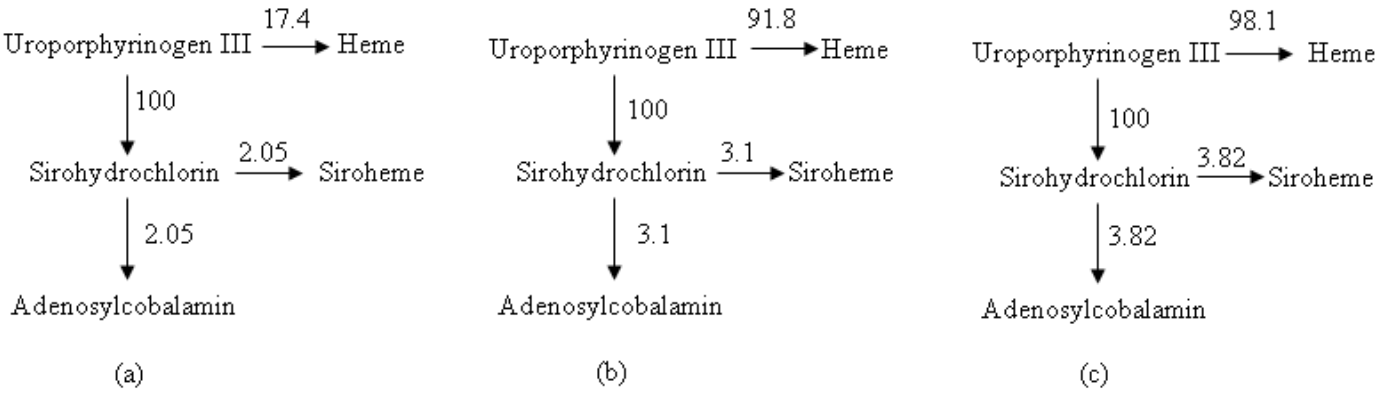

Figure 6

Metabolic flux distribution at uroporphyrinogen III node. (a) Batch fermentation; (b) Fed-batch fermentation; (c) Coupled fermentation.

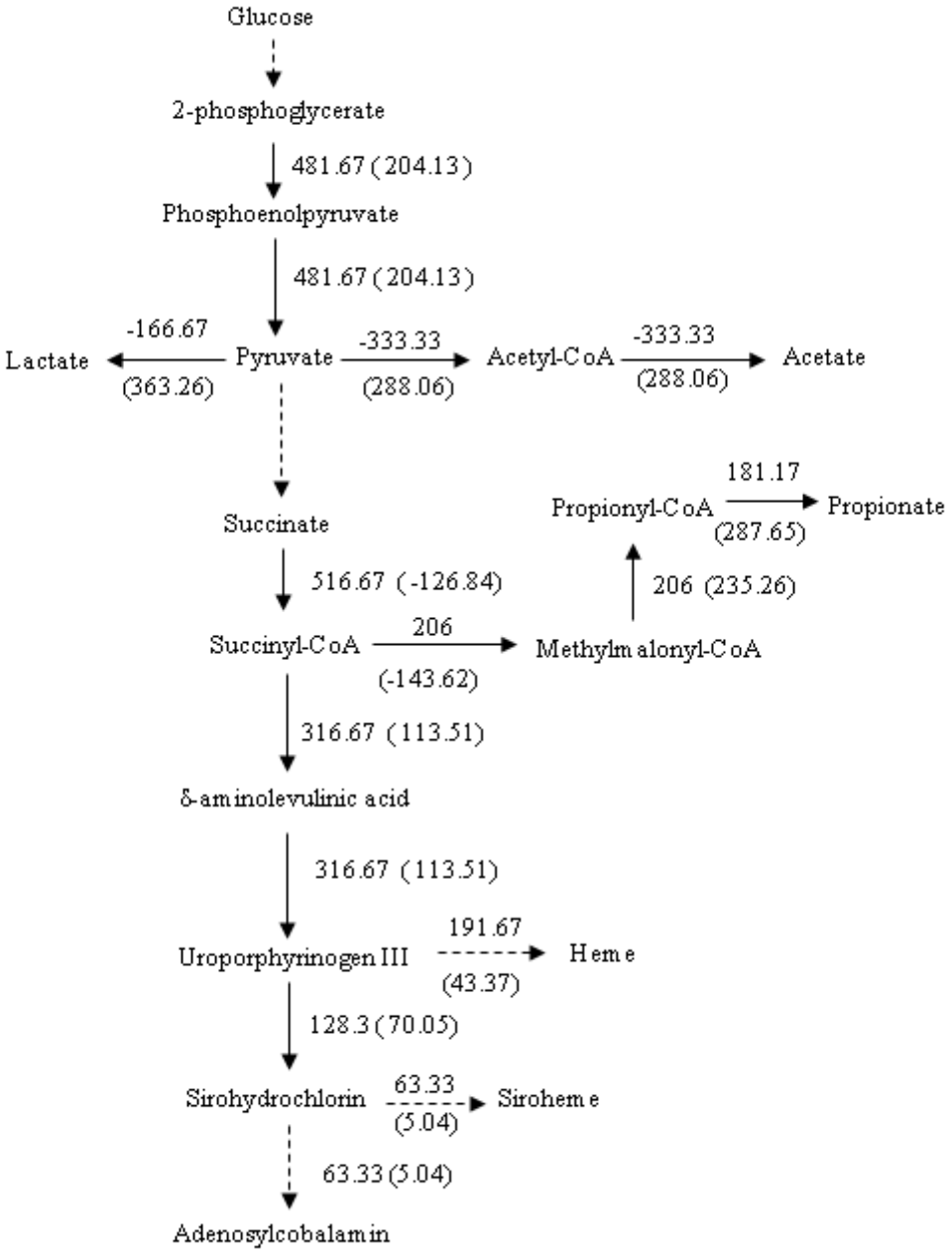

Figure 7

Metabolic flux distribution of Propionibacterium freudenreichii in the coupled fermentation process. The value in brackets was the distribution of 0-84 h, and the value without brackets was the distribution of 84-132 h.