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Expression and Characterization of a Novel Propionyl-CoA Dehydrogenase Gene from *Candida rugosa* in *Pichia pastoris*

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Abstract The propionyl-CoA dehydrogenase (PACD) gene was firstly cloned from *Candida rugosa* by the cDNA RACE technique. The 6× His-tagged recombinant PACD gene was expressed in *Pichia pastoris* GS115 and purified with Ni-NTA affinity chromatography. SDS-PAGE analysis and Western blotting revealed that the molecular mass of the purified PACD was 49 kDa. The results showed that the recombinant protein had the activity of catalyzing propionyl-CoA to acrylyl-CoA. The K_m, k_{cat}, and V_{max} values of the purified PACD were calculated to be 40.86 μM, 0.566 s^{-1} and 0.693 U mg^{-1} min^{-1}. The optimal temperature and pH of the purified PACD were 30 °C and 7.0, respectively. The recombinant PACD maintained 76.3%, 30.1%, and 4.3% of its original activity after 2 h incubation in standard buffer at 30, 40, and 50 °C, respectively. Mg^{2+} had an activating effect on the enzyme, while Mn^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+} had weak inhibition. Since PACD catalyzed the key step (from propionyl-CoA to acrylyl-CoA) in the modified β-oxidation pathway from glucose to 3-hydroxypropionic acid (3-HP), the integration of recombinant PACD could benefit the engineered strains for effective production of 3-HP from the most abundant biomass – sugars.

Keywords Propionyl-CoA dehydrogenase · Gene expression · Characterization · *Candida rugosa* · *Pichia pastoris*

Introduction

3-Hydroxypropionic acid (3-HP), the isomer of lactic acid, is a promising platform chemical for the production of industrially important polymer materials. In a report from the United States Department of Energy, it is listed among the top 20 value added chemical building blocks manufactured from biomass [1]. Currently, 3-HP is mainly produced through the petrochemical synthesis routes. Confronted with the scarcity of fossil fuel

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supplies, the fermentative production of this valuable chemical is gaining increasing attention. The biosynthetic pathway for 3-HP by microbial fermentation has shown good prospect for its mild operation conditions, few by-products, and reduced environmental load as compared with the chemical process [1–5].

3-HP is a naturally existing metabolite in the biological organism. It is a key intermediate of the 3-HP cycle [6, 7]. A variety of microorganisms have been reported to be able to accumulate 3-HP as an end product, while glycerol, propionic acid, or acrylic acid serves as the fermentative substrates [8]. Many efforts have also been done on metabolic engineered *Escherichia coli* for 3-HP production [3, 4, 9–11]. A pathway starting from glycerol (glycerol→3-hydroxypropionaldehyde→3-HP) was established in engineered *E. coli* [4, 11]. On the other hand, researchers are developing new engineered strains to produce 3-HP from the most abundant biomass sugars. Mainly seven metabolic pathways from glucose to 3-HP have been proposed in microorganism (Fig. 1) [8, 10, 12–14], among which, the modified β-oxidation pathway (namely propionic acid (PA) pathway, glucose→propionic acid→propionyl-CoA→acrylyl-CoA→β-hydropropionyl-CoA→3-HP) is proposed as the most promising one. This route is thermodynamically favorable and proposed to be existed in several 3-HP producing microorganisms [8]. All the enzymes in the modified β-oxidation pathway have not been characterized except the propionyl-CoA transferase which was used to construct engineered *E. coli* to produce 3-HP from glucose to lactoyl-CoA and then to 3-HP [10].

We obtained a mutant of *Candida rugosa* which could directly produce 3-HP from glucose with PA as a selective inducer. The productivity of 3-HP was up to 18 g/L when 1% PA was added in the fermentation media at the initial stage, while the control strain only produced 5 g/L of 3-HP. Previous studies [15, 16] have proposed that the metabolic flux to 3-HP in *C. rugosa* could be strengthened by two key enzymes (propionyl-CoA

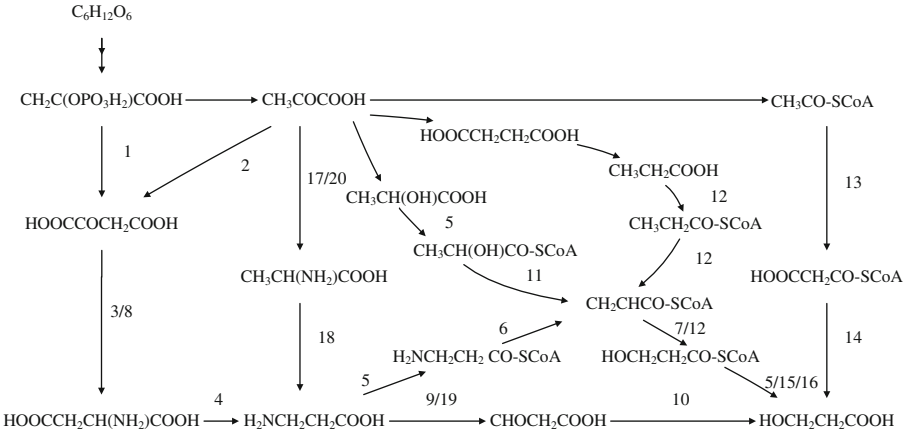

Fig. 1 Overview of seven fermentation pathways for the production of 3-HP from glucose. Enzymes involved 1 phosphoenolpyruvate carboxylase, *2* pyruvate carboxylase, *3* aspartate aminotransferase, *4* aspartate decarboxylase, *5* CoA transferase, *6* β-alanine-CoA ammonia lyase, *7* 3-HP-CoA dehydratase, *8* glutamate dehydrogenase, *9* 4-aminobutyrate aminotransferase, *10* 3-hydroxyisobutyrate dehydrogenase, *11* lactyl-CoA dehydratase, *12* OS17 enzyme (consists of three functional domains: CoA synthetase, propionyl-CoA dehydrogenase, 3-HP dehydratase), *13* acetyl-CoA carboxylase, *14* malonyl-CoA reductase, *15* 3-hydroxypropionyl-CoA hydrolase, *16* 3-hydroxyisobutryl-CoA hydrolase, *17* pyruvate-glutamate transaminase, *18* alanine 2,3-aminomutase, *19* β-alanine-2-oxoglutarate aminotransferase, *20* alanine dehydrogenase

dehydrogenase and 3-HP dehydrogenase) in the modified β-oxidation pathway; however, the two enzymes have not been cloned and identified. As a series of research, we first cloned the full-length cDNA of propionyl-CoA dehydrogenase using 5′ and 3′ RACE-PCR from the mutant *C. rugosa* that could directly produce 3-HP from glucose according to the conserved motifs of acyl-CoA dehydrogenase (BlastX result was about 70%) and compared the transcription levels of PACD gene in different stages in *C. rugosa* with and without propionate as inducer in fermentation using Dot blotting hybridization [17].

In this study, the PACD from *C. rugosa* was further expressed and purified. Molecular mass was analyzed using SDS-PAGE and Western blotting. The activity of the enzyme was measured and the effects of temperature, pH, and ions on the enzyme were studied respectively.

Materials and Methods

Strains, Plasmids, and Culture Conditions

C. rugosa BS-2 was obtained from China Center of Industrial Culture Collection (CICC), which was a mutant directly from *C. rugosa* CICC 31280 by treating with chemical mutagen. The full-length cDNA encoding PACD of *C. rugosa* BS-2 was cloned using 5′ and 3′ RACE-PCR and submitted to the GenBank (GenBank number GU338397). Plasmid pMD18-T (Takara, China) was used as the cloning vector. Yeast expression vector pPIC9K and host strain *Pichia pastoris* GS115 were purchased from Invitrogen. To construct a recombinant expression vector containing PACD gene, the forward primer Ff (GCGGCCGCGATGTCGATTAAGGACGACATCC) and the reverse primer Rf1 (GCGGCCGCATGATGATGATGATGATGCAACTTGGCCAACTTCGCCT) were used to amplify cDNA–PACD. The *Not* I-digested pPIC9k containing signal peptide itself was dephosphorylated by calf intestinal alkaline phosphatase (Takara, China) and ligated with the *Not* I-digested PCR product. The resulting PACD-pPIC9k plasmid was transformed into *E. coli* JM109. The positive clone was confirmed by sequencing, and the recombinant plasmid's correct orientation was confirmed by loading the sequence on the NCBI/BLAST/BlastX site. The resulting plasmid was named pPIC9k-PACD-6His. The recombinant vector pPIC9k-PACD-6His and pPIC9k were both linerized with *BspE* I and electrotransformed into *P. pastoris* GS115 in 2 mm cuvette (1,500 V, 200 Ω, 25 μF). The transformants were selected on RDB medium (1 M sorbitol, 2% dextrose, 1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, 0.005% amino acids) without histidine for His^+ GS115 and MM medium (1.34% YNB, 4×10^{-5}% biotin, 0.5% (*v*/*v*) methanol) for Mut^+ characteristics. BMGY and BMMY medium (1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% YNB; 4×10^{-5}% biotin; 1% (*v*/*v*) glycerol for BMGY and 0.5% (*v*/*v*) methanol for BMMY) were used for expression.

Expression of PACD in *Pichia pastoris* GS115

Transformants GS115/pPIC9k-PACD-6His and negative control GS115/pPIC9k were cultivated following the instruction of *P. pastoris* Expression Kit. Firstly, a colony was inoculated in 25 ml of BMGY in a 250-ml baffled flask; secondly, it was grown at 28–30 °C in a shaking incubator (250–300 rpm) until culture reached an OD_{600}=2–6 (approximately 16–18 h); thirdly, the cells were harvested by centrifuging at 1,500×*g* for 5 min at room temperature; fourthly, the supernatant was decanted and the cell pellet was resuspended to OD_{600} of 1.0 in

BMMY medium for further protein expression; finally, 100% methanol was added to a final concentration of 0.5% every 12 h to maintain induction.

PACD Purification, SDS-PAGE Analysis, and Western Blotting

The culture supernatant of GS115 (pPIC9k/pPIC9k-PACD-6His) was collected after 72 h induction by centrifugation (5,000 rpm, 10 min). The obtained cell-free medium containing secreted recombinant protein was ten times concentrated by ultrafiltration (Millipore; 4 °C, 5,000 rpm, 30 min) which could cut off the proteins larger than 30 kDa. Then the concentrated proteins (about 100 mg) were applied onto a 5 ml Ni-NTA spin column (Ni Sepharose™ 6 Fast Flow, GE Healthcare). The purification of PACD was carried out according to the instruction offered by the manufacturer. Briefly, the column was firstly equilibrated with 15 ml of binding buffer (100 mM NaH_2PO4, 10 mM Tris–HCl, 10 mM imidazole) and then the concentrated proteins obtained above were loaded onto the column. Then, the column was washed twice with 15 ml of washing buffer (100 mM NaH_2PO4, 10 mM Tris–HCl, 20 mM imidazole) to remove non-specific proteins. The 6×His-tagged PACD was eluted with 15 ml of elution buffer (100 mM NaH_2PO4, 10 mM Tris–HCl, 250 mM imidazole). Protein concentration of the collected eluates was determined using the Bradford protein assay (Bio-Rad, USA) [18]. The eluted 6×His-tagged protein was analyzed by SDS-PAGE [11] and further confirmed by Western blotting [19, 20]. The primary and secondary antibodies used for Western detection were rat anti-His (Tiangen, China) and sheep anti-rat IgG-HRP (Horse Radish Peroxidase; Tiangen, China) at the working concentrations of 5×10^{-4} and 2×10^{-3} mg/ml, respectively.

Assay of PACD Activity

The activity of purified propionyl-CoA dehydrogenase was determined using a method as previously described [15, 21]. Typically, the assay buffer contained 0.4 ml of 50 mM Tris–HCl (pH 7.0) containing 200 μM propionyl-CoA, 700 μM $Fc^{+}PF6^{-}$ (ferricenium hexafluorophosphate) and 500 μM *N*-ethylmaleimide. Propionyl-CoA dehydrogenase activity was determined by the initial decrease in absorbance at 300 nm upon the reduction of the ferricenium ion (Cary 50 ultraviolet spectrophotometer, USA). The standard assay was started by addition of the purified enzyme (about 50 μg) to the buffer. All the assays were done in triplicate.

Effects of pH and Temperature on Purified PACD Activity

The effect of pH on the enzyme activity was determined in 50 mM Tris–HCl buffers with various pH from 5.0 to 9.0. The purified PACD was firstly incubated in the buffer for 5 min and then the standard assay conditions were employed except pH change. The effect of temperature on the PACD activity was determined from 20 to 60 °C under standard assay conditions except that the enzyme was incubated for 5 min at various temperatures before determination. The thermostability of the purified PACD was further investigated by incubating the enzyme in the standard buffer for 2 h at 30, 40, and 50 °C prior to the assay of the remaining activity at the corresponding temperatures.

The Kinetics of Purified PACD

The enzyme activity was assayed with various concentrations of propionyl-CoA from 100 to 350 μM in 50 mM Tris–HCl (pH 7.0, 30 °C) containing 700 μM $Fc^{+}PF6^{-}$

and 500 μM *N*-ethylmaleimide. All the assays were done in triplicate. Different concentrations of propionyl-CoA [S] and the corresponding reaction rates V were reversed to be $[S]^{-1}$ and V^{-1}, which had linear relationship with each other and double reciprocal plot was performed. Then K_m, k_{cat}, and V_{max} values were calculated according to the formula:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}}$$

Effect of Metal Ions on Purified PACD Activity

The purified PACD was incubated at 30 °C (pH 7.0) in the standard buffers for 5 min with various metal ions at the final concentrations of 1 and 5 mM, respectively. Then, the enzyme assay was performed under standard conditions as described above. The metal ions tested included Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+}.

Results

Expression and Purification of PACD in *P. pastoris*

The full-length cDNA–PACD gene amplified with the primers Ff and Rf1 was cloned into the expression vector pPIC9K and the positive recombinant plasmid was identified. The recombinant plasmid was linearized and electrotransformed into *P. pastoris* GS115 to get the positive transformant named GS115/pPIC9K-PACD-6His, which was cultured following the instruction of *P. pastoris* Expression Kit and induced with methanol. After 72 h induction, about 0.13 mg/ml total protein in the medium was obtained. The culture was then centrifuged and resultant supernatant was concentrated and changed buffer by ultrafiltration. The concentrated protein was then purified by affinity chromatography. SDS-PAGE showed the total protein of the supernatant expressed in recombinant GS115/pPIC9k-PACD-6His cells at 30 °C (lane 1 in Fig. 2a), the total protein of the supernatant expressed in control GS115/pPIC9k (lane 2 in Fig. 2a), and the purified protein of the supernatant expressed in GS115/pPIC9k-PACD-6His with a molecular mass of about 49 kDa (lane 2 in Fig. 2b). No corresponding protein (about 49 kDa) was purified from the control GS115/pPIC9k (lane 1 in Fig. 2b). The protein was further confirmed using Western blotting (lane 3 in Fig. 2b).

Effect of pH and Temperature on Purified PACD Activity

The purified PACD activity was measured at various pH values and temperatures. As shown in Fig. 3, when pH value was higher than 7.5 and lower than 6.0, the enzyme activity decreased significantly and an optimal pH was observed at 7.0. When the temperature was higher than 40 °C or lower than 25 °C, the enzyme activity decreased greatly and an optimal temperature was observed at 30 °C. The thermostability for the recombinant PACD was studied at temperatures varied from 30 to 50 °C. As shown in Fig. 4, the PACD maintained 76.3%, 30.1%, and 4.3% of its original activity after 2 h incubation, respectively.

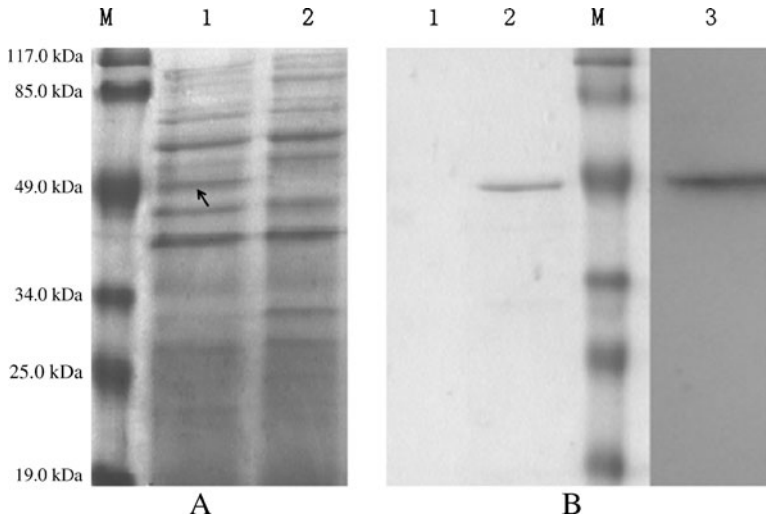

Fig. 2 Expression of the cDNA–PACD in *Pichia pastoris* GS115. **a** *Lane 1* the total protein of the supernatant expressed in recombinant GS115/pPIC9k-PACD-6His cells at 30 °C, *lane 2* the total protein of the supernatant expressed in control GS115/pPIC9k. **b** *Lane 1* the purified protein expressed in control GS115/pPIC9k, *lane 2* the purified PACD expressed in recombinant GS115/pPIC9k-PACD-6His cells at 30 °C for 72 h, *lane 3* Western blotting analysis of the expressed fusion protein PACD-6His. *Lane M* protein markers (117.0, 85.0, 49.0, 34.0, 25.0, 19.0 kDa from top to bottom, respectively). In **a**, the recombinant protein band was arrowed

The Kinetics of Purified PACD

The kinetic parameters of recombinant PACD were determined under the optimal conditions at 30 °C and pH of 7.0. The K_m, k_{cat}, and V_{max} were calculated by the double reciprocal method with the value of 40.86 μM, 0.566 s^{-1}, and 0.693 Umg^{-1} min^{-1} (Fig. 5).

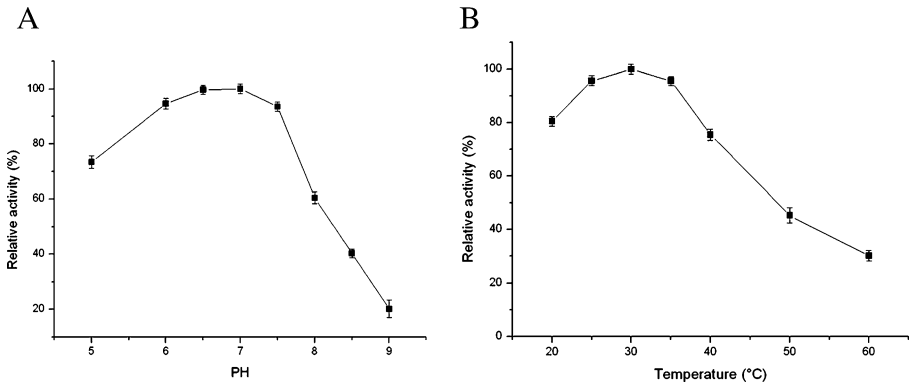

Fig. 3 The effects of pH and temperature on the relative activities of PACD (means±SD, n=3). **a** PACD was incubated at pH ranging from 5.0 to 9.0 (30 °C) in 50 mM Tris–HCl buffer. **b** PACD was incubated at temperatures ranging from 20 to 60 °C in 50 mM Tris–HCl buffer (pH 7.0)

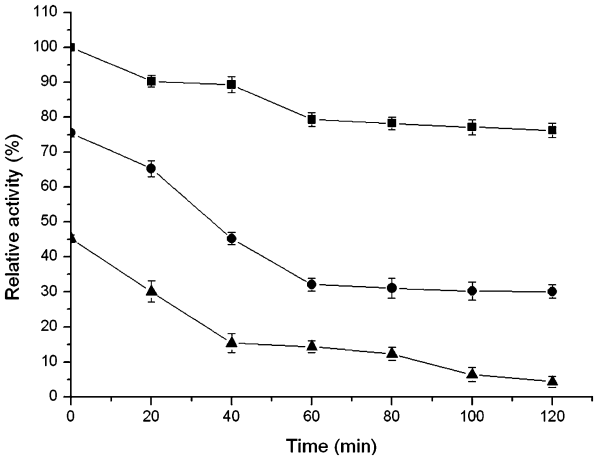

Fig. 4 Thermostability of purified PACD (means±SD, n=3). PACD was incubated in 50 mM Tris–HCl buffer (pH 7.0) under the standard assay conditions except the assay temperatures at 30 (*black squares*), 40 (*black circles*), and 50 °C (*black triangles*), respectively

Effect of Metal Ions on Purified PACD Activity

As indicated in Table 1, the effects of several metal ions on the activity of PACD were studied. Mg^{2+} had an activating effect on the enzyme, while other ions such as Mn^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+} could weakly inhibit the PACD activity at high concentrations.

Discussion

Although propionyl-CoA dehydrogenase activity had been described in many microorganisms such as *Salmonella typhimurium*, *Prototheca zopfii*, and *Moraxella lwoffl*, the dehydrogenase activity was demonstrated only in cell-free extracts and propionyl-CoA

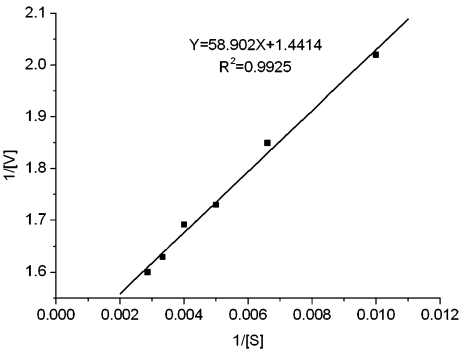

Fig. 5 The kinetics of purified PACD. *1/[S]* the reverse of different concentrations of propionyl-CoA. *1/[V]* the reverse of reaction rates of different concentrations of propionyl-CoA. The enzyme activity was assayed with various concentrations of propionyl-CoA from 100 to 350 μM in 50 mM Tris–HCl (pH 7.0, 30 °C) containing 700 μM $Fc^{+}PF6^{-}$ and 500 μM *N*-ethylmaleimide as described in the method. All the assays were done in triplicate

Table 1 Effect of metal ions on the relative activity of PACD

Metal ions	Concentrations (mM)	V (U mg^{-1} min^{-1})	Relative activity (%)
No		0.576	100
MgSO4	1	0.587±0.01	101.9±1.74
	5	0.64±0.015	111.11±2.6
MnSO4	1	0.563±0.019	97.743±3.29
	5	0.558±0.021	96.875±3.64
CaCl2	1	0.55±0.009	95.486±1.56
	5	0.528±0.01	91.667±1.74
ZnCl2	1	0.571±0.017	99.097±2.95
	5	0.561±0.016	97.396±2.78
CuSO4	1	0.566±0.013	98.264±2.25
	5	0.573±0.015	99.479±2.6

Data were given as means±SD, n=3

dehydrogenases from these organisms had neither been isolated nor characterized [16]. In the present study, the PACD gene obtained from *C. rugosa* was first successfully expressed in *P. pastoris*, and the purified recombinant enzyme had propionyl-CoA dehydrogenase activity, which demonstrated that the PACD produced by *P. pastoris* was in the active form. The purified recombinant PACD had a molecular mass of about 49 kDa, which had similar size as estimated from the deduced amino acid sequence of this gene. The enzyme was characterized with K_m (40.86 μM), k_{cat} (0.566 s^{-1}), and V_{max} (0.693 Umg^{-1} min^{-1}). The optimal temperature and pH of the recombinant PACD were 30 °C and 7.0, respectively. Thermostability was studied and the results showed that it maintained 76.32%, 30.11%, and 4.32% of its original activity when PACD was incubated for 2 h in the standard buffer at 30, 40, and 50 °C. The effects of metal ions on the activity of PACD were studied, which showed that Mg^{2+} had an activating effect on the enzyme, while other ions such as Mn^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+} could weakly inhibit the PACD activity at high concentrations.

The propionyl-CoA dehydrogenase which was purified from *Clostridium propionicum* exhibited propionyl-CoA and butyryl-CoA dehydrogenase activity (K_m=50 μM, V_{max}=0.79 U mg^{-1} min^{-1}) [22]. Butyryl-CoA dehydrogenase was purified from *Streptomyces collinus* with K_m of 18 μM [23]. Compared with former research, the PACD enzyme from *C. rugosa* had a good binding capacity with propionyl-CoA.

The propionyl-CoA dehydrogenase purified from *C. propionicum* composed of a propionyl-CoA dehydrogenase (α_2×40 kDa) and an electron-transferring flavoprotein (Etf; β, 38 kDa; γ, 29 kDa), which could catalyze propionyl-CoA to acrylyl-CoA in anaerobic conditions [22]. The butyryl-CoA dehydrogenase from *Clostridium kluyveri* had three subunits with molecular mass of 41 kDa (butyryl-CoA dehydrogenase), 38 kDa (Etfα), 26 kDa (Etfβ), as revealed by SDS-PAGE and could catalyze butyryl-CoA to crotonyl-CoA [24]. These results suggested that acyl-CoA dehydrogenases from different organisms showed different amino acid compositions and structures, and the propionyl-CoA dehydrogenase reported here had one 49 kDa subunit. The primary sequence of this propionyl-CoA dehydrogenase showed about 70% identity to other yeast acyl-CoA dehydrogenases, and these enzymes had similar conserved motifs. In addition, based on an assumption that this enzyme works following the same mechanism as the above

dehydrogenases, we added $Fc^{+}PF6^{-}$ instead of Etf as electron-transferring acceptor in the in vitro enzyme assay medium, and the activity of the enzyme was confirmed.

In conclusion, the *C. rugosa* PACD gene was successfully characterized and analyzed, which provided evidence that the mutant *C. rugosa* could produce 3-HP through the modified β-oxidation pathway. This could benefit the engineered strains for effective production of 3-HP from the most abundant biomass – sugars.

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