

Production of 3-hydroxypropionic acid by recombinant *Klebsiella pneumoniae* based on aeration and ORP controlled strategy

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Abstract—A biosynthetic pathway for the production of 3-hydroxypropionic acid (3-HP) from glycerol was established in recombinant *Klebsiella pneumoniae* by introducing the aldehyde dehydrogenase gene from *Escherichia coli*. The activity of aldehyde dehydrogenase, which oxidized 3-hydroxypropionaldehyde (3-HPA) to 3-HP, was detected and 3-HP was produced by the recombinant strains. Three different oxygen supply strategies, associated with measuring the oxidoreduction potential (ORP) during the fermentation under these conditions, were adopted for higher production of 3-HP by the recombinant cells. About 0.8 g/l 3-HP and more 1,3-propanediol production by the recombinant *Klebsiella pneumoniae* were obtained under completely aerobic conditions. Under micro-aerobic conditions, 3-HP production could be increased to 2.2 g/l and 1,3-propanediol production was almost the same as in the original strain. Under the anaerobic conditions, 1,3-propanediol was the main product and about 1.3 g/l 3-HP was produced. Finally, 3-HP production of the recombinant strain was increased to 2.8 g/l under micro-aerobic condition with a further two-stage ORP controlled strategy.

Key words: 3-Hydroxypropionic Acid, 1,3-Propanediol, *Klebsiella pneumoniae*, Aldehyde Dehydrogenase, Glycerol Dehydratase

INTRODUCTION

Bio-based chemicals have recently attracted increasing attention. 3-hydroxypropionic acid (3-HP), as well as 1,3-propanediol (1,3-PD), is an important chemical intermediate in the manufacture of other chemical products. They both can be used as the feedstock for the production of polymeric materials such as polytrimethylene terephthalate (PTT) and poly[3-hydroxypropionic acid] (P[3-HP]) [1,2]. Another promising large-volume application of 3-HP is for the production of acrylic acid, a component in acrylic-based paints and super absorbent polymers, and many other bulk chemicals such as 1,3-propanediol, acrylamide and malonic acid [3]. 3-HP will also become the important target for fermentative production from sugars on bulk industry scales owing to the diminishing petroleum reserves and the failure of its large-scale chemical production [4]. However, the applications of 3-HP have not yet been fully developed, due to the limited availability of 3-HP (International conference communication with Cargill technocrat, Beijing, 2005). Recently studies in the field of industrial biotechnology have focused on constructing novel strains for highly efficient production of 3-HP. Gene modification and biochemical process optimum have been applied to develop a host microorganism that may enable such a fermentation process.

However, only some special microorganisms in nature could use different substrates to produce 3-HP. For example, *Hansenula miso* could transform 1,3-propanediol to 3-HP [5], *Fusarium merismoides* could transform 1,4-butanediol to 3-HP [6], and *Candida ugosae*

could transform propionic acid to 3-HP [7]. But for more large-scale biological production of 3-HP, it is desirable to use cheaper and more abundant technical substrates such as glycerol and glucose.

As a metabolic intermediate in the novel autotrophic pathway-3-HP circulation pathway, 3-HP could be little detected in some certain facultative aerobic, phototrophic bacterium such as *Chloroflexus aurantiacus* [8]. However, as a metabolic intermediate, it could not be accumulated to a high amount because the circular metabolic pathway would be disturbed by excessive accumulation. No other natural microorganisms so far have been found that could efficiently convert glucose or glycerol to 3-HP. Two approaches, in principle, can be used according to utilizing different substrates such as glucose and glycerol. They both used *E. coli* as the host cell for 3-HP production. These studies were reported by Suthers and Selifonova. Suthers et al. cloned the two key genes encoding glycerol dehydratase (GDHt) and aldehyde dehydrogenase (AldA) into *E. coli* to achieve the production of 3-HP from glycerol [9]. In this metabolic pathway (Fig. 1(a)), glycerol was first dehydrated to 3-hydroxypropionaldehyde (3-HPA) by exogenous GDHt. Then the 3-HPA was oxidized to 3-HP by exogenous AldA. Another approach used by Selifonova was to redesign the metabolic pathway from glucose to 3-HP via lactate or alanine pathway by introducing several other key enzyme genes (listed in Fig. 1(b)) [3]. But the exogenous enzyme activities and the production of 3-HP of the recombinant *E. coli* with the above two strategies were always low. No 1,3-PD was produced by the recombinant *E. coli* because no enzymes for the reduction of 3-HPA existed and 1,3-PD was usually produced in anaerobic or micro-aerobic conditions.

In this study, we used *Klebsiella pneumoniae* as the host cell for

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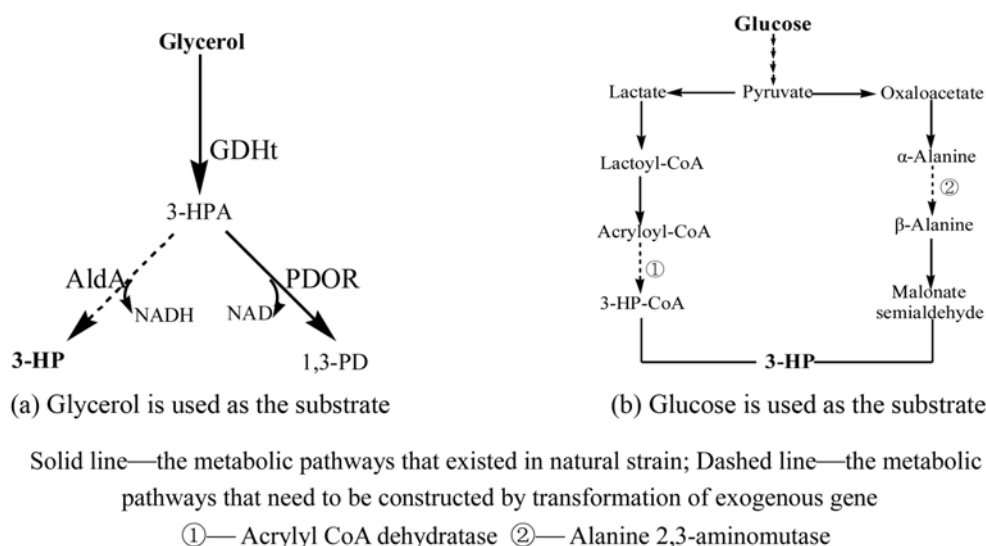


Fig. 1. The metabolic pathway of 3-HP using different substrate with gene modification technology.

Table 1. Bacterial strains and plasmids

Strain and plasmid	Relevant characteristics	Source
<i>Klebsiella pneumoniae</i> ME-308	Host of recombinant plasmid	Lab collection
<i>E. coli</i> DH 5α	Host of recombinant plasmid	Lab collection
<i>E. coli</i> JM109	Source of <i>aldA</i> gene	Lab collection
pDK6	Kan ^R	Donated by Prof. Merrick MJ
pET22b	Tet ^R , used for protein purification	Lab collection
pMD18- simple-T	Amp ^R , used for sequencing	Takara company
pMD18- simple-T- aldA	1.44 kb DNA fragment with <i>aldA</i> gene in T-vector used for sequencing	This work
pDK6/aldA	Kan ^R , 1.44 kb DNA fragment with <i>aldA</i> gene in pUC18 used for expression	This work
pET22b/aldA	Tet ^R , 1.44 kb DNA fragment with <i>aldA</i> gene in pUC18 used for purification	This work

Kan^R: kanamycin resistance, Amp^R: ampicillin resistance, Tet^R: tetracycline resistance

3-HP production with only exogenous expression of *aldA*. Less gene was transformed compared with the traditional gene modification methods. *Klebsiella pneumoniae* was more tolerant of high concentration of the substrate glycerol than other host cells. The GDHt in native strain was less sensitive to higher dissolved oxygen. The previous research about constructing recombinant *E. coli* for 3-HP production all indicated that both the substrate glycerol and O₂ could quickly inactivate the GDHt of recombinant *E. coli* [10]. Subsequently, little activity of GDHt was detected when *E. coli* was used as the host for GDHt expression [11]. The reason for low activity of GDHt in recombinants may be that the fermentation of the recombinant strain was performed under aerobic conditions and the *E. coli* could not synthesize coenzyme B₁₂ or vitamin B₁₂, which was the indispensable reactive cofactor for the GDHt activity [12,13]. Only expression of GDHt in *E. coli* without any other optimization may not be proper for the target production. So GDHt in native strains would catalyze the glycerol to 3-HPA more efficiently than in recombinant *E. coli*. To our knowledge, this is the first report showing that the engineering *Klebsiella pneumoniae* has the ability for 3-

HP production.

EXPERIMENTAL

1. Strains, Plasmids and Culture Media

The strains and plasmids used in this study are listed in Table 1. LB medium was used as the seed culture of *E. coli*, *K. pneumoniae* and their derivatives. When *K. pneumoniae* was used for electroporation, EDTA was added to the germ medium to a final concentration of 0.7 mM.

After the electroporation, the seed culture for activating the recombinant *K. pneumoniae* was SOC medium [14]. When necessary, 50 µg/ml kanamycin was added to the medium as the selection marker.

The fermentation medium for recombinant *K. pneumoniae* was yeast extract 5 g/l, glycerol 30 g/l, K₂HPO₄·3H₂O 10 g/l, KH₂PO₄ 2 g/l, NH₄Cl 1 g/l, NaCl 0.5 g/l, MgSO₄·7H₂O 0.1 g/l, FeCl₃·6H₂O 30 mg/l, CoCl₂·6H₂O 5 mg/l, VB₁₂ 5 mg/l. For controlling the aeration and measuring the value of ORP more easily, a batch culture was carried out in a 3 L bioreactor (NBS, New Brunswick, USA)

with a working volume of 2 L under different aeration conditions. The cultivation was carried out at 37 °C, 5% (v/v) inoculation volume, 300 rpm agitation speed and initial pH 7.0. An anaerobic or aerobic environment in the bioreactor was maintained by aerating nitrogen or air at 0.5 vvm. The one without aeration is considered as a micro-aerobic condition. The ORP was controlled (−100 mV–40 mV) in this work by adjusting the flow rates of the air.

2. Construction of Plasmids and Strains

The *aldA* gene was amplified by PCR using total genome DNA of *E. coli* JM109 as template DNA and primers A1 and A2, which were designed by using the sequence information of *aldA* gene (gene ID 6058212, GeneBank) from *E. coli* K12.

A1: 5-AAAAGAATTCCATGTCAGTACCCGTTCAACA-3

A2: 5-CCCGGGTTAAGACTGTAAATAAACACCT-3

The PCR product was purified and connected into cloning vector pMD18-simple-T (products of Takara) for sequencing. After the sequencing, *aldA* gene was connected with the expression vector pDK6 and cloning vector pET-22b using the restriction endonuclease sites *Eco*RI and *Sma*I that were introduced by the primers used (underlined), yielding plasmid pDK6/*aldA*, pET-22b/*aldA*, respectively. The PCR programs were performed on a thermocycler (Eppendorf) and consisted of: 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, as well as final extension step of 72 °C for 10 min. The PCR mixture consisted of 1 ng each template, 200 mM each dNTP, 20 mM each of the primers, 2.5 µl Taq PCR buffer and 0.3 µl Ex Taq DNA polymerase (purchased from Takara) in 25 µl. The recombinant plasmid pDK6/*aldA* was purified and transformed into *E. coli* and *K. pneumoniae* ME-308 by standard transformation protocol by the method of electroporation [15,16].

3. Polyacrylamide Gel Electrophoresis and Characterization of Recombinant Protein

The SDS-PAGE was conducted on a 12% polyacrylamide gel by the method of Joseph et al. Operation was performed on the Mini-ProteinIII electrophoresis system (Bio-Rad). Protein on the gel was stained with 0.2% (w/v) Coomassie brilliant blue R-250. Marker proteins with molecular weights ranging from 14.3 to 97.2 kDa were used to estimate the molecular weight of the expression products. The concentration of protein was measured according to the Bradford method with bovine serum albumin (BSA) as standard protein.

Aldehyde dehydrogenase was partially purified for biochemical characterization. The *aldA* was connected to vector pET-22b for AldA purification. *E. coli* cells carrying recombinant vectors pET-22b/*aldA* were harvested, suspended with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, 2 mM DTT, pH 7.4), and disrupted by sonication. The supernatant obtained by centrifugation was loaded onto a Histrap HP (purchased from Pharmacia Biotech) column equilibrated in binding buffer, and washed with the same buffer. Then it was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 30–300 mM imidazole gradient, and 2 mM DTT, pH 7.4). The enzyme purified as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for biochemical characterization.

4. Preparation of Cell-free Extracts and Assay of the Key Enzyme Activity

IPTG was added into the culture to a final concentration of 1.0 mM to induce recombinant protein expression when recombinant

cells grew until optical density at 600 nm reached 0.4–0.6. The cells were harvested (at 4 h after IPTG induction for AldA activity measurement, at 12 h after IPTG induction for GDHt activity measurement) by centrifugation from the fermentation culture. After the cell pellet was washed with 0.035 mol/l potassium phosphate buffer (pH 8.0), the cells were resuspended with the same buffer in 3.0 ml. The cells were disrupted ultrasonically at 4 °C for 10 min by a cell sonicator (SCIENITZ JY92-II, China). Cell debris was removed by centrifugation, resulting in a cell-free extract, which was subsequently used for the enzyme activity assay.

To decrease the errors, Glycerol dehydrogenase (GDHt) activity was determined quickly after being sampled from the fermentation broth by the modified 3-methyl-2-benzothiazolinone hydrazone (MBTH) method according to Toraya [17]. The reaction mixture (3.5 ml) was composed of 0.05 M KCl (0.1 ml), 0.2 M 1,2-propanediol (0.1 ml), 50 mM ATP (0.1 ml), 10 mM Mg²⁺ (0.1 ml), 10 µM coenzyme B₁₂ (0.1 ml) and 0.7 ml cell extraction. After incubation at 37 °C for 10 min, the enzyme reaction was stopped by adding 1.0 ml of 0.1 M potassium citrate buffer and 0.5 ml of 0.1% MBTH solution. After 15 min at 37 °C, 1.0 ml of water was added and the absorbance was measured at 305 nm. One unit of GDHt activity (1 U) is defined as the amount of enzyme required to form 1 µmol of proportionaldehyde per minute at 37 °C.

The AldA activity of crude extracts can be measured by the formation speed of NADH from NAD⁺ from the oxidization of 3-HPA. The reaction was carried out at 37 °C in the presence of 1 mM DTT, 2 mM 3-HPA, 2 mM NAD⁺ and an appropriate volume of the enzyme solution in a 2 ml final potassium phosphate buffer (pH 7.0). One unit of AldA activity (1 U) was defined as the amount of enzyme required to reduce 1 µmol of NAD⁺ to NADH per minute. Since 3-HPA here used is not available commercially, it was synthesized chemically from acrolein using the method described by Hall and Stern.

5. Analysis of Biomass, Metabolic Products and ORP

Biomass was determined by measuring the value of optical density at 600 nm with appropriate dilution with a UV-visible spectroscopy system (DU-640, Beckman, USA). The value of the optical density was converted to cell dry weight (CDW) by using a calibration equation ($CDW = 4.183 \times OD_{600} - 0.117$).

The samples from fermentation were always prepared into two completely identical parts. One was used for 3-hydroxypropionic acid concentration determination, and the other was used for alcohol and acetate concentration determination. 3-HP concentration was determined by HPLC with ultraviolet (UV) detector. Ethanol and 1,3-PD were measured by HPLC with refractive index (RI) detector (Summit P 680 HPLC, Dionex, USA; Shodex RI-101 Refractive Index detector, Showa Denko, Japan; Dionex UVD 170U detector; Aminex HPX-87H ion exclusion column 300 mm × 7.8 mm, Bio-Rad, USA) under the following conditions: sample volume 20 µl; mobile phase 0.005 M H₂SO₄; flow rate 0.8 ml·min^{−1}; column temperature 65 °C. However, glycerol cannot be quantified exactly with the two detectors above because no absorption peak in HPLC appeared with a UV detector and the peaks of glycerol and 3-HP in HPLC coincided with the RI detector in this experiment.

ORP was measured by a redox combination electrode that consisted of a pH electrode and a redox controller (pH2100e, Mettler

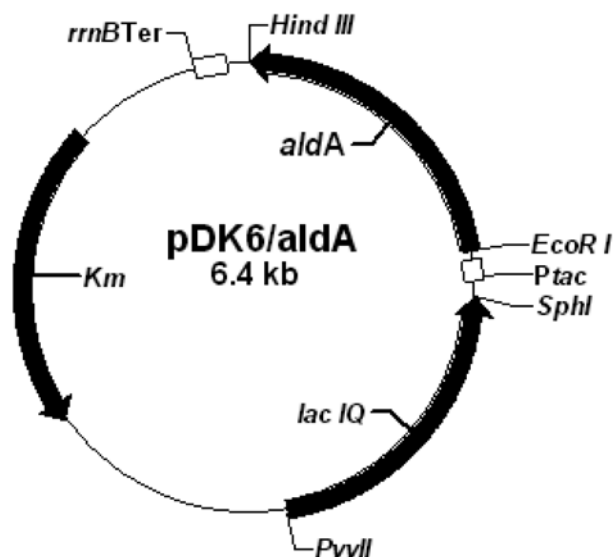


Fig. 2. Structure of the recombinant plasmids constructed in this study.

Toledo Instruments Company, Shanghai). Before each measurement, the electrode was treated in electrode cleaning solution for no less than 2 h and then calibrated with redox solution.

RESULTS AND DISCUSSION

1. Construction of Recombinant Strains

The recombinant plasmid from the transformed strain was purified and analyzed. The structure of the recombinant plasmids is shown in Fig. 2. The results of agarose gel electrophoresis of PCR products showed that the DNA fragments (*aldA*) were about 1.44 kb. A fragment was inserted into vector pDK6 and pET-22b, yielding recombinant plasmid pDK6/*aldA*, pET-22b/*aldA*, respectively. The recombinant plasmids were transformed into *K. pneumoniae* ME-308 and *E. coli* DH5 α successfully. The positive colonies were screened with kanamycin resistance markers or tetracycline resistant markers.

The amplified *aldA* gene was connected to plasmid pMD18-simple-T for sequencing. DNA sequence analysis showed that the *aldA* gene was 1,440 bp, which was in accordance with *E. coli* *aldA* gene sequence announced by NCBI gene bank, encoding 479 amino acid residues with predicted molecular weight of about 53 kDa.

Table 2. Kinetic properties of AldA on different aldehydes

Aldehyde	Cofactor	K _m (mM)	V _{max} ($\mu\text{M}\cdot\text{min}^{-1}$)
Formaldehyde	NAD ⁺	0.92	10.4
Acetaldehyde	NAD ⁺	0.84	20.2
Propanaldehyde	NAD ⁺	0.42	26.4
3-Hydroxypropionaldehyde	NAD ⁺	0.31	28.4
	NADP ⁺	0.34	10.3
Butaraldehyde	NAD ⁺	0.29	27.1
Valeraldehyde	NAD ⁺	0.32	29.4

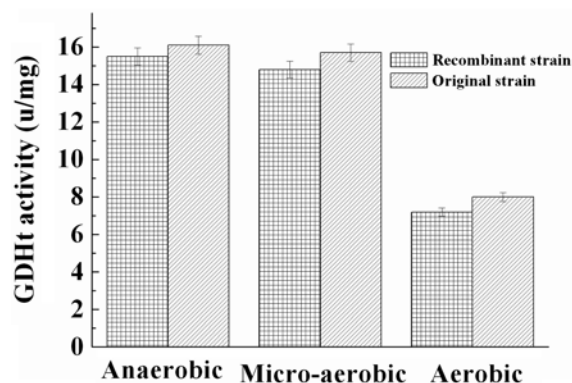
Cofactors NAD⁺ or NADP⁺ was constant at 2 mM, and the aldehydes varied between 0.006 and 0.6 mM

2. Characterization of Recombinant Enzyme

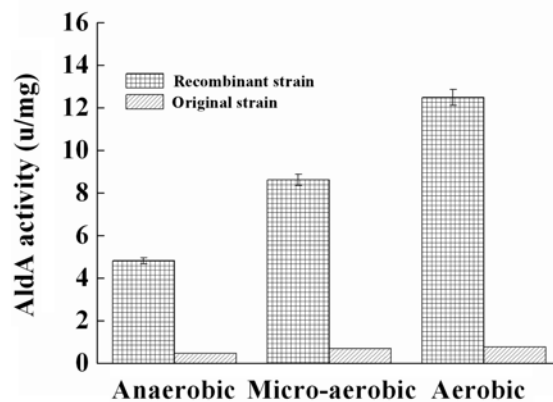
The enzyme was purified for further study. The purified recombinant enzyme exhibited its Michaelis-Menten kinetic properties as described in Table 2. The apparent K_m values for 3-HPA, butaraldehyde and valeraldehyde were lower than that for other substrates. These suggested that the activities of AldA to the different substrates might be relation to the length of aldehyde chain and showed higher affinity of the long chain aldehydes to be linked to the recombinant AldA. The kinetic parameters of the 3-HPA oxidation using NAD⁺ and NADP⁺ were also measured. The maximum reaction rates (V_{max}) for the oxidation of 3-HPA to 3-HP were 28.4 and 10.3 $\mu\text{M}\cdot\text{min}^{-1}$ protein when using NAD⁺ and NADP⁺ as respective cofactors. Although the V_{max} for the 3-HPA oxidation reaction was about 2.6-fold higher with NAD⁺ than with NADP⁺, the half-saturation constant (K_m) to 3-HPA substrate when using fixed concentration of NAD⁺ (0.5 mM) was almost the same as with the fixed concentration of NADP⁺ (0.5 mM). According to the definition of K_m and V_{max}, it can be concluded that the AldA could have had high activity for 3-HPA when NAD⁺ was used as the cofactor. Jo et al. [18] also reported that AldH (another aldehyde dehydrogenase derived from *E. coli*) preferred NAD⁺ to NADP⁺ with broad substrate specificities.

3. Key Enzyme Activities in Crude Extracts under Different Aerations

The effect of aeration conditions on GDHt and recombinant AldA activities of the recombinant strain and the parent strain during the



(a) Effect of aeration conditions on GDHt activity



(b) Effect of aeration conditions on AldA activity

Fig. 3. The key enzyme activities of the recombinant and the original strains under different aerations in batch cultures.

fermentation is shown in Fig. 3.

There was no distinct difference of GDHt activities between the recombinant and the original strains under the same aeration condition. This showed that the gene modification to the *Klebsiella pneumoniae* did not impair the native GDHt activities. The GDHt activities of both the recombinant and the original strain under the micro-aerobic condition were almost the same as that under anaerobic condition, but the one under the aerobic condition was 46% lower than that under the anaerobic condition. This may be due to the fact that the original strain, which was derived from *Klebsiella pneumoniae* ATCC25955, was not sensitive to the low level oxygen [19]. But under the high level dissolved oxygen conditions, the GDHt activities would still decrease more or less.

The original *Klebsiella pneumoniae* strain showed very low AldA activity for the reaction from 3-HPA to 3-HP lower than 0.8 U/mg, which was often not efficiently enough for the oxidation of the aldehydes. AldA activity could be significantly increased up to 4.8-12.4 U/mg crude protein by plasmid-based over-expression of the *aldA*, which was 6-15 times greater than that of the wild strain. This was necessary and enough for the oxidation of 3-HPA. It was also observed that the activities of the AldA under different aeration culture conditions were much different. In anaerobic culture, the AldA activity was the lowest, while in aerobic culture the AldA activity was the highest. The AldA activity in micro-aerobic condition was between anaerobic and aerobic condition. This showed that the recombinant AldA might be sensitive to the dissolved oxygen level. Thus, the ability for the oxidation of 3-HPA would be relevant to the aerations.

4. 3-HP Production by Recombinant Strains under Completely Aerobic Conditions

Batch cultures of the recombinant *K. pneumoniae* harboring pDK6/*aldA* and the original strain were carried out for about 28 h with 30 g/L glycerol as a single carbon source under completely aerobic conditions. Differences between fermentation parameters of the original strain and the recombinant strain are shown in Table 3. The difference of biomass indicated that the cell growth of recombinant strain was impaired by the expression of the exogenous gene. The over-expression of plasmid-encoded protein can be a factor to impose a heavy metabolic burden on the host cell because a large amount of energy is consumed during protein synthesis. It was also obvious that no 3-HP was produced by the original *Klebsiella pneumoniae* ME-308. Little 1,3-propanediol (6.2 g/l) was obtained under this condition, which was far lower than that under the micro-aerobic

conditions [19]. Thus the fermentation by original *K. pneumoniae* under completely aerobic conditions was nonsense for the industrial production. However, 0.81 g/l 3-HP was obtained by the recombinant *Klebsiella pneumoniae* and 11.3 g/l 1,3-propanediol was obtained, which were higher than that of the original strain. Certainly, the 3-HP production by the recombinants was due to the introduction of exogenous AldA. The production of 2,3-butanediol was increased (72.2%) obviously in recombinant cells, while the final acetate concentration was only a bit higher than that of the parent strain. These might have resulted from the increased NADH level due to the oxidation of 3-HPA to 3-HP. Both of the metabolic pathways for the synthesis of 2,3-butanediol and 1,3-PD were NADH-consumed processes. This is the first report that we know of that utilized the *Klebsiella pneumoniae* as the host cell for co-production of 1,3-PD and 3-HP under the completely aerobic conditions, although the production of 3-HP was still low. From the combination analysis of the enzyme activities under different aerations with the cofactors that the enzymes utilized in the metabolic pathway, it could be concluded that 3-HP production during the fermentation would be impacted by the oxygen supply. Thus, the experiments for 3-HP production under different aerations were investigated to obtain a suitable aeration strategy for higher 3-HP production.

5. 3-HP Production, ORP Measurement under Three Different Aerations by the Recombinants

As shown in Fig. 4, the profile of extracellular of ORP and the metabolites (1,3-PD and 3-HP) production under aerobic, micro-aerobic and anaerobic conditions were investigated. While the ORP value (10-30 mV, above zero) was the highest under the aerobic conditions, it could draw to between -140 mV and -180 mV quickly under the anaerobic conditions. The cell growth under aerobic with high ORP value was faster than that under the micro-aerobic and anaerobic. However, more 1,3-PD and 3-HP were produced under micro-aerobic conditions. Under anaerobic condition, the lowest AldA activity (Fig. 3) might impair the transformation of 3-HPA to 3-HP. The supply of supernumerary NADH resulting from the oxidation of 3-HPA was thus decreased. So the production of 1,3-PD and 3-HP was both impaired. Under aerobic condition, more substrate was distributed to glycerol oxidation pathway, which produces more energy for cell growth. Thus, both the aerobic and anaerobic conditions might be not suitable for the accumulation of 3-HP. It could also be found that 3-HP was accumulated only in a short time during the fermentation. Exclusive of these times, 3-HP was not produced. Under the aerobic and anaerobic conditions, 3-HP accumulated in a different fermentation stage.

6. 3-HP Production with ORP Controlled Strategy

As described in Fig. 4, 3-HP was produced at different times by the recombinant strain under different aerations, associated with the different ORP values. This meant that the preferable ORP range for 3-HP production might be different at various stages during the fermentation. However, under the micro-aerobic condition, it was always difficult to optimize the fermentation process due to lack of proper parameters that can be used to monitor the fermentation process. Extracellular ORP was shown to affect the synthesis or stability of certain enzymes, which in turn could change metabolic fluxes and/or ATP yield [20]. Also, the sensitivity of the ORP sensor to the red-ox state was higher than that of the dissolved oxygen (DO) probe, while it was difficult to monitor the micro-aerobic fermentation

Table 3. Comparing of the fermentation of the Recombinant *K. pneumoniae* with those of the parent strain under completely aerobic conditions

Strains	Recombinant strain	Parent strain
Fermentation time (h)	28	28
Biomass (g/l)	2.48	3.51
Glycerol consumption (g/l)	30	30
1,3-PD (g/l)	11.3	6.2
3-HP (g/l)	0.81	-
Acetate(g/l)	0.74	0.68
Lactate (g/l)	0.30	0.32
2,3-Butanediol (g/l)	3.1	1.8

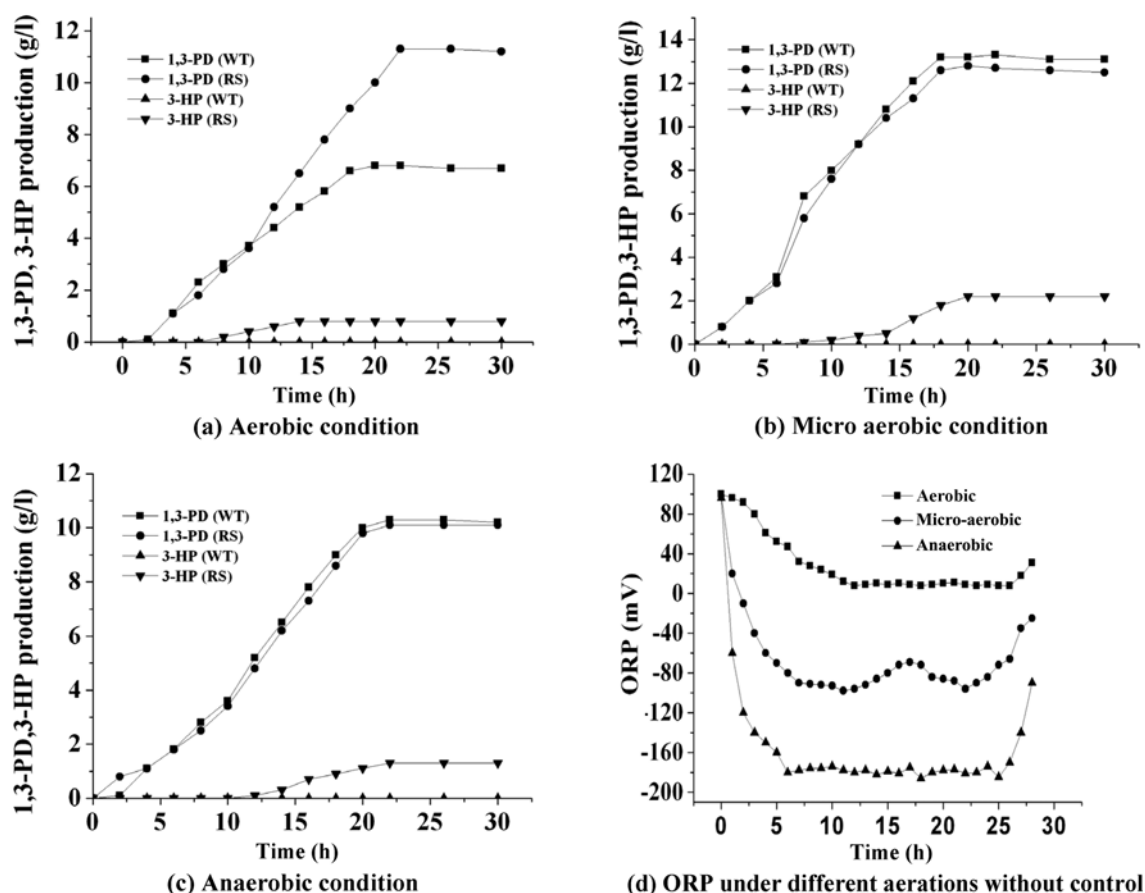


Fig. 4. Time course profile of recombinant and wild *Klebsiella pneumoniae* under different aerations.

WT: wild type strain RS: recombinant strain

process. Based on this analysis, a two-stage ORP control strategy was therefore proposed to achieve a further optimal production of 3-HP. In this strategy, the ORP was controlled at 10–20 mV in the first 14 h (the first stage) to maintain a high ORP level by proper aeration, and then switched to $-70 \sim -100$ mV to maintain a lower ORP level by lowering the amount of aeration and even stopping aerating after 14 h (the second stage). The time course of the two-stage ORP control strategy for 3-HP fermentation is shown in Fig. 5.

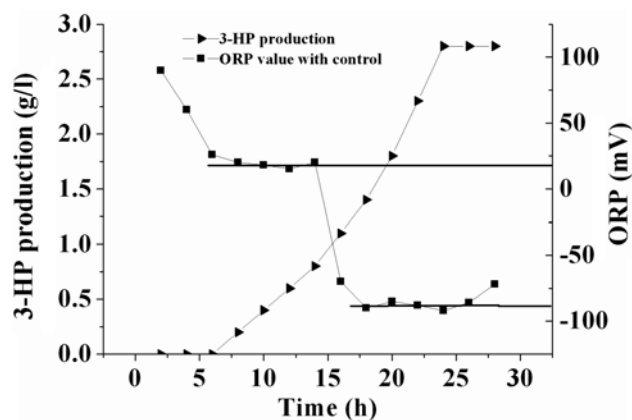


Fig. 5. Time course of the two-stage ORP control strategy for 3-HP fermentation.

It was observed that by using the two-stage ORP control strategy the final concentration of 3-HP reached 2.8 g/l. 1,3-PD production was 9.8 g/l, which was a bit lower than that under the micro-aerobic conditions. 3-HP accumulation both at the first stage and at the second stage was higher than the results with the constant aeration strategies. The duration of 3-HP accumulation was extended to over 12 h.

SUMMARY

In this work, aldehyde dehydrogenase of *E. coli* was introduced into *Klebsiella pneumoniae*. The *Klebsiella pneumoniae* acquired the ability for 3-HP production. The production of 1,3-PD and 3-HP varies under different aerations. Under the micro-aerobic conditions, the concentration of 3-HP reached 2.2 g/l, which was the highest among the three constant aeration strategies. Finally, 3-HP concentration was improved to 2.8 g/l with a further two-stage ORP controlled strategy. The results showed that the metabolic flux could be redistributed by transforming the exogenous gene. Although the production of 3-HP was slight in this work compared with the other production of organic acid, the implementation for co-production of 3-HP and 1,3-PD in *Klebsiella pneumoniae* was significant. Maybe gene knock-out and fed-batch fermentation were better approaches for improving the production of 3-HP in the recombinant strain. Deactivating *dhaT* gene, which encodes the enzyme for catalyzing

the reduction of 3-HPA to 1,3-PD, would block the 1,3-P D metabolic pathway. More substrate would redistribute to 3-HP metabolic pathway. But high titer of 3-HP was harmful to growth of the host strain. We also should seek to improve the acid-resistance ability for the *Klebsiella pneumoniae* strains while 3-HP titer is high. As glycerol production has increased rapidly in recent years due to the tremendous growth of biodiesel industries [21], this study is mainly encouraging for further development of a bioprocess to produce 1,3-propanediol and/or 3-hydroxypropionic acid from crude glycerol by *Klebsiella pneumoniae*.

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