Effect of culture conditions on 3-hydroxypropionaldehyde detoxification in 1,3-propanediol fermentation by Klebsiella pneumoniae

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Abstract

1,3-Propanediol (1,3-PD) production is favored by high amount of glycerol which also contributes to the lethal 3-hydroxypropionaldehyde (3-HPA) accumulation. The influences of three culture conditions including initial glycerol concentration, stirring rate, and oxidoreduction potential (ORP) regulation on 3-HPA accumulation and 1,3-PD production were investigated. 3-HPA detoxification was available at initial glycerol concentration, aeration rate and stirring rate of up to 40 g l\textsuperscript{-1}, 0.5 vvm air flow and 250 rpm, respectively. Alternatively, the stirring rate of 300 rpm was sufficient to fulfill the fermentation with initial 50 g glycerol l\textsuperscript{-1}. In addition, 3-HPA accumulation could be avoided at the initial 50 g glycerol l\textsuperscript{-1} under the regulated reducing conditions (\textasciitilde200 and \textasciitilde400 mV). However, ORP regulation was not convenient and feasible for the industrial production in that more carbon was distributed to weak organic acids. Consequently, two-stage fed-batch strategy was presented to achieve 3-HPA detoxification and maintain the expected high productivity of 1,3-PD, in which the batch culture stage was conducted at initial glycerol concentration and stirring rate of 40 g l\textsuperscript{-1} and 250 rpm, respectively, and the feeding culture stage was performed at stirring rate of 300 rpm. 74.07 g 1,3-PD l\textsuperscript{-1} was produced in 24 h with the yield and productivity of 0.62 mol mol\textsuperscript{-1} and 3.08 g l\textsuperscript{-1} h\textsuperscript{-1}.

Keywords: Detoxification; Fermentation; 3-Hydroxypropionaldehyde; Klebsiella pneumoniae; 1,3-Propanediol

1. Introduction

1,3-Propanediol (1,3-PD), a valuable bifunctional molecule, is a promising chemical for many synthetic reactions, particularly as a monomer for the synthesis of polytrimethylene polyesters (PTT)\textsuperscript{[1]}. The metabolic overflow leads to 1,3-PD excretion at the excess glycerol substrate. The conversion process is carried out in two enzymatic steps by several microorganisms including\textit{Clostridia, Citrobacter, Lactobacillus} and\textit{Klebsiella}\textsuperscript{[2,3]}. The glycerol dehydratase removes a water molecule from glycerol in presence of coenzyme vitamin B\textsubscript{12} to form 3-hydroxypropionaldehyde (3-HPA)\textsuperscript{[4]}. Subsequently, 1,3-PD oxidoreductase transfers a reducing equivalent from NADH to 3-HPA, giving 1,3-PD.

Biotechnologically produced 3-HPA known as Reuterin could be used to protect food against microbial spoilage and also to produce acrylic acid\textsuperscript{[5,6]}. However, as observed by Barbirato et al.\textsuperscript{[7]}, the accumulation of 3-HPA caused the fermentation premature cessation at the initial glycerol concentration of above 40 g l\textsuperscript{-1}. The characteristic physiological property of this abnormal cessation is glycerol uptake and cell growth cease, and pH value of the fermentation broth increases.

In our practices with\textit{Klebsiella pneumoniae} AC15, the fermentation ceases when 3-HPA concentration is over 10.7 mM. While the occurrence of the inhibitory phenomenon could be avoided by controlling pH 8\textsuperscript{[8]}, we have to explore new way to overcome this problem in that the electro dialysis was employed to remove the salt ions in the downstream processing\textsuperscript{[9]}. The present study provided our investigation of initial glycerol concentration, stirring rate and oxidoreduction potential (ORP) regulation with the aim to avoid the toxic 3-HPA accumulation and maintain higher 1,3-PD productivity of by\textit{K. pneumoniae} AC15.
2. Materials and methods

2.1. Microorganism

*K. pneumoniae* AC 15 (GenBank accession number DQ185604) was isolated from the soil samples. The culture was maintained on Luria–Bertani (LB) agar slant at 4°C.

2.2. Growth medium and aerobic culture conditions

The basic culture media contained: glycerol, 20–60 g l⁻¹; K₂HPO₄, 0.69 g l⁻¹; KH₂PO₄, 0.25 g l⁻¹; (NH₄)₂SO₄, 4 g l⁻¹; MgSO₄·7H₂O, 0.2 g l⁻¹; yeast extract, 1.5 g l⁻¹; 1 ml of trace element solution. The composition of the trace element solution was: MnSO₄·4H₂O, 100 mg l⁻¹; ZnCl₂, 70 mg l⁻¹; Na₂MoO₄·2H₂O, 35 mg l⁻¹; H₃BO₃, 60 mg l⁻¹; CoCl₂·6H₂O, 200 mg l⁻¹; CuSO₄·5H₂O, 29.28 mg l⁻¹; NiCl₂·6H₂O, 25 mg l⁻¹; 0.9 ml 10 M HCl.

Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with *K. pneumoniae* and incubated at 37°C to prepare the inoculum. A 1.5% (v/v) prepared inoculum was added aseptically to the 5 l B. Braun Biostat B Plus fermenter (B. Braun, Germany) with 4 l working volume. A 10 M NaOH was used to maintain the pH of the broth at 7. The fermentation was terminated until glycerol was exhausted or 3-HPA accumulation caused premature cessation occurred. The fermentation was incubated at 37°C, 0.5 vvm and 150–350 rpm. The glycerol fed-batch fermentation was performed at 37°C, 0.5 vvm, and pH 7.

In fed-batch experiments, the glycerol feeding was started when the glycerol concentration in broth was below 10 g l⁻¹. The glycerol concentration was controlled to 10–15 g l⁻¹ at 250 rpm and 15–20 g l⁻¹ at 300 rpm separately.

2.3. Measurement of oxidoreduction potential

ORP was measured with a redox meter (6308OT, RKKT, Shanghai, China) with a redox-combined electrode. Before each measurement, the electrode was polished with a fine alumina powder to restore the platinum surface. 1% Sodium borohydride in 1% sodium hydroxide solution was employed to manipulate ORP of the culture broth.

2.4. Assays

Dry cell mass was computed from the optical density calibration curved determined at 650 nm (OD₆₅₀). Glycerol, 1,3-PD, 2,3-butanediol (2,3-BD), lactate, succinate, acetate and ethanol were determined by a Shimadzu 10A VP HPLC system using an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad, Palo Alto, CA, USA) as described[10]. 3-HPA was determined using the colorimetric method described by Circle et al.[11]. Specific production rate of 1,3-PD calculated according to Kastner et al. [2].

3. Results and discussion

3.1. Influence of initial glycerol concentration on 3-HPA accumulation

Batch cultures were performed at an initial concentration of glycerol from 20 to 50 g l⁻¹, 0.5 vvm air flow and 250 rpm. The premature fermentations occurred at the initial 50 g glycerol l⁻¹ (Fig. 1A). The specific rate of 1,3-PD formation (q₁,₃-PD) reached its peak value ahead of the maximum 3-HPA concentration (3-HPA_max). By contrast, 3-HPA concentration progressed with the same evolution behavior as q₁,₃-PD in cultures with 20 g glycerol l⁻¹ (Fig. 1B). In absence of oxygen or if the rate of carbon consumption is greater than the capacity to reoxidize the reduced equivalents generated, the microbial response is metabolite overflow. Together with acetate excretion, 1,3-PD overflow were observed in early-exponential growth phase even at the initial 20 g glycerol l⁻¹. Reason for this phenomenon could be that the metabolic overflow reduced the metabolic intermediates accumulation and maintained the intercellular redox state via limiting the tricarboxylic acid cycle, regeneration NAD⁺ and recycling coenzyme A [12]. It was also noticed that q₁,₃-PD increased slightly prior to the premature cessation, suggesting that the glycerol dehydratase may be more subjective to 3-HPA than 1,3-PD oxidoreductase.
As summarized in Table 1, the batch cultures at initial concentration of glycerol from 20 to 40 g l\(^{-1}\) achieved the similar 1,3-PD yields \(Y_{1,3\text{-PD/glycerol}}\), while a lower \(Y_{1,3\text{-PD/glycerol}}\) value was obtained at 50 g glycerol l\(^{-1}\) due to the premature cessation. 1,3-PD productivity and 3-HPA\(_{\text{max}}\) were enhanced gradually by the increasing initial glycerol concentration. This was consistent with the observations that the fast growing cells produced more 1,3-PD at the excess glycerol substrate\([13,14]\). However, the high concentration of 3-HPA would repress the glycerol uptake and cell growth. Thus the maximum concentration of glycerol could be 40 g l\(^{-1}\) at 0.5 vvm air flow, 250 rpm based on the present data.

3.2. Influence of stirring rate on 3-HPA accumulation

The stirring rate ranged from 200 to 350 rpm was applied to batch fermentations at the initial glycerol and aeration of 50 g l\(^{-1}\) and 0.5 vvm air flow, respectively. The fermentations at 200 and 250 rpm resulted in the unexpected cessation of culture. And \(q_{1,3\text{-PD}}\) peak came earlier than did 3-HPA\(_{\text{max}}\). What was different from the profile in Fig. 1A was \(q_{1,3\text{-PD}}\) at 200 rpm decreased smoothly (Fig. 2A), indicating that such a high concentration 3-HPA of 12.45 mM may cause inactivation of both glycerol dehydratase and 1,3-PD oxidoreductase. The batch fermentations could be fulfilled at 300 and 350 rpm. And 3-HPA\(_{\text{max}}\) occurred at the same time with \(q_{1,3\text{-PD}}\) peak (Fig. 2B).

It was reported that \(Y_{1,3\text{-PD/glycerol}}\) at the aerobic conditions was lower than that at anaerobic conditions\([15]\). The availability of oxygen and the nature and quantity of the carbon source dictate the status of the TCA cycle\([16]\). If the oxygen utilization is sufficient, the reduced cofactors generated by glycerol consumption would be reoxidized in the electron transport chain and the redox balance could be maintained. The high stirring rate enhances oxygen transfer rate to a culture and facilitates diffusion. The enterobacteria could adapt their metabolism to oxygen availability via a group of global repression regulators, which include the one-component Fnr protein, and the two-component Arc system. The main targets for repression are genes related to the electron transfer chain and the TCA cycle\([17]\). Moreover, it was found that the most significant role of ArcA functions under microaerobic conditions, while that of FNR functions under more strictly anaerobic conditions. Oxygen could relieve cell of ArcA inhibitory influence\([18]\). Thus, more carbon flow was distributed to TCA cycle, promoting NAD\(^+\) regeneration and weakening 1,3-PD synthesis. Accordingly, 3-HPA\(_{\text{max}}\) declined at higher stirring rate (Table 2). Therefore, the higher productivity of 1,3-PD could be obtained at the medium stirring rate (250 and 300 rpm). The stirring rate of 300 rpm was sufficient to fulfill the fermentation with initial 50 g glycerol l\(^{-1}\).

3.3. ORP evolution characteristics during 1,3-PD fermentation

ORP has been used as a parameter to control, optimize, and scale-up fermentations. It has been reported that each species, or even each strain, has a preferable redox potential range\([19,20]\). We directed our attention to the effect of ORP on the metabolic end product spectrum and 3-HPA accumulation. As shown in
Table 2
The impact of stirring rates on 1,3-PD production and 3-HPA accumulation

<table>
<thead>
<tr>
<th>Stirring rate (rpm)</th>
<th>$Y_{1,3-PD/glycerol}$ (mol mol$^{-1}$)</th>
<th>3-HPA$_{\text{max}}$ (mM)</th>
<th>1,3-PD productivity (g l$^{-1}$ h$^{-1}$)</th>
<th>Premature cessation of fermentation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>0.40 ± 0.09</td>
<td>12.45 ± 0.16</td>
<td>0.33 ± 0.05</td>
<td>+</td>
</tr>
<tr>
<td>250</td>
<td>0.49 ± 0.02</td>
<td>11.25 ± 0.13</td>
<td>0.42 ± 0.03</td>
<td>+</td>
</tr>
<tr>
<td>300</td>
<td>0.56 ± 0.06</td>
<td>6.70 ± 0.03</td>
<td>0.42 ± 0.04</td>
<td>−</td>
</tr>
<tr>
<td>350</td>
<td>0.50 ± 0.01</td>
<td>2.59 ± 0.01</td>
<td>0.39 ± 0.07</td>
<td>−</td>
</tr>
</tbody>
</table>

The fermentation was performed at 0.5 vvm air flow, 50 g glycerol l$^{-1}$.

$^a$ "+": occurrence of premature fermentation cessation; "−": normal fermentation.

3.4. Metabolic adaptation by K. pneumoniae in response to ORP regulation

The initial ORPs were regulated to −200 and −400 mV in the ORP regulation trials, respectively. The controls were the experiments without ORP regulation. As listed in Table 3, biomass and $Y_{1,3-PD/glycerol}$ remained unaffected at the regulated ORPs. However, the drastic change in final by-product concentrations was observed. The synthesis of 2,3-BD was strongly inhibited at the reducing conditions. Concerning the ethanol/acetate ratio, on average it was equal to 0.6 in the control experiments, and it decreased to 0.24 and 0.48 at −200 and −400 mV, respectively. Redistribution of flux to ethanol over acetate was accompanied by a decreased 1,3-PD productivity, resulting in a prolonged cycle. Under low ORP conditions, we notably showed 3-HPA$_{\text{max}}$ decreased from 7.18 to 2.08 and 0.13 mM, respectively. Moreover, succinate and lactate production were enhanced at the reducing conditions. Redox potential has been shown to significantly alter the carbon flux and change the resultant end products in fermentations [22,23]. Such a modification might be explained by an overall activation or inhibition of the microbial physiology in different oxidoreduction conditions.

Table 3
Yields of the end products, the 3-HPA accumulation and the biomass

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Result obtained at a ORP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial ORP −200 mV</td>
</tr>
<tr>
<td>3-HPA$_{\text{max}}$ (mM)</td>
<td>2.08 ± 0.01</td>
</tr>
<tr>
<td>Biomass concentration (g l$^{-1}$)</td>
<td>2.62 ± 0.09</td>
</tr>
<tr>
<td>$Y_{1,3-PD/glycerol}$ (mol mol$^{-1}$)</td>
<td>0.55 ± 0.01</td>
</tr>
<tr>
<td>$Y_{2,3-BD/glycerol}$ (mol mol$^{-1}$)</td>
<td>0.06 ± 0.001</td>
</tr>
<tr>
<td>$Y_{\text{succinate/glycerol}}$ (mol mol$^{-1}$)</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>$Y_{\text{lactate/glycerol}}$ (mol mol$^{-1}$)</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>$Y_{\text{ethanol/glycerol}}$ (mol mol$^{-1}$)</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>1,3-PD productivity (g l$^{-1}$ h$^{-1}$)</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>Carbon recovery$^a$ (%)</td>
<td>88.83 ± 3.02</td>
</tr>
</tbody>
</table>

The fermentation was performed at 0.5 vvm air flow, 300 rpm, and 50 g glycerol l$^{-1}$.

$^a$ Exclusion of carbon dioxide.
mechanism. The batch culture could be performed at the initial stage (C). Symbols: biomass (■), 1,3-PD concentration (▲), and 1,3-PD concentration (◇).

3.5. Glycerol fed-batch fermentation with the two-stage strategy

It was indicated that ORP was a valuable parameter to monitor and control anaerobic fermentation of 1,3-PD production [24], more carbon, however, was distributed to weak organic acids and synthesis of 2,3-BD, an important byproduct, was inhibited at the regulated reducing conditions. This would influences the fed-batch fermentation and increase cost of the downstream purification processes. Therefore, ORP regulation was not taken into consideration for further fed-batch culture. Based on the fact that 1,3-PD productivity and glycerol tolerance was enhanced at stirring of 300 rpm and the bacterial growth was slower at lower initial glycerol and stirring rate, the two-stage fed-batch strategy was presented correspondingly to realize 3-HPA detoxification and maintain expected high productivity of 1,3-PD. The batch culture stage was conducted at initial glycerol concentration and stirring rate of 40 g l\(^{-1}\) and 250 rpm, respectively, and the feeding culture was performed at stirring rate of 300 rpm. The control experiments were performed at initial 40 g glycerol l\(^{-1}\) and 250 rpm, or initial 50 g glycerol l\(^{-1}\) and 300 rpm. In all fed-batch fermentations the premature cessation due to 3-HPA accumulation was prevented. As shown in Fig. 4, 74.07 g 1,3-PD l\(^{-1}\) was produced in 24 h with the yield of 0.62 mol mol\(^{-1}\) in the two-stage culture, while the 60.78 or 63.26 g 1,3-PD l\(^{-1}\) with the yield of 0.58 and 0.55 mol mol\(^{-1}\), respectively, were obtained in 24 h at initial 50 g glycerol l\(^{-1}\) and 300 rpm or initial 40 g glycerol l\(^{-1}\) and 250 rpm, respectively. Such a high 1,3-PD concentration and productivity of 3.09 g l\(^{-1}\) h\(^{-1}\) made it comparable with the previously reported works [25,26].

4. Conclusions

This work aimed at elucidating 3-HPA detoxification by controlling major operational parameters for the 1,3-PD fermentation and obtaining the fermentation strategy. 1,3-PD production is favored by high amount of glycerol via overflow mechanism. The batch culture could be performed at the initial glycerol concentration, aeration, and stirring rate at 40 g l\(^{-1}\), 0.5 vvm air flow, and 250 rpm. In addition, the stirring rate of 300 rpm was sufficient to fulfill the fermentation at initial 50 g glycerol l\(^{-1}\) and 0.5 vvm. 3-HPA accumulation also could be avoided at the initial 50 g glycerol l\(^{-1}\) under the regulated reducing conditions. However, the carbon flux was redistributed to organic acid while the important byproduct 2,3-BD synthesis was inhibited at the regulated conditions. In the undissociated or acidic form, the weak organic acids easily permeate membranes, uncoupling the transmembrane pH gradient. Once across the membrane, the proton acidifies the cytoplasm, while the anion increases the internal osmotic pressure and interferes with methionine biosynthesis [27]. Therefore, ORP regulation was not taken into consideration for further fed-batch culture. The two-stage fed-batch strategy was presented to avoid the lethal 3-HPA accumulation. The subsequent fed-batch fermentations resulted in 74.07 g 1,3-PD l\(^{-1}\) in 24 h. As far as we known, such a high productivity of 3.09 g l\(^{-1}\) h\(^{-1}\) is among the most competent lists of 1,3-PD production. The concrete physiological mechanism of effect of 3-HPA on the enzymes of glycerol catabolism is under way in our laboratory.

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References


