Improvement of Ethanol Concentration and Yield by Initial Aeration and Agitation Culture in Very High Gravity Fermentation*

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Abstract  Effect of oxygen and agitation on ethanol production by *Saccharomyces cerevisiae* in a very high gravity (VHG) fermentation was studied. The results indicated that agitation was the most significant factor to improve ethanol concentration and yield. Compared to static culture, agitation led to a 69.0 % increase in ethanol titre (from 75.8 to 128.1 g L⁻¹) under non-aeration condition, and ethanol concentration increased by 68.7 % (from 85.2 to 143.8 g L⁻¹) under initial aeration condition. An initial aeration strategy and agitation culture were identified as an optimal condition for VHG in the fed-batch fermentation, that allowed a final ethanol concentration to achieve 143.8 g L⁻¹ in 54 h, in a double feeding fermentation, and the yield of ethanol on substrate (Yₚ/s) reached 0.471 g/g (The stoichiometric yield was 92.2 %) which very few studies achieved. These outstanding performances resulted from higher biomass and enhanced cell activities which were essential for VHG because of aeration and agitation. Fig 5, Tab 1, Ref 12

Keywords very high gravity fermentation (VHG); ethanol fermentation; initial aeration; agitation culture; oxygen

Abstract  研究了氧气和震荡条件对酿酒酵母高浓度乙醇发酵的影响。结果表明, 震荡是提高发酵液乙醇浓度和产率的最重要因素。与静止培养相比, 在不通气情况下震荡培养使乙醇浓度提高了69% (从75.8 g L⁻¹提高到128.1 g L⁻¹), 在通气条件下乙醇浓度提高了68.7% (从85.2 g L⁻¹提高到143.8 g L⁻¹)。在最优条件下, 两次补料, 经54 h发酵, 发酵液中乙醇浓度达到143.8 g L⁻¹, 乙醇产率与理论产率的比值为0.471 g/g (即92.2%)。分析, 通气和震荡条件提高了发酵液中酿酒酵母的生物量和细胞活力。图5 表1 参12

Keywords 高浓度发酵; 乙醇发酵; 初期通气; 震荡培养; 氧气

With the rapid consumption of oil, people have to cast about for substitutes for it. The production of bio-ethanol as one of the alternative fossil fuel energy resources has been a subject of great interest [1]. The technology of very high gravity fermentation (VHG) for fuel alcohol production is intensively concerned because of its particular advantages, such as less energy consumption in distilling, more efficient utilization of equipments and less wastewater to be processed[2]. However, researchers only concerned the ethanol concentration in the fermentation liquid in VHG, but usually ignored other parameters such as reducing glucose, fermentation time and the yield of ethanol production on substrate (Yₚ/s), which was another determining factor for ethanol production costs. Improvement of Yₚ/s by optimization was quite prospective to reduce production costs.

Among the factors affecting fermentation time, ethanol concentration and Yₚ/s, oxygen was crucial, which affected cell growth, reproduction and activity, and also formation of by-products including glycerol and ace acetate. The growth of *Saccharomyces cerevisiae* would be inhibited when oxygen was absent. In particular, *S. cerevisiae* required a certain supply of elemental oxygen in order to synthesize unsaturated fatty acids and sterols, which were important constituents of its cell envelopes[3]. However, if oxygen was provided too much, Yₚ/s would decrease sharply because of aerobic respiration. Under full aeration, yeast would consume more glucose to produce CO₂ and H₂O.

The impacts of oxygen supply on VHG have been reported in some articles. In continuous cultures, at a given dilution rate, biomass concentration, biomass/glucose yield and viability were enhanced by increasing air supply while ethanol concentration decreased both under oxygen-limited and oxygen-unlimited continuous cultures. The inhibition of growth by ethanol decreased under microaerobic conditions, where
oxygen limitation occurred even though aeration was carried out, compared to anaerobic conditions where oxygen was totally absent. Specific ethanol productivity was stimulated when the percentage of oxygen in the feed increased. In fed-batch process, higher ethanol concentration (147 g L\(^{-1}\) in 45 h) and average productivity were obtained in cultures without oxygen limitation. Compared to micro-aerobic culture, full aeration led to 23% increase in viable cell mass, but the yield of ethanol on glucose was only 0.43 g/g.\(^{1-7}\).

On the other hand, in VHG fermentation, agitation affecting fermentation system state was another important factor for cell growth and ethanol production by yeast. Under VHG, the strategy of agitation or supplying air to form a roll fermentation system has been brought into effect in some articles.\(^{8-10}\) But few articles investigated its impacts specially.

The aim of this work was to explore the effects of oxygen supply and agitation on dynamic yeast behaviour in batch VHG alcohol fermentations, especially from the aspects of the parameters of ethanol concentration, reducing glucose, and the yield of ethanol on substrate (Y\(_{\text{Eth}}\)).

## 1 Material & Methods

### 1.1 Microorganism

*S. cerevisiae* strain CCTCC M 206111, preserved in China Center for Type Collection, was separated from wine lees in our laboratory.

### 1.2 Medium and batch fermentation

 Yeast strain CCTCC M 206111 was maintained on malt wort agar medium at 4 °C. Before fermentation, the yeast was incubated at 30 °C at rotary shaker (200 r/min) in 250-mL Erlenmeyer flasks with 50 mL medium containing in g L\(^{-1}\): glucose 100, yeast extract 8.5, (NH\(_4\))\(_2\)SO\(_4\) 1.3, MgSO\(_4\)\(_7\)H\(_2\)O 0.1 and CaCl\(_2\) 0.06. After 16 h of growth, the yeast was used as an inoculum for fermentation, so that the biomass concentration at the start was 2.0–3.5 g dry weight L\(^{-1}\). Fermentation medium except glucose contained in g L\(^{-1}\): Yeast extract 8.5, peptone 5, (NH\(_4\))\(_2\)SO\(_4\) 1.5, KH\(_2\)PO\(_4\) 1.5, MgSO\(_4\)\(_7\)H\(_2\)O 0.65, CaCl\(_2\) 2.8, inositol 80 mg, pantothenic acid 60 mg, biotin 250 µg, thiamine 10 mg, pyridoxine 30 mg, nicotinic acid 30 mg and para-aminobenzoic 6 mg. Before inoculation, aeration was done until dissolved oxygen concentration reached 4.0–9.0 mg L\(^{-1}\) in initial aeration fermentation process. Fermentations were provided in 250-mL flasks with 60 mL media at the start, where glucose was about 150 g L\(^{-1}\) after inoculation, then glucose with concentration of 562.5 g L\(^{-1}\) was fed twice when fermented for 8 h and 24 h, respectively. Finally, the total additive glucose was 305 g L\(^{-1}\). Then the fed-batch fermentation was performed in a 10-L fermentor with 8-L medium.

### 1.3 Sampling and analysis

Samples from the fermentation media were taken regularly. Ethanol concentration was determined by gas chromatography, using a FID detector. The samples taken from flasks were centrifuged at 5 000 r/min, and then dried at 105 °C to constant weight. Glucose concentration was determined using the DNS method (3,5-dinitro salicylic acid). Viable-cell counts were determined by a direct microscopic method at a magnification of 400 with the methylene blue technique.

## 2 Results

### 2.1 Impact of aeration on fermentation parameters

Four tests were designed for the factors of aeration and agitation (Table 1, Fig. 1-A–C). From Table 1, the effects of oxygen were a little different, compared agitating culture with static culture. Under static culture, aeration slightly improved both the final ethanol titre (85.2 vs 75.8 g L\(^{-1}\)) and cell concentration (7.71 vs 7.58 g L\(^{-1}\)). On the contrast, aeration influenced fermentation process more strongly (143.8 vs 128.1 g L\(^{-1}\)) under agitating culture. Aeration improved the final ethanol titre by 12.3%, biomass from 13.05 g L\(^{-1}\) to 13.82 g L\(^{-1}\), and the stoichiometric yield from 82.2% to 92.2%.

![Cell viability was also quantified. In fermentations 2 and 4, the yeast cells were shapely and strong. Finally, over 98% of viable cells were measured even until the ethanol concentration reached up to 143.8 g L\(^{-1}\).](image)

### Table 1 Effects of the initial aeration strategy and agitation on ethanol production, residual glucose and other fermentation parameters

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ethanol] final (ρ/g L(^{-1}))</td>
<td>75.8</td>
<td>128.1</td>
<td>85.2</td>
<td>143.8</td>
</tr>
<tr>
<td>Residual glucose (ρ/g L(^{-1}))</td>
<td>136.0</td>
<td>42.6</td>
<td>124.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Y(_{\text{Eth}}) (ρ/g g(^{-1}))</td>
<td>0.249</td>
<td>0.420</td>
<td>0.279</td>
<td>0.471</td>
</tr>
<tr>
<td>Stoichiometric yield (%/w%)</td>
<td>48.7</td>
<td>82.2</td>
<td>54.6</td>
<td>92.2</td>
</tr>
<tr>
<td>Glucose utilization (%/w%)</td>
<td>55.4</td>
<td>86.0</td>
<td>59.2</td>
<td>98.4</td>
</tr>
<tr>
<td>Biomass(_{\text{max}}) (ρ/g L(^{-1}))</td>
<td>7.58</td>
<td>13.05</td>
<td>7.71</td>
<td>13.82</td>
</tr>
<tr>
<td>Average rate of the glucose consumption during 4–8 h (ρ/g L(^{-1}) h(^{-1}))</td>
<td>5.13</td>
<td>13.43</td>
<td>5.58</td>
<td>15.00</td>
</tr>
<tr>
<td>Average rate of the ethanol formation during 4–8 h (ρ/g L(^{-1}) h(^{-1}))</td>
<td>3.48</td>
<td>7.30</td>
<td>3.88</td>
<td>7.12</td>
</tr>
<tr>
<td>Average ethanol productivity (ρ/g L(^{-1}) h(^{-1}))</td>
<td>1.40</td>
<td>2.37</td>
<td>1.58</td>
<td>2.66</td>
</tr>
</tbody>
</table>

Total glucose concentration was 305 g L\(^{-1}\). The initial aeration strategy made the saturation of oxygen reaching 4.0–9.0 mg L\(^{-1}\). Stirring rate was 200 r/min under agitating culture. Y\(_{\text{Eth}}\), the yield of ethanol on substrate; Stoichiometric yield, the ratio of produced mass of ethanol to theoretical mass of ethanol calculated from total mass of glucose; Glucose utilization, the ratio of the consumed mass of glucose to total mass of glucose.

1. Non-aeration and static culture; 2. Non-aeration and agitating culture; 3. Aeration and static culture; 4. Aeration and agitating culture
The initial aeration strategy, which led to dissolved oxygen concentration reaching 4.0~9.0 mg L\(^{-1}\), was used in VHG in our research. The consuming rate of oxygen by yeast was rapid. After inoculation for 3~4 hours, oxygen concentration decreased sharply below 0.5 mg L\(^{-1}\) (Fig. 2).

### 2.2 Impact of agitation on fermentation parameters

From Table 1, agitation played an important role in ethanol production. It strongly improved the final ethanol titre and biomass concentration. Under non-aeration condition, agitation led to a 69.0 % increase in ethanol titre (from 75.8 to 128.1 g L\(^{-1}\)). Under aeration condition, the final ethanol titre increased by 68.7 % (from 85.2 to 143.8 g L\(^{-1}\)). Among the four fermentations, distinction of the stoichiometric yield was obvious, too.

It was no doubt that agitation would strongly improve ethanol concentration from the results (Table 1, Fig. 1-A~C). To understand whether agitation affected fermentation by enhancing dissolved oxygen of the system, we replaced the oxygen of surface atmosphere with nitrogen. The result indicated that no obvious difference appeared (Data not shown). So agitation mainly affected the fermentation process by forming a symmetrical system.

In order to prove the effect of agitation on fermentation process, different stirring rates (0, 50, 200, 300 r/min) were presented. At 0 r/min, biomass was 7.81 g L\(^{-1}\), and ethanol concentration 84.9 g L\(^{-1}\), and at 50, 200, 300 r/min, biomass was approximately 13.5~14.0 g L\(^{-1}\), and ethanol concentration 143~144 g L\(^{-1}\). So, different stirring rates almost had no obvious difference in biomass and ethanol concentration. Therefore, stirring rate had no fatal effect on fermentation system, and agitation mainly formed a homogeneous system.

### 2.3 Difference among four fermentations

Ethanol is one of the products from yeast metabolism. Fermentation capability of yeast was correlated with biomass and cell activities. Different biomasses brought into different fermentation capabilities (Table 1, Fig. 1-A~C). Generally speaking, enough biomass in the broth was an essential factor

![Fig. 1 Time courses of ethanol, glucose and biomass concentrations during fermentations with S. cerevisiae](image)

\(\text{\bullet: 1, Non-aeration and static culture; } \text{\blacklozenge: 2, Non-aeration and agitating culture; } \text{\blacktriangle: 3, Aeration and static culture; } \text{\blacktriangledown: 4, Aeration and agitating culture} \)

![Fig. 2 Change in dissolved oxygen concentration in initial aeration culture at the first fermentation phase](image)

![Fig. 3 Active enzymes under different fermentation conditions](image)

\(\text{a, Non-aeration and static culture; b, Non-aeration and agitating culture; c, Aeration and static culture; d, Aeration and agitating culture. In the picture (from left to right), No. 1 cell is the control, No. 2–20 are corresponding to 19 kinds of enzymes. Colored wells was positive, and deeper colour ones mean higher activity of enzymes. No. 2, Alkaline phosphatase (+, violet); No. 3, Esterase (C4) (+, violet); No. 4, Esterase lipase (C8) (+, violet); No. 6, Leucine arylamidase (+, orange); No. 11, Acid phosphatase (+, violet); No. 12, Naphthol-AS-BI-phosphohydrolase (+, blue); No. 13, α-galactosidase (+, violet); No. 16, α-glucosidase (+, violet)}\)
for fast conversion to ethanol. More cells, more ethanol acquired in definite time and volume. In fermentation 4, the amount of cells was found the most and the ethanol titre the highest. From our research, biomass was the most direct factor leading to different fermentation capabilities of yeast in term of ethanol concentration.

In the VHG technique of our research, appropriate oxygen and feeding time were taken to regulate biomass. As a result, the yield of ethanol on substrate \( Y_{p/s} \) reached 0.471 g/g (The stoichiometric yield was 92.2%) in the best fermentation (4).

Then, to identify the characteristics of the different states in the four fermentations, through analyzing 4 groups of tests by enzymatic detection kit (Obtained from biomerieux in France) which was a semi-micromethod designed for the research of enzymatic activities, the results indicated that the kinds of active enzymes were discrepant, moreover, the activity of the same enzyme was different. In positive assay, enzyme activity was higher in deeper color (Fig. 3). For example, the \( \alpha \)-glucosidase appeared in fermentations 2 and 4 in, in which ethanol concentration was higher, and there was also difference in enzyme activity. The results showed that \textit{S. cerevisiae} revealed different fermentation performances in different states.

2.4 Kinetic parameters under aeration and agitation condition in 10-L fermentation

The fed-batch experiment was performed in a 10-L fermentor with 8-L medium (Fig. 4-A-B). There was a short lag phase for yeast after inoculation, while they grew slowly and consumed glucose slowly, too. Then after about four hours, the yeast came into log phase apparently, while the yeast consumed glucose quickly. From Fig. 4, the speed of consuming glucose was slow during the period from 0 to 4 h, and then it became fast. After 8 h, glucose concentration descended from 146 g L\(^{-1}\) to 85 g L\(^{-1}\). The average speed of consuming glucose achieved 7.6 g L\(^{-1}\) h\(^{-1}\), and the maximal speed was about 18 g L\(^{-1}\) h\(^{-1}\), slower than that of 100-mL fermentation, and the biomass was lower than that of 100-mL fermentation, too. After the second feeding, because the ethanol concentration reached 13%, the circumstance was bad for yeast. The fermentation speed slowed down gradually, especially after 48 h. At the end, the biomass decreased slightly.

From Fig. 5, pH value descended from 6.5 to 3.5 when the fermentation was conducted for 10 hours, and it was then kept at the same level for all the rest time. The lower pH value was propitious to restrain other bacteria’s growth.

3 Discussion

The effects of initial aeration and agitation on ethanol concentration and yield in VHG ethanol fermentation were investigated in this study. Four kinds of tests were designed for the investigation (Table 1). The results showed the initial aeration strategy shortened the lag phase and increased the amount of cells quickly. Oxygen was one of crucial factors for the growth of \textit{S. cerevisiae}, and it could accelerate its growth and reproduction, especially in the VHG fermentation, where oxygen became a limiting factor\(^{(1)}\). Appropriate oxygen could promote biosynthesis of plasma membrane, and poly-unsaturated fatty acid and lipid in chondriosome to protect the integrity of cell membrane from the poisoning by high concentration ethanol. However, oxygen would destroy the process of anaerobic metabolism. In that case, the yeast produced less ethanol, which was not expected. So apropos oxygen was an important factor in ethanol production for final ethanol concentration and stoichiometric yield. In our
research, initial aeration was taken, and the initial aeration strategy achieved appropriate dissolved oxygen concentration (4.0–9.0 mg L\(^{-1}\)). Within the range of oxygen concentration, this strategy not only provided suitable oxygen for yeast growing quickly, but also avoided aerobic respiration to a certain extent.

From the research, agitating culture would make ethanol production well performed. It could be related with the status of the broth, which affected yeast growth and ethanol production. Agitation could create a symmetrical fermentation system. Moreover, it did avail to release CO\(_2\) which was an inhibitor to yeast\(^{[10–12]}\), and to accelerate the consumption of nutrition by yeast. Under this condition, yeast would grow well and cells were vigorous, so that the cells had good performance on ethanol production and endurance to bad circumstance including higher concentration of residual sugars, ethanol poisoning, by-products and nutrition lacking, especially in the last period of fermentation.

Whereas, in static culture, the yeast cells would subside to the bottom of the fermentor, thus they could not absorb nutrient well. As a result, the biomass was so low and the cell size was so small that they were weak in ethanol production.

The glucose concentration in the broth of VHG was much higher than that in common fermentation. Correspondingly, the biomass in VHG should be, therefore, higher. But if the yeast cells were very excessive, they would consume more sugars for growth, which would lower the stoichiometric yield. Moreover, the yeast cells would act on each others. Therefore, it was a required way to control optimized biomass in order to ferment sugars to ethanol fast and efficiently.

Considering the results of the 10-L fermentation, the biomass was lower than that in 100-mL fermentation at the initial phase, and the time was prolonged. From Fig. 4-A, the speeds of glucose consumption and ethanol production were slower. The reason must be the lower amount of yeast cells, which was related with weak transfer capability of material elements in the 10-L fermentor.

In this study, the initial aeration strategy and agitating culture increased the final ethanol concentration (up to 143.8 g L\(^{-1}\)), and made the stoichiometric yield (up to 92\%) higher than other general VHG ethanol fermentation processes, that was to say, the equal raw material would obtain more ethanol under that condition. The increased stoichiometric yield could decrease the bio-ethanol production cost effectively. Furthermore, the fermentation time was shortened for only 54 hours. So, it became a highly competitive technique.

In conclusion, oxygen and agitation were two important factors in VHG. Oxygen was mainly related with yeast growth, quick accumulation of biomass and cell activities. On the other hand, agitation led to fermentation liquid mixing well, CO\(_2\) releasing and nutrition consuming, etc. To summarize, the two factors affected ethanol fermentation by biomass and cell activities. Therefore, it was necessary to accelerate accumulation of biomass, acquire optimal cell amount, and keep higher cell activities for fermentation by some means, so as to achieve high concentration and yield of ethanol.

References
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