Streptococcus bovisによる豆腐廃液(TLW)を用いた生キャッサバのL-乳酸発酵における生成物阻害の影響

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Effect of Product Inhibitions on L-Lactic Acid Fermentation From Fresh Cassava Roots in Tofu Liquid Waste by *Streptococcus bovis*

Suripto Dwi YUWONO, Abdul GHOFAR and Takao KOKUGAN†

Department of Chemical Engineering, Tokyo University of Agriculture and Technology, 2-24-16, Naka-machi, Koganei-shi, Tokyo 184-8588, Japan

The effects of product inhibition on lactic acid fermentation from fresh cassava roots in tofu liquid waste by *Streptococcus bovis* were studied in batch culture. The lactic acid production and growth rate gradually decrease with increase of inhibitor (lactic acid) concentration. The effects of product inhibition on productivity and specific growth rate were confirmed by the Lineweaver–Burk plot to noncompetitive inhibition. The product inhibition was evaluated by two inhibitions constants: inhibition constant for productivity ($K_I$) and inhibition constant for specific growth rate ($K_{sI}$). They decrease with increasing of inhibitor concentration. The values of $K_I$ and $K_{sI}$ in a rich media are smaller than those in a poor media and the $K_I$ is larger than the $K_{sI}$ in those media. The experiment results on batch fermentation agree with the noncompetitive inhibition model for about four days, but they deviate from the inhibition model after four days due to high inhibitor concentration. It is supposed that accumulation of lactic acid in broth decidedly causes damage on viable cell.

**Key words:** Product inhibition, lactic acid fermentation, fresh cassava roots, tofu liquid waste, *Streptococcus bovis*.

1. INTRODUCTION

To reduce the raw material cost of lactic acid fermentation, fresh cassava roots (FCR) was used in tofu liquid waste (TLW) without glucose addition [1]. However, the lactic acid productivity and specific growth rate were very low in compare with standard media (Glucose-TSB). In order to increase specific growth rate and the production of lactic acid by *Streptococcus bovis*, the concentrated maguro waste (CMW) as nitrogen source was added to the FCR-TLW media (new media). The productivity and specific growth rate in the new media were comparable to those in standard media [2]. Furthermore, to improve fermentative efficiency such optimum conditions have been studied for lactic acid fermentation from the new media as temperature [3] and pH [4]. The maximum productivity and specific growth rate of *S. bovis* in these media were obtained at temperature of 39°C and pH 5.5.

The problems in fermentation process are the existence of such inhibitor that inhibit cell growth and reduce their activity for product formation, such as substrates inhibition and product inhibition. The batch process for lactic acid production is known to be limited with product inhibition. The inhibition by product (lactic acid) may be competitive or noncompetitive inhibition [5]. Aiba *et al.* [6] have reported the kinetic of product inhibition in ethanol fermentation. The effects of product on specific productivity and growth rate were confirmed to noncompetitive inhibition by Lineweaver–Burk plot. Ishizaki and Otha [7] have reported batch culture kinetics of L-lactate fermentation by *Streptococcus* sp. IO-1. They carried out numerical analyses by uncompetitive inhibition at different initial substrate concentration. Kinetic study of product inhibition has been studied in the chemostat of lactic acid fermentation from glucose by *Streptococcus faecalis*. The inhibition for specific growth rate and specific production rate of lactic acid were regarded to noncompetitive inhibition [8].

The purpose of the present paper is to clarify the effect of product inhibition on lactic acid fermentation from fresh cassava roots (FCR) by *Streptococcus bovis* in batch culture. Experimental results were arranged by Lineweaver–Burk plot. Inhibition models for productivity and specific growth rate were applied to Michaelis–Menten and Monod–type equations, respectively. The experiment results were evaluated by inhibition constants and compared with those inhibition models.
2. Materials and Methods

2.1 Microorganism and medium

The microorganism used in this study was Streptococcus bovis (RIKEN, Japan). Cultivation was in MRS (Difco, USA), which contained (per liter of distilled water) 10 g of peptone, 10 g of meat extract, 5 g of yeast extract, 20 g of glucose, 2 g of K₂HPO₄, 5 g of sodium acetate, 2 g of dianmonium citrate, 0.1 g of MgSO₄·7H₂O, 0.05 g of MnSO₄·H₂O and 1 g of Tween 80. After incubation at 37°C for 18 h, the culture was used as the inoculum for the fermentation process.

2.2 Fermentation

Substrate, medium and microorganism were used same as those in the previous study [4]. To evaluate the product inhibition, the inhibition experiments were carried out with the various initial inhibitor (lactic acid) concentrations (I₀) of 0, 10, 30, and 50 g/L and various sugar substrate concentrations (S₀) of 10, 30, 50 and 100 g/L, prior to the fermentation experiments. The fermentation experiments were conducted in a 1 L fermentor (ABLE, Japan) and controlled at a temperature 37°C. The culture pH was maintained at pH 5.5 by CaCO₃.

2.3 Analytical methods

The concentrations of lactic acid and glucose were measured by a biosensor (Oji Scientific Instruments, Ltd, Himeji, Japan). Aliquot of the fermentation broth sample were collected aseptically. Viable cell of S. bovis (CFU/mL) in dilute sample were counted by count method using bromocresol purple agar medium (Nissui Pharmaceutical, Tokyo) after anaerobic incubation at 37°C for 72 h.

3. Results and Discussions

In many studies, product inhibition by lactic acid bacteria was described as a competitive; uncompetitive and non-competitive function of lactic acid [5-7,10]. In order to elucidate the type of product inhibition, the batch culture was carried out at various inhibitor concentrations. Figs. 1 and 2 show relationships between total lactic acid (inhibitor+...
fermented lactic acid) concentrations or viable cell count and fermentation time, taking inhibitor concentration ($I_0$) as parameter. Although the lactic acid production gradually decreases with increase of $I_0$, total lactic acid concentrations are equivalent level at the final fermentation for various inhibitor concentrations ($I_0$). On the other hand the maximum viable cell count levels decreases with increase of $I_0$ because cell growth was inhibited by lactic acid.

The lactic acid productivity ($V$) at each $I_0$ was obtained from the maximum slope of the lactic acid concentration. Specific growth rate ($\mu$) was calculated from the maximum slope of the relation between logarithmic viable cell count and fermentation time. Figs. 3 and 4 show Lineweaver–Burk plot for the productivity and specific growth rate, where initial substrate concentration ($S_0$) was substituted for substrate concentration ($S$), because the maximum productivity and specific growth rate arise early in fermentation period. The linear least squares fits are shown by straight lines in Figs. 3 and 4. For product inhibition, the inhibition is noncompetitive, because the curves cross at same interaction on horizontal axis for various $I_0$. These results in type of noncompetitive inhibition are same as those by Aiba et al., [6], Ohara et al., [8] and Yamane [10].

The reaction mechanism for noncompetitive inhibition can be described as follows [5]:

$$E + S \xrightleftharpoons{K_m} ES \rightarrow E + P$$

$$+ I \quad \quad +$$

$$\begin{array}{c}
\text{ES} \\
\text{EI + S} \xrightarrow{K_m} \text{ESI}
\end{array}$$

(1)

where $K_m$ is Michaelis–Menten constant, with definition of

$$K_m = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[ESI]}$$

(2)

and $K_i$ is the inhibition constant for productivity,

$$K_i = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]}$$

(3)

where $E$, $S$, $P$ and $I$ are the enzyme, substrate, product and inhibitor, respectively. The $ES$, $EI$ and $ESI$ are complexes with the product and inhibitor. For product inhibi-
tion, I is equivalent to P. We can develop the following rate equation:

$$V = \frac{V_{m, app} [S]}{K_m + [S]}$$  \hspace{1cm} (4)

where

$$V_{m, app} = \frac{V_m}{1 + \frac{[I]}{K_i}}$$  \hspace{1cm} (5)

The parameters in this expression, $V_{m, app}$, $V_m$ and $[I]$ are maximum productivity for each $I$, maximum productivity without inhibitor and inhibitor concentration, respectively. Lineweaver–Burk plot for the productivity is given by following equation:

$$\frac{1}{V} = \frac{1}{V_{m, app}} + \frac{K_m}{V_{m, app}} \frac{1}{[S]}$$  \hspace{1cm} (6)

Calculation of specific growth rate could be derived by a Monod–type equation:

$$\mu = \frac{\mu_{m, app} [S]}{K_s + [S]}$$  \hspace{1cm} (7)

where $K_s$ is saturation constant and the $\mu_{m, app}$ is the maximum specific growth rate at $I$ and it can be expressed by following equation:

$$\mu_{m, app} = \frac{\mu_m}{1 + \frac{[I]}{K_i}}$$  \hspace{1cm} (8)

in which $\mu_m$ is the maximum specific growth rate without inhibitor and $K_i$ is inhibition constant for specific growth rate, equivalently to $K_i$ for productivity. Lineweaver–Burk plot for specific growth rate is expressed by following equation:

$$\frac{1}{\mu} = \frac{1}{\mu_{m, app}} + \frac{K_i}{\mu_{m, app}} \frac{1}{[S]}$$  \hspace{1cm} (9)

The kinetic parameters of product inhibition for productivity and specific growth rate are summarized in Table 1. The values $V_m$, $K_m$, $\mu_m$ and $K_s$ in these correlations can be directly calculated from Figs. 3 and 4, respectively. Maximum values of productivity ($V_{m, app}$) and specific growth rate ($\mu_{m, app}$) could be calculated from the intercepts of the straight line at each $[I_0]$ in Figs 3 and 4. The values of maximum productivity ($V_m$) and specific growth rate ($\mu_m$) were 1.53 g/Lh and 0.99 h$^{-1}$ in glucose-TSB media, respectively. Ruiz et al. [12] has reported the value of $\mu_m$ (0.94 h$^{-1}$) for S. bovis in glucose-MRS media. Present result is nearly equal to theirs in standard media. The $K_i$ and $K_{si}$ can be calculated from Eqs. (5) and (8) for each $[I]$, respectively. We used the $[I_0]$ instead of $[I]$, because the maximum values of $V$ and $\mu$ arise on early time of fermentation. When inhibition constant is small, the damage on cell is large. The $K_i$ and $K_{si}$ decrease with increase of $[I]$. The inhibition constants in a rich media, new media and glucose-TSB are smaller than in a poor media, FCR–TLW, and the $K_i$ is larger than the $K_{si}$ in those media. These results are agree with those obtained by Ohara et al. [8] the values $K_i=11.0$ g/L and $K_{si}=9.5$ g/L for Streptococcus faecalis and Aiba et al., [6] have reported the value $K_i=66.7$ g/L and $K_{si}=35.7$ g/L for ethanol production from glucose. It is suggested that inhibitor concentration causes stronger damage on the specific growth rate than on the productivity generally.

Figs. 5 and 6 show the comparison of inhibition experimental results with calculations from inhibition models using average inhibition constants, $K_{i, av}$ and $K_{si, av}$ respectively. The productivity and specific growth rate for experimental values agree with the values from those models at low $[I_0]$, but smaller than models at high $[I_0]$. It may be related experimental values of $K_i$ and $K_{si}$ that are lower than values of $K_{i, av}$ and $K_{si, av}$ at high inhibitor concentration. Schepers et al., [9] reported that lactic acid bacteria are more sensitive to product inhibitor concentration.

Fig. 7 shows the fermented lactic acid concentration ($C_{la}$), residual sugar concentration ($C_r,s$) during fermentation in various media. In batch fermentation, lactic acid

<table>
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<th>Specific growth rate</th>
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<tr>
<td></td>
<td></td>
<td>$V_m$ [g/L]</td>
<td>$K_m$ [g/L]</td>
</tr>
<tr>
<td>(a) FCR–TLW</td>
<td>10</td>
<td>0.78</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.39</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.18</td>
<td>10.5</td>
</tr>
<tr>
<td>(b) FCR–TLW–CMW2</td>
<td>10</td>
<td>1.34</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.55</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.30</td>
<td>14.2</td>
</tr>
<tr>
<td>(c) Glucose–TSB</td>
<td>10</td>
<td>1.53</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.62</td>
<td>20.4</td>
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<tr>
<td></td>
<td>50</td>
<td>0.32</td>
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Effect of Product Inhibition on L-Lactic Acid Production

Fig. 5 Comparison of experimental results with inhibition model for productivity using average inhibition constant (K_{i, av}). Symbols are same as those in Fig. 1

Fig. 6 Comparison of experimental results with inhibition model for specific growth rate using average inhibition constant (K_{i, av}). Symbols are same as those in Fig. 1

Fig. 7 Lactic acid concentration (---) and residual sugar concentration (---) during batch fermentation (S_0=100 g/L). The media: △, FCR-TLW; ○, FCR-(TLW+CMW2); ★, glucose-TSB.
concentration increases with fermentation time. To estimate the productivity and specific growth rate from the inhibition models at a fermentation time, we used the average lactic acid concentration \( C_{l,a,m} \) during the fermentation, as inhibitor concentration \( I \). For example at 72 h fermentation time, the fermented lactic acid concentration \( C_{l,a} \) is 60 g/L for FCR–(TLW+CMW2). The viable cell was not inhibited by the inhibition concentration \( I = 60 \) g/L but it is inhibited by \( I = C_{l,a,m} \) is 35 g/L. Where, the \( C_{l,a,m} \) is average lactic acid concentration during the fermentation time. In Fig. 7, etching area (A) is equal to etching area (B) at the level of average lactic acid concentration \( C_{l,a,m} \).

Figs. 8 and 9 show comparison of productivity and specific growth rate from experiment results with those from the inhibition models. The fine line in Figs. 8 and 9 show the calculated curves by inhibition models for productivity (Eq. 5) and specific growth rate (Eq. 8) using \( I = C_{l,a,m}, K_i = K_{i,av} \) and \( K_m = K_{m,av} \), respectively. Fermentation experiment results fairly agree with the non-competitive product inhibition models, but deviated from models at long time fermentation because viable cell is more damaged at high inhibitor concentration than those from the models.

4. Conclusions

The product inhibition experiment was performed to clarify the effect of product inhibition on lactic acid fermentation from fresh cassava roots (FCR) by *Streptococcus bovis* in batch culture. It can be certified from Lineweaver-Burk plot that the inhibition is noncompetitive. The product inhibition was evaluated by two inhibition constants: inhibition constant for productivity \( (K_i) \) and inhibition constant for specific growth rate \( (K_m) \). They are not constant, but decrease on the increase of inhibitor concentration. The values of \( K_i \) and \( K_m \) in a rich media are smaller than those in a poor media and the \( K_i \) is larger than the \( K_m \) in these media. We compared the batch fermentation experimental results with those from the non-competitive model using average product concentration. They agreed with those from models, but they deviated from the inhibition models at high inhibitor concentration.

References


Streptococcus bovis による豆腐廃液（TLW）を用いた 生キャッサバのL-乳酸発酵における生成物阻害の影響

スリプトドゥ ウオノ, アブドール ゴファル, 国眼孝雄

東京農工大学工学部化学システム工学科

Streptococcus bovis による豆腐廃液（TLW）を用いた 生キャッサバのL-乳酸発酵における生成物阻害の影響を、回分培養により検討した。

乳酸発酵における生成物と増殖速度は、阻害物（乳酸）濃度の増加により、徐々に低下した。生成物と増殖速度への生成物阻害の影響を、非拮抗阻害のLineweaver-Burkプロットによって確認した。生成物阻害の影響は次の2つの阻害定数によって評価した。すなわち、生成阻害定数（$K_I$）と比増殖速度阻害定数（$K_a$）である。それらは阻害物濃度の増大により減小した。高栄養培地の$K_I$値と$K_a$値は、栄養欠乏培地の$K_I$値と$K_a$値よりも小さく、そしてそのような培地の中では、$K_I$値は$K_a$値よりも大きくなった。回分培養の結果は、約4日間は非拮抗阻害モデルに合致したが、4日以降は高濃度の阻害物のために、阻害モデルに合致しなくなった。これにより、培地中への乳酸の蓄積が生存細胞へのダメージを引き起こすことが明らかになった。