1. Introduction

Lignocellulosic biomass has excellent potential for the production of alternative biofuels to solve future environmental problems. Lignocellulosic biomass is abundant in Japan and mainly used for heat generation, but has other potential uses. The present study focused on ethanol production from lignocellulosic biomass. Ethanol can be used as a fuel for internal combustion engines, and a small percentage of ethanol mixed with gasoline is now used as fuel for cars in Japan.

Lignocellulosic biomass tends to be hard, because lignin binds to cellulose and hemicellulose. Therefore, lignocellulosic biomass requires several processing steps for the production of ethanol. First, the hard lignocellulosic biomass is physically and chemically crushed (pretreatment). After pretreatment, enzymes such as cellulase and β-glucosidase can break down the cellulose by enzymatic hydrolysis to form sugars, which are finally converted to ethanol by fermentation by yeast.

This process must be optimized for industrial applications, and a cost-effective method is essential. Simultaneous saccharification and fermentation (SSF) is a candidate cost-effective method based on enzymatic hydrolysis and fermentation in the same reactor. However, the optimum temperatures for enzymatic hydrolysis and fermentation differ, so ethanol production is less efficient using SSF than with separate hydrolysis and fermentation (SHF). In general, the optimum temperature for yeast growth and fermentation is 30°C, and that for enzymatic hydrolysis is approximately 50°C. Moreover, high temperatures (36°C or 37°C) may cause the yeast to activate several biochemical pathways for heat tolerance.

To solve this problem, SSF methods that employ temperature changes have been proposed. However, such temperature changes are difficult to regulate in practice. For practical applications, the simplest possible method for ethanol production is desirable. Recent studies have investigated thermotolerant yeasts. The optimum temperature for the thermotolerant yeast Kluveyromyces marxianus is approximately 45°C, whereas that of Saccharomyces cerevisiae is approximately 30°C. However, Kluveyromyces sp. is more easily inhibited, compared to S. cerevisiae, under certain conditions.

Furthermore, pretreatment is important for the success of the following steps in ethanol production from lignocellulosic biomass. For example, hydrothermal pretreatment uses compressed hot water and is widely employed as an environmentally friendly method. However, this method produces fermentation inhibitors, such as furfural. These fermentation inhibitors affect yeast growth and fermentation. The inhibition mechanism of 5-HMF has been investigated, especially the expression of genes related to inhibitor tolerance.

**Keywords**
Simultaneous saccharification and fermentation, Preculture condition, Ethanol production, Lignocellulosic biomass, Fermentation inhibitor
However, genetic modification of yeast is difficult for practical applications. Moreover, \textit{S. cerevisiae} has shown temperature adaptation in long-term culture\textsuperscript{10}. Therefore, we focused on preculture of yeast to generate temperature-adapted yeast. Yeast is usually precultured in test tubes in volumes of 5 mL, so this method is easy to adapt to practical applications. In this study, we investigated the application of yeast preculture to generate strains that are adapted for efficient ethanol production.

2. Materials and Methods

A strain of \textit{S. cerevisiae} type 2 (Sigma-Aldrich, Japan) was cultured in yeast extract dextrose medium (YPD; yeast extract, peptone, dextrose; Difco, Japan). Before preculture, 100 \( \mu \)L of cultured yeast was added to 5 mL of YPD in a test tube and was cultured at 30 \( ^\circ \)C, overnight. The yeast was then incubated at 30 \( ^\circ \)C or 35 \( ^\circ \)C with shaking at 120 rpm for preculture. The precultured cells (1 mL) were used for SSF. The SSF mixture consisted of 50 mL of 0.1 M (1 M = 1 mol L\(^{-1}\)) acetic buffer, and 77.52 U/g-glucan of cellulase and 111.25 U/g-glucan of \( \beta \)-glucosidase for enzymatic hydrolysis in a flask. The substrate was 5 g of cellulose (sigma cell 20 \( \mu \)m, Sigma Aldrich, Japan). The SSF incubation temperature ranged from 30 to 35 \( ^\circ \)C. The inhibitor 5-HMF (5 mM) was added during the preculture and SSF. Samples of 2 mL were taken at each time point. The ethanol concentration was measured by high-performance liquid chromatography (HPLC) using a SUGAR KS-802 (Shodex, Japan) column operated at 60 \( ^\circ \)C with water as the eluent at 0.8 cm\(^3\)/min.

3. Results and Discussion

Figure 1 shows the effect of increasing the pre-culture temperature on ethanol production in SSF with 5-HMF. Generally, the optimum temperature for growth of \textit{S. cerevisiae} is 30 \( ^\circ \)C. However, ethanol production was higher using yeast cultured at 35 \( ^\circ \)C than with yeast cultured at 30 \( ^\circ \)C. Unfortunately, the SSF temperature was 30 \( ^\circ \)C in this case, which is too low for the enzyme reaction. Therefore, we investigated the effect of increasing the SSF temperature. Figure 2 shows that increasing the preculture temperature resulted in higher ethanol production (Fig. 2). Furthermore, final ethanol production was slightly higher than with the SSF temperature of 30 \( ^\circ \)C. Therefore, increasing the preculture temperature raises ethanol production by SSF, even in the presence of 5-HMF.

5-HMF is a fermentation inhibitor which affects yeast growth and fermentation\textsuperscript{7}. These fermentation inhibitors are generated by some pretreatments of lignocellulosic biomass. This study showed that adaptation to high temperatures increases ethanol production and 5-HMF tolerance in SSF. In a previous study, yeasts were selected by long-term adaptation\textsuperscript{10}. The selected yeasts showed higher ethanol production at 39 \( ^\circ \)C than wild-type yeast. However, only fermentation was investigated. The present study showed that yeast preculture at 35 \( ^\circ \)C selects for adaptation to high temperature and that such adaptation improves the efficiency of SSF.

Higher preculture temperature increased ethanol production. Therefore, we investigated the effect of 5-HMF on preculture. Higher preculture temperature in the presence of 5-HMF increased ethanol production of SSF without 5-HMF (Fig. 3). Moreover, the final ethanol concentration was markedly higher using SSF with preculture at 35 \( ^\circ \)C than for SSF with preculture at 30 \( ^\circ \)C.

Addition of 5-HMF in preculture had a strong effect on SSF at 30 \( ^\circ \)C, so we investigated ethanol production via SSF at 35 \( ^\circ \)C using the same preculture conditions. Interestingly, higher SSF temperature did not increase ethanol production (Fig. 4). We investigated the
Effect of 5-HMF and temperature on ethanol production in both preculture and SSF. Addition of 5-HMF to SSF did not increase ethanol production. However, we tested only one concentration (5 mM HMF). Different concentrations in preculture and SSF might increase ethanol production.

Yeast is known to acquire thermotolerance through cultivation at high temperature and regulate many of the gene expressions. In our study, ethanol production was higher using precultured yeast at 35 °C than with precultured yeast at normal temperature in SSF. Therefore, we also showed that yeast acquired thermotolerance by cultivation at high temperature. Furthermore, the growth rate of yeast at 35 °C was lower than that of yeast at 30 °C in the preculture (data not shown). Nevertheless, ethanol productivity using yeast precultured at 35 °C was higher than that of yeast precultured at normal temperature. Therefore, the ethanol productivity per cell of thermotolerant yeast might be higher than that of normal yeast.

4. Conclusion

Higher preculture temperature with 5-HMF increases the production of ethanol in SSF. Because preculture temperature in this study was not a severe condition for the yeast, the yeast adapted to SSF with 5-HMF. Furthermore, in the presence of 5-HMF on preculture, the final ethanol concentration was higher by SSF with preculture at 35 °C with 5-HMF than by SSF with preculture at 30 °C. Therefore, modifying the preculture conditions can be used to increase the ethanol concentration produced by SSF.

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要 旨

同時糖化発酵を用いた高効率エタノール生産のための酵母前培養条件

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木質系バイオマスは，国内の賦存量も豊富なことから，その
有効利用が期待されている。木質系バイオマスから生産される
バイオエタノールは，ガソリンと混合し，内燃機関の燃料とし
て用いられている。バイオエタノール生産工程のコストを低減
させるために，本研究では反応器の削減が期待される同時糖化
発酵法を用いた。同時糖化発酵法を効率良く行うためには，前
処理で生成する発酵阻害物質，および高温に対する耐性を持っ
た酵母を用いないければならない。そこで我々は，同時糖化発酵
で使用する酵母を増殖させる前培養に着目した。前培養温度を
35 ℃ に上昇させた酵母は発酵阻害物質の一つである5-HMF 存
在下での同時糖化発酵において，通常の培養温度である30 ℃
と比べ，エタノール生産量が高かった。また，前培養時に
5-HMF を加えた酵母においても，5-HMF 非存在下のSSF 時に
高温前培養の効果が見られた。本研究では，前培養温度を変化
させることにより，同時糖化発酵において発酵阻害物質存在下
においてもエタノール生産効率を上昇させた。