High-temperature Fermentation Technology for Low-cost Bioethanol

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Considering its advantages including reduction of cooling cost and saving water during the fermentation process, which consequently cut down the total running cost, high-temperature fermentation with thermotolerant microbes is expected to be one of next-generation fermentation technologies. We focused on the establishment of high-temperature fermentation technology for ethanol production from biomass in Thailand, for which thermotolerant microbes suitable for various types of biomass were selected and advanced fermentation processes including a temperature-uncontrolled fermentation and a simultaneous fermentation and distillation under a low pressure were investigated.

Key Words

High-temperature fermentation, Thermotolerant microbes, Bioethanol, Kluyveromyces marxianus, Zymomonas mobilis

1. Introduction

Global temperatures have been increasing due to increases in the amounts of carbon dioxide emission by utilization of fossil fuels including coal, petroleum and natural gas. Reduction of carbon dioxide emission has thus become an urgent issue, and biofuels derived from biomass have attracted attention as an alternative to fossil fuels. Since the worldwide demand for bioethanol has been increasing, the development of energy-saving, efficient ethanol production technology is required.

Since the ethanol fermentation process is exothermic, the cooling of fermenter is indispensable for stable fermentation with microbes. The cooling, however, becomes difficult in hot seasons, so that fermentation industries in tropical countries and even in Japan stop their fermentation process for 1-2 months during such seasons. Thus, high-temperature fermentation with thermotolerant microbes is expected to be developed. High-temperature fermentation of ethanol has several advantages including high fermentation rate, decreased risk of contamination, reduction of cooling and operating costs. High-temperature fermentation could thus achieve the running cost reduction of fermentation process as well as a stable fermentation.

For high-temperature fermentation, thermotolerant microbes that are stable under high temperature conditions and efficient in useful material production are essential. In this study, we introduced two ethanologenic microbes, Kluyveromyces marxianus and Zymomonas mobilis. The former is thermotolerant yeast and is able

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to efficiently produce ethanol at high temperatures, and it has the potential to assimilate a wide variety of substrates \(^9\) \(^6\). On the other hand, the latter as a bacterium has a unique metabolic pathway that enables an approximately 3-times higher rate of ethanol production than that of *Saccharomyces cerevisiae* \(^9\) \(^6\). *Z. mobilis* with thermotolerance is thus expected to perform high-speed and high-temperature fermentation. We screened thermotolerant microbes suitable for various types of biomass and bred more thermotolerant and/or stress-resistant microbes that are useful for high-temperature fermentation of ethanol. One of such thermotolerant yeasts was applied for ethanol fermentation under temperature-uncontrolled conditions. We also developed the new process of simultaneous fermentation and distillation under a low pressure. This process may have some benefits, for example, in ethanol recovery from a low concentration of ethanol, which occurs when raw garbage or cellulosic materials are used as raw materials for ethanol fermentation.

2. Experimental

2.1 Strains

*K. marxianus* DMKU3-1042 was obtained from soil and water samples from sugar cane plantations and sugar factories in four provinces, namely Phra Nakhon Si Ayutthaya, Ratchaburi, Suphanburi and Uthaithani, Thailand \(^7\). Isolation was carried out at 35 °C by an enrichment technique using sugar juice medium to be incubated for 3 days under shaking condition, and then streaked on agar plates containing the same medium and incubated at 35 °C. *K. marxianus* DMKU3-1042 derivatives (which will be reported elsewhere) were isolated under various stress conditions from DMKU3-1042 as a parental strain, and of those, one strain that was isolated as an iron-resistant on KMYPD plates (see below) containing 17 mM FeSO\(_4\) at 37 °C, was used in this study. *Z. mobilis* TISTR548 was obtained from the TISTR culture collection (Bangkok MIRCEN), and ZM4 (NRRL B-14023) was provided by E. Yanase. Thermo-adapted strains (which will be reported elsewhere) were isolated from TISTR548 as a parental strain by repeated-cultivation more than 80 times at high temperatures (from 37 °C to 40 °C) in ZMYPD medium (see below) under a static condition, and one of the most thermotolerant strains was used in this study.

2.2 Media and cell growth condition

*K. marxianus* cells were grown in KMYPD medium containing 1 % (w/v) yeast extract, 2 % (w/v) peptone and 3 % (w/v) glucose at temperatures indicated under a shaking condition at 160 rpm, and *Z. mobilis* cells were grown in ZMYPD medium containing 0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone and 3 % (w/v) glucose at temperatures indicated under a static condition. Cell growth of *K. marxianus* and *Z. mobilis* was determined by measuring an optical density at OD\(_{660}\) and OD\(_{550}\), respectively. The media for the process of simultaneous fermentation and distillation were KMYP-20 % rice medium containing 1 % (w/v) yeast extract, 2 % (w/v) peptone and 20 % (w/v) rice hydrolysate (equivalent to 13 % glucose) for *K. marxianus* cells and ZMYP-20 % rice medium containing 0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone and 20 % (w/v) rice hydrolysate (equivalent to 13 % glucose) for *Z. mobilis* cells. Minimum-20 % rice medium for fermentation contained 20 % (w/v) rice hydrolysate (equivalent to 13 % glucose), 0.2 % urea, 0.05 % potassium dihydrogen phosphate and 0.1 % magnesium sulfate heptahydrate. Rice was hydrolyzed into glucose by Yuniase S containing α-amylase and glucoamylase (2000 U/g rice; Yakult Pharmaceutical Industry Co, Tokyo) at 55 °C for 24 h. Cell growth in rice medium was not analyzed due to the strong turbidity of the medium.

2.3 Fermentation with rice hydrolysate

*K. marxianus* was pre-cultivated in 50 ml of KMYP-5 % rice medium at 30 °C for 18 h under a shaking condition at 100 rpm. The pre-culture was inoculated at 10 % into KMYP-20 % rice medium. Fermentation was carried at 100 rpm for 72 h at 45 °C. At the times indicated, the culture medium was collected for analysis of cell growth, ethanol concentration and sugar concentration.

2.4 Simultaneous fermentation and distillation

After rice hydrolysis, the hydrolysate was transferred into a fermentation and distillation tank, and fermentation was performed at 41 °C by *K. marxianus* DMKU3-1042 under a shaking condition at 160 rpm or by a thermo-adapted strain of *Z. mobilis* under a static condition. After fermentation, the vapor pressure of the fermentation and distillation unit was decreased to 70 mbar and ethanol was collected as the first recovery ethanol into a primary ethanol recovery bottle for about 12 h. After the first distillation under the low air pressure, ethanol from the primary ethanol recovery bottle was collected as the secondary recovery ethanol at 70 mbar. Some leaking ethanol was trapped in a drain tank.

2.5 Measurement of glucose and ethanol concentrations in culture medium

Glucose and ethanol concentrations in culture medium were determined at 60 °C by an HPLC system consisting of an L-2130 Pump, L-2490 Refractive Index Detector, L-2200...
Autosampler, L-2350 Column oven, and Hitachi Model D-2000 Elite HPLC System Manager, equipped with a GL-C610-S Gelpack column (Hitachi Chemical, Tokyo, Japan) using distilled water from an RFD240NA Water Distillation Apparatus (Aquarius, ADVANTEC, Japan) as a mobile phase at a flow rate of 0.3 ml/min.

3. Results and Discussion

3.1 K. marxianus as a thermotolerant and ethanologenic yeast

3.1.1 Yeasts suitable for biomass

K. marxianus is able to utilize various sugars including glucose, mannose, galactose, xylose and arabinose and possesses many sugar transporters encoded by its genome. We performed screening of yeasts for biomass of molasses, sugar cane juice, cassava starch and lignocellulose hydrolysate at high temperatures, and we found highly efficient ethanol fermenting species suitable for each biomass (Table 1). These yeasts listed in Table 1, however, exhibited not always good efficiencies when different biomasses were tested. We thus utilized K. marxianus DMKU3-1042 in the following experiments because it showed a good performance with all biomasses tested.

3.1.2 Improvement of stress-tolerance of thermotolerant K. marxianus

We also developed several derivatives under various stress conditions from K. marxianus DMKU3-1042 as a parent, which was isolated in Thailand as the most thermotolerant and efficiently ethanol-producing yeast. Fermentation abilities of an iron-resistant DMKU3-1042 derivative, DMKU3-1042 as a parental strain and Saccharomyces cerevisiae as a yeast generally utilized in the fermentation industry were examined in minimum-20% rice medium under a shaking condition at 100 rpm and under a static condition at 45 °C (Fig. 1). The derivative showed high levels of glucose utilization and ethanol production compared to those of the other two strains under both conditions. On the other hand, S. cerevisiae could not

Table 1 Yeast strains suitable for various biomasses at high temperatures

| Biomass          | Microorganism          | Growth Temperature |
|------------------|------------------------|--------------------|
| Molasses         | Saccharomyces cerevisiae DMKU 3-S087 | 40 °C              |
| Sugar cane       | Kluyveromyces marxianus 3-KS07     | 40 °C              |
| Cassava starch*  | Pichia kudriavzevi KU-ET1          | 40 °C              |
| Lignocellulose*  | Candida tropicalis DMKU3-K2260     | 40 °C              |

* The biomass was used as hydrolyzate

Fig. 1 Comparison of ethanol fermentation ability (The K. marxianus DMKU3-1042 derivative, K. marxianus DMKU3-1042 and S. cerevisiae were grown in 50 ml of minimum-20% rice medium at 45 °C under a shaking condition at 100 rpm (a, b) and a static condition (c, d). Concentrations of glucose (a, c) and ethanol (b, d) in the culture medium were determined by HPLC. Open squares, open circles and open triangles were represent the K. marxianus DMKU3-1042 derivative, K. marxianus DMKU3-1042 and S. cerevisiae, respectively)
utilize glucose in the hydrolysate under conditions. These experiments indicate the possibility that thermotolerant strains isolated from tropical environments can be improved to be more thermotolerant or efficient ethanol-producing strains.

3.1.3 Ethanol fermentation under a temperature-uncontrolled condition

Due to the availability of thermotolerant strains, one may expect that a temperature-uncontrolled fermentation can be performed and is economical under high-temperature circumstance like tropical countries or hot seasons. Ethanol fermentation with *K. marxianus* DMKU3-1042 was thus compared under temperature-uncontrolled and -controlled (40°C) conditions in YPD medium containing 9% glucose by using a 5-L fermenter. As a result, cell growth and ethanol production at 7 h under temperature-controlled condition were better than those under temperature-uncontrolled, and the ethanol concentration reached a maximum at 12 h under both tested conditions (Fig. 2). Under the temperature-uncontrolled condition, temperature was approximately 25°C at the beginning of fermentation and increased gradually to 35°C at 11 h. The final temperature at 48 h was 33°C. Optimal temperature for this yeast strain was around 37°C which is the average temperature during summer time in Thailand. Furthermore, such a temperature-uncontrolled ethanol fermentation with sugar cane juice was successfully performed even in 3,000 L scale in Thailand, where the initial and maximum temperatures were 30°C and 39°C, respectively (data not shown). These findings and facts suggest the possibility of temperature-uncontrolled ethanol fermentation in industry, which could be an economical process under high-temperature circumstances because it may cut down the cost of cooling units and for cooling fermenter.

3.1.4 Application of distillation under a low pressure at 40°C

Since the saturation vapor pressure of ethanol is 177.8 mbar at 40°C as a theoretical value, ethanol is recoverable from the culture tank by reducing the vapor pressure below that value. DMKU3-1042, which is able to efficiently produce ethanol even at a relatively high temperature, was thus used for the simultaneous fermentation and distillation
test at 40 °C in KMYP-20 % rice medium. For this test, a system consisting of a fermentation and distillation tank and a distillation apparatus, primary and secondary ethanol recovery units, a vacuum pump and a drain unit was constructed (Fig. 3). Due to the constitution of this system, the air in the tank was discharged outside during vacuum distillation, some of which was trapped in the drain unit. The operation procedure of this system is described in Experimental. Simultaneous fermentation and distillation under a low pressure was repeated three times, and glucose consumption and ethanol production were monitored (Fig. 4a). The reproducible results suggest that \textit{K. marxianus} can survive under a low pressure. The maximum concentration of ethanol in the medium was about 5 % (Fig. 4a). The ethanol concentrations in primary and secondary bottles were about 35 % and 60 %, respectively (Fig. 4b).

Considering the concentration of ethanol, the technology of the simultaneous fermentation and distillation under a low pressure could be connected to the technology of membrane separation of ethanol and water.

3.2 Thermotolerant ethanologenic \textit{Z. mobilis}

3.2.1 Potential of \textit{Z. mobilis} as an ethanol producer

\textit{Z. mobilis} as a Gram-negative and facultative anaerobe is known to be an efficient ethanol producer, achieving an approximately 3-times higher rate of ethanol production than that of yeast by its strong Entner-Doudoroff pathway \textsuperscript{5,6}, and it is generally recognized as having a safe (GRAS) status. The bacterium, however, can assimilate only three sugars, glucose, fructose and sucrose. Thermotolerant \textit{Z. mobilis} can thus be used for high-speed and high-temperature fermentation and is suitable for biomass such as sugar cane juice. We thus compared the cell growth and ethanol productivity among six \textit{Z. mobilis} strains from the TISTR culture collection in ZMYPD medium at 30 °C and 39 °C under a static condition, and it was found that TISTR548 showed highest optical density and the most abundant ethanol in the medium at 39 °C, suggesting that TISTR548 is the most thermotolerant strain.

3.2.2 Ethanol production from acid hydrolysate of Jerusalem artichoke roots

The selection and characterization of \textit{Z. mobilis} for ethanol production from acid hydrolysate of Jerusalem artichoke (\textit{Helianthus tuberosus} L.) roots were first investigated \textsuperscript{10}. Growth and ethanol production of four \textit{Z. mobilis} strains isolated in Thailand, TISTR405, TISTR548, TISTR550 and TISTR551, were compared with those of the type strain \textit{Z. mobilis} ZM4 (NRRL B-14023) at different temperatures. Among the strains tested, TISTR548 gave the highest optical density and ethanol concentration at 39 °C. Therefore, this strain was chosen for ethanol production from acid hydrolysate of Jerusalem artichoke roots, called Jerusalem artichoke juice.

To optimize the medium for ethanol production from Jerusalem artichoke juice, effects of several factors were examined and the results showed that the maximum ethanol concentration (95.9 g/L) with 98 % of the theoretical ethanol yield was obtained when the fermentation was carried out in a medium containing 250 g/L total sugars, pH 5.0, inoculation size at 10 % and using 0.5 g/L
diammonium phosphate as a nitrogen source. The maximum ethanol yield obtained in this study was higher than yields previously reported.

3.2.3 Improvement of the thermotolerance of thermotolerant Z. mobilis

In order to elevate thermotolerance of thermotolerant Z. mobilis, we conducted repeated cultivation at high temperatures and isolated a thermo-adapted strain as a TISTR548 derivative as described in Experimental. The derivative showed about 4-times higher optical density than that of the parent at 40°C in ZMYPD medium. The growth and ethanol production capacity of the derivative were then compared with those of S. cerevisiae and K. marxianus DMKU3-1042 (Fig. 5). The Z. mobilis TISTR548 derivative, K. marxianus DMKU3-1042 and S. cerevisiae were grown in KMYPD medium at 30°C and at 41°C. As a result, cell growth, glucose consumption and ethanol accumulation of the derivative were shown to be faster than those of the others at both 30°C and 41°C. Therefore, it is likely that the TISTR548 derivative is the most efficient ethanol producer among the three strains even at 41°C under conditions tested.

3.2.4 Application of distillation under a low pressure at 40°C

To evaluate fermentation ability from rice, the TISTR548 derivative was subjected to fermentation experiments in ZMYP-10% rice medium at 40°C (Fig. 6). In three repetitions of the fermentation and distillation process, patterns of glucose consumption and ethanol production showed periodic changes (Fig. 6a), suggesting that the derivative can grow under a reduced pressure. Although the maximum concentration of ethanol in the medium was almost the same as that in the experiments with yeast, an average time for fermentation was about 3 h shorter than that with K. marxianus DMKU3-1042 (Fig. 4a and Fig. 6a). Therefore, it is likely that the simultaneous fermentation and

![Fig. 4 Ethanol production by DMKU3-1042 (After saccharification in KMYP-20% rice medium, fermentation with K. marxianus DMKU3-1042 was initiated. The initiation times (straight arrows) of the 1st, 2nd and 3rd fermentation were 18 h, 72 h and 132 h, respectively, and the finishing times (dotted lines) were 36 h, 98 h and 148 h, respectively. The initiation times (straight arrows with 1) of the first recovery of ethanol under a low pressure were 36 h, 98 h and 148 h, respectively, and finishing times (dotted lines with 1) were 52 h, 110 h and 160 h, respectively. The initiation times (straight arrows with 2) of the second recovery of ethanol were 52 h, 110 h and 160 h, respectively. Other experimental conditions are described in Experimental. (a) Open circles and open squares indicate concentrations of glucose and ethanol, respectively, in the culture. (b) Open circles and open squares indicate ethanol concentrations in the primary and secondary recovery bottles, respectively. Concentrations of glucose and ethanol were measured by HPLC at the times indicated.)

![Fig. 5](image-url)
Fig. 5 Fermentation activities of a TISTR548 derivative and DMKU3-1042 (The Z. mobilis TISTR548 derivative, K. marxianus DMKU3-1042 and S. cerevisiae were grown in KMYPD medium at 30°C (a) and at 41°C (c) and their growth was monitored by measuring OD_{660}. Concentrations of glucose and ethanol in the culture at 30°C (b) and at 41°C (d) were measured by HPLC. Open circles, open squares and open triangles represent the Z. mobilis TISTR548 derivative, K. marxianus DMKU3-1042 and S. cerevisiae, respectively. Open symbols and closed symbols in b and d represent concentrations of glucose and ethanol in the culture, respectively.)

distillation process with the TISTR548 derivative is more efficient than that with the yeast. Ethanol concentrations recovered by primary and secondary recovery processes were 25-35% and about 60%, respectively (Fig. 6b).

4. Conclusions

In this study, we selected thermotolerant yeasts suitable for various biomasses (Table 1). We also showed the possibility that the thermostolerance of naturally isolated thermotolerant microbes can be further improved and the possibility of temperature-uncontrolled fermentation with K. marxianus DMKU3-1042 (Fig. 2). Z. mobilis is able to perform high-speed fermentation compared to yeast [6, 7], and thus high-speed and high-temperature fermentation is expected with thermotolerant Z. mobilis. A thermo-adapted derivative from thermotolerant Z. mobilis TISTR548 showed significantly high levels of performance in growth and ethanol fermentation at a high temperature compared to those of the parent and also to K. marxianus DMKU3-1042 and S. cerevisiae (Fig. 5). Further improvement for stable high-temperature fermentation might be possible by the addition of sorbitol because its supplementation promotes cell growth and increases the ethanol fermentation capability of Z. mobilis under heat, ethanol, and osmotic stress conditions [11].

The thermostolerance of K. marxianus DMKU3-1042 and the thermo-adapted derivative of Z. mobilis TISTR548 were applied for a new technology of simultaneous fermentation and distillation under a low pressure at a high temperature. Both microbes were shown to be applicable to the new technology. Notably, the Z. mobilis TISTR548 derivative showed much better performance than that of K. marxianus DMKU3-1042 in the simultaneous fermentation and distillation. It is expected that high-temperature fermentation or simultaneous fermentation and distillation at high temperatures will become widely used in the fermentation industry in the near future, and thermotolerant microbes isolated from tropical countries or further thermo-adapted or stress-adapted mutants may be key factors for such application.

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Ethanol production by a TISTR548 derivative (After saccharification in ZMYP-20 % rice medium, fermentation with Z. mobilis TISTR548 derivative was initiated. The initiation times (straight lines) of the 1st, 2nd and 3rd fermentation were 18 h, 72 h, and 112 h, respectively, and the finishing times (dotted lines) were 42 h, 84 h and 130 h, respectively. The initiation time (straight lines with 1) of the first recovery of ethanol under a low pressure were 42 h, 84 h and 130 h, respectively, and finishing times (dotted lines with 1) were 60 h, 110 h and 142 h, respectively. The initiation times (straight lines with 2) of the second recovery of ethanol were 60 h, 110 h and 142 h, respectively. Other experimental conditions are described in Experimental. (a) Open circles and open squares indicate concentrations of glucose and ethanol, respectively, in the culture medium. The arrows and dotted arrows indicate the start and finish time of fermentation, respectively. (b) Open circles and open squares indicate ethanol concentrations in the primary and secondary bottles, respectively. The arrows and dotted arrows indicate the start time of first and second recovery of ethanol, respectively. Concentrations of glucose and ethanol were measured at the times indicated by HPLC).

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