SELECTION AND IDENTIFICATION OF THERMOTOLERANT ETHANOL PRODUCING YEAST STRAINS

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ABSTRACT: Among eight yeast strains isolated from starters in Vietnam, only one strain coded as ITB3 could grow well at 45°C with optical density (OD_{660nm}) of 3.8 that was equal to *Kluyveromyces marxianus* DMKU3-1042 strain (OD_{660nm} of 4). At 30°C, the strain ITB3 fermented 10% (w/v) glucose to 4.45% (v/v) ethanol in 14 hours at a yield of 0.38 g ethanol/g glucose, or 74.9% of theoretical yield that was slightly lower than that of *K. marxianus* DMKU3-1042 (produced 4.61 % ethanol (v/v), 0.39 g ethanol/g glucose, or 77% of theoretical yield). However, at 45°C, the strain ITB3 fermented 10% (w/v) glucose to 4.54% (v/v) ethanol at a yield of 0.39 g ethanol/g glucose, or 75.6% of theoretical yield that was slightly higher than that of *K. marxianus* DMKU3-1042, (produced 4.39 % (v/v), 0.37 g ethanol/g glucose, or 72.3% of theoretical yield). The strain ITB3 was identified as *K. marxianus* ITB3 based on the results of ITS rDNA sequencing and yeast mating. These results demonstrated that the *K. marxianus* ITB3 can be used for industrial ethanol production at high temperature in order to reduce energy consumption, especially in simultaneous saccharification and fermentation processes for production of ethanol from starchy and cellulosic materials that decreases the amount of saccharification enzymes and investment costs for a plant.

Keywords: *Kluyveromyces marxianus*, ethanol fermentation, thermotolerant yeasts.

INTRODUCTION

In tropical countries, the ingenious alcoholic production has been done at high temperature because the day-time temperatures are high throughout the year ranging from 30 to 37°C. The advantages of traditional ethanol making at high temperature are rapid saccharification and fermentation, that decreased risk of contamination [5].

The starters that are used for traditional alcoholic make from rice or glutinous rice, called “Men” in Vietnamese, are produced in solid state tablets which combine two groups of microorganisms, such as, moulds and yeasts. The moulds are the major producers of amylases that degrade starch into fermentable sugars, and the yeasts convert the sugars to alcohol.

In industrial ethanol production, heat is generated at the rate of 140.2 cal/g glucose during fermentation that is controlled by cooling system. Cooling costs during the process of ethanol production are expensive. Therefore, there have been some reports on isolation, improvement and application of

thermotolerant yeast strains in ethanol production at high temperature [1, 2, 5].

Kluyveromyces marxianus is a well-known thermotolerant yeast species that can produce ethanol at high temperature and use a wide range of carbon sources [3]. *K. marxianus* DMKU3-1042 was found to grow and produce ethanol at 45°C and use carbon sources, such as, cellobiose, xylose, xylitol, arabinose, glycerol, and lactose [5].

The objectives of this study are to select yeast strains capable of growth and ethanol production at elevated temperature from starters and to identification them to species.

MATERIALS AND METHODS

Yeast strains

The strains ITB1, ITB2, ITB3, ITB4, ITB5, ITB6, ITB7 and ITB11 were isolated from starters in Vietnam; the *Kluyveromyces marxianus* DMKU3-1042 (KM) was isolated in Thailand [5]; and the *K. marxianus* leu2⁻ mutant (RAK 4088) and *K. marxianus* ura5⁻ mutant

(RAK 4084) were selected by Yamaguchi University.

Culture media

YPD medium (1% yeast extract, 2% peptone and 2% glucose), and YPD10 (1% yeast extract, 2% peptone and 10% glucose), minimal medium (MM: yeast nitrogen base without amino acids and ammonium sulfate (1.7 g/l), ammonium sulfate (5 g/l), glucose (20 g/l), uracil (0.08 g/l), and 5-Fluoroorotic Acid (5-FOA) medium.

Selection of thermotolerant yeast strains

Yeasts that isolated from starters (Men) were selected both on YPD agar at 45°C (the growth levels assessed as following: no-growth, weak-growth, and good-growth), and in liquid YPD at 45°C (the cell growth was recorded automatically by a TVS.062CA Bio-Photorecorder (Toyo Seisakusho, Ltd., Chiba, Japan).

Fermentation for ethanol production

Forty milliliters of YPD10 medium was inoculated with an overnight YPD culture to an initial optical density at 660 nm (OD_{660}) of 0.2 at 30°C and 45°C with shaking at 100 rpm and without shaking. Supernatant samples were collected by centrifugation and were analyzed directly by Biosensor BF-5 (Oji Scientific Instruments, Hyogo, Japan) to determine sugar and ethanol concentrations.

Yeast mating technique for identification of yeast

First of all, the strain ITB3 was grown overnight in 2 ml of YPD, centrifuged, washed once with sterile water, and resuspended in 1 ml of sterile water. Aliquots (200 μ l) of cells were then spread on 5-FOA plates and exposed to UV for 30 s. Colonies were picked after 2 days at 28°C and tested for a uracil requirement by assessing growth on MM medium with and without uracil. Then, the uracil-requiring mutants were transformed with *ura3* and *ura5* gene fragment by a lithium acetate method [2] to select the ITB3 *ura3*⁻ mutant (mutation in the *ura3* gene). Next, the ITB3 *ura3*⁻ mutant was

cross-bred with *K. marxianus* DMKU 3-1042 *leu2*⁻ mutant and *K. marxianus* NBRC 1777 *ura5*⁻ mutant by streaking perpendicular on 2% glucose medium, the plates incubated at 28°C for 2 days and replicated to the MM medium without uracil by replica plating using the sterile velveteen pads. Lastly, if the colonies appear on the MM medium without uracil, the strain ITB3 is the same species to identified strains.

Identification of selected strain by sequencing of the rDNA of internal transcribed spacer (ITS) region

The ITS rDNA was amplified from genomic DNA (extracted from yeast cells) by PCR using a KOD Plus kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions with forward primer ITS1 and reverse primer ITS4. The PCR was initiated at 94°C for 1 min, followed by 30 cycles of the following program: 94°C for 20s, 60°C for 30s, and 68°C for 2 min. The PCR product was checked by agarose gel electrophoresis, purified by using ExoSAP-IT according to manufacturer's protocol and sequenced using ITS2 and ITS3 primers according to protocol of Macrogen in Japan. The ITS sequence was submitted to BLAST analysis in the National Center for Biotechnology Information nucleotide database. Yeast identification was assumed if the query sequence showed 99% identity with DNA sequences from nucleotide database.

Creation of the phylogenetic tree. A sequence file of each species was aligned using Clustalx 2.0.11 program for multiple alignment mode. The multiple-alignment file was then used to create a neighbor-joining rectangular cladogram with Treeview X 0.5.0 program.

RESULTS AND DISCUSSION

Selection of thermotolerant yeast strains

The thermotolerant growth levels of the isolates were comparable to that of KM strain on YPD agar at 45°C. Among the isolates, the strain ITB3 was good-growth at 45°C (fig. 1).

Fig. 1. The growth levels of isolates (a) and KM at 45°C (b)

Fig. 2. Cell growth of isolates in comparison with KM strain at 45°C

The cell growth of the isolates was also tested in liquid YPD at 45°C. The result showed that the strain ITB3 could grow well at 45°C with (OD_{660nm}) of 3.8 that was equal to KM (OD_{660nm} of 4) (fig. 2).

Fermentation for ethanol production by thermotolerant yeasts

The strain ITB3 was tested for ethanol production in YPD10 medium at 30 and 45°C with shaking at 100 rpm and compared to KM strains

Fig. 3. Ethanol production by the strain ITB3 in YPD10 medium at 30°C (a) and at 45°C (b)

Fig. 4. Ethanol production by the strain KM in YPD10 medium at 30°C (a) and at 45°C (b)

At 30°C, the strain ITB3 produced 4.45% (v/v) ethanol (36.31 g/l) in 14 hours at a yield of 0.38 g/g, or 74.9% of theoretical yield that was slight lower than that of KM (produced 4.61 % (v/v) ethanol (36.9 g/l), 0.39 g/g, or 77% of theoretical yield). However, at 45°C, the ITB3

produced 4.54% (v/v) ethanol (36.31 g/l) at a yield of 0.39 g/g, or 75.6% of theoretical yield that was slight higher than that of KM (produced 4.39 % (v/v) ethanol (35.11 g/l), 0.37 g/g, or 72.3% of theoretical yield) (fig. 3, fig. 4 and table 1).

Table 1. Ethanol production of the strain ITB3 and KM in YPD10 medium at 30°C and 45°C with shaking at 100 rpm in 14 hours

Strains	at 30°C			at 45°C		
	Ethanol (g/l)	g ethanol/g glucose	% of theor. yield	Ethanol (g/l)	g ethanol/g glucose	% of theor. yield
ITB3	36.31	0.38	74.9	36.31	0.39	75.58
KM	36.9	0.39	77	35.11	0.37	72.91

Table 2. Ethanol production by the strain ITB3 and KM in YPD10 medium at 30°C and 45°C without shaking in 24 hours

Strains	At 30°C			At 45°C		
	Ethanol (g/l)	g ethanol/g glucose	% of theor. yield	Ethanol (g/l)	g ethanol/g glucose	% of theor. yield
ITB3	21.57	0.23	46	27.5	0.3	59
KM	24.54	0.28	54.43	28.9	0.32	62

The strain ITB3 was also tested for ethanol fermentation in YPD10 at 30 and 45°C without shaking at 100 rpm and compared with KM strain

The results of static ethanol fermentation of both ITB3 and KM showed that the ethanol concentration was lower and the fermentation time was longer than those of shaking condition (table 2). This indicated that oxygen is a

necessary factor for shorting of ethanol fermentation time of *K. marxianus*.

The yeast mating for identification of unknown yeast strain

The uracil-requiring mutants were isolated from the strain ITB3 by UV mutagenesis on 5-fluoroorotic (FOA) medium (fig. 5) and the mutation was in the *ura3* gene that confirmed by *ura5* gene recombination (fig. 6). The ITB3

ura3⁻ mutant mated both *K. marxianus* leu2⁻ and *K. marxianus* ura5⁻, as a result the colonies appeared on the MM medium without uracil

(fig. 7). These results confirmed that the strain ITB3 was the same species with the identified mutants that belong to *K. marxianus*.

Fig. 5. The ITB3 mutants were isolated by UV mutagenesis on FoA medium (a) and required uracil for growth (b)

Fig. 6. The mutant was ITB3 ura3⁻ mutant because it only recombined with ura5 fragment

Fig. 7. The ITB3 ura3⁻ mated with both *K. marxianus* leu2⁻ and *K. marxianus* ura5⁻, as a result the colonies appeared on the MM medium without uracil

Identification of selected thermotolerant yeast strain by ITS rDNA sequencing

The IBT3 strain was also identified by ITS rDNA sequencing. The results of ITS rDNA

sequencing showed that the strain ITB was 100% homology to *Kluyveromyces marxianus* strain. Therefore, the strain ITB3 was identified as *K. marxianus* ITB3 (fig. 8).

Fig. 8. Phylogenetic tree of the strain ITB3

CONCLUSION

From Vietnamese starters isolated a thermotolerant yeast (coded as ITB3) that could grow well at 45°C on YPD agar. The strain ITB3 was identified as *K. marxianus* ITB3 based on the results of ITS rDNA sequencing and yeast mating.

At 30°C, the ITB3 strain produced 4.45% (v/v) ethanol in 14 hours at a yield of 0.38 g/g, or 74.9% of theoretical yield that was slight lower than that of KM (produced 4.61 % (v/v) ethanol, 0.39 g/g, or 77% of theoretical yield).

At 45°C, the ITB3 produced 4.54% (v/v) ethanol at a yield of 0.39 g/g, or 75.6% of theoretical yield that was slight higher than that of KM (4.39% (v/v) ethanol, of 0.37 g/g, or 72.3% of theoretical yield).

These results shown that *K. marxianus* ITB3 can be used for industrial ethanol production at high temperature in order to reduce production cost.

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TUYỂN CHỌN VÀ ĐỊNH DANH CÁC CHỦNG NẤM MEN CHỊU NHIỆT LÊN MEN ETHANOL

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TÓM TẮT

Trong số tám chủng nấm men phân lập được từ men rượu, chỉ có chủng ITB3 sinh trưởng tốt ở 45°C, với mật độ quang học (OD 660 nm) tương đương với chủng *Kluyveromyces marxianus* DMKU3-1042. Ở 30°C, chủng ITB3 lên men 10% (w/v) glucose thành 4,45% (v/v) ethanol sau 14 giờ lên men, với hiệu suất 0,38% g ethanol/g glucose, hoặc 74,9% hiệu suất lý thuyết, thấp hơn không đáng kể so với chủng KM (4,61% (v/v) ethanol, 0,39 g ethanol/g glucose, hoặc 77% hiệu suất lý thuyết). Ở 45°C, chủng ITB3 lên men 10% (w/v) glucose thành 4,54% (v/v) ethanol, với hiệu suất 0,39 g ethanol/g glucose, hay 75,6% hiệu suất lý thuyết, cao hơn một chút so với chủng KM (4,39 % (v/v) ethanol, 0,37 g/g, hoặc 72,3% hiệu suất lý thuyết). Dựa trên kết quả so sánh trình tự rDNA ITS với ngân hàng dữ liệu DNA và lai nấm men, chủng ITB3 được định danh là *Kluyveromyces marxianus* ITB3.

Từ khóa: *Kluyveromyces marxianus*, nấm men chịu nhiệt.

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