High-ethanol-producing yeast *Saccharomyces cerevisiae* KKU 6M4.1 for efficient industrial ethanol production

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**Abstract** *Saccharomyces cerevisiae* KKU 6M4.1 is a high-ethanol-producing yeast isolated from sugarcane juice. The characteristics of KKU 6M4.1 include rapid growth and operation over a wide range of pH from 5-8. KKU 6M4.1 was tested for its potential performance against *Saccharomyces cerevisiae* XP in an ethanol production plant at Khon Kaen Alcohol Co., Ltd with an ethanol production potential for the batch fermentation of molasses of 150,000-160,000 L/d. Cell biomass cultivations were performed in the ethanol production plant in four successive culture vessels and a yeast activation tank. The same quality molasses was used in tests for XP in the production period 1-24 August 2015 and for KKU 6M4.1 during 1-24 November 2015. The molasses had a high microbial contamination (over 1x10⁶cfu/mL) and volatile acids (over 5,000 ppm). XP required about 120 kg sulphuric acid per day for the adjustment of the media pH to 4.2-4.6. However, KKU 6M4.1 did not require sulphuric acid in the factory tests. The production of cell biomass and ethanol were controlled at a cell concentration level of 2x10⁹ cells/mL in the tests. The biomass cultivation time of KKU 6M4.1 was less than XP by about 3 h. KKU 6M4.1 and XP produced highest ethanol concentrations at 36 h of 9.75 ± 0.29 and 9.10 ± 0.38 % (v/v), respectively. KKU 6M4.1 produced 7.14% more ethanol than XP. The yield efficiency for industrial alcohol production from KKU 6M4.1 was higher than XP by about 6.46%. KKU 6M4.1 may overcome the high microbial contamination and volatile acids, obtaining stability in batch fermentation and subsequently to distillation operations and resulting high efficiency for industrial ethanol production.

**Key words** *Saccharomyces cerevisiae*, industrial ethanol production, batch fermentation, molasses

**INTRODUCTION**

Many industrial ethanol production plants in Thailand are based on imported technology, including fermentation technology using industrial yeast strains. The Khon Kaen Alcohol Company used ethanol-production technology from Praj Industries, including using *Saccharomyces cerevisiae* XP for fermentation from molasses. The batch fermentation process in Khon Kaen Alcohol can produce 150,000-160,000 L/d. In addition, the waste water from the ethanol production process is used to produce bio-methane for energy production. The optimum pH for growth of XP is 4.2-4.6, so 120 kg sulfuric acid is required per day to adjust the pH of the media.

Fadel et al. (2013) report that *S. cerevisiae* F-514, an industrial strain for ethanol production from sugarcane molasses, can grow in a medium that is adjusted to pH 4.6 by H₂SO₄. Normally, yeast grows well in the range of pH 4.5-6.5 (Walker 1998; Okolo et al. 2004). However, microbial contaminations are normally found in molasses and some produce volatile acids such as acetic, formic and butyric acid that affect the growth of yeast and fermentation activity at high concentrations of the volatile acids (Olbrich 2006).

*Saccharomyces cerevisiae* KKU 6M4.1, isolated from sugar cane juice in Thailand by Milintawisamai et al. (2008), showed high invertase activity and a high growth rate. It had a sucrose consumption rate of 23 g/L/h in complete medium containing 10% (w/v) sucrose, incubated by shaking 160 rpm at 30°C. In our recent research, KKU 6M4.1 produced ethanol superior in some aspects to other high-ethanol-producing strains, including TISTR 5339 and TISTR 5596 (Thailand Institute of Scientific and Technological Research) (Saelee and Milintawisamai 2015). KKU 6M4.1 can grow over a wide range of pH from 5-8 (data not shown).

Here, we compare the ethanol production potential of KKU 6M4.1 and XP in a production plant of Khon Kaen Alcohol Company.
MATERIALS AND METHODS

Yeast strains

We used *S. cerevisiae* KKU 6M4.1 from Milintawisamai et al. (2008) and *S. cerevisiae* XP from Khon Kaen Alcohol Company.

Media

The media for cell biomass or inoculum production was undertaken in successive culture vessels (CV1 – CV4) that contained heated molasses diluted by water to obtain a specific gravity of 1.045 and supplemented with di-ammonium phosphate (DAP), peptone and yeast extract. The media for cell biomass production in the yeast activation tank was molasses diluted by UV-treated process water and supplemented with DAP. The media for cell biomass production of XP strains was adjusted to pH 4.2-4.5 by 98% H₂SO₄. The media for ethanol fermentation was molasses diluted by UV-treated process water to obtain a specific gravity of 1.045 and supplemented with urea. The molasses used for ethanol production was 50.65-51.54% (w/w) total reducing sugars, 45.64-46.33% (w/w) fermentable sugars, 1.40-1.42 specific gravity, 5,307-5,451 ppm volatile acidity, pH 4.85-4.87, Brix 75.12-78.60, and microbial contamination of higher than 1x10⁸ cfu/mL.

Ethanol production at laboratory scale was performed in a 250 mL flask containing 100 mL molasses media starting with 2.0 × 10⁷ cells/mL for final cell concentration, incubated at 30°C at static conditions for 48 h. The experiments were replicated three times. Ethanol was detected by the head-space technique and gas chromatography with an FID detector.

Industrial ethanol production process

Ethanol fermentation was performed by batch fermentation during 1-24 August 2015 for XP and 1-24 November 2015 for KKU 6M4.1. Cell biomass was produced aerobically at 30-32°C in four culture vessels, CV1 - CV4, (starting from a working volume of 60 L to 72 m³) and activation tanks 305A and 305B (working volume of 630 m³) of cell culture, to obtain at least 1.8 × 10⁸ cells/mL in each culture vessel. The cell culture prepared in the yeast activation tanks of 305A and 305B were transferred and separated to three batch-fermentation tanks in each line A and B (six batches in total). The new molasses media was applied to obtain working volume of 630 m³ for ethanol fermentation. The batch fermentations were controlled by agitation at 30-32°C and lasted 36 h. The fermented media, containing ethanol, was separated from the yeast residue by gravity and successively subjected to distillation. The ethanol production was approximately 150,000-160,000 L/d. All other protocols and analyses followed standard methods of Praj Industries Limited.

Data analysis

We compared the cultivation times of cell biomass production (six batches) and the time course of ethanol production (10 batches) for both KKU 6M4.1 and XP, fermentation efficiency and % yield efficiency of industrial alcohol production (IA) for 20 batches of KKU 6M4.1 and 13 batches of XP. The % yield efficiency of industrial alcohol was calculated from the raw material of molasses, cell biomass production, fermentation and distillation processes. Statistical analysis of treatments was done by SPSS statistics version 17.0. A one-way ANOVA was used for testing the equality of the means, and a multiple comparison using Duncan’s method determined differences between groups at a significance level of 0.05.

RESULTS AND DISCUSSION

At a laboratory scale, both *S. cerevisiae* KKU 6M4.1 and XP produced ethanol, with a highest ethanol concentration at 48 h of 5.40 ± 0.24 and 5.24 ± 0.20 % (v/v), respectively. KKU 6M4.1 produced higher amounts of ethanol than XP by 3.05%, but this was not significantly different at the level of 0.05 (Fig. 1).
Fig. 1. Time course of ethanol production of *Saccharomyces cerevisiae* KKU 6M4.1 and XP in 100 mL of molasses media (specific gravity 1.045) supplemented with 100 ppm DAP in 250 mL flask, incubated at 30°C at static conditions for 48 h.

To determine cell biomass production by KKU 6M4.1 and XP, they were cultured in CV1 - CV4 and an activation tank in which cell concentration should be more than $1.8 \times 10^8$ cfu/mL before being transferred to the fermentation tank. The total cultivation times (CV1 - CV4) of KKU 6M4.1 and XP were 39.9 ± 2.30 and 41.7 ± 1.9 h, respectively, which was not significantly different at the level of 0.05 (Fig. 2). However, KKU 6M4.1 reached $1.8 \times 10^8$ cfu/mL before XP, after about 3 h in the activation tank (305A/B).

Fig. 2. Cultivation times of culture vessels CV1 – CV4 and activation tank (305A/B) for *Saccharomyces cerevisiae* KKU 6M4.1 and XP.

In addition, cell biomass production of XP requires 120 kg of H$_2$SO$_4$ per day for media pH adjustment to 4.2-4.6. Sulphuric acid (H$_2$SO$_4$) may have an additional effect on bio-methane production and hydrogen sulphide (H$_2$S) removal in the gas purification process for bio-energy in Khon Kaen Alcohol Company. However, cultivation of KKU 6M4.1 does not require pH adjustment at all (media pH 4.8-4.9) because of the wide optimum pH of media for growth of KKU 6M4.1 is pH 5-8. The application of KKU 6M4.1 can reduce the use of H$_2$SO$_4$, preserving the equipment used.

For industrial ethanol production, KKU 6M4.1 and XP in 10 batch fermentations produced the highest ethanol at 36 h of 9.75 ± 0.29 and 9.10 ± 0.38 % (v/v), respectively (Fig. 3). KKU 6M4.1 showed 7.14% ethanol production more than XP, which was significantly different at the level of 0.05. Fermentation efficiencies of KKU 6M4.1 and XP were 90.55 ±
1.83% and 90.08 ± 2.93%, respectively. In addition, % yield efficiency of KKU 6M4.1 and XP were 105.94 ± 12.69% and 99.51 ± 7.07%, respectively (Fig. 4). Both fermentation efficiency and % yield efficiency were not significantly different at the level of 0.05. However, the fermentation efficiency and % yield efficiency were calculated from 24 days of ethanol production (approximately 3,600,000 L). So, the % yield difference of KKU 6M4.1 was more than XP by about 6.46%, which may affect the profit from ethanol production.

**Fig. 3.** Time course of ethanol production by *Saccharomyces cerevisiae* KKU 6M4.1 and XP at an industrial scale.

![Graph showing time course of ethanol production](image1)

**Fig. 4.** Fermentation efficiency and % yield efficiency of ethanol production by *Saccharomyces cerevisiae* KKU 6M4.1 and XP.

![Graph showing fermentation efficiency and % yield efficiency](image2)

The important character of KKU 6M4.1 is that it is a high invertase activity strain, with high invertase activity with both intracellular (clued cell extract) and extracellular (culture media) activities of 91 and 1 U/mg, respectively (Milintawisamai et al. 2008). This indicates that it is good for both cell biomass production and ethanol production. Jimenez and Benitez (1986) also reported that yeasts having good invertase activity are also important in converting sucrose to ethanol. This high invertase character of KKU 6M4.1 may support the lower cultivation time (3 h) and the higher ethanol production (7.14%) than XP.

KKU 6M4.1 may also compete with microbial contamination in the fermentation process because of the high acidity of 5,307-5,451 ppm of volatile acid in molasses. Fermented wash quality from KKU 6M4.1 and XP showed high volatile acid of 1,745-1,959 ppm and lactic acid of 3,020-3,599 ppm. In addition, the microbial contamination population was also higher than the standard level of $1 \times 10^8$ cfu/mL. Microbial contamination, especially lactic acid bacteria, can affect the efficiency of the fermentation and lead to obstruction of the fermentation process (Beckner et al. 2011). Organic acid (e.g. acetic acid, lactic acid) can inhibit yeast growth because it can lower intracellular pH following translocation across the yeast plasma membrane (Walker 1998). Microbial contamination, especially lactic acid bacteria which are found in industrial
ethanol fermentation, could inhibit yeast activity and utilization of sucrose. However, the capability of high growth efficiency of KKU 6M4.1 may compete with such microbial contamination.

CONCLUSIONS

Saccharomyces cerevisiae KKU 6M4.1 is a useful strain for industrial ethanol production plant by batch fermentation. The advantage of S. cerevisiae KKU 6M4.1 is that it does not require H₂SO₄ as does XP. The % yield efficiency of industrial alcohol production from KKU 6M4.1 was higher than XP by about 6.46%. KKU 6M4.1 may also overcome the high microbial contamination and volatile acids, obtaining stability in batch fermentation and subsequently to distillation operations and resulting high efficiency for industrial ethanol production.

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REFERENCES


La levadura altamente productora de etanol Saccharomyces cerevisiae KKU 6M4.1 para una produccion industrial eficiente de etanol

RESUMEN. La Saccharomyces cerevisiae KKU 6M4.1 es una levadura altamente productora de etanol aislada del jugo de la caña de azucar. Las caracteristicas de la KKU 6M4.1 incluye rapido crecimiento y operacion sobre un amplio rango de pH de 5-8. La KKU 6M4.1 fue probada por su desempeño potencial comparada contra la Saccharomyces cerevisiae XP en una planta de produccion de etanol en Khon Kaen Alcohol Co., Ltd con una produccion de etanol potencial para la fermentacion de melaza en batch de 150.000-160.000 L/d.
Cultivos de células de biomasa fueron realizadas en la planta de producción de etanol en cuatro tanques sucesivos de cultivo y en un tanque de activación de levadura. La misma calidad de melaza fue utilizada en las pruebas de XP en el periodo de producción del 1 al 24 de agosto 2015 y para la KKKU 6M4.1 durante el 1 al 24 de noviembre 2015. Las melazas tuvieron una alta contaminación microbiana (arriba de $1 \times 10^8$ cfu/mL) y ácidos volátiles (arriba de 5.000 ppm). La XP necesita cerca de 120 kg de ácido sulfurico por día para el ajuste de la media de pH a 4.2-4.6. Sin embargo, la KKKU 6M4.1 no necesitó ácido sulfurico en las pruebas de fabrica. La producción de células de biomasa y etanol fueron controladas a un nivel de concentración de células de $2 \times 10^8$ células/mL en las pruebas. El tiempo de cultivo de la biomasa de la KKKU 6M4.1 fue menos que la xp por cerca de 3 horas. La KKKU 6M4.1 y la XP produjeron concentraciones más altas de etanol a 36 horas de 9.75 ± 0.29 y 9.10 ± 0.38% (v/v), respectivamente. La KKKU 6M4.1 produjo 7.14% más etanol que la XP. La eficiencia en rendimiento para la producción industrial de alcohol a partir de la KKKU 6M4.1 fue mayor que la XP por cerca de 6.46%. La KKKU 6M4.1 puede superar la alta contaminación microbiana y los ácidos volátiles, obteniendo estabilidad en la fermentación batch y después en las operaciones de destilación y resultar de alta eficiencia para la producción industrial de etanol.

**Palabras clave:** Saccharomyces cerevisiae, produccion industrial de etanol, fermentacion batch, melaza