Bioethanol Production from Sugar Cane Syrup by Thermo-tolerant Yeast, *Kluyveromyces marxianus* DMKU3-1042, using Fed-batch and Repeated-batch Fermentation in a Nonsterile System

Podchamarn Pimpakan1, Wichien Yongmanitchai1 and Savitree Limtong1,2,*

ABSTRACT

Ethanol production from sugar cane syrup was carried out to compare the efficiency of batch-, fed-batch and repeated-batch fermentation using *Kluyveromyces marxianus* DMKU3-1042. The sugar cane syrup medium contained 18% total sugar, 0.1% (NH4)2SO4, 0.1% KH2PO4, and 0.1% MgSO4.7H2O, with the pH adjusted to 4.5. Experiments were carried out in a 2.5 L jar fermenter at a controlled temperature of 35 °C and an agitation speed of 300 rpm without aeration. All experiments were carried out in a nonsterile system that resembled the process used in the bioethanol fermentation industry in Thailand. The ethanol concentration from batch fermentation was lowest among the three processes where 6.35% w/v was achieved in 72 hr resulting in an ethanol productivity of 0.88 g.L⁻¹.hr⁻¹ and the ethanol yield corresponded to 82.73% of the theoretical yield. For fed-batch fermentation, the exponential feeding scheme barely improved the ethanol yields while sigmoidal feeding gave a considerably higher final ethanol concentration reaching 7.42% w/v which was equivalent to ethanol productivity at 1.03 g.L⁻¹.hr⁻¹ and 90.40% of theoretical yield. In the repeated-batch experiment, a rather high ethanol concentration of 7.91% w/v at 72 hr was obtained in the first batch with ethanol productivity of 1.10 g.L⁻¹.hr⁻¹ and 93.40% of the theoretical yield. However, the final ethanol contents of the two successive batches were relatively low at 6.01% w/v and 6.66% w/v, respectively. The results from this research demonstrated that in Thailand, sugar cane syrup could be employed as an alternative renewable carbon source for ethanol production using sigmoidal fed-batch fermentation with an efficient thermo-tolerant yeast strain (*Kluyveromyces marxianus* DMKU3-1042).

Keywords: ethanol production, sugar cane syrup, thermo-tolerant yeast, *Kluyveromyces marxianus* DMKU3-1042, fed-batch fermentation, repeated-batch fermentation, nonsterile

INTRODUCTION

Currently, the depletion of fossil fuel reserves has caused an increase in petroleum costs. A major policy in Thailand involves the encouragement of bioethanol use as an alternative energy source for automobiles. Although cane molasses is the preferable raw material, its supply is becoming limited due to its use in many fermentation industries in the country. Hence in recent years, the price of molasses has inevitably increased (Department of Alternative Energy...
Development and Efficiency, 2012). In addition, it has been reported that molasses might contain some inhibitors, such as hydroxymethylfurfural, hexanol and heptanol, which interfere with yeast metabolisms (Glacet et al., 1985; Fattohi, 1994). Therefore, the juice from sugar cane (Saccharum officinarum), a high biomass tropical crop, seems to be an attractive renewable carbon source for the ethanol fermentation industry (Limtong et al., 2007). However, due to its relatively low sugar content at approx. 12–17% (Wheals et al., 1999), it is susceptible to microbial spoilage during storage. To resolve this problem, the juice needs to be concentrated to increase the sugar concentration which consequently reduces the water activity and restricts bacterial growth. The sugarcane syrup is then available all year round as a raw material for the ethanol industry.

The thermo-tolerant yeast Kluyveromyces marxianus DMKU3-1042 has been reported to be a candidate strain to replace the popular Saccharomyces cerevisiae that is widely employed in the ethanol fermentation industry in Thailand (Limtong et al., 2007). The former yeast strain has the advantage of tolerating an elevated fermentation temperature which is a very common phenomenon in a tropical country such as Thailand, especially in the summer. Abdel-Banat et al. (2010) reported that K. marxianus DMKU3-1042 was found to be the most suitable strain for high temperature growth and ethanol production at 45 °C when compared with the Brazilian S. cerevisiae strain. In addition, its ability to use a wide range of carbon sources make this species attractive for industrial applications (Fonseca et al., 2008).

Ethanol fermentation can be carried out in batch, fed-batch or continuous mode. The choice depends on the advancements made by the fermentation industry in each country. In Thailand, the batch and fed-batch processes are more common. The development of an efficient ethanol fermentation process is critical due to the relatively low price of bioethanol (Bai et al., 2004). Typically, fermentation processes are often conducted in the batch mode. However, the batch process has many disadvantages, particularly when the microorganisms are either slow growing or strongly affected by product inhibition (Najafpour et al., 2004). The fed-batch culture is quite advantageous because the inhibition, generally caused by high concentrations of substrate and product in the medium on the metabolic cell performance, is reduced or eliminated at best. In addition, the ethanol fermentation process is normally conducted under nonsterile conditions to reduce the production cost. An increase in the number of yeast cells during the start up of the fed-batch system will enhance the competitiveness of the yeast against bacterial contamination. Several authors attained an increase in ethanol productivity between 10 and 14% by adding sucrose according to the yeast’s linear or exponential growth mode (Echegaray et al., 2000; Laopaiboon et al., 2007). Repeated-batch fermentation involves withdrawing a portion of the fermentation broth at time intervals and the residual part of the broth is used as an inoculum for the next batch. This process aims to increase the productivity, and it is interesting because it has several advantages compared to the conventional batch fermentation such as no new inoculum requirement for each batch and long term productivity. Other advantages of this operation are that there is no nonproductive, idle time required for cleaning and re-sterilization, and not much control is required compared to a continuous mode (Laopaiboon et al., 2007). Moreover, cell adaptation to very high osmotic pressure (for example, high substrate or product concentration) may take place during the repeated-batch fermentation (Anastassiadis and Rehm, 2006).

The objectives of the present study were to quantify the production of ethanol from sugar cane syrup under fed-batch and repeated-batch fermentation in a nonsterile system using the thermo-tolerant yeast, K. marxianus DMKU3-1042.
MATERIALS AND METHODS

Yeast strain

The thermo-tolerant yeast strain, *Kluyveromyces marxianus* DMKU3-1042, isolated by Limtong *et al.* (2007) and maintained at the Department of Microbiology, Kasetsart University was used in this study. The culture was stored on yeast extract-malt extract (YM) agar slant containing (per liter) 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g glucose and 1.5 g agar and was kept at 4°C and renewed every month.

Sugarcane syrup

Sugarcane syrup was generously supplied by the Sahakarn Sugar Mill, Chonburi. It consisted of 57.4% total sugars (53.6% sucrose, 2.3% glucose and 1.5% fructose) as determined by high performance liquid chromatography (HPLC). The syrup was light brown and contained moderate suspended solids—mostly sugarcane debris.

Medium and inoculum preparation

The yeast strain was streaked on YM plates and incubated at room temperature for 48 hr. Then the yeast cells were transferred into a 250 mL flask containing 50 mL yeast-peptone-glucose (YPD) medium (1% yeast extract, 2% peptone and 2% glucose). They were then incubated at ambient temperature on a rotary shaker at 150 rpm for 24 hr. After incubation, this *K. marxianus* culture was used as the inoculum for all experiments.

Experimental system

All fermentations were carried out in a 2.5 L jar fermenter (Marubishi; Tokyo, Japan) with a 1.5 L working volume using 5% inoculum size, an agitation speed of 300 rpm, without aeration and a controlled temperature at 35°C.

1. Batch fermentation

The sugar cane syrup medium was prepared by diluting the syrup to attain 18% total sugar and supplemented with 0.1% (NH$_4$)$_2$SO$_4$, 0.1% KH$_2$PO$_4$, 0.1% MgSO$_4$7H$_2$O and the initial pH was adjusted to 4.5, as predetermined in the shaking flask experiments. During the course of 72 hr fermentation, samples were taken at 6 hr intervals.

2. Fed-batch fermentation

Two feeding schemes—namely, exponential feeding and sigmoidal feeding (Lee and Kim, 2001)—were employed. Starting fermentation volumes were prepared depending on the calculation of each feeding protocol. The initial sugar concentration was adjusted to 4% which was predetermined to minimize the effect of a high concentration of substrate on yeast growth. The total sugar used was based on 18%, similar to batch fermentation. The initial substrate volume was 600 mL. The feeding volume was calculated using Equation 1:

$$V_f = \frac{e^{\mu_{max}}}{\sum e^{\mu_{max}}} \cdot V_a$$

where $V_f$ is the feeding volume (mL), $\mu_{max}$ is the predetermined maximum specific growth rate (0.296 hr$^{-1}$) and $V_a$ is substrate accumulated volume at feeding time (mL). A preliminary study in 500 mL flasks revealed that an exponential fed-batch with three incremental feedings improved the final ethanol content compared to batch fermentation (data not shown). Therefore, the experiment in the 2.5 L jar fermenter was repeated. The calculated feeding volumes at 6, 12 and 18 hr after inoculation are shown in Table 1.

For the sigmoidal feeding scheme, the feeding volume was calculated using Equation 2:

$$V_f = \frac{\mu_{max}}{Y_{x/s}} \cdot X V_a \cdot e^{\mu t}$$

where $V_f$ is the feeding volume (mL), $\mu_{max}$ is the predetermined maximum specific growth rate (0.296 hr$^{-1}$), $Y_{x/s}$ is the cell yield coefficient.
(0.494 g cell dry weight/g substrate), X is cell dry weight at feeding time (g·L⁻¹), \( V_a \) is substrate accumulated volume at feeding time (mL) and \( \mu_t \) is specific growth rate per hour at feeding time. Similarly, an experiment at flask level indicated that a sigmoidal fed-batch fermentation with two incremental feedings produced the best ethanol yield. The initial substrate volume was 1,100 mL. The calculated feeding volumes are shown in Table 2.

3. Repeated-batch fermentation

Repeated-batch fermentation was performed for three consecutive batch cycles. The first batch fermentation was executed under the same conditions as batch fermentation for 72 hr. At the end of fermentation, 80% of the broth (1,200 mL) was withdrawn and the same amount of fresh medium containing 18% total sugar was fed in. For the second and third batches, the fermentation time was shortened to 48 hr.

Analysis of fermentation parameters

Yeast growth was evaluated by measuring the optical density at 600 nm with a spectrophotometer (Spectrophotometer 258; Corning; New York, USA) after washing twice and resuspending with 0.85% NaCl. A direct viable cell count was carried out using a hemacytometer after staining with 0.1% methylene blue (Borzani and Vario, 1958).

The ethanol concentration in the medium was determined by a gas chromatograph (GC-9A; Shimadzu; Kyoto, Japan) equipped with a flame ionization detector using a glass column packed with PEG-20M (Shimadzu; Kyoto, Japan).

The total sugar concentration (sucrose, glucose and fructose) in the fermentation broth was analyzed by HPLC (Agilent 1100; Agilent Technologies; Palo Alto, USA) with a refractive index detector and a sugar column (ULTRON PS-80; Shinwa Chemical Industries; Kyoto, Japan). The sugar content in the medium was calculated as the total fermentable hexose sugars by summing the concentrations of glucose and fructose from sucrose hydrolysis and the residual glucose and fructose in the syrup.

RESULTS AND DISCUSSION

Batch fermentation

The maximum ethanol concentration of 6.35% w/v was achieved in 72 hr resulting in an ethanol productivity of 0.88 g·L⁻¹·hr⁻¹ and an ethanol yield corresponding to 82.73% of the theoretical yield during fermentation in the

### Table 1
Feeding volume (\( V_f \)) and substrate accumulated volume at feeding time (\( V_a \)) at 6, 12 and 18 hr after inoculation.

<table>
<thead>
<tr>
<th>Feeding time (hr)</th>
<th>( V_a ) (mL)</th>
<th>( V_f ) (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>600</td>
<td>220</td>
</tr>
<tr>
<td>12</td>
<td>820</td>
<td>300</td>
</tr>
<tr>
<td>18</td>
<td>1,120</td>
<td>420</td>
</tr>
<tr>
<td>Final volume</td>
<td>1,540</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2
Calculated feeding volume (\( V_f \)) and substrate accumulated volume at feeding time (\( V_a \)) at 6, 12 and 18 hr after inoculation.

<table>
<thead>
<tr>
<th>Feeding time (hr)</th>
<th>( V_a ) (mL)</th>
<th>( V_f ) (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1,100</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>1,195</td>
<td>306</td>
</tr>
<tr>
<td>Final volume</td>
<td>1,501</td>
<td></td>
</tr>
</tbody>
</table>
2.5 L batch fermentation (Figure 1). During fermentation, the yeast growth and ethanol concentration increased gradually until the end of fermentation. Sucrose was rapidly hydrolyzed to glucose and fructose from the beginning by the inulinase present in *K. marxianus* DMKU3-1042 (Lertwattanasakul *et al.*, 2011) leading to an increase in the concentrations of glucose and fructose. Utilization of sucrose was exhausted within 24 hr. Glucose was completely and continually utilized until the end of fermentation, while a small amount of fructose (2.45%) remained in the medium. The results indicated that the organism can use sucrose as a carbon source and glucose repression was not observed. Dhaliwal *et al.* (2011) studied the production of ethanol from sugar cane juice by galactose adaptation with *Pichia kudriavzevii*. They found that sucrose was completely utilized within 9 hr and only a limited amount of fructose was available after 12 hr. Lertwattanasakul *et al.* (2011) reported that inulinase was involved in the hydrolysis of sucrose, raffinose and inulin in *K. marxianus* DMKU3-1042 and the effect of glucose repression was observed on the utilization capability of sucrose in *S. cerevisiae* but not in *K. marxianus*. The strain DMKU3-1042 produced the highest biomass of 4.03 g.L\(^{-1}\) at 54 hr and this decreased slightly toward the end of fermentation. It was possible that the high ethanol concentration led to the loss of yeast cells resulting in autolysis. Zafar and Owais (2006) reported that the growth rate of *K. marxianus* decreased due to the accumulation of ethanol in the broth leading to product inhibition. Diminishing amounts of nutrients coupled with unfavorable culture conditions such as low pH might also have contributed to the reduction in cell biomass (Pramanik, 2003).

**Fed-batch fermentation**

Sugar consumption, ethanol accumulation and biomass production during exponential

![Figure 1](image-url)  
**Figure 1** Time course of ethanol concentration (percent weight by volume; %w/v), sugar concentration (percent weight by volume; %w/v) and cell dry weight (CDW) from sugarcane syrup in batch fermentation in 2.5 L jar fermenter using thermo-tolerant yeast, *K. marxianus* DMKU3-1042, from nonsterile medium containing 18% total sugar, 0.1% \((\text{NH}_4)_2\text{SO}_4\), 0.1% KH\(_2\)PO\(_4\), 0.1% MgSO\(_4\).7H\(_2\)O, adjusted to an initial pH of 4.5 and controlled temperature at 35 C.
feeding fed-batch fermentation of *K. marxianus* DMKU3-1042 are shown in Figure 2. The initial sugar concentration in the medium at 4% was rapidly consumed and approached zero (only 0.39% of fructose remained) after 6 hr. The first feed of 220 mL was then added followed by 300 and 420 mL in the second (at 12 hr) and third feeds (at 18 hr), respectively. The maximum ethanol attained in this experiment was 6.59% w/v after 72 hr of fermentation which was equivalent to ethanol productivity at 0.92 g.L⁻¹.hr⁻¹ and 92.8% of the theoretical yield. It appeared that the exponential fed-batch process slightly improved the final ethanol concentration, although low yeast growth (3.3 g.L⁻¹ of cell dry weight) was observed after completing the feeding scheme. Consequently, a relatively high level of sugar (4.83 g.L⁻¹) remained in the medium at the end of fermentation. This was probably due to the late supply of sugar in the first feed (0.39% of fructose at 6 hr), suppressed continuation of yeast growth and weakening in the activity of the culture.

In the sigmoidal fed-batch fermentation, maximum ethanol concentration was reached (7.42% w/v) after 72 hr with a productivity of 1.03 g.L⁻¹.hr⁻¹ and 90.4% of the theoretical yield (Figure 3). All ethanol production parameters were considerably improved compared to batch fermentation. *K. marxianus* DMKU3-1042 grew rapidly in this fed-batch fermentation, especially in the first 18 hr. The biomass of 4.41 g.L⁻¹ was achieved at 12 hr which was the highest among the three techniques employed. It should be noted that the sugar content of the medium at 6 and 12 hr was presumably at appropriate levels of 2.93 and 2.46%, respectively. Hence, the yeast could maintain its growth throughout the fermentation course. In batch fermentation, similar growth was attained but it occurred at 54 hr. It is possible that the high sugar concentration at the beginning of fermentation might retard yeast growth. Ozmihci and Kargi (2007) reported that the ethanol and biomass yield decreased when the feed sugar content was 200 g.L⁻¹ because of the high osmotic

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**Figure 2**  Time course of ethanol concentration (percent weight by volume; %w/v), sugar concentration and cell dry weight (CDW) from sugarcane syrup in exponential feeding fed-batch fermentation in 2.5 L jar fermenter using the thermo-tolerant yeast, *K. marxianus* DMKU3-1042, from nonsterile medium containing 18% total sugar, 0.1% (NH₄)₂SO₄, 0.1% K₂HPO₄, 0.1% MgSO₄·7H₂O, adjusted to an initial pH of 4.5 and controlled temperature at 35 °C.
pressure and maintenance requirements at a high sugar concentration. Similar results were also observed by Pramanik (2003). Cell growth slightly decreased after the addition of sugar at 12 hr until it was relatively stable from 42 hr till the end of fermentation. Similarly, most of the sugar remaining in the fermented broth was fructose which indicated *K. marxianus* DMKU3-1042 showed a higher affinity for glucose than fructose. Najafpour *et al.* (2004) reported that the batch process has many disadvantages, particularly when the microorganisms are either slow growing or strongly affected by product inhibition. In contrast, the advantages of the fed-batch process are the reduction of substrate inhibition, higher productivity, higher dissolved oxygen in the medium, decreased fermentation time and reduced toxic effects of the medium components, which are present at high concentrations (Stanbury and Whitaker, 1984).

**Repeated-batch fermentation**

The fermentation time course of repeated-batch fermentation by *K. marxianus* DMKU3-1042 is shown in Figure 4. The kinetics in the first batch of the three parameters—namely, cell mass, sugar utilization and ethanol formation—resembled those obtained in the batch process (Figure 1). Surprisingly, the final ethanol concentration was noticeably higher at 7.91% w/v after 72 hr with productivity of 1.10 g.L⁻¹.hr⁻¹ and 89.6% of the theoretical yield. The sugar concentration was almost completely utilized in the first batch (1.43% of residual sugar at 72 hr), in particular, the sucrose concentration which had disappeared by 18 hr. The first batch started with only 0.21 g.L⁻¹ of yeast biomass, while the second and third batches had substantially higher cell dry weights of 0.88 and 1.10 g.L⁻¹, respectively. However, determination of yeast viability by methylene blue staining and observation under a light microscope revealed that the initial viable cells of the three batches were similar and in the range of $1.35 \times 10^7$ to $5.00 \times$

![Figure 3](image)

**Figure 3** Time course of ethanol concentration (percent weight by volume; %w/v), sugar concentration and cell dry weight (CDW) from sugarcane syrup in sigmoidal fed-batch fermentation in 2.5 L jar fermenter using the thermo-tolerant yeast, *K. marxianus* DMKU3-1042, from nonsterile medium containing 18% total sugar, 0.1% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.1% MgSO₄.7H₂O adjusted to an initial pH of 4.5 and controlled temperature at 35 °C.
10^7 cells.mL^-1 which implied that most of the yeast cells in the later batches were nonviable. It was plausible that yeast cells in the second and third batches might not have been very active due to the effect of the high ethanol concentration at the completion of the first batch. This phenomenon resulted in low ethanol contents of 6.01% w/v and 6.66% w/v in the fermented broth of the two later batches, respectively. Hence, the amounts of residual sugars at the end of fermentation (48 hr) were considerably high at 4.92% and 3.85%, respectively. Similar results were observed by Laopaiboon et al. (2007) who reported that the lower initial cell concentration could directly affect the ethanol productivity. Alfenore et al. (2002) also mentioned that an increase in vitamins and exponential feeding of the vitamins slowed down the decline in cell viability at high ethanol concentrations. Anastassiadis and Rehm (2006) reported that repeated-batch fermentation aimed to increase the productivity and it had several advantages compared to the conventional batch fermentation such as no new inoculum requirement for each batch, long-term productivity and cell adaptation to osmotic pressure (for example, high substrate or product concentration) might take place during the repeated-batch fermentation.

CONCLUSION

The successful operation of ethanol fermentation depends on the viability of the yeast strains used with regard to a number of stress factors occurring during the process. Thus, the selection process is important for efficient ethanol fermentation. In addition to ethanol production from cane molasses by K. marxianus DMKU3-1042 (Limtong et al., 2007), the results from this research demonstrated that sugar cane syrup could be employed as an alternative renewable carbon source for ethanol production using this efficient thermo-tolerant yeast strain as well. K. marxianus can use sucrose as a carbon source as the inulinase (an enzyme present in the yeast

![Figure 4](image-url) Figure 4 Time course of ethanol concentration (percent weight by volume; %w/v), sugar concentration and cell dry weight (CDW) from sugarcane syrup in three consecutive repeated-batch fermentation in a 2.5 L jar fermenter using the thermo-tolerant yeast, K. marxianus DMKU3-1042, from nonsterile medium containing 18% total sugar, 0.1% (NH_4)_2SO_4, 0.1% KH_2PO_4, 0.1% MgSO_4.7H_2O, adjusted to an initial pH of 4.5 and controlled temperature at 35 °C.
K. marxianus DMKU3-1042 produced the highest ethanol concentration of 7.42% w/v in sigmoidal fed-batch fermentation with ethanol productivity of 1.03 gL⁻¹.hr⁻¹ and 90.40% of the theoretical yield, while the batch fermentation gave a lower biomass, ethanol concentration and ethanol productivity. However, repeated-batch fermentation also gave a high ethanol concentration and ethanol productivity in the first batch after which decreases in the ethanol concentration, ethanol productivity and biomass were observed in the second and third batches which were probably due to reduced activity by the yeast cells. It should be also emphasized that all experiments were carried out in a nonsterile system to resemble the process used in the bioethanol fermentation industry in the country.

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LITERATURE CITED


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