Chiang Mai J. Sci. 2016; 43(1) : 32-44
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Very High Gravity Ethanol Fermentation by the Newly Isolated Osmotolerant *Saccharomyces cerevisiae* Isolate G2-3-2

Patcharaporn Hoondee [a], Vasana Tolieng [b], Somboon Tanasupawat [c], Vichien Kitpreechavanich [d] , Ancharida Akaracharanya [a]*

[a] Department of Microbiology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, Thailand.

[b] Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, Thailand.

[c] Department of Microbiology and Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, Thailand.

[d] Department of Microbiology, Faculty of Science, Kasetsart University, 50 Paholyothin Road, Bangkok 10900, Thailand.

*Author for correspondence; e-mail: sanchari@chula.ac.th

Received: 27 May 2014
Accepted: 4 August 2014

ABSTRACT

The high yield ethanol fermenting osmotolerant yeast G2-3-2 was obtained after screening 147 ethanol fermenting yeasts that had been isolated from six sugar factories in Thailand. It was found that high capability of osmotic tolerance did not indicate high fermentation efficiency under that high osmotic condition. The yeast G2-3-2 was identified as *Saccharomyces cerevisiae* according to its colony and cell morphology, biochemistry, and sequence analysis of the variable D1/D2 region of the large subunit of the rRNA gene. Optimal conditions for ethanol production under a very high gravity (VHG) condition were: (i) inoculum grown in 150 g/L glucose containing medium; (ii) inoculation of late log phase cells to a final concentration of 1×10^9 cells/mL; (iii) key nutrient concentrations of (all (g/L)) glucose, 280; polypeptone, 5; yeast extract, 7.5; $(NH_4)_2HPO_4$, 1; $MgSO_4.7H_2O$, 1.5; KH_2PO_4 3; and (iv) incubation at 30 °C, pH 5.0 under oxygen limitation for 120 h. Under these conditions the maximum ethanol produced obtained was 134.7 g/L (0.48 g ethanol/g glucose utilized) and the ethanol productivity was 1.12 g/L/h. Removal of carbon dioxide from, and the relief of oxygen-stress to, the optimized VHG fermentation decreased the maximum ethanol produced to 125.1 g/L (0.45 g/g glucose) and 122.5 g/L (0.44 g/g glucose), respectively, but increased the maximum ethanol productivity to 1.73 and 1.70 g/L/h, respectively.

Keywords: Ethanol, osmotolerant yeast, very high gravity, *Saccharomyces cerevisiae*, carbon dioxide stress

1. INTRODUCTION

Very high gravity (VHG) ethanol fermentation is one of the improvement technologies used to increase the concentration of ethanol produced in the ferment [1] through the fermentation of mash containing a very high concentration of fermentable sugar (at least 27 g dissolved solids per 100 g mash) [2]. Since two molecules each of ethanol and carbon dioxide (CO_2) are converted from one molecule of glucose (Eq. (1)), then this means that theoretically 1 g of glucose gives 0.51 g of ethanol.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$
$$180\ g \qquad (2 \times 46)g\ (2 \times 44)g \qquad \text{Eq.(1)}$$

However, the actual ethanol yield obtained is less than the theoretical yield due to the fact that some of the glucose is assimilated for alternative microbial metabolisms, including cell growth [3]. The use of VHG ethanol fermentation has several advantages, including the reduction of subsequent distillation costs, a lower process water requirement, and a lower contamination risk [4]. However, successful (efficient) fermentation under VHG conditions is limited by the tolerance of yeast cells to both the high osmotic pressure at the beginning of the fermentation process and to the high ethanol concentration at the end of the fermentation. High osmotic pressures and ethanol concentrations both have detrimental effects on the yeast viability and growth and on the ethanol production [1]. Therefore, a high ethanol producing yeast strain that is both osmotolerant and ethanol tolerant is required for efficient VHG fermentation [5]. Several investigations have observed that the inclusion of suitable levels of Mg^{2+}, yeast extract, peptone, and potassium phosphate buffers in the ferment medium can relieve to some extent the detrimental effects to the yeast cells of the VHG fermentation conditions [6-8]. In contrast, an excessive ammonium level has a negative effect on the ethanol production level due to the increased production level of higher alcohols, acetic acid or hydrogen sulfide [7]. Not only does the high osmotic pressure and ethanol concentration under VHG fermentation conditions stress the yeast cells, but the cells themselves also cause other environmental stresses, such as inducing an oxygen deficiency, CO_2 build up, oxidative stress, and a low pH in the ferment, which all have an adverse affect on the ethanol production performance of yeast [8]. Note that although ethanol fermentation is anaerobic, yeast requires a small amount of oxygen during the fermentation process to synthesize ergosterol and unsaturated fatty acids for the maintenance of its membrane integrity [9]. In this work, an osmotolerant yeast isolate that is potentially suitable for VHG ethanol fermentation was selected following isolation of 147 yeast colonies from industrial sources. The fermentation medium was then optimized for this isolate and the effect of CO_2 stress and oxygen deficiency on the ethanol yield attained under the optimized VHG condition was compared.

2. MATERIALS AND METHODS

2.1 Isolation and Screening for Ethanol Fermenting Yeast Isolates

Yeasts were isolated from the sugarcane juices and process-sediments collected from the following six sugar factories in Thailand: Rajburi sugar Co., Ltd.; Thai Multi Sugar Industry Co., Ltd.; Thai Sugar Industry Co., Ltd.; Khonburi Sugar Pub. Co., Ltd.; E-san Sugar Industry Co., Ltd.; and Buriram Sugar Factory Co., Ltd. One g (sediment) or 1 mL (juice) of each sample was inoculated into 5 mL of selective medium (SM; all (g/L): glucose, 20; peptone, 3; yeast extract, 3; chloramphenicol, 0.1; plus 30 mL/L ethanol;

pH 5.6) and incubated at 30 °C, under an oxygen limited condition (the cotton plug of the test tube was tightly sealed with parafilm) for 72 h. The obtained cultures were purified by the streak plate method using isolation medium (IM; all (g/L): glucose, 100; peptone, 3; yeast extract, 3; chloramphenicol, 0.1; agar, 20; pH 5.6) and incubated at 30 °C for 48 h in an oxygen limited condition using the candle jar method. The resultant pure cultures were cultivated on a yeast-peptone-dextrose (YPD) slant (all (g/L): glucose, 100; yeast extract, 10; peptone, 20; agar, 20; pH 5.6) at 30 °C for 48 h and then stored at 4 °C.

The isolated yeasts were further screened for their ethanol fermenting capability by inoculating a single colony, selected from those grown on the IM at 30 °C for 48 h, into 50 mL of fermentation medium (FM; all (g/L): glucose, 150; yeast extract, 6; polypeptone, 9; pH 5.0) and incubating at 30 °C, 200 rpm for 24 h. The cultures were then transferred at 0.5 mL to 50 mL of fresh FM and incubated under the same conditions. After 24 h, they were transferred at 4.25 mL to 42.5 mL of fresh FM in a 50-mL Erlenmeyer flask and incubated at 30 °C with oxygen limitation for 72 h. The cultures were then harvested by centrifugation at 4 °C, 20,440 × g, for 5 min, and the resultant supernatants analyzed for the ethanol concentration. The *S. cerevisiae* TISTR 5596 obtained from the Thailand Institute of Scientific Technological Research was used as the reference control.

2.2 Screening for Osmotolerant Yeast Isolates for Ethanol Production

Osmotolerant yeasts were screened from the selected ethanol fermenting yeasts based on their ability to grow in YPD broth supplemented with glucose to 240 g/L after incubation at 30 °C with shaking at 150 rpm for 24 h. Growth was determined from the cell turbidity of the inoculated medium by measuring the optical density at 660 nm (OD_{660nm}). Cultures which attained a higher OD_{660nm} than the control (*S. cerevisiae* TISTR 5596), were selected for further evaluation of their ability to grow in YPD broth containing 280 g/L glucose.

Isolates which were able to grow in YPD broth containing 280 g/L glucose were then further tested for their ethanol fermenting efficiency under VHG condition at 30 °C for 72 h. Modified ethanol production medium (EPM; all (g/L): glucose, 280; yeast extract, 3; polypeptone, 6; $MgSO_4.7H_2O$, 0.025; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1; $(NH_4)_2HPO_4$, 1; $MnSO_4.4H_2O$, 0.5; $ZnSO_4.7H_2O$, 0.02; pH 5.0) [10] was used for both ethanol production and inoculum preparation, performed as described above. The culture supernatants, obtained after centrifugation of the resultant cultures, were analyzed for ethanol and residual glucose levels.

2.3 Selection of The Glucose Concentration in Medium for Inoculum Preparation

A single colony of the selected ethanol fermenting osmotolerant yeasts was inoculated into yeast extract malt extract (YM) broth (all (g/L): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; pH 5.0) except that the glucose concentration was supplemented to 150, 240, or 280 g/L final concentration, and incubated at 30 °C, 200 rpm, for 24 h. The cultures were transferred to 50 mL of fresh YM broth at an initial $OD_{660\ nm}$ of 0.05 and incubated as above. The $OD_{660\ nm}$ was monitored every 30 minutes and the cell viability was determined every 3 h by the direct counting method using a haemacytometer and staining with methylene blue [11]. The YM broth with the glucose concentration that supported the maximum increase in yeast cell number and biomass yield was selected as the

inoculum preparation medium for all further experiments.

2.4 VHG Ethanol Fermentation Inoculum Preparation

A single colony of *S. cerevisiae* G2-3-2 was inoculated into 50 mL of YEM broth containing 150 g/L glucose and incubated at 30 °C, 200 rpm for 24 h. The culture was transferred to 50 mL of fresh YEM broth at an initial seeding $OD_{660\ nm}$ of 0.05 and incubated as above until it reached late log phase (15 h). The late log phase cells were then harvested by centrifugation at 4 °C, 9, 820 × g, for 15 min, and used as the inoculum.

Optimization of The Conditions for Ethanol Production under VHG Condition

The inoculum was added at a final cell concentration of 1×10^8 cells/mL in 46.75 mL of EPM in a 50-mL Erlenmeyer flask and incubated at 30 °C under oxygen limitation for 72 h. After centrifugation at 4 °C, 20,440 × g, for 5 min, the resultant supernatant was analyzed for ethanol and for residual glucose levels. Optimization of the culture conditions was performed by a sequential univariate approach, varying (in order) the concentration of the polypeptone, yeast extract, $MgSO_4$, and KH_2PO_4 concentrations in the EPM formulation, followed by the inoculum size and fermentation period. Initial conditions were based upon the basic EPM as above and then modified by sequentially incorporating the most optimal found condition in terms of the highest ethanol yield and production.

2.5 VHG Ethanol Fermentation under Oxygen or CO_2 Stress-relief Condition

Oxygen deficiency and CO_2 stress under the VHG fermentation condition were separately relieved by growth in a slow shaking flask at 100 rpm with an air space (35 mL medium in a 50-mL Erlenmeyer flask without a parafilm seal) and fitting the flask with perforated rubber stopper with a copper (II) sulfate-lock (to permit CO_2 release while avoiding the entrance of air), respectively. Late log phase cells of *S. cerevisiae* isolate G2-3-2 were inoculated at a final cell concentration of 1×10^9 cells/mL into the optimized EPM containing 280 g/L glucose (35 mL in a 50-mL Erlenmeyer flask) and incubated at 30 °C under the slow shaking or the CO_2-entrapped condition.

2.6 Identification of The Selected Ethanol Fermenting Osmotolerant Yeast

Selected ethanol fermenting osmotolerant yeast were identified and compared with the standard description of type strains [12] by molecular characterization based on a comparative analysis of the variable D1/D2 domain of the large-subunit (LSU) rDNA sequence. The phylogenetic tree was constructed from the evolutionary distance using the neighbour-joining (NJ) method [13], performed with the MEGA 5.05 software [14] and Kimura's two-parameter model [15], whilst 1,000 random re-sampling Bootstrap analysis was performed [16].

2.7 Analytical Procedures

Ethanol Analysis

Ethanol was quantified as previously reported [17] by gas chromatography (Hewlett-Packard, HP5890 series, USA) with a flame ionization detector at 150 °C using a Porapak QS (cabowax 20 M) column (2 m × 0.32 m) at an oven temperature of 175 °C. Helium at a flow rate of 35 mL/min was used as the carrier gas. Ethanol yield and ethanol productivity were a calculation of an ethanol produced (g) per g of glucose utilized, and an ethanol concentration obtained within

1 h, respectively.

Glucose Analysis

Glucose was analyzed by the Somogyi-Nelson method [18] using glucose as the standard sugar. The concentration of glucose was determined by reference to the standard curve. The amount of glucose that had been utilized was calculated as the difference between the initial and residual glucose levels in the culture broth.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening for Ethanol Fermenting Yeast

A total of 147 yeast colonies were isolated from 83 samples (55 isolates from 33 samples of sugarcane juice and 92 isolates from 50 samples of process-sediment) collected from six sugar factories by culturing on SM supplemented with ethanol (30 mL/L). All of the isolated yeasts could ferment glucose to ethanol, but of these 23 isolates produced a higher ethanol yield than the reference control strain, *S. cerevisiae* TISTR 5596 (data not shown) and so were selected for further study. The ethanol fermentation capability of yeasts has previously been shown to be related to their ethanol tolerance [19]. For example, thermotolerant ethanol fermenting yeasts have previously been isolated using medium containing 40 mL/L ethanol and incubating at 40 or 45 °C [20], Similarly, YPD medium containing 50 mL/L ethanol was used to isolate ethanol fermenting yeasts [19].

3.2 Screening for High Ethanol Fermenting Osmotolerant Yeasts

The 23 selected high level ethanol fermenting yeasts were then grown in YPD broth containing 240 g/L glucose and the cell turbidity ($OD_{660\ nm}$) of the cultures were measured. Twelve isolates produced a cell turbidity that was greater than that of the reference *S. cerevisiae* TISTR 5596 strain (data not shown) and so were further tested for their ability to grow in YPD medium containing 280 g/L glucose. After incubation, it was found that all 12 isolates could still grow well in the YPD medium containing 280 g/L glucose (data not shown) and so these 12 yeast isolates were categorized as osmotolerant yeasts.

These 12 osmotolerant yeast strains were then further tested for their ethanol fermentation efficiency in EPM containing 280 g/L glucose. The same EPM was used for inoculum preparation and the obtained inoculum was inoculated at 100 mL/L. Fermentation cultures were incubated at 30 °C under an oxygen limited condition for 72 h. Although all 12 isolates could utilize glucose and produce ethanol under these conditions (Table1), they varied in their ethanol production efficiency from 37.0 to 73.2 g/L. With respect to the top three ethanol producing strains, isolate G2-4-1 produced the highest ethanol (73.2 g/L) and the highest utilized glucose level (67.0 ± 0.7%), whilst isolates G1-5-1 and G2-3-2 produced slightly less ethanol (73.0 and 71.9 g/L) and utilized 67.0 ± 0.8 and 66.7 ± 1.1% of the net glucose, respectively. This gave an ethanol yield of 0.39 ± 0.01, 0.39 ± 0.02, and 0.38 ± 0.01 g/g for isolates G2-4-1, G1-5-1, and G2-3-2, respectively. Note, that since the growth of these three isolates in the EPM containing 280 g/L glucose were different, then the number of cells inoculated was different for each yeast isolate. These three isolates (G2-4-1, G1-5-1, and G2-3-2) were selected as ethanol fermenting osmotolerant yeasts for further study.

Table 1. Ethanol production and glucose utilization of the 12 osmotolerant yeasts when grown in EP medium containing 280 g/L of glucose.

Yeast isolation No.	Ethanol (g/L)[a]	Glucose utilized (%)[a]
G1-5-1	73.0 ± 4.5	66.7 ± 1.1
G1-8-1	65.2 ± 4.6	60.4 ± 3.4
G2-3-2	71.9 ± 1.2	67.0 ± 0.8
G2-4-1	73.2 ± 2.0	67.0 ± 0.7
G2-5-2	69.1 ± 1.5	65.9 ± 0.6
G2-10-1	56.4 ± 4.5	56.0 ± 4.3
G2-15-1	64.2 ± 3.5	60.2 ± 2.6
G3-3-1	70.3 ± 1.3	65.5 ± 0.9
G6-11-2	62.7 ± 1.0	60.4 ± 2.9
G6-11-3	67.0 ± 1.7	42.5 ± 2.3
G6-12-2	37.0 ± 0.6	44.1 ± 0.7
G8-1-2	44.9 ± 0.8	55.1 ± 1.3

[a]Data are displayed as the mean ± SD, and are derived from triplicate experiments.

3.3 Inoculum Preparation Medium and Ethanol Production by Selected Isolates

The three selected ethanol fermenting osmotolerant yeasts (G2-4-1, G1-5-1 and G2-3-2) were then grown in YM broth containing 150, 240, or 280 g/L glucose. The increase in the cell number and glucose utilization of all three yeast isolates were highest in the YM broth containing 150 g/L glucose and decreased with increasing glucose levels to the lowest values in the YM broth with 280 g/L glucose (Table 2). Isolate G2-4-1 had the highest level of cell number increase (9.1×10^6 cells /g glucose utilized) and isolate G2-3-2 utilized the largest proportion of available glucose (59.4 ± 1.99 %). The YM broth containing 150 g/L glucose was, therefore, selected as the inoculum preparation medium for subsequent experiments.

Table 2. Effect of the glucose concentration in the inoculum preparation medium (YM) on the yeast cell number and glucose utilization of the three selected ethanol fermenting osmotolerant yeasts.

Yeast isolate	Glucose concentration (g/L)	Initial cell concentration (cells/mL)[a]	Final cell concentration (cells/mL)[a]	Glucose utilized (%)[a]	Increase of cell number (Cells /g glucose utilized)[a]
G2-4-1	150	1.6×10^6	7.5×10^8	55.0 ± 0.54	9.1×10^6
	240	1.6×10^6	7.0×10^8	38.0 ± 0.47	7.7×10^6
	280	1.5×10^6	6.6×10^8	35.3 ± 0.83	6.3×10^6
G1-5-1	150	1.3×10^6	7.0×10^8	51.8 ± 0.96	9.0×10^6
	240	1.2×10^6	5.0×10^8	39.2 ± 1.16	5.3×10^6
	280	1.1×10^6	4.0×10^8	30.9 ± 0.56	4.6×10^6
G2-3-2	150	1.2×10^6	7.5×10^8	59.4 ± 1.99	8.4×10^6
	240	1.0×10^6	6.5×10^8	41.4 ± 1.04	6.5×10^6
	280	1.1×10^6	6.3×10^8	36.3 ± 0.80	6.1×10^6

Cells were grown in YM broth containing (150, 240, or 280 g/L) glucose at 30 °C, 200 rpm, for 24 h. aData are displayed as the mean ± 1 SD, and are derived from triplicate experiments.

Ethanol production of the three selected isolates was further examined in EPM containing 150, 240, or 280 g/L glucose. All three isolates produced a higher (~1.3-fold) ethanol in the EPM containing 240 g/L glucose than with 150 g/L glucose (Figure 1). Increasing the glucose concentration to 280 g/L increased the ethanol produced by isolate G2-3-2, but that produced by isolates G1-5-1 and G2-4-1 was the same or decreased (1.04-fold), respectively. The level of residual glucose in the fermentation broth increased with increasing initial glucose concentrations in the EPM in all cases, revealing incomplete glucose utilization. In the EPM medium containing 280 g/L glucose, isolate G2-3-2 gave the highest ethanol concentration (84.6 g/L) and utilized the highest proportion of available glucose (~82%). Accordingly, isolate G2-3-2 was deemed to be likely to be the most suitable strain for ethanol fermentation under VHG conditions and so was selected for VHG ethanol fermentation experiments.

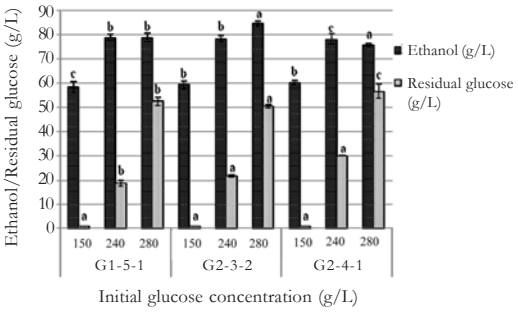

Figure 1. Effect of the initial glucose concentration in the EPM on the ethanol produced and residual glucose level in the ferments of the three selected ethanol fermenting osmotolerant yeasts. Inocparation medium contained 150 g/L glucose. The fermentation was performed at 30 °C under an oxygen limited condition for 72 h. Ethanol (■), residual glucose (■). The data are displayed as the mean ± 1 SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$; Duncan's MMT).

3.4 Identification of The Three High Yield Ethanol Fermenting Osmotolerant Yeast Isolates

Analysis of D1/D2 domain of the rDNA (LSU) indicated that all three isolates were *S. cerevisiae*, with 100% sequence identity for isolates G1-5-1 and G2-3-2 to *S. cerevisiae* strain D3C (Accession no. JF715188.1), and 100% sequence identity to *S. cerevisiae* strain Y5-3 (Accession no. HQ711330.1) for isolate G2-4-1. From the 566 bp D1/D2 amplicon sequence obtained for these three isolates, 541 bp could be unambiguously aligned with the BLASTn derived homologs and the two outgroup species. The NJ-derived phylogenetic tree and position of these isolates is shown in Figure 2. The yeasts, G1-5-1, G2-3-2, and G2-4-1 have been deposited in the WDCM 511 culture collection (Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand) with collection numbers MSCU 0676 0641 and 0677, respectively.

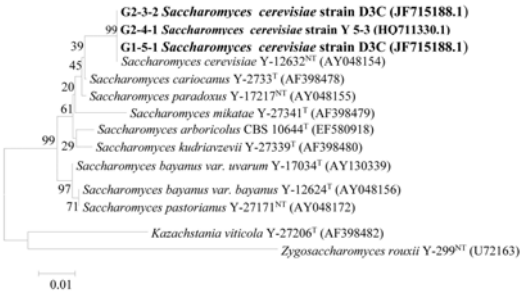

Figure 2. Phylogenetic tree showing the position of yeast isolates G2-3-2, G2-4-1, and G1-5-1 (Bold). The tree was constructed by the NJ distance based method using 541 bp of the D1/D2 region of the rRNA LSU gene. Bootstrap values shown at each node are based on 1,000 replications. GenBank

accession codes are shown in parenthesis after each species. Scale bar represents 0.01% sequence divergence.

3.5 Optimal Conditions for VHG Ethanol Fermentation by *S. cerevisiae* G2-3-2

The ethanol fermentation ability of *S. cerevisiae* G2-3-2 in EPM under VHG conditions was then evaluated. Late log phase cells grown in YEM broth containing 150 g/L glucose were used as the inoculum at a final cell concentration of 1×10^8 cells/mL. The fermentation culture was incubated at 30 °C under an oxygen limited condition for 72 h.

Optimal Glucose Concentration for Ethanol Production

The EPM containing various concentrations of glucose (240, 280, 300, 320, or 340 g/L) was used for the VHG fermentation medium. The highest ethanol production (84.3 g/L) by G2-3-2 was obtained in EPM containing 280 g/L glucose, where further increasing the glucose concentration caused a slight reduction in the obtained ethanol concentration, decreasing to 73.0 g/L at a glucose concentration of 340 g/L (Figure 3). Increasing the glucose concentration above 280 (g/L) led to a significant and almost corresponding increase in the residual glucose levels, with essentially the same net amount of glucose utilization (210-219 g/L) being observed at 300-340 g/L glucose (Figure 3).

Optimal Initial Cell Concentration for Ethanol Production

When the EPM containing 280 g/L glucose was inoculated at various initial G2-3-2 cell concentrations (1×10^8, 2.5×10^8, 5×10^8, 1×10^9, or 2.5×10^9 cells/mL) the final ethanol concentration obtained increased with increasing initial cell concentration up to 1×10^9 cells/mL at 103.3 g/L, but did not markedly increase further at higher initial cell levels (103.6 g/L at 2.5×10^9 cells/mL) (Figure 4). Likewise, the residual glucose level in the fermentation medium decreased with increasing initial cell concentration, with a glucose utilization level of 248.9 and 262.2 g/L for an initial G2-3-2 cell concentration of 1×10^9 and 2.5×10^9 cells/mL, respectively. The ethanol yield was maximal (0.42 g ethanol/g glucose utilized) when the initial cell concentration was 1×10^9 cells/mL, and so this initial cell concentration level was selected. In accord, it was previously reported that increasing the inoculum size two-fold increased the ethanol production level of *S. cerevisiae* in a sucrose rich (280 g/L) EPM-like media, whilst increasing the inoculum size 3.3-fold reduced the optimal fermentation time from 72 h to 48 h [21].

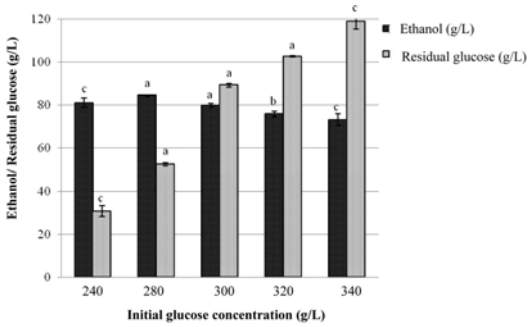

Figure 3. Effect of the initial glucose concentration in the EPM on the ethanol production and residual glucose level in the fermentation by *S. cerevisiae* G2-3-2. Initial cell concentration was 1×10^8 cells/mL. The fermentation was performed at 30 °C under an oxygen limited condition for 72 h. Ethanol (■), residual glucose (■). Data are displayed as the mean ± 1 SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$; Duncan's MMT)

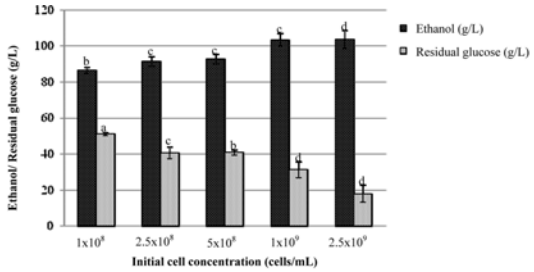

Figure 4. Effect of the initial cell concentration of *S. cerevisiae* G2-3-2 on the ethanol production and residual glucose level in the fermentation of EPM containing 280 g/L glucose. The fermentation was performed at 30 °C under an oxygen limited condition for 72 h. Ethanol (■), residual glucose (■). Data are displayed as the mean ± 1 SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$; Duncan's MMT).

Optimal Fermentation Time for Ethanol Production

Glucose fermentation in the EPM containing 280 g/L glucose and an initial G2-3-2 cell concentration of 1×10^9 cells/mL was evaluated for the ethanol and residual glucose concentrations every 24 h over a 168 h period. The ethanol increased with increasing fermentation time up to a maximum at 120 to 144 h, with an ethanol production of 113.3 and 113.7 g/L, respectively, whilst the residual glucose level decreased with fermentation time to a minimum at 144 to 168 h (Figure 5). The glucose utilization level was maximal at 120 to 144 h at 276.3 and 277.8 g/L, respectively, giving a maximal ethanol yield of 0.41 g /g glucose at 120 h and ethanol productivity was 0.94 g/L/h.

Optimal Concentration of Nutrients for Ethanol Production

The optimal nutrient levels for ethanol fermentation by *S. cerevisiae* isolate G2-3-2 was evaluated in EPM containing 280 g/L glucose by a sequential univariate variation in the level of the polypeptone, yeast extract, $MgSO_4$, and KH_2PO_4, maintaining the same initial cell concentration (1×10^9 cells/mL) and fermentation period (120 h).

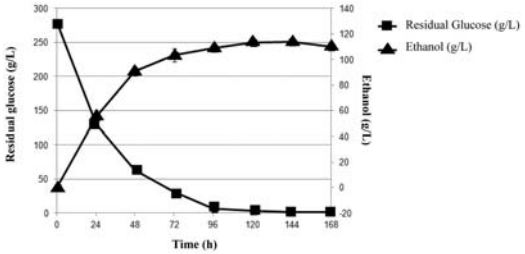

Figure 5. Effect of the fermentation time on the ethanol and residual glucose levels of ferments with an initial *S. cerevisiae* G2-3-2 cell concentration of 1×10^9 cells/mL in EPM containing 280 g/L glucose. The fermentation was performed at 30 °C under an oxygen limited condition. Ethanol (▲), residual glucose (■). The data are displayed as the mean ± 1 SD, and are derived from triplicate experiments.

Increasing the concentration of polypeptone in the EPM (0, 2.5, 5, 7.5, and 10 g/L), increased the ethanol concentration to a maximum (115.6 g/L) at a polypeptone concentration of 5 g/L, and this then slightly decreased at higher polypeptone concentrations (Figure 6A). Then, with 5 g/L polypeptone in the EPM, increasing the concentration of yeast extract (0, 3, 6, 7.5, 9, and 12 g/L) was found to increase the obtained ethanol to a numerical maximum (116.9 g/L) at a yeast concentration of 7.5 g/L (Figure 6B). These results agreed well with a previous study that reported a two-fold increase in the peptone and yeast extract concentration in peptone-yeast extract-nutrient (PYN) medium (all (g/L): glucose, 300; peptone, 3.5; yeast extract, 3; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 1; pH 5.6)

resulted in an increased ethanol produced by *S. cerevisiae* [8]. Likewise, the ethanol obtained from the VHG fermentation of sweet sorghum juice supplemented with sucrose or sugarcane molasses to a final sugar content of 280 g/L at 30 °C was increased when 50 g/L polypeptone and 3 g/L yeast extract were added. However, replacement of the polypeptone and yeast extract with ammonium sulfate to an equivalent nitrogen content led to a reduced ethanol production [7], since excessive ammonium ions promoted the synthesis of more by products, such as higher alcohols, acetic acid, or hydrogen sulfide.

Within the modified EPM containing 280 g/L glucose, 5 g/L polypeptone, and 7.5 g/L yeast extract, increasing the concentration of $MgSO_4.7H_2O$ (0, 0.025, 0.075, 0.5, 1.5, and 2.5 g/L) increased the ethanol concentration to a numerical maximum of 132.9 g/L at a $MgSO_4.7H_2O$ concentration of 1.5 g/L and then decreased at the highest $MgSO_4$ level evaluated of 2.5 g/L (Figure 6C). It was previously reported that the ethanol production increased when the concentration of $MgSO_4.7H_2O$ in the PYN medium containing 200 g/L glucose was increased from 1 to 3 g/L [8], which is likely to be due to the importance of Mg^{2+} as a cofactor (as $MgATP^{2-}$) for many glycolytic enzymes [22] and its capability to relieve ethanol toxicity during yeast fermentation [23]. Finally, when the KH_2PO_4 concentration in the modified EPM (now with 1.5 g/L $MgSO_4.7H_2O$) was increased (0, 0.5, 1.5, 3, 6, and 9 g/L), the ethanol concentration increased to a maximum of 134.7 g/L at a KH_2PO_4 concentration of 3 g/L (Figure 6D). The ethanol productivity and ethanol yield at this final optimized EPM condition were 1.12 g/L/h and 0.48 g/g glucose utilized, respectively. Increasing the KH_2PO_4 concentration from 2 to 6 g/L in PYN medium containing 200 g/L glucose was reported to result in an increased ethanol production [8], due to the increased buffering capacity of the fermentation medium. Likewise, the supplementation of corn flour hydrolyzate with Mg^{2+}, yeast extract, glycine, biotin, and peptone were all reported to increase the obtained ethanol under VHG fermentation conditions, with the concentration of Mg^{2+} and peptone being reported as the critical factors [5].

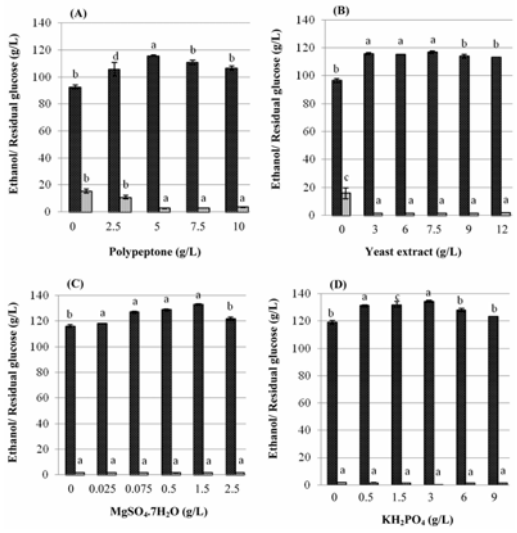

Figure 6. Effect of the (A) polypeptone, (B) yeast extract, (C) $MgSO_4.7H_2O$ and (D) KH_2PO_4 concentration in the EPM containing 280 g/L glucose on the ethanol and residual glucose levels of ferments with *S. cerevisiae* G2-3-2. Initial cell concentration was 1×10^9 cells/mL and incubated at 30 °C under an oxygen limited condition for 120 h. Ethanol (■), residual glucose (■). Data are displayed as the mean ± 1 SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$; Duncan's MMT).

3.6 VHG Ethanol Production under Oxygen or CO_2 Stress-relief Conditions

When the fermentation of the optimized EPM (oEPM) (EPM but with 280 g/L glucose, 5 g/L polypeptone, 7.5 g/L yeast extract, 1.5 g/L $MgSO_4.7H_2O$, and 3 g/L KH_2PO_4) was performed by reducing medium volume in 50 mL flask from 46.75 to 35 mL and shaking at 100 rpm to relief oxygen stress, isolate G2-3-2 produced maximum ethanol at 122.5 g/L (0.44 g ethanol /g glucose utilized) after 72 h (Figure 7A), which was lower than that obtained (134.7 g/L; 0.48 g ethanol/g glucose utilized) when fermented under the oxygen limited condition. However, the ethanol productivity was 1.70 g/L/h, which was 1.5-fold higher than that obtained when fermented under the oxygen limited condition (1.12 g/L/h). When the CO_2 released from fermentation was removed from the culture by trapping with copper (II) sulfate, the maximum ethanol production decreased to 125.1 g/L after 72 h, giving an ethanol yield of 0.45 g ethanol /g glucose (Figure 7B), while the ethanol productivity was 1.73 g/L/h, some 1.54-fold higher than those of fermentation under the oxygen limited condition.Yeast requires some oxygen during fermentation for the synthesis of ergosterol and unsaturated fatty acids to maintain their membrane integrity [21], which is essential for cells to counteract the ethanol toxicity. While, pyruvic acid synthesized by the glycolytic pathway is converted to acetaldehyde, CO_2, and ethanol by the anaerobic alcoholic fermentation pathway in the absence of oxygen. Removal of CO_2 (released from the decarboxylation of pyruvic acid to acetaldehyde) relieved the oxygen mass transfer stress and resulted in an increased cell viability and ethanol production.

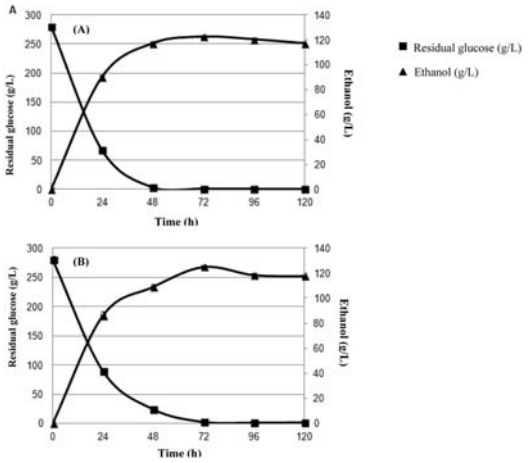

Figure 7. Effect of (A) oxygen stress-relief and (B) CO_2 removal on the ethanol and residual glucose levels of VHG ferments with *S. cerevisiae* G2-3-2 in the optimized EPM. Initial cell concentration was 1×10^9 cells/mL and incubated at 30 °C. Ethanol (▲), residual glucose (■). The data are displayed as the mean ± 1 SD, and are derived from triplicate experiments.

4. CONCLUSION

S. cerevisiae isolate G2-3-2, a high ethanol fermenting osmotolerant yeast, was isolated from sugarcane juice by a selective culture method using medium containing 30 mL/L ethanol. The *S. cerevisiae* G2-3-2 isolate grew in YM broth containing 150 g/L glucose better than in YM broth containing 240 or 280 g/L glucose, but late log phase cells of *S. cerevisiae* G2-3-2 grown in the YM broth containing 150 g/L glucose produced a maximum ethanol level in EPM containing 280 g/L glucose. Based on this result, osmotic tolerance did not directly relate to fermentation efficiency under osmotic condition was concluded. The initial cell inoculum level of the *S. cerevisiae* G2-3-2 was found to be optimal at 1×10^9 cells/mL. The use of *S.*

cerevisiae G2-3-2 at the optimal cell inoculum level produced a maximum ethanol concentration of 113.3 g/L after 120 h of fermentation. The ethanol production was increased to 134.7 g/L (0.48 g ethanol/g glucose utilized) in the optimized EPM medium and VHG fermentation conditions. In the VHG fermentation condition, the removal of CO_2 from the system was found to promote a higher ethanol yield and ethanol productivity by *S. cerevisiae* G2-3-2 more than the oxygen stress-relief to maintain the cell membrane integrity.

ACKNOWLEDGEMENT

The authors thank Dr. Robert Butcher for critical reading of the manuscript and the Office of the Cane and Sugar Board (OCSB) for kindly providing samples from sugar factories in Thailand. This study was financially supported by the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University RES56-0530-035-EN).

REFERENCES

[1] Kawa-Rygielska J. and Pietrzak W., *Biomass Bioeng.*, 2014; **60**: 50-57. DOI 10.1016/j.biombioe. 2013.10.028.

[2] Bayrock D.P. and Ingledew W.M., *J. Ind. Microbiol. Biot.*, 2001; **27**: 87-93. DOI 10.1038/sj.jim.7000167.

[3] Badger P.C., Ethanol from Cellulose: A General Review; in Janick J. and Whipkey A., eds., *Trends in New Crops and New Uses*, ASHS Press, Alexandria, Verginia, 2002: 17-21.

[4] Pereira F.B., Guimaraes P.M.R., Teixeira J.A. and Domingues L., *Biotechnol. Lett.*, 2010; **32**: 1655-1661. DOI 10.1007/s10529-010-0330-9.

[5] Liu J.J., Ding W.T., Zhang G.C. and Wang J.Y., *Appl. Microbiol. Biotechnol.*, 2011; **91**: 1239-1246. DOI 10.1007/s00253-011-3404-2.

[6] Wang F.Q., Gao C.J., Yang C.Y. and Xu P., *Biotechnol. Lett.*, 2007; **29**: 233-236. DOI 10.1007/s10529-006-9220-6.

[7] Laopaiboon L., Nuanpeng S., Srinophakun P., Klanrit P. and Laopaiboon P., *Bioresour. Technol.*, 2009; **100**: 4176-4182. DOI 10.1016/j.biortech. 2009.03.046.

[8] D'Amore T., Panchal C.J., Russell I. and Stewart G.G., *J. Ind. Microbiol.,* 1988; **2**: 365-372.DOI 10.1007/BF01569575.

[9] Zheng D.Q., Wu X.C., Tao X.L., Wang P.M., Li P., Chi X.Q., Li Y.D., Yan Q.F. and Zhao Y.H., *Bioresour. Technol.*, 2011; **102**: 3020-3027. DOI 10.1016/j.biortech. 2010.09.122.

[10] Laopaiboon L., Nuanpeng S., Srinophakun P., Klanrit P. and Laopaiboon P., *Biotechnology*, 2008; **7**: 493-498. DOI 10.3923/biotech.2008. 493.498.

[11] Zoecklien B.W., Fugelsang K.C., Gump B.H. and Nury F.S., Laboratory Procedures. In: Wine Analysis and production, Chapman and Hall, New York, 1995; 374-375.

[12] Kurtzman C.P., Fell J.W. and Boekhout T., *The Yeasts: A Taxonomic Study* (5th ed.), Elsevier, USA, 2011.

[13] Saitou N. and Nei M., *Mol. Biol. Evol.*, 1987; **4**: 406-425.

[14] Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S., *Mol. Biol. Evol.*, 2011; **28**: 2731-2739. DOI 10.1093/molbev/mst197.

[15] Kimura M., *J. Mol. Evol.*, 1980; **16**: 111-120. DOI 10.1007/BF 01731581.

[16] Felsenstein J., *Evolution*, 1985; **39**: 738-791. DOI 10.2307/2408678.

[17] Jutakanoke R., Leepipatpiboon N., Tolieng V., Kitpreecavanich V.,

Srinorakutara T. and Akaracharanya A., *Biomass Bioeng.*, 2012; **39**: 283-289. DOI 10.1016/j.biomBioe.2012.01.018.

[18] Somogyi M., *J. Biol. Chem.*, 1952; **195**: 19-23.

[19] Choi G.W., Um H.J., Kim Y., Kang H.W., Kim M. and Chung B.W., *Biomass Bioeng.*, 2010; **34**: 1223-1231. DOI 10.1016/j.biombioe.2012.03.019.

[20] Limtong S., Sringiew C. and Yongmanitchai W., *Bioresour.Technol.*, 2007; **98**: 3367-3374. DOI 10.1016/j.biortech.2006.10.044.

[21] Breisha G.Z., *Biomass Bioeng.*, 2010; **34**: 1243-1249. DOI 10.1016/j.biombioe.2010.03.017.

[22] Garfinkel L. and Garfinkel D., *Magnesium,* 1985; **4(2-3)**: 60-72.

[23] Dombek K.M. and Ingram L.O., *Appl. Environ. Microbiol.*, 1986; **52**: 975-981.