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Effect of initial cell, yeast extract and sugar concentrations on ethanol production from molasses by thermotolerant yeast *Saccharomyces cerevisiae* RMU Y-10

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Abstract

Effect of fermentation factors, i.e., initial cell (5-15%), yeast extract (3-9 g/l) and initial sugar concentrations (120-220 g/l) on ethanol production from molasses using thermotolerant yeast *Saccharomyces cerevisiae* RMU Y-10 were investigated. An L_9 (3^4) orthogonal array design was used to optimize the fermentation factors, and the fermentation experiment was carried out at 37°C using batch mode. Results showed that initial sugar concentration had a major effect on ethanol production, followed by yeast extract and initial cell concentrations, respectively. Optimum initial cell, yeast extract and initial sugar concentrations for ethanol production by thermotolerant yeast *S. cerevisiae* RMU Y-10 were 15% (v/v), 3 and 120 g/l. Highest ethanol concentration (56.62 g/l), ethanol yield (0.50) and ethanol productivity (1.57 g/l/h) were achieved under optimum fermentation conditions. Results also showed that ethanol concentrations obtained under the optimum conditions were 25.82, 94.04 and 64.55% greater than those in YM medium (control 1), molasses only (control 2) and molasses supplemented with YM medium (control 3) experiments, respectively with fermentation time reduced to 36 h.

Keywords: Ethanol, Thermotolerant yeast, Orthogonal array design, Molasses

1. Introduction

Ethanol production of fuel alcohol from renewable biomass is a good source of energy because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity [1]. It is an alternative to petroleum-based fuels and can be made from very common crops that contain sugar and starch such as sugarcane, potato and corn by fermentation of microorganisms. Use of ethanol fuels results in clean emissions and reduces greenhouse gases from vehicles [2]. Generally, ethanol is produced by mesophilic microorganisms at optimum temperature range of 25-30°C. However, during ethanol production temperature may increase by up to 5-10°C above normal. Therefore, to avoid problems resulting from overheating in the fermentation process, thermotolerant yeasts that can tolerate high temperatures are necessary [3 & 4].

High temperature fermentation for ethanol production has several advantages including increasing the rate of catalytic reactions related to fermentation, reducing problems of contamination by other microorganisms and saving energy in cooling systems and operating costs [4 & 5].

Several yeast species have been characterized and classified as thermotolerant such as *Kluyveromyces marxianus, Saccharomyces cerevisiae* and *Pichia kudriavzevii* [1, 6-7]. These strains can produce ethanol at high temperatures ranging from 37-45°C.

Sugarcane molasses is a by-product of the sugar industry which has high fermentable sugar and is suitable for renewable energy production. In addition, it is cheap compared to other agricultural feedstock used for renewable energy production [8].

Initial cell, nitrogen and sugar concentrations are important parameters for growth and ethanol production of yeast. Here, these three main parameters were investigated for high ethanol production from sugarcane molasses by thermotolerant yeast RMU Y-10 under batch mode using a statistical L_9 (3^4) orthogonal array design. Ethanol production from a synthetic medium (YM medium) for sugarcane molasses only and for sugarcane molasses supplemented with YM medium were performed as the control experiments.

2. Materials and methods

2.1 Microorganism and inoculum preparation

Thermotolerant yeasts were isolated from local fruits collected in Maha Sarakham Province, Thailand [9]. The selected thermotolerant yeast was identified based on the morphological and D1/D2 domain of the 26S rDNA gene sequencing analysis [10]. Genomic DNA of the yeasts was performed according to the procedure followed by Harju et al. [11] with slight modifications. The D1/D2 domain was amplified by PCR with specific primers NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) [12]. Homology analysis was performed using the BLAST program by comparing pairwise sequences.

The selected yeast was transferred to YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2% glucose) and grown at 30°C with shaking speed of 150 rpm for 16-18 h. The 10% (v/v) enriched cultures were moved to YM broth (10%, w/v of glucose) and incubated at 30°C with an agitation rate of 150 rpm for 6-8 h before use as inoculum for ethanol production.

2.2 Raw material

Sugarcane molasses was obtained from Mitr Phu Viang Sugar Co., Ltd., Thailand and kept at -20°C until required for use.

2.3 Ethanol production medium and batch ethanol production

The molasses was adjusted to the desired concentration, then supplemented with yeast extract following the levels in Table 1 and supplemented again with nutrients from YM medium (except glucose). The pH of the medium was adjusted to 5 by 4 N NaOH. Batch fermentation was performed in 500 ml air-locked flasks with 250 ml working volume. After sterilization, the flasks were inoculated with active inoculums between 5-15%, v/v (Table 1) and incubated at 37°C under static condition. During fermentation, samples were collected for analysis. YM medium (control 1), sugarcane molasses (control 2) and sugarcane molasses supplemented with nutrient from YM medium (except glucose) (control 3) containing 100 g/l of total sugar [13 & 14] were also prepared as the control treatments. Table 1 shows the L_9 (3^4) orthogonal design. Levels 1, 2 and 3 refer to the lowest, middle and highest values of the parameters tested, respectively. All experimental runs were performed in triplicate. The blank factor was a dummy and used for error evaluation. Analysis of variance (ANOVA) was used as the statistical analysis tool to estimate the effects of a factor on characteristic properties [15].

2.4 Analytical methods

Total sugars were assayed using the phenol sulfuric acid method [16]. Yeast cell numbers and total soluble solids were determined using a hemocytometer and a hand-held refractometer, respectively [17]. The pH was measured using a pH meter. Ethanol concentration was measured by gas chromatography (GC) (Shimadzu GC-14B, Japan) using a polyethylene glycol (PEG-20 M) pack column with a flame ionization detector (FID). Nitrogen (N_2) was used as a carrier gas, and 2-propanol was used as an internal standard [18].

Ethanol yield ($Y_{p/s}$) was calculated as the ethanol produced and expressed as g ethanol per g sugar utilized (g/g). Volumetric ethanol productivity (Q_p, g/l/h) was calculated by ethanol concentration produced (P, g/l) divided by the fermentation time (t, h) giving the greatest ethanol concentration: $Q_p = P/t$.

3. Results and discussion

3.1 Identification of the selected yeast strain

Phylogenetic analysis is shown in Figure 1. Sequences of the thermotolerant yeast strain RMU Y-10 were 99% identical to the sequence of *S. cerevisiae.* Therefore, thermotolerant yeast strain RMU Y-10 was identified as *S. cerevisiae*.

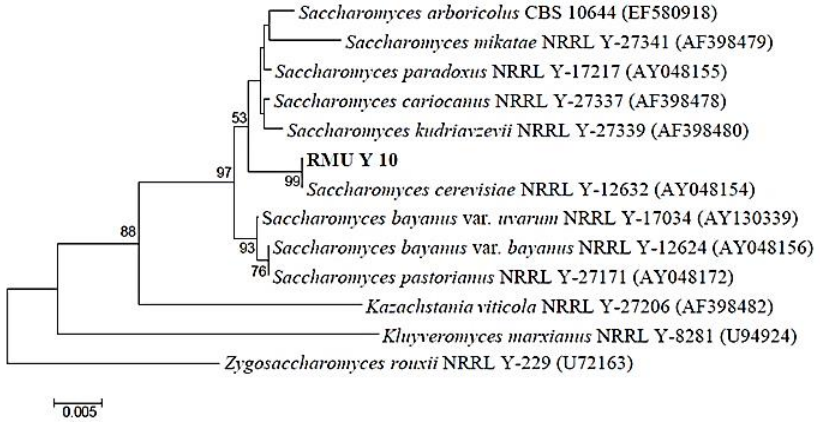

Figure 1 Phylogenetic tree of the D1/D2 domain of 26S rDNA from neighbor-joining depicting relationships among type strains of the selected yeast species.

3.2 Ethanol fermentation from sugarcane molasses by thermotolerant yeast S. cerevisiae RMU Y-10 using an L_9 (3^4) orthogonal array design

Batch ethanol fermentations of experimental Runs 1-9 (Table 1) were carried out. In Run 1 (initial cells, 15%; yeast extract, 3 g/l and total sugar, 220 g/l), pH of the fermentation broth decreased from 5.00 to 4.63 at 24 h (Figure 2), indicating carbon dioxide (CO_2) production. Then, pH increased to 4.85 at 48 h and remained constant until the end of fermentation. Results implied that fermentation occurred. Total sugar concentration decreased from 210.87 to 121.78 g/l at 36 h and sugar utilized was about 89.09 g/l (Figure 2). In addition, highest cell concentration (6.91×10^7 cells/ml) was presented at 36 h fermentation time (Figure 2). Results showed that thermotolerant yeast *S. cerevisiae* RMU Y-10 could grow under this condition. Ethanol increased with increasing fermentation time. In addition, sugar consumption began immediately after the yeast was inoculated into the ethanol production medium, implying that a lag phase did not occur under this condition. Concentration of ethanol (*P*) was 43.68 g/l at 36 h, corresponding to $Y_{p/s}$ and Q_p at 0.49 and 1.22 g/l/h, respectively (data not shown)**.**

Table 1 Orthogonal experimental results of ethanol fermentation from sugarcane molasses by thermotolerant yeast *S. cerevisiae* RMU Y-10

Run	Factor A	Factor B	Factor C	Factor D	Response
	Initial cells (%, v/v)	Yeast extract (g/l)	Total sugar (g/l)	Blank	(P: ethanol,
1	15	3	220	(level 2)	43.86±0.35[c]
2	15	6	120	(level 3)	55.57±0.41[e]
3	10	9	120	(level 2)	50.79±0.39[d]
4	5	9	220	(level 3)	32.10±0.72[a]
5	10	6	220	(level 1)	34.80±0.44[a]
6	10	3	170	(level 3)	45.71±0.62[c]
7	15	9	170	(level 1)	41.40±0.31[b]
8	5	6	170	(level 2)	40.35±0.45[b]
9	5	3	120	(level 1)	52.83±0.55[d]

[a, b, c, d and e] Means followed by the same letter within the same column are not significantly different using Duncan's multiple range test at level of 0.05

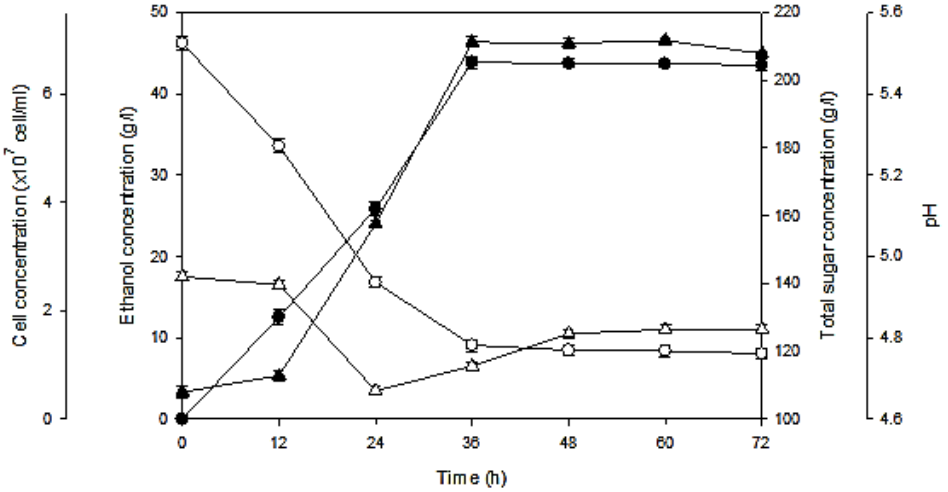

Figure 2 Batch profiles of ethanol fermentation of experimental Run 1 from sugarcane molasses by thermotolerant yeast *S. cerevisiae* RMU Y-10 (●, ethanol; ○, total sugar; ▲, cell and Δ, pH)

Batch culture profiles of the parameters measured during ethanol production of the eight remaining runs were similar to Run 1 (data not shown). Table 1 summarizes batch fermentation results of Runs 1 to 9. *P* values ranged from 32.10-55.57 g/l. In addition, cell concentrations ranged from 1.56×10^7-8.00×10^7 cells/ml, while sugar utilization ranged from 87.34-113.95 g/l (data not shown). Different amounts of parameters affected ethanol fermentation efficiencies. Highest ethanol concentration was obtained from Run 2. Ethanol concentration decreased with increasing sugar concentration, indicating that higher initial sugar adversely affected ethanol production with a negative effect on cell growth, resulting in reduction of cell numbers and final ethanol concentration [1 & 6]. When 6 g/l of yeast extract was supplemented in the medium, ethanol concentration was slightly higher than when yeast extract was added at 3 and 9 g/l. Our results contrasted with Nuanpeng et al. [19] who reported that maximum ethanol concentration (106.82 g/l) by *S. cerevisiae* NP 01 from sweet sorghum juice at 37°C was achieved when yeast extract at 9 g/l was supplemented into the fermentation medium. Increasing initial cell concentrations led to a high ethanol concentration. This result concurred with Charoensopharat et al. [1] and Nuanpeng et al. [6].

3.3 Range analysis of L_9 (3^4) orthogonal array design experiment for ethanol concentrations

P values were used as response values for analysis of optimum conditions of orthogonal array design. Table 1 shows the orthogonal experimental results of ethanol fermentation with concentrations in the range of 32.10-55.57 g/l. These results were used to analyze the order of influence on ethanol concentration.

Table 2 Range analysis of L_9 (3^4) orthogonal array design experiment for ethanol concentration

	P: Ethanol concentration (g/l)		
	Initial cells	Yeast extract	Total sugar
K_1	125.28	142.40	159.19
K_2	131.30	130.72	127.46
K_3	140.83	124.29	110.76
k_1	41.76	47.47	53.06
k_2	43.77	43.57	42.49
k_3	46.94	41.43	36.92
R	5.18	6.04	16.14
Q	A_3	B_1	C_1

Range analysis was used to clarify the consequence of initial cells (*Factor A*), yeast extract (*Factor B*) and total sugar (*Factor C*). A larger *R* value of the factor represents a greater effect on final *P*. The *k* values are used to clarify the optimum level of each factor to promote the highest ethanol concentration. Results showed that the order of influence for ethanol concentration was sugar, yeast extract and initial cell concentrations, respectively and optimum levels of the factor were $A_3B_1C_1$, corresponding to initial cells 15% (v/v), yeast extract 3 and sugar 120 g/l, respectively. Analysis of variance (ANOVA) was applied to verify the order of the effects of these three

parameters on ethanol concentration (data not shown). According to the *F* values in our study, order of influence on ethanol concentration was as follows: $F_{Total\ sugar} = 63.50$, $F_{Yeast\ extract} = 8.84$ and $F_{Initial\ cells} = 6.45$, which was a similar result to the *R* values. Correlation coefficient or R^2 of the predicted and actual results for *P* was 0.9875 (98.75%) (data not shown). These results confirmed an acceptable fit of the model to the data [20]. Finding showed that ethanol concentration increased with increasing initial cell concentration (Table 2). Highest ethanol concentration was achieved at 15% of the initial cells. Generally, high initial cells reduced the lag phase of growth and increased sugar utilization and ethanol fermentation rate, leading to high ethanol concentration and volumetric ethanol productivity [6]. When yeast extract was used as the nitrogen source, results showed that yeast was not stimulated by higher yeast extract concentrations (9 g/l). Tesnière et al. [21] reported that an excess of various nitrogen sources enhanced cell death under lipid limitation conditions.

Molecular analyses indicated that target of rapamycin (TOR) nitrogen cellular signaling is involved in triggering cell death in such conditions. Laopaiboon et al. [18] reported that capability of nitrogen utilization or nitrogen requirement by yeast might depend on other factors such as yeast strain. Ethanol concentration from different initial sugar concentrations (120 to 220 g/l) decreased with increased total sugar concentration. High sugar concentrations may inhibit cell growth and enzymes involved in the metabolic pathway and reduce the viability of yeast cells, the conversion rate of substrate and ethanol yield [22].

3.4 Verification experiments

YM medium, sugarcane molasses and sugarcane molasses supplemented with nutrient from YM medium (except glucose) were used as control 1, 2 and 3, respectively. As shown in Table 3, ethanol concentration under optimum conditions was 56.62 g/l at 36 h corresponding to $Y_{p/s}$ and Q_p at 0.50 and 1.57 g/l/h, respectively. Ethanol concentrations from control experiments (1, 2 and 3) were 45.00, 29.18 and 34.41 g/l, respectively at 48 h of fermentation time. Results indicated that ethanol concentrations obtained under the optimum condition were 25.82, 94.04 and 64.55% greater than those in control 1, control 2 and control 3 experiments, respectively. In addition, fermentation time was reduced to 36 h (Table 3).

Table 3 Ethanol fermentation from sugarcane molasses by thermotolerant yeast *S. cerevisiae* RMU Y-10 under the optimum condition

Fermentation results	Optimum condition ($A_3B_1C_1$)	Control		
		1	2	3
Ethanol (g/l)	56.62±0.64[d]	45.00±0.22[c]	29.18±0.61[a]	34.41±0.52[b]
Initial sugar (g/l)	116.67±1.31[b]	110.32±0.92[a]	111.23±0.83[a]	112.61±1.12[a]
Sugar utilized (g/l)	112.36±1.02[d]	95.74±0.91[c]	72.95±0.65[a]	81.93±1.11[b]
Fermentation time (h)	36	48	48	48
$Y_{p/s}$	0.50±0.01[c]	0.47±0.00[b]	0.40±0.01[a]	0.42±0.01[a]
Q_p (g/l/h)	1.57±0.01[d]	0.94±0.01[c]	0.67±0.00[a]	0.72±0.01[b]
Cell concentration (10^7 cells/ml)	6.79±0.21[c]	5.49±0.31[b]	3.44±0.44[a]	2.18±0.71[a]
pH	4.51±0.10[a]	4.41±0.10[a]	4.65±0.10[a]	4.70±0.05[a]

[a, b, c and d] Means followed by the same letter within the same row are not significantly different using Duncan's multiple range test at the level of 0.05.

4. Conclusions

Our results revealed that thermotolerant yeast *S. cerevisiae* RMU Y-10 showed ability to produce ethanol from sugarcane molasses. In addition, order of influence for high ethanol concentration were sugar, yeast extract and initial cell concentrations, respectively, and optimum levels of the factors were $A_3B_1C_1$, corresponding to initial cell concentration 15% (v/v), yeast extract 3 and sugar 120 g/l, respectively. Under this condition, ethanol concentration, $Y_{p/s}$, Q_p and cell concentration were significantly improved.

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6. References

[1] Charoensopharat, K., Thanonkeo, P., Thanonkeo, S., Yamada, M. Ethanol production from Jerusalem artichoke tubers at high temperature by newly isolated thermotolerant inulin utilizing yeast *Kluyveromyces marxianus* using consolidated bioprocessing. Antonie van Leeuwenhoek. 2015;108:173-190.

[2] Ariyajaroenwong, P., Laopaiboon, P., Laopaiboon, L. Capability of sweet sorghum stalks as supporting materials for yeast immobilization to produce ethanol under various fermentation processes. J. Taiwan Inst. Chem. Eng. 2015;49:79-84.

[3] Kiran, S. N., Sridhar, M., Suresh, K., Banat, I. M., Venkateswar, R. L. Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. Bioresour. Technol. 2000;72(1):43-46.

[4] Limtong, S., Sringiew, C., Yongmanitchai, W. Production of fuel ethanol at high temperature from sugar cane juice by newly isolated *Kluyveromyces marxianus*. Bioresour. Technol2007;98:3367-3374.

[5] Sootsuwan, K., Irie, A., Murata, M., Lertwattanasakul, N., Thanonkeo, P., Yamada, M. Thermotolerant *Zymomonas mobilis*: comparison of ethanol fermentation capability with that of an efficient type strain. Open Biotechnol J. 2007;1:59-65.

[6] Nuanpeng, S., Thanonkeo, S., Yamada, M., Thanonkeo, P. Ethanol production from sweet sorghum juice at high temperatures using newly isolated thermotolerant yeast *Saccharomyces cerevisiae* DBKKU Y-53. Energies. 2016;9:253.

[7] Chamnipa, N., Thanonkeo, S., Klanrit, P., Thanonkeo, P. The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high-temperature ethanol production. Braz J Microbiol. 2018;49:378-391.

[8] Wechgama, K., Laopaiboon, L., Laopaiboon, P. Enhancement of batch butanol production from sugarcane molasses using nitrogen supplementation integrated with gas stripping for product recovery. Ind. Crops Prod. 2017;95:216-226.

[9] Thipawan, P., Nanthachad, T., Yuntayam, T., Paimarat, N., Phuduangchit, S. Isolation of thermotolerant yeasts isolated from local fruits for ethanol production. [dissertation]. Maha Sarakham: Rajabhat Maha Sarakham University; 2015

[10] Kurtzman, C.P., Robnett, C.J. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit ({26S}) ribosomal {DNA} partial sequences. Antonie van Leeuwenhoek. 1998;73(4):331-371.

[11] Harju, S., Fedosyuk, H., Peterson, K.R. Rapid isolation of yeast genomic DNA: Bust n' Grab. BMC Biotechnol. 2004;4:8.

[12] O'Donnell, K. *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW, editors. The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International. Wallingford; 1993

[13] Shafaghat, H., Najafpour, D.G., Rezaei1, S.P., Sharifzadeh-Baei, Mazyar. Ethanol production with natural carbon sources in batch and continuous fermentation using free and immobilized *Saccharomyces cerevisiae*. J Sci Ind Res (India) 2011;70:162-169.

[14] Charoensopharat, K., Wechgama, K. Isolation and Selection of Newly Thermotolerant Yeast from Edible Local Fruits for Ethanol Production from Cassava Starch. Prawarun Agr J. 2018;15 Suppl 1: 29-39.

[15] Farzaneh, A., Ehteshamzadeh, M., Mohammadi, M. Corrosion performance of the electroless Ni–P coatings prepared in different conditions and optimized by the Taguchi method. J Appl Electrochem 2011;41:19-27.

[16] Dubois, M., Gilles, K.A., Hamilton, J.R., Robers, P.A., Smith, F. Colourimetric Method for Determination of Sugar and Related Substances. Anal. Chem. 1956;28:350-356.

[17] Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., Nury, F.S. Wine Analysis and production. New York: Chapman & Hall; 1995

[18] Laopaiboon, L., Nuanpeng, S., Srinophakun, P., Klanrit, P., Laopaiboon, P. Ethanol production from sweet sorghum juice using very high gravity technology: Effects of carbon and nitrogen supplementations. Bioresour Technol. 2009;100(18):4176-4182.

[19] Nuanpeng, S., Laopaiboon, L., Srinophakun, P., Klanrit, P., Jaisil, P., Laopaiboon, P. Ethanol production from sweet sorghum juice under very high gravity conditions. Electron Journal of Biotechnol. 2011;14:1.

[20] Jangchud, A. Product optimization In: Statistics for product development and application. Kasetsart University, Bangkok; 2006

[21] Tesnière, C., Delobel, P., Pradal, M., Blondin, B. Impact of Nutrient Imbalance on Wine Alcoholic Fermentations: Nitrogen Excess Enhances Yeast Cell Death in Lipid-Limited Must. PLoS One. 2013;8(4):61645.

[22] Ozmihci, S., Kargi, F. Effects of feed sugar concentration on continuous ethanol fermentation of cheese whey powder solution (CWP). Enzyme Microb. Technol. 2007;41:876–880.