Screening Useful Isolated Yeasts for Ethanol Fermentation at High Temperature

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Abstract

Due to a current challenge of increasing global temperature, thermo-ethanologenic yeasts receive considerably interest nowadays. In this study, 11 of 31 yeast isolates selected from the previous research based on their well growth at temperature up to 45°C were tested for fermentative capacity and ethanol tolerance at high temperatures. Five isolates BM2, BM3, HX1, C2 and V2 performed significantly well (at the 95% confidence level) in fermentation at 40°C. Of which BM3, BM2, HX1 and C2 were able to tolerate up to 15% v/v ethanol. After 5 days of fermentation at high temperature (40^{o}C), the highest fermentation yield of yeasts could reach up to 76% and this was found in fermentation from saccharified glutinous rice liquid, indicating the further application feasibility of these yeasts for ethanol production at high temperature. Four selected target yeasts were characterized as follow: HX1: Candida tropicalis, BM3: Torulaspora globosa, both C2 and BM2: Pichia kudriavzevii.

Key words: Ethanol, ethanol tolerance, fermentation, thermo-tolerance, yeast.

1. Introduction

Ethanol is an important industrial chemical with emerging potential as a bio-fuel to replace non-renewable fossil fuels (Alfenore *et al.*, 2002). The increasing demand of ethanol production for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives, has necessitated increased production of this alcohol. Developing and utilizing of ethanologenic microorganisms can obviously lead to success in large-scale ethanol manufacture. Ethanol production is usually accomplished by chemical synthesis of petrochemical substrates and microbial conversion of carbohydrates present in agricultural products by yeasts. The tolerant ability of yeasts under the conditions of high temperature and high ethanol levels (Ansanay-Galeote *et al.*, 2001; Osho *et al.*, 2005) during fermentation process is becoming an increasingly important characteristic that attracts many researchers as there has been a mixed variety of benefits which could be exploited through the use of thermophilic yeasts for ethanol production. Thermo-tolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries and under tropical climates as well. Cooling costs during the process of ethanol production are expensive; hence, by using thermo-tolerant yeasts, cooling and distillation costs can be reduced. Besides, higher saccharification and fermentation rates, continuous ethanol removal and reduced contamination have stimulated a search for routes to thermo-tolerant yeasts. With the aim to pave the way for application of useful thermo- and ethanol tolerant yeasts for ethanol production, selected yeast isolates were tested and characterized for their fermentative performance at high temperature and their ethanol tolerance.

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2. Materials and Methods

2.1 Cultures and media

The yeast: eleven strains that were isolated from samples of ripe fruits, flowers of fruit-tree, alcoholic starter, sugarcane bagasse, sawdust and selected from the preliminary screening experiments (Dung et al., unpubl. data) based on their well growth at temperature up to 45°C.

The mould: *Amylomyces rouxii* strain no. 20.3 (CBS 111757) (Dung *et al.*, 2006).

Culture media: YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar), YPD broth (1% yeast extract, 2% peptone, 2% glucose) (Messenguy *et al.*, 2008), PGA (20% potato, 2% glucose, 2% agar) and Oxytetracycline-glucose-yeast agar (OGYA, Merck).

2.2 Screening for ethanol fermentation ability at high temperature

Ability of ethanol fermentation at high temperature was determined by measuring the gas production in Durham test tubes. Pre-culture yeasts (on YPD broth for 24 hours at 30°C) was inoculated into Durham tubes containing liquid of glucose 2%, and then incubated at 30, 35, 40, and 45°C, respectively. The gas production was measured after 12, 24, 36, and 48 hours of incubation.

2.3 Challenge tests with different ethanol-supplemented levels

Pure ethanol was added to YPD broth at levels of 0, 3, 6, 9, 12 and 15% v/v, and this was inoculated with 10^4 yeast cells per ml (microscopic count). Numbers of viable yeast cells at start and after 3 days were measured, by plate counting on OGYA medium.

2.4 Ethanol fermentation ability from saccharified glutinous rice liquid and medium of molasses

Saccharifield glutinous rice liquid was prepared by using *Amylomyces rouxii*. Mould was first cultured on the PGA medium for 3 days at 30°C and then inoculated into steamed glutinous rice. After 4 days at 30°C of incubation, saccharified glutinous rice collected by centrifuging at 7,000 rpm for 20 minutes and finally adjusted to 20°Brix. Molasses from Phung Hiep sugar factory (Hau Giang province, Vietnam) was prepared by adjusted with distilled water to 20°Brix and sterilized at 115°C for 10 minutes before used.

The 150 ml volume of saccharifield glutinous rice liquid or molasses inoculated with 3 ml of selected yeast isolates (10^6 cells/ml), and the fermentative capacity of yeasts was evaluated after 5 days of incubation at 40°C. The pH was measured with a digital pH meter WTW pH525. The total sugar was determined by hand refractometer (Euromex-Holland). Ethanol was determined by distillation method. Glucose was analyzed by glucose oxidase kit (GAGO-20, Sigma).

2.5 Identification of the selected target yeasts

The target yeast isolates were identified based on their morphological, physiological and biochemical characteristics. Besides, amplified the D1/D2 domain of the 26S rDNA of selected yeasts were analyzed by PCR with the 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'). Nucleotide sequence was aligned and compared with the data obtained from Gene Bank. (http://www.ncbi.nlm.nih.gov/).

2.6 Statistical analysis

Experimental data were statistically analyzed using Statgraphics Plus Version 5.0, Manugistics, Inc., Rock-ville, USA.

3. Results and Discussion

3.1 Screening of yeast for ethanol fermentation at high temperature

The ethanol fermentation capacity of 11 yeast isolates at different temperatures was evaluated based on the levels of CO_2 produced in Durham test tubes after 48 hours of incubation (Table 1).

Overall, all tested yeast isolates could grow well and ferment at 30, 35, and 40°C by measuring the rate of gas discharges; however, no gas production was observed at 45°C. The difference levels of the gas production were recorded among these isolates.

After 12 hours, the fermentation rates were still slowly due to low CO_2 production (average about 1.65 to 2.03 mm). After 36 hours, the average height of CO_2 ranged from 15.0 to 19.1 mm, particularly, some yeast isolates reached the maximum height of Durham tubes (42 mm) including BM3 (at 30°C), HX1 (at 35°C) and V2 (at 40°C). After 48 hours, two more isolates generated CO_2 up to the maximum value, namely HN3 (at 30°C) and BM3 (at 40°C). Statistical analysis revealed that among 11 yeast isolates, five isolates BM3, BM2, HX1, C2 and V2 could ferment well at high temperature (40°C) and distinguished at a statistical significance of 95% confidence compared to other isolates. Consequently, these five isolates were selected for further experiments.

3.2 Tolerance to ethanol present in liquid medium (challenge test)

In this challenge test (see materials and methods), numbers of viable yeast cells at the start and after 3 days of fermentation at 40°C were determined by plate counting and were reported as log CFU/ml (Table 2).

The plate counting was used to evaluate the growth and the decline of yeast cells exposed to different ethanol-supplemented levels. After 3 days of incubation at 40°C, all tested yeasts, except strain V2, grew very well in medium containing 0, 3, 6 or 9% v/v ethanol, resulting in higher levels of viable yeast cells compared with the non-incubated control. In ethanol supplied media, density of yeast cells decreased correlatively with the increase of ethanol concentrations. Four yeast isolates namely C2, BM2, BM3, and HX1 showed the ability to be able to tolerate up to 15% v/v ethanol based on lower levels of these yeasts were found in comparison with the inoculation level. When we would define the ethanol tolerance as the level where yeast levels are static, it could be concluded from Table 2 that the ethanol tolerance of the yeasts C2, BM2, BM3, and HX1 was approximately 12-15% v/v. These four yeasts were further tested for their fermentative capacity from the materials of saccharifield glutinous rice or molasses at high temperature (40°C).

3.3 Fermentative capacity of selected yeasts during ethanol fermentation from saccharified glutinous rice liquid or medium of molasses

In this study of ethanol fermentation by a role of yeast, as popular regional ingredients for fermented rice wine processing and industrial ethanol production, the saccharified glutinous rice liquid and medium of molasses were employed and compared. The performance of fermentative capacity of yeasts was monitored for their fermentation rate. This was estimated by the gas production rate as measured in a calibrated water lock (the average volume of a gas bubble was 0.8 ml). In Figure 1, the results of gas production rate by the tested yeasts HX1, C2, BM2 and BM3 during the fermentation were described. These yeasts were able to ferment rapidly from the beginning of incubation time. A sudden increase in gas production rate was observed after 1 day of fermentation and a significant decrease after 5 days. The difference of gas production was found between two different kinds of media. In most cases of tested yeasts, the gas discharge through numbers of bubbles produced in saccharified glutinous rice liquid was higher in comparison with those in medium of molasses.

The results of yeast performance during ethanol fermentation from saccharifield glutinous rice and from molasses were described in Table 3 and Table 4, respectively. The levels of pH, Brix, remained glucose and produced ethanol were remarkably noted.

During the fermentation from saccharifield glutinous rice, pH after fermentation was lower than initial pH level, at a range of 4.03 to 4.12 which could show the successful fermentation process. This decrease caused by glucose oxidation into gluconic or acetic acid. During fermentation process, yeasts converted glucose into alcohol and other intermediate products, including organic acids. Consequently, Brix levels were also decreased from initial level at 20 to 10.11-12.30 after 5 days of incubation. Ethanol concentrations were produced from 4.5 to 7.0% v/v. The significantly highest ethanol concentrations were found in cases of yeasts C2 (7% v/v) and BM2 (6.83% v/v). These results had good correlation to the concentrations of glucose remaining which almost completely consumed by yeast to achieve high ethanol concentration after fermentation.

Following the same principle of fermentative performance, during the fermentation from medium of molasses, pH value after fermentation was lower than initial pH level, at a range 4.63 to 4.91. Levels of Brix after fermentation were also reduced from initial level at 20 to 12.42-12.76 after 5 days of incubation. Results of produced ethanol concentration at 20°C (% v/v) showed negligibility varied. The same ethanol level (3.00% v/v) was produced in case of yeasts including HX1, C2 and BM2 whereas the significantly higher ethanol concentration (3.83% v/v) was found in case of BM3.

The fermentation yield was used as an indicator to express the capacity to consume glucose and produce ethanol. A yield of 100% is used as a theoretical reference, based on two molecules of ethanol being produced from one molecule of glucose. Therefore, the fermentation yield was calculated by actual output divided by theoretical output, multiplied with 100%. In the ethanol fermentation from saccharified glutinous rice liquid, the ethanol production yield could reach up to 76% whereas there was significantly lower yield in case of medium of molasses (44%). In practice, the actual observed ethanol yield in successful ethanol fermentation can vary from 75% to 93% depending on raw materials and inoculation sources as well as the technological process (Thuong and Hang, 2000).

Considering the exploring of useful yeasts for ethanol fermentation at high temperature condition, the results of the certain high ethanol yields (74-76%) obtained in some testing cases in the present study, in which the incubation temperature (40°C) is not the optimum condition for ethanol fermentation, this could indicate the further promising application of selected target yeasts for ethanol fermentation at high temperature processing.

3.4 Identification of the selected target yeasts

Morphological characteristics of four selected target yeast cells were round and ellipse shape; budding cells were present and developed. The colonies of all four isolates grown after 3 days of incubation at 30°C on YPD medium were round, disjointed, milky color, 0.1 mm thick, 2-3 mm in diameter, smooth surfaces, and steady edge. According to the methods of Kurtzman and Fell (1997), ascospore formation is use for indication of the ascomycetous yeasts. Ascospore formed on agar medium after 3 weeks at 30^{o}C. Asci were persistent form 1-2 spheroidal ascospore (Figure 2).

In addition, yeast genus could identify preliminary based on their morphological, physiological and biochemical characteristics (Dung, 1998; Luong, 2003). Typical physiological and biochemical characteristics of these four isolates were described in Table 5, in which the results demonstrated that all four yeast isolates were *Ascomycota*. The nucleotide sequence of D1/D2 domain of 26S rDNA of these 4 selected yeasts were analyzed and compared with NCBI database in gene bank that reached 100% similarity. The identification results of four selected yeast isolates showed that HX1 and BM3 were *Candida tropicalis* and *Torulaspora globosa*, respectively and; both C2 and BM2 belonged to *Pichia kudriavzevii*. *Candida tropicalis* is a potentially useful organism for the commercial production of ethanol as it is capable of fermenting starch at an effective rate. *C. tropicalis* does not require the saccharification step. Furthermore, fed-batch fermentation by free *C. tropicalis* cells increased the final concentration of ethanol, reaching published values for *Saccharomyces cerevisiae* recombinant strains expressing both alpha-amylase and glucoamylase (Jamai *et al*., 2006). According to Limtong *et al.* (2002), *T. globosa* is one of yeast strains capable to produce high levels of ethanol, about 6.03% v/v. In another recent study (Dhaliwal *et al.*, 2011) it has indicated that fermentation in a laboratory fermenter with galactose adapted *P. kudriavzevii* cells at 40°C, resulted in an ethanol concentration of 7,19% w/v from sugarcane juice composed of about 14% w/v sucrose, 2% w/v glucose and 1% w/v fructose.

4. Conclusions

The five yeast isolates HX1, V2, C2, BM2 and BM3 were selected from 11 tested isolates based on their good fermentative ability at high temperature (40°C). They could also perform their ethanol tolerance capacity, of which, four isolates HX1, C2, BM2, and BM3 could tolerate up to 15% v/v ethanol concentration. These four yeasts could consume almost completely the glucose contents to produce ethanol. The higher ethanol yield was found in fermentation from saccharifield glutinous rice liquid compared to medium of molasses. Based on the nucleotide sequence of D1/D2 domain of 26S rDNA analysis and comparison with NCBI database in gene bank that reached 100% similarity, the identification results of 4 selected target yeast isolates were as follow: HX1: *Candida tropicalis*, BM3: *Torulaspora globosa*, both C2 and BM2 belonged to *Pichia kudriavzevii*.

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Table 1. Gas production rate performed in height of CO_2 (mm) in Durham tubes

Isolates	30°C				35°C				40°C			
	12h	24h	36h	48h	12h	24h	36h	48h	12h	24h	36h	48h
HX1	5.0[1]	30.3	38.7	40.0	1.7	27.3	42.0	42.0	3.7	11.7	16.0	35.3[a2]
C2	0.0	2.7	8.0	35.7	2.7	4.7	6.3	15.3	0.0	5.7	17.0	39.3[a]
HDD2	0.0	1.7	8.7	8.7	1.3	2.7	5.7	9.3	0.0	3.0	11.3	24.0[bc]
V2	0.0	4.0	8.3	19.7	0.0	0.0	9.7	25.0	7.0	24.0	42.0	42.0[a]
V3	0.0	2.0	7.7	20.7	0.7	3.3	5.7	7.7	0.7	7.0	12.7	28.7[b]
HN3	1.7	25.0	36.0	42.0	0.0	0.0	3.3	6.3	0.0	0.0	1.7	4.7[c]
HN4	0.0	2.7	13.3	15.7	0.0	0.0	5.3	12.0	2.7	4.7	9.3	28.0[b]
BM2	0.7	4.3	14.3	21.3	3.7	7.3	16.7	26.0	0.0	5.7	18.0	40.7[a]
BM3	12.3	38.7	42.0	42.0	2.7	20.7	28.7	32.7	5.0	20.0	22.0	42.0[a]
MO	0.0	2.7	9.7	31.3	0.0	0.0	9.3	10.3	0.0	1.3	11.7	19.0[c]
CC	2.7	9.3	23.7	40.7	5.3	16.3	32.7	38.0	0.0	7.3	13.3	27.3[b]

[1]Values are means of triplicates; [2]means with different subscripts within a column are statistically different at the 95% confidence level.

Table 2. Viable yeast cells (log CFU/ml) exposed to various concentrations of ethanol

Isolates	Ethanol concentration (% v/v)	Initial	After 3 days fermentation
HX1	0	4.21[1]	6.89
	3	4.29	5.74
	6	4.32	4.91
	9	4.30	4.56
	12	4.21	4.11
	15	4.23	3.01
V2	0	4.46	6.72
	3	4.28	5.61
	6	4.22	4.34
	9	4.17	3.55
	12	4.25	2.45
	15	4.22	0.70
C2	0	4.38	6.55
	3	4.34	4.57
	6	4.19	5.68
	9	4.27	5.45
	12	4.21	4.79
	15	4.18	3.17
BM2	0	4.26	6.99
	3	4.23	6.24
	6	4.24	5.56
	9	4.22	5.27
	12	4.19	4.68
	15	4.19	3.13
BM3	0	4.33	6.78
	3	4.30	5.43
	6	4.28	5.39
	9	4.23	5.43
	12	4.22	3.96
	15	4.26	3.05

[1]*Values are means of triplicates*

Table 3. Results of Brix, pH, remained glucose and produced ethanol in the fermentation from saccharifield glutinous

Isolates	Fermentation in saccharifield glutinous at 40°C						
	°Brix		pH		Glucose (% w/v)		Ethanol at 20°C (% v/v)
	Initial	Final	Initial	Final	Initial	Final	
1.HX1	20[1]	12.30	4.22	4.06	18.01	3.33	4.50[c2]
2.C2	20	10.11	4.22	4.03	18.01	1.13	7.00[a]
3.BM2	20	10.13	4.22	4.03	18.01	1.16	6.83[a]
4.BM3	20	11.12	4.22	4.12	18.01	1.30	5.83[b]

[1]*Value in the table was average value of triplication;* [2]*the average values with the same letter were not significant at 95% probability.*

Table 4. Average Brix, pH, glucose remaining and ethanol concentration in molasses

Isolates	Fermentation in molasses at 40°C						
	°Brix		pH		Glucose (% w/v)		Ethanol at 20°C (% v/v)
	Initial	Final	Initial	Final	Initial	Final	
HX1	20[1]	12.58	5.17	4.91	17.28	3.61	3.00[b2]
C2	20	12.42	5.17	4.63	17.28	3.60	3.00[b]
BM2	20	12.48	5.17	4.66	17.28	3.60	3.00[b]
BM3	20	12.76	5.17	4.71	17.28	3.44	3.83[a]

[1]Value in the table was average value of triplication; [2]the average values with the same letter were not significant at 95% probability.

Table 5. Physiological and biochemical properties of the selected target yeasts

Yeast isolates	Biofilm-forming	Sediment-forming	Acospores	Urease	Starch formation	Gelatine
HX1	-	+	-	-	-	-
C2	+	-	+	-	-	-
BM2	+	-	+	-	-	-
BM3	-	+	+	-	-	-

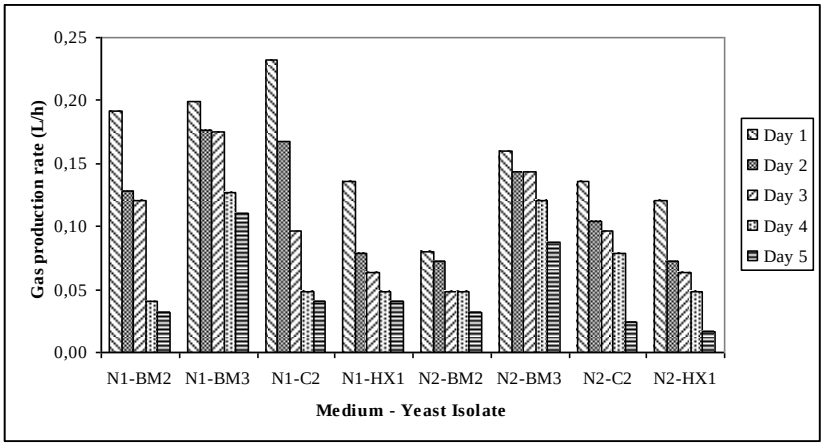

Figure 1. Gas production by yeasts HX1, C2, BM2, BM3 in saccharified glutinous rice (N1) or in molasses (N2) after 5 days of fermentation at 40°C

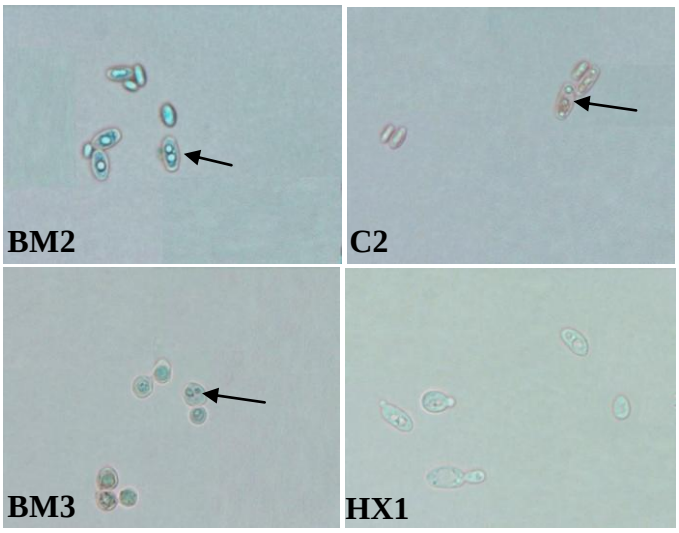

Figure 2. Ascospore of yeasts BM2, C2, BM3 and non-ascospore HX1 (E100)