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SUMMARY
BACHELOR OF SCIENCE THESIS
THE ADVANCED PROGRAM IN BIOTECHNOLOGY

SELECTION AND STUDY ON ETHANOL FERMENTATION CONDITIONS BY THERMO-TOLERANT YEASTS

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Abstract

In this study, 44 yeast isolates were tested for their thermo-tolerant ability at 30, 36, 39, 42, 43, 44, and 45ºC, and for their ethanol tolerant ability at 4, 8, 10, and 12% (v/v) of supplemented ethanol. The yeast isolates with high capacity of thermo- and ethanol tolerance were further tested for ethanol fermentation ability in 2% glucose liquid and ethanol fermentation ability at high temperatures (room temperature, 35, 40 and 45ºC). The selected yeast isolate was screened for ethanol production in different conditions consisting of inoculum concentration (10^4, 10^5, and 10^6 cells/mL), initial sugar concentration (15, 20, 25 and 30ºBrix), fermentation time (3, 5, and 7 days) and pH of medium (pH4, pH4.66 (natural), pH5, and pH6) in molasses. Seven yeast isolates (C2, CC, BM2, V2, V3, L04-2, and L07-2) were found to be able for growth at 42ºC, in which BM2 could grow at 43ºC. There were 25 in a total of 44 yeast isolates performing their growth in the medium containing 12% ethanol. Among them, V2 isolate had high capacity of ethanol fermentation in 2% glucose medium than others. Favorable conditions for V2 isolate to produce ethanol in molasses at 40ºC were determined as follow: 10^5 cells/mL of inoculum level, 25ºBrix of initial sugar concentration, 5 days of fermentation time, and pH of natural medium (pH4.66). The result of molecular analysis of ITS1, 5.8S rDNA, and ITS2 sequence showed that the V2 yeast isolate belonged to Pichia kudriavzevii with 100% homogeneous level.

Key words: *ethanol fermentation, ethanol tolerant ability, Pichia kudriavzevii, thermo-tolerant ability, thermo-tolerant yeast*

CONTENTS

1. INTRODUCTION

Ethanol is an important industrial chemical with various applications, like bio-fuel, industrial solvents, cleansing agents, preservatives… Producing ethanol by microorganisms in large scale has obtained certain achievements (Dung et al., 2012).

Temperature is one of the major factors effecting on the ethanol fermentation of yeasts. The study on yeasts capable of tolerating high temperature attracts many researchers due to a number of potential benefits in ethanol production including reducing cost of cooling under thermal conditions, enhancing saccharification and fermentation rates, and reducing contamination. Besides, ethanol is also one of the major factors effecting on the development of yeasts. Selecting the yeasts with thermo- and ethanol tolerant ability is necessary in industrial production of ethanol.

Nowadays, with the increase in the prices of fossil fuels, ethanol is promising alternative liquid fuel. Ethanol can be produced from agricultural wastes, in particular molasses. Cane molasses is a low-cost source of sugar, and in contrast to other agricultural by-products, it does not require hydrolysis (Ghorbani et al., 2011).

Recent studies have been carried out on isolation of thermo-tolerant yeasts (Nguyen Van Anh et al., 2011), testing for ethanol tolerant ability of yeasts (Nguyen Thi Ngoc Mai, 2011) and testing thermo-tolerant ability of yeasts (Nguyen Huu Tuong et al., 2012). This study is to follow up in term of applying thermo-tolerant yeasts to produce ethanol in molasses.

Objectives

To determine thermo- and ethanol tolerant ability of yeast isolates, and to test ethanol fermentation ability at high temperature in molasses as well as favorable conditions for the fermentation of selected yeasts.

The study was followed:

- Testing thermo-tolerant ability of 44 yeast isolates
- Testing ethanol tolerant ability of 44 yeast isolates
- Study on ethanol fermentation ability of selected yeast isolates in glucose liquid
- Study on ethanol fermentation ability of selected yeast isolates at high temperature
- Study on ethanol fermentation conditions for selected yeast isolates
- Effects of inoculation levels and sugar concentrations
- Effects of fermentation time and pH of molasses medium
- Identification of the selected thermo-tolerant yeast isolates

2. MATERIALS AND METHODS

2.1. Materials

- Molasses (bought from Phung Hiep Sugar Factory, Nga Bay Town, Hau Giang Province)

- Potato, agar

- 44 yeast isolates from Biotechnology R&D Institute, Can Tho University, Vietnam and Faculty of Technology, Khon Kaen University, Thailand: C2, CC, BM2, BM3, HN3, HN4, HDD2, V2, V3, HX1, N1, MO, T, 14, 17, 26, 30, 57, 65, 66, 87, 89, 92, 96, 107, 110, 122, 135, MR15, MR19, N23, VIII, 20/5, 29/3, 126.5, Y1c, Y5c, Y10, Y11, Y58, Y64, Y65, L04-2 and L07-2

- Chemicals: C_2H_5OH, D-glucose, NaOH, citric acid…

- Media: YM agar, PGY

- Devices: oven, microscope, electric stove, flasks, pH meter, water-lock, refractometer…

2.2. Methods

2.2.1. Testing thermo-tolerant ability of 44 yeast isolates

Yeasts were inoculated into PGY medium for 24 hours. Thermo-tolerant ability of yeast isolates was determined by culturing the yeasts on YM agar medium and incubating at 30, 36, 39, 42, 43, 44, and 45ºC in two days. The isolates forming colonies at high temperatures were recorded.

2.2.2. Testing ethanol tolerant ability of 44 yeast isolates

Yeasts were inoculated into PGY medium for 24 hours. Ethanol tolerant ability of yeast isolates was determined by

culturing the yeasts on YM agar medium supplemented with pure ethanol at levels of 4, 8, 10, and 12% (v/v) and incubating at 30ºC in two days. The isolates forming colonies on high ethanol concentration supplemented medium were recorded.

2.2.3. Study on ethanol fermentation ability of selected yeast isolates in glucose liquid

Ethanol fermentation ability of yeasts was determined by measuring the CO_2 height in Durham test tubes produced by yeasts. Yeasts were inoculated into PGY medium for 24 hours. Then, inoculated suspension of yeast cells into Durham tubes containing liquid of glucose 2%, and incubated at room temperature. Measure the accumulation of CO_2 gas in the inner Durham tubes at 4, 8, 12, 16, 20, and 24 hours.

2.2.4. Study on ethanol fermentation ability of selected yeast isolates at high temperature

Yeasts were inoculated into PGY medium for 24 hours. The 1 mL of pre-culture yeasts with 10^8 cells/mL (microscopic count) was inoculated into 99 mL of 20ºBrix molasses medium sterilizing at 121ºC for 15 minutes. Incubated the molasses medium anaerobically at room temperature, 35, 40, and 45ºC for five days. Ethanol after fermentation was determined by distillation method.

2.2.5. Study on ethanol fermentation conditions for selected yeast isolates

2.2.5.1. Effects of inoculation levels and sugar concentrations on ethanol fermentation of yeast isolate

The 1 mL pre-culture yeasts (on PGY medium for 24 hours) was inoculated into 99 mL of sterilized molasses medium adjusted to different levels of inoculum (10^4, 10^5 and 10^6 cells/mL) and sugar concentration (15, 20, 25, and 30ºBrix). Incubated the molasses medium anaerobically at the temperature selecting from 2.2.4 experiment for five days. Ethanol after fermentation was determined by distillation method.

2.2.5.2. Effects of fermentation time and pH of molasses medium on ethanol fermentation of yeast isolate

The 1 mL pre-culture yeasts (on PGY medium for 24 hours) was inoculated into 99 mL of sterilized molasses medium adjusted to different levels of pH (4.0, 5.0, 6.0 and natural pH). Inoculum levels and sugar concentration were selected from 2.2.5.1 experiment. Incubated the molasses medium anaerobically at the temperature selecting in 2.2.4 experiment. Ethanol was determined by distillation method after 3, 5 and 7 fermentation days, respectively.

2.2.6. Identification of the selected thermo-tolerant yeast isolates

The target yeast isolates were identified based on molecular technique. The DNA of yeast isolates was extracted using simple DNA extraction method. The regions of ITS1, 5.8 rDNA, and ITS2 of selected yeast isolates were amplified by PCR with universal primers ITS 1 and ITS 4. DNA sequence of yeast was determined by DNA sequencing machine. Nucleotide sequence was aligned and compared with the data obtained from Gene Bank (http://www.ncbi.nlm.nih.gov/).

Sequences of ITS 1 and ITS 4 primer as follow:

ITS1: 5’-TCCGTAGGTGAACCTGCGG-3’

ITS4: 5’-TCCTCCGCTTATTGATATGC-3’

2.2.7. Statistical data analysis

The statistical data were analyzed by Microsoft Office Excel 2007 and Statgraphics centurion XV (USA) software.

3. RESULTS AND DISCUSSION

3.1. Thermo-tolerant ability of 44 yeast isolates

The thermo-tolerant ability of yeasts was determined based on the growth of forming colonies at high temperature after 48 hours. The results were presented in Table 3.

Table 3. Thermo-tolerant ability of 44 yeast isolates

No	Yeast isolates	Temperature						
		30°C	36°C	39°C	42°C	43°C	44 °C	45°C
1	14	+	+	+	–	–	–	–
2	17	+	+	–	–	–	–	–
3	20/5	+	+	+	–	–	–	–
4	26	+	+	–	–	–	–	–
5	29/3	+	+	+	–	–	–	–
6	30	+	+	+	–	–	–	–
7	57	+	+	+	–	–	–	–
8	65	+	+	+	–	–	–	–
9	66	+	+	–	–	–	–	–
10	87	+	+	+	–	–	–	–
11	89	+	+	+	–	–	–	–
12	92	+	+	+	–	–	–	–
13	96	+	+	+	–	–	–	–
14	107	+	+	+	–	–	–	–
15	110	+	+	+	–	–	–	–
16	122	+	+	–	–	–	–	–
17	126.5	+	+	–	–	–	–	–
18	135	+	+	–	–	–	–	–
19	BM2	+	+	+	+	+	+	–
20	BM3	+	+	+	+	–	–	–
21	C2	+	+	+	+	+	–	–
22	CC	+	+	+	+	+	–	–
23	HDD2	+	+	+	+	–	–	–

Table 3. Thermo-tolerant ability of 44 yeast isolates *(continued)*

No	Yeast isolates	Temperature						
		30ºC	36ºC	39ºC	42ºC	43ºC	44 ºC	45ºC
24	HN3	+	+	+	+	–	–	–
25	HN4	+	+	+	+	–	–	–
26	HX1	+	+	+	+	–	–	–
27	MO	+	+	+	+	–	–	–
28	MR15	+	+	+	–	–	–	–
29	MR19	+	+	+	–	–	–	–
30	N1	+	+	+	+	–	–	–
31	N23	+	+	+	–	–	–	–
32	T	+	+	+	–	–	–	–
33	V2	+	+	+	+	+	–	–
34	V3	+	+	+	+	+	–	–
35	VIII	+	+	–	–	–	–	–
36	Y10	+	+	–	–	–	–	–
37	Y11	+	+	+	+	–	–	–
38	Y1c	+	+	+	+	–	–	–
39	Y58	+	+	+	–	–	–	–
40	Y5c	+	+	+	–	–	–	–
41	Y64	+	+	+	–	–	–	–
42	Y65	+	+	+	–	–	–	–
43	L04-2	+	+	+	+	+	–	–
44	L07-2	+	+	+	+	+	–	–
Total		44	44	36	16	7	1	0

**Note: "+": forming colonies, "–": no forming colonies*

After 48 hours incubating, all 44 yeast isolates formed colonies at 30 – 36ºC. The number of yeast isolates forming colonies at 39ºC, and 42ºC were 36 and 16, respectively. At 43ºC, there were seven isolates forming colonies, notated as C2, CC,

BM2, V2, V3, L04-2 and L07-2 (Figure 7). Only, BM2 yeast isolate could form colonies at 44ºC.

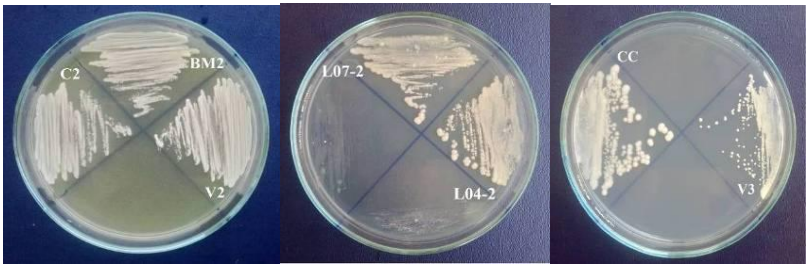

Figure 7. Colonies of the seven yeast isolates BM2, C2, CC, V2, V3, L04-2 and L07-2 at 43ºC

It is shown that temperature is one of the major factors effecting on the development of yeasts. The higher the temperature is, the less numbers of yeast colony forming are.

3.2. Ethanol tolerant ability of 44 yeast isolates

Ethanol tolerant ability of yeasts was determined based on the growth of forming colonies on different ethanol supplemented medium after 48 hours. The results were presented in Table 4.

Table 4. Ethanol tolerant ability of 44 yeast isolates

No	Yeast isolates	Percentage of supplemented ethanol			
		4%	8%	10%	12%
1	14	+	+	+	+
2	17	+	+	+	–
3	20/5	+	+	+	+
4	26	+	+	+	+
5	29/3	+	+	+	+
6	30	+	+	+	–
7	57	+	+	+	+
8	65	+	+	+	+

Table 4. Ethanol tolerant ability of 44 yeast isolates *(continued)*

No	Yeast isolates	Percentage of supplemented ethanol			
		4%	8%	10%	12%
9	66	+	+	+	–
10	87	+	+	+	+
11	89	+	+	+	+
12	92	+	+	+	–
13	96	+	+	+	–
14	107	+	+	+	+
15	110	+	+	–	–
16	122	+	+	+	–
17	126.5	+	+	+	–
18	135	+	+	+	–
19	BM2	+	+	+	–
20	BM3	+	+	–	–
21	C2	+	+	+	+
22	CC	+	+	+	+
23	HDD2	+	+	+	–
24	HN3	+	+	–	–
25	HN4	+	+	+	–
26	HX1	+	+	–	–
27	MO	+	+	+	+
28	MR15	+	+	+	+
29	MR19	+	+	+	+
30	N1	+	+	+	+
31	N23	+	+	+	+
32	T	+	+	–	–
33	V2	+	+	+	+
34	V3	+	+	+	+
35	VIII	+	+	+	+
36	Y10	+	+	+	+

Table 4. Ethanol tolerant ability of 44 yeast isolates *(continued)*

No	Yeast isolates	Percentage of supplemented ethanol			
37	Y11	+	+	+	–
38	Y1c	+	+	–	–
39	Y58	+	+	+	+
40	Y5c	+	+	+	–
41	Y64	+	+	+	+
42	Y65	+	+	+	+
43	L04-2	+	+	+	+
44	L07-2	+	+	+	+
Total		44	44	38	25

**Note: "+": forming colonies, "–": no forming colonies*

All 44 yeast isolates formed colonies in the medium containing 4% and 8% of ethanol. In 10% ethanol medium, there were 38 isolates forming colonies. The number of yeast isolates forming colonies decreased when the supplemented ethanol concentration increased. As the results, 25 isolates could form colonies in the medium containing 12% ethanol.

Ethanol is also one of the major factors effecting on the development of yeasts. Ethanol inhibits the growth of yeasts, and at high concentration ethanol could causes poison for yeast.

Both seven yeast isolates forming colonies at 43ºC (C2, CC, BM2, V2, V3, L04-2 and L07-2) could form colonies in the medium containing 12% of ethanol. These seven yeast isolates were used in further experiments.

3.3. Ethanol fermentation ability of selected yeast isolates in glucose liquid

Ethanol fermentation ability of seven yeast isolates was determined based on their CO_2 production in Durham test tubes after 24 hours of fermentation. The results were presented in Table 5.

Table 5. Average height of CO_2 (mm) in Durham tubes

Yeast isolates	CO_2 appearance time (hrs)	Average height of CO_2 (mm) in Durham tube				
		8 hrs	12 hrs	16 hrs	20 hrs	24 hrs
C2	7	1.00^{b}	4.67^{c}	10.00^{c}	20.33^{cde}	30.00^{a}
CC	7	1.00^{b}	5.33^{bc}	10.67^{c}	23.33^{bcd}	30.00^{a}
BM2	7	1.00^{b}	4.00^{c}	9.67^{c}	20.00^{de}	27.33^{b}
V2	6	2.00^{a}	9.33^{a}	20.67^{a}	30.00^{a}	30.00^{a}
V3	7	0.67^{b}	3.67^{c}	8.33^{c}	18.67^{e}	27.67^{b}
L04-2	7	0.67^{b}	7.67^{ab}	15.67^{b}	24.67^{bc}	30.00^{a}
L07-2	10	0.00^{c}	4.00^{c}	10.67^{c}	27.33^{ab}	30.00^{a}
cv%		69,07	44,57	39,09	19,13	4,72

**Note: The maximum height of CO_2 trapped in Durham tube is 30 mm. Value in the table was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.*

In the first stage of fermentation, yeasts mainly increased biomass, the fermentation process was slow. After 6 hours, CO_2 appeared in tubes containing V2 isolate then in others tubes (7 and 10 hours). At 8, 12, 16 and 20 hours, height of CO_2 in tubes containing V2 isolate was always higher than others. After 20 hours, V2 isolate reached the maximum height of Durham tubes (30 mm). At 24 hours, almost isolates generated CO_2 up to the maximum value. The ethanol fermentation ability of V2 isolate in 2% glucose medium was faster and stronger than other six yeast

isolates. Thus, the V2 yeast isolate was selected for further experiments to find favorable conditions for yeast producing ethanol from molasses at high temperature.

3.4. Ethanol fermentation ability of V2 yeast isolate at high temperature

Ethanol fermentation ability at high temperature of V2 yeast isolate was determined by measuring the ethanol concentration after five fermentation days at room temperature, 35, 40, and 45ºC. The results were presented in Figure 8.

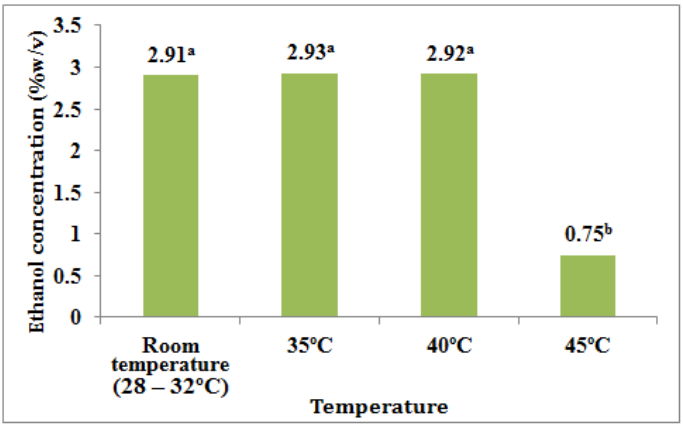

Figure 8. Effect of temperature on ethanol concentration

**Note: Value in the figure was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.*

The ethanol concentrations at three temperature 35ºC, 40ºC and room temperature (28 – 32ºC) were not significantly different at the 95% confidence level. The highest ethanol concentration produced by V2 isolate was 2.93% (w/v) at 35ºC. There was a sharp drop of ethanol concentration from 2.92% to 0.75% (w/v) when temperature increased from 40ºC to 45ºC.

Temperature is one of the major factors effecting on the development of yeasts. The higher the temperature, the deeper the inhibitory effect of ethanol and the higher the maximal intracellular alcohol concentration (Navarro and Durand, 1978). It made yeasts stop to grow rapidly. Thus, ethanol concentration decreased when the temperature increased.

The suitable temperature for thermo-tolerant experiments of V2 yeast isolate was determined as 40ºC.

3.5. Ethanol fermentation conditions for V2 yeast isolate at 40ºC

3.5.1. Effects of inoculum levels and sugar concentrations on ethanol fermentation of V2 yeast isolate

The effects of inoculum levels and sugar concentrations on ethanol fermentation of V2 yeast isolate were determined by measuring the ethanol concentration after five fermentation days at 40ºC. The results were presented in Figure 9.

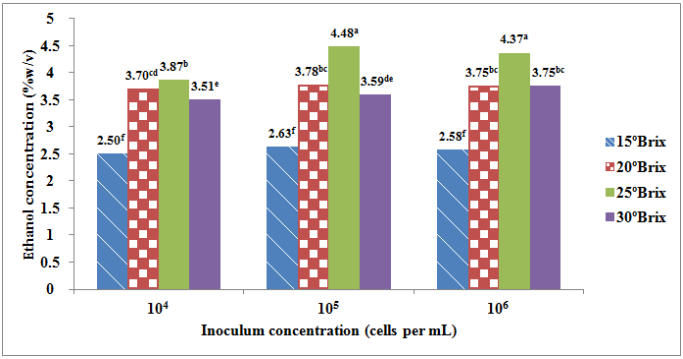

Figure 9. Effects of inoculum levels and sugar concentrations on ethanol concentration

**Note: Value in the figure was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.*

The ethanol concentration of 10^5 cells per mL – 25ºBrix treatment was the highest value (4.48% w/v); and it was not significantly different from 10^6 cells per mL – 25ºBrix one (4.37% w/v). The V2 yeast isolate produced the lowest ethanol concentration with 10^4 cells per mL of inoculum level and 15 degrees of initial Brix concentration (2.50% w/v). The ethanol concentration of the treatment with the highest inoculum level (10^6 cells per mL) and sugar concentration (30ºBrix) was not the highest value (only 3.75% w/v). It showed that high inoculum level and sugar concentration would limit partly the ethanol fermentation ability of yeasts.

In general, ethanol concentration changed with the same trend at all levels of inoculum: when changing sugar concentration from 15 to 30ºBrix, the ethanol concentration increased; sugar concentration was increased to 30ºBrix, ethanol concentration dropped down. In both three cases of inoculum levels, ethanol concentration was always at high value when sugar concentration was 25ºBrix. Initial sugar concentration is one of important factors effecting on ethanol fermentation ability of yeasts: low sugar concentration makes fermentation productivity decrease while the high sugar content in the fermentation medium causes an increase in the osmotic pressure, which has a damaging effect on yeast cells (Pereira et al., 2010).

With the same sugar concentration, the ethanol concentration had the same change, except 30ºBrix: the highest ethanol concentration was in 10^5 cells/mL treatment. In fermentation process, initial inoculum level affects on ethanol

productivity: With higher inoculum levels of yeasts, the initial fermentation rate was improved, after which the fermentation could be prevented (Luong Duc Pham, 2006).

Thus, the chosen inoculum level and sugar concentration were 10^5 cells per mL and 25ºBrix, respectively.

Sugar utilization

In general, utilized sugar increased with inoculum level. The yeasts used sugar the most in treatment 10^6 cells per mL – 25ºBrix (Figure 10), but all utilized sugar was not converted to ethanol. Because the V2 isolate could not use all kinds of sugar in molasses for fermenting, sugar utilization rate was low.

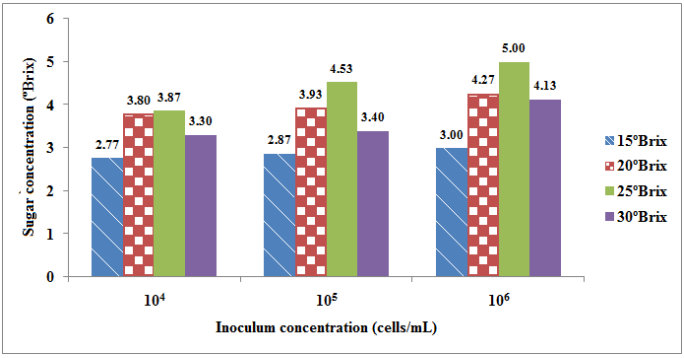

Figure 10. Sugar utilization in fermentation

**Note: Value in the figure was average value of 12 repetitions.*

2.5.2. Effects of fermentation time and pH of molasses medium on ethanol fermentation of V2 yeast isolate

The effects of fermentation time and pH of molasses medium on ethanol fermentation of V2 yeast isolate were determined by measuring the ethanol concentration after 3, 5, and

7 fermentation days at 40ºC. The results were presented in Figure 11.

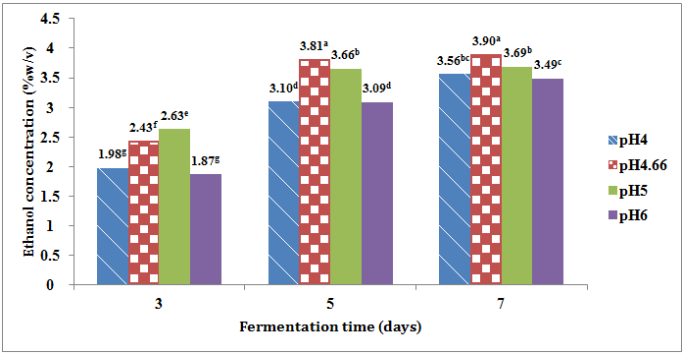

Figure 11. Effects of fermentation time and pH on ethanol concentration

**Note: Value in the figure was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.*

After 5 and 7 fermentation days, the V2 isolate produced maximal ethanol concentrations in natural pH (4.66) of molasses medium (3.81% and 3.90% w/v, respectively). In pH 6 medium, the lowest ethanol concentration was obtained from V2 after 3 days of fermentation (1.87% w/v). The longer fermentation time was, the higher ethanol concentration got, but ethanol concentrations of 5 and 7 fermentation days were slightly different.

Ethanol concentrations of 5 and 7 fermentation days changed similarly: when pH decreased from 6 to 4.66, ethanol concentration increased, but when pH dropped to 4, ethanol concentration decreased. The V2 isolate produced highest concentration of ethanol in natural pH medium. It is illustrated

that pH of medium is one of the main factors effecting on the growth of yeasts.

When fermentation time increased, ethanol concentration raised correlatively. At the first stage, yeasts improved biomass, ethanol fermentation rate was slow. Thus, V2 produced low concentration of ethanol in 3 first days. After 5 and 7 days, ethanol concentrations were not significantly different due to two causes: (1) death phase of yeasts made fermentation rate slow, and (2) produced ethanol inhibited fermentation of yeasts.

Changes of pH in fermentation

In fermentation, pH of medium had a trend to come to optimal pH level suitable to yeast fermentation (Figure 12).

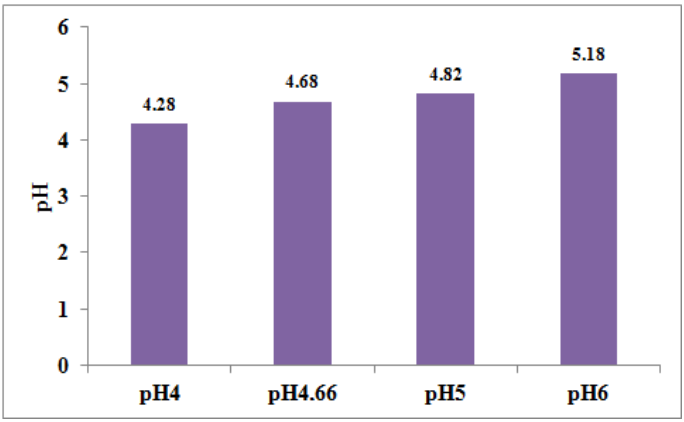

Figure 12. pH of medium after fermentation

In natural molasses medium, the change of pH was not significant (from 4.66 to 4.68). In other treatments, pH changed to the optimal value. In this study, optimal pH value for ethanol fermentation of V2 isolate was about 4.66.

3.6. Identification of the V2 yeast isolate

On PGY agar medium, V2 isolate could form creamy white dry serrated, wrinkled, irregular dome colonies. The colonies were about 3 – 3.5 mm of size and 0.1 mm of height (Figure 13).

Microscope images showed that the diameter of the V2 cells ranged from 2.5 to 6 µm, and the cells' shape varied from oval to ellipsoidal. The V2 cells were single budding cells (Figure 14).

Figure 13. V2 colonies on PGY agar medium

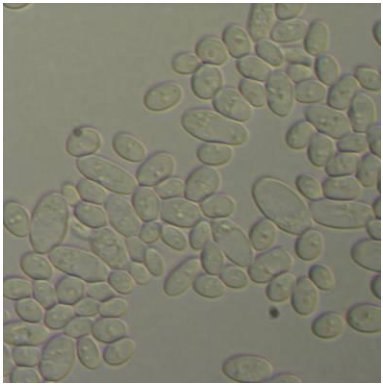

Figure 14. V2 cells under microscope X100

The selected sequences were homologous with ITS1, 5.8 rDNA, and ITS2 sequences of *Pichia kudriavzevii* species with 100% homogeneous level. Thus, V2 belonged to the *Pichia kudriavzevii*.

Pichia kudriavzevii cells could assimilate sugars like glucose, sucrose, galactose, fructose, and mannose. The yeast cells could tolerate up to 40% glucose and 5% NaCl concentrations but their growth was inhibited at 1% acetic acid

and 0.01% cyclohexamide concentrations. *Pichia kudriavzevii* produced about 35 and 200% more ethanol than the conventional *Saccharomyces cerevisiae* cells at 40 and 45ºC, respectively (Oberoi et al., 2012).

According to Yuangsaard et al. (2013), *Pichia kudriavzevii* can produce ethanol from cassava starch hydrolysate at a high temperature up to 45ºC, but the optimal temperature for ethanol production was at 40ºC.

4. CONCLUSIONS AND SUGGESTIONS

4.1. Conclusions

- Seven from 44 yeast isolates, notated as C2, CC, BM2, V2, V3, L04-2, and L07-2 could grow at 43ºC. Among them, BM2 could grow at 44ºC.

- 38 yeast isolates could grow on 10% ethanol supplemented medium, and 25 isolates expressed their ethanol tolerant ability on medium containing 12% ethanol.

- Seven yeast isolates C2, CC, BM2, V2, V3, L04-2, and L07-2 were selected from 44 isolates due to their thermo- and ethanol tolerant ability.

- V2 yeast isolate had fast and strong fermentation ability in 2% glucose medium.

- V2 produced 2.92% (w/v) of ethanol in molasses medium at 40ºC. It was not significantly different from 35ºC (2.93%) and room temperature (2.91%) at the 95% confidence level.

- Favorable conditions for V2 to produce ethanol at 40ºC in molasses were determined as 10^5 cells per mL of inoculum level, 25ºBrix of sugar concentration, 5 fermentation days, and pH 4.66.

- Molecular analysis showed that V2 belonged to the *Pichia kudriavzevii*.

4.2. Suggestions

- Study on factors effecting on fermentation ability of yeasts in details: nitrogen content, $MgSO_4$ content…

- Testing ethanol ability of V2 yeast isolate in other kinds of medium: fruit juices, sugar cane juice…

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