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# ETHANOL PRODUCTION FROM MOLASSES AT HIGH TEMPERATURE BY THERMOTOLERANT YEASTS ISOLATED FROM COCOA

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**ABSTRACT**

*Thermotolerant ethanologenic yeasts have highly attracted many scientists due to the current challenges of increasing global temperature and the benefits associated with processing at high temperature as well as reducing cooling cost. In this study, 50 yeast strains were isolated from 35 different materials of cocoa. Based on characteristics of morphology, physiology and biochemistry, 50 yeast isolates were preliminarily classified into genera as Candida, Hanseniaspora, Kluyveromyces, Pichia, and Saccharomyces. There were 17/50 isolates showing their ethanol tolerant ability up to 12% (v/v) of ethanol and 23/50 isolates could grow at 45°C. The two selected isolates (CT2.5D and PD1.6H) were tested for the ethanol fermentation from molasses (22°Brix) at different temperatures. The results of ethanol concentrations obtained as follow: 7.36% (v/v) at 30°C, 4.15% (v/v) at 40°C, 1.45% (v/v) at 42°C by CT2.5D and 7.4% (v/v) at 30°C, 2.93% (v/v) at 40°C, 1.43% (v/v) at 42°C by PD1.6H. The identification results showed that CT2.5D and PD1.6H were Saccharomyces cerevisiae.*

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## 1 INTRODUCTION

Nowadays, the demand of industrial ethanol with high purity has become higher than ever because it is believed that ethanol is a safe bio-fuel and has the possibility to replace fossil fuels. Temperature is one of factors significantly affecting the fermentation ability of yeast. In summer, temperature of the South of Vietnam dramatically increases and it tends to go up with the global warming. Thus, selection of thermotolerant yeast strains is an essential solution for dealing with climate change. Furthermore, high temperature fermentation has several advantages such as cost reduction associated with cooling fermentation vats, obtaining higher yields in saccharification, continuous removal of ethanol, and risk reduction of bacterial contamination (Banat *et al*., 1998; Roehr, 2001; Limtong *et al*., 2007; Abdel-Banat *et al*., 2010). Therefore, the selection of thermotolerant yeast strains applied in ethanol production contributes to decrease the manufacturing expenses.

Cocoa, *Theobroma cacao*, is one among local plants located in the South and Western Central Highland of Vietnam. The recent global studies have concentrated essentially on microorganisms in cocoa fermentation in which yeast is significantly considered. Cocoa fermentation is a spontaneous microbiological process and yeast is the most dom-

inant and abundant microorganism at the onset of fermentation, beside of filamentous fungi, lactic acid bacteria, acetic acid bacteria and spore-forming bacteria (Nielsen *et al*., 2007; Dung *et al*., 2013). In the cocoa fermenting process, the temperature always reaches to 45-50℃ so the yeasts involved in this process are able to grow and ferment at high temperatures (Ardhana and Fleet, 2003; Schwan and Wheals, 2004). The aim of the present study is to isolate yeasts from cocoa and study their ethanol fermentation from molasses at high temperature.

## 2 MATERIALS AND METHODS

### 2.1 Materials and media

– There were 35 samples of cocoa collected in 7 different places in Can Tho city, Tien Giang and Ben Tre provinces, including cocoa leaves, flowers, fruits, fruit peels after 2 days of seeding, dried fruit peels, dried fermented beans, fermented beans after 1, 4 and 6 days.

– Microbiological media: Yeast extract 5 g/L, peptone 5 g/L, D-glucose 20 g/L (YPD broth), and 20 g/L of agar was added for YPD agar medium.

### 2.2 Research Methods

#### *2.2.1 Isolation of yeast strains*

Five grams of cocoa samples were added in 50 mL of YPD broth and incubated at 35℃, 150 rpm for 24-48 hours. Yeast colonies were selected and streaked on YPD agar, and incubated at 35°C. Purified yeast cultures were kept on YPD agar slants and stored at 4°C.

#### *2.2.2 Examination of morphological, physiological and biochemical characteristics*

*Morphological characteristic:* Shapes and dimension of colonies and cells were observed by microscope and recorded.

*Saccharose and maltose fermentation ability:* Suspension of 24 h inoculated yeast cells was inoculated into Durham tubes containing 2% (w/v) of saccharose or maltose solution and incubated at 35°C. The gas production by accumulating $CO_2$ in the inner Durham tubes was measured at every 2-hour intervals up to 24 hours.

*Urea anabolism:* Yeast isolates were inoculated into tubes containing 3 mL of Stuart Urea broth, the color change of medium was recorded after incubating at 35°C for 48 hours.

*Gelatin liquefaction:* Yeast isolates were inoculated into tubes containing 3 mL gelatin medium and then incubated at 30°C in 48 hours. Tubes were immediately cooled down and recorded for gelatin liquefaction.

#### *2.2.3 Screening for ethanol fermentation ability of yeast isolates*

Test was carried in Durham tubes containing 2% (w/v) glucose solution and incubated at 35°C. The gas production by accumulating $CO_2$ in the inner Durham tubes was measured at every 2-hour intervals up to 24 hours.

#### *2.2.4 Testing ethanol- and thermo-tolerant ability of yeast isolates*

*Ethanol tolerance*: Yeast isolates were streaked onto YPD agar supplemented with 3, 6, 9, and 12% (v/v) of ethanol and then incubated at 35°C for 1-4 days. The formation of colonies appeared on medium was recorded.

*Thermo-tolerance:* Yeast isolates were streaked onto YPD agar and then incubated at 37°C, 40°C, 43°C, and 45°C for 1-4 days. The formation of colonies appeared on medium was recorded.

#### *2.2.5 Testing ethanol fermentation from molasses at different temperatures*

The selected yeast isolates were inoculated into YPD broth and incubated for 24 hours. Yeast cell suspension (1 mL, $10^6$ cells/mL) was inoculated into 99 mL of 22°Brix molasses medium and then incubated at 30°C, 35°C, 37°C, 40°C and 42°C. The results of pH, °Brix and ethanol concentration were determined.

#### *2.2.6 Identification of selected yeast isolates*

The DNA of selected yeast isolates were extracted and used for nucleotide sequencing. The divergent D1/D2 domain of the LSU rRNA gene was amplified with specific primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG) (O'Donnell, 1993). Nucleotide sequences were aligned and compared with the database on NCBI website. The identification was done at Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan.

#### *2.2.7 Analytical methods and statistical analysis*

pH was measured with a digital pH meter (Sartorius PB-20). Total dissolved solids content of saccharified liquid (°Brix) was measured by manual refractometer (FG102/112, Euromex-Holland). Alcoholic content was determined using the distillation method. Ethanol yields: Yps = ethanol produced/SCg*100, where SCg = SC x SG x 10 (SCg: sugar concentration in g/L, SC: sugar concentration in °Brix, SG: specific gravity under different °Brix, 10: conversion factor) (Bruce *et al*., 1995). Experimental data were statistically analyzed using Statgraphics Centurion XV, Manugistics Inc., USA.

## 3 RESULTS AND DISCUSSION

### 3.1 Morphological, physiological and biochemical characteristics of yeast isolates

There were 50 yeast strains isolated and purified from 35 cocoa samples. Based on cell morphology, physiological and biochemical characteristics, 50 yeast isolates were divided into 6 groups (according to the classification study of Barnett *et al*., 1983; Kurtzman *et al*., 2011; Pham, 2006) (Table 1). They were preliminarily classified into genera as *Candida, Hanseniaspora, Kluyveromyces, Pichia*, and *Saccharomyces.*

**Table 1: Summary of yeast cell shape and preliminary classification**

| Group | Cell conformation | Name of yeast isolate | No. of isolate | Preliminary classification |
|---|---|---|---|---|
| 1 | Spherical | CL1.3A, CL2.1B, CL2.8C, CT1.3B, CT2.5A, CT2.5D, PD1.6H | 7 | *Saccharomyces* |
| 2 | Large ovoid | CL1.2A, CL1.3C, CL2.1A, CL2.4B, CT1.2A, CT1.2B, CT2.2A, CT2.4A, CT2.4G, PD1.6B | 10 | *Saccharomyces* |
| 3 | Small ovoid | CL1.2G, CL2.2A, CL2.2E, CT2.3B, PD1.1B | 5 | *Pichia* |
| 4 | Apiculate ellipse | CL2.7B, CL2.8E, CT1.1B, MC1.3C, PD1.2A, PD1.2B, PD1.3A | 7 | *Hanseniaspora* |
| 5 | Elongated ellipse | CL1.5G, CL2.4A, CL3.4A, CL3.4F, CT2.2C, MC1.4C, PD1.4A, PD1.4B, PD1.5C, PD1.7B | 10 | *Kluyveromyces* |
| 6 | Cylinder | CL2.5A, CL2.5D, CL2.6A, CL2.7A, CL3.3A, CL3.3B, CT2.3D, PD1.1D, PD1.3B, PD1.5D, PD1.7A | 11 | *Candida* |
| Total number | | | 50 | |

*Colony morphology of yeast isolates:* The colonies of yeast isolates grew onto medium surface by 1-4 mm in diameter and 0.1 mm in height. Some colonies had smooth surface while others owned rough one. The margin of colonies was also diverse such as entire, undulate, serrated, filiform and lobate form. The colonies of yeast isolates had creamy white and white color. Some representative colonies were illustrated in Figure 1.

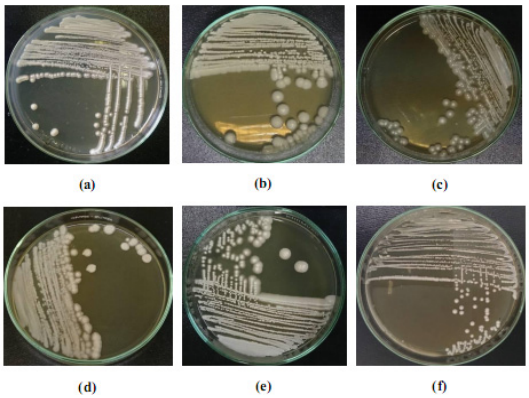



**Fig. 1: Some representative colony forms of yeasts**

**Note: (a): PD1.1D, (b): CL3.3B, (c): CL1.2A, (d): CL2.5A, (e): CL1.3C, (f): PD1.6H*

*Cell morphology of yeast isolates:* Cell shape of yeast isolates was diverse but they fell into 4 main forms including spherical, ovoid, ellipse and cylinder shape. There was also a difference between yeast isolates in term of dimension but generally cells' length was about 3-11 µm and width of cells was approximately 2-6 µm.

*Budding and endospore formation:* Yeast isolates in group 1, 2, 3, 5 and 6 were multilateral budding while isolates in group 4 were bipolar budding. All of yeast isolates had the ability of sporulation in nutritional deficiency condition. Although the endospore dimensions between yeast isolates were not homogeneous, the number of endospore inside the cell was similar with 1-4 endospores. Yeast tends to form endospores so as to survive in harsh living conditions because these endospores basically were constructed by a solid wall with good ability of thermotolerance (Neiman, 2005).

*Saccharose and maltose fermentation ability:* 24/50 yeast isolates were capable of using saccharose as carbon sources for fermentation after 24 hours. Most of strains in groups 1, 2, 4, 5 and 6 were capable of fermenting saccharose while all members in group 3 could not ferment this sugar. Most yeast isolates (43/50) were able to ferment maltose. Sugar fermentation capacity of yeast strains is variety that is assessed through the ability to generate $CO_2$ in fermentation (Kurtzman *et al*., 2011). Thus, testing the ability of sugar consumption is one of criteria for classification of yeast and also the step to pick out appropriate yeast strains for fermenting of different substrates.

*Urea assimilation:* 5/50 yeast isolates including CL3.4A, CT1.1B, CL3.3A, PD1.1B and CL3.3A were able to employ urea as a source of nitrogen. All yeast isolates in groups 1 and 2 were not capable of urea resolution. The majority of members belonging to Acogenous species less likely resolved of urea while species of the Basidiomycetous genera had this capability (Barnett *et al*., 1983).

*Gelatin liquefaction:* 4/50 yeast isolates consisting of CL1.2G, CL3.4A, CT2.5D and PD1.5D possessing gelatin liquefaction capacity by gelatinase. This ability of yeasts was also often associated with protease activity but just some yeast species were capable of produce protease (Kurtzman *et al*., 2011).

### 3.2 Glucose fermentation ability of yeast isolates

The results showed that 43/50 yeast isolates performing to be able to ferment glucose after 24 h. Eight yeast isolates including CL2.7A, CT1.3B, CT2.5D, PD1.1D, PD1.3B, PD1.5D, PD1.6B and PD1.6H had faster and stronger fermentation capacity. These 8 isolates could show the maximum gas production in the inner Durham tubes (30 mm) within 10 h of fermentation and were not statistically significance different at 95% confidence level (data not shown).

### 3.3 Ethanol- and thermo-tolerant ability of yeast isolates

*Ethanol tolerant ability:* When increasing the ethanol concentration in culture medium, the number of yeast colonies developed in medium gradually decreased, this can be explained for causing affect to the yeast growth. There were 17/50 isolates showing their ethanol tolerant ability up to 12% (v/v) of ethanol concentration. Among 8 yeast isolates giving the active fermentation capacity (CL2.7A, CT1.3B, CT2.5D, PD1.1D, PD1.3B, PD1.5D, PD1.6B and PD1 .6H) noted in the previous test of glucose fermentation, 2/8 yeast isolates (PD1.5D and PD1.6H) were capable of good colony formation on medium supplemented by 12% (v/v) ethanol and 4/8 strains (CL2.7A, CT2.5D, PD1.1D and PD1.6B) grew in medium containing 9% (v/v) ethanol. Remaining two isolates were CT1.3B and PD1.3B tolerated at 6% (v/v) of ethanol after 48 hours of incubation.

*Thermotolerant ability*: All yeast isolates could grow well in a range of temperatures at 30-37°C. There were 23/50 yeast strains showing the high heat resistance by growing at 45°C; however, the number of yeast colonies generally decreased when increasing the incubation temperature. Six isolates (CL2.7A, CT1.3B, PD1.1D, PD1.3B, PD1.5D, PD1.6B) could grow at temperature of 45°C while CT2.5D and PD1.6H were able to grow at 40°C after 48 h of incubation.

### 3.4 Ethanol fermentation by selected yeast isolates at high temperature

The results of ethanol fermentation ability of 8 selected yeast isolates (CL2.7A, CT1.3B, CT2.5D, PD1.1D, PD1.3B, PD1.5D, PD1.6B and PD1.6H) were presented in Table 2. The highest ethanol production was found in PD1.6H and CT2.5D isolates were 7.40% and 7.36% (v/v) in ethanol concentration and 66.41% and 64.76% in ethanol yield at 30°C, respectively.

**Table 2: Ethanol production capacity of 8 selected yeast isolates at 30 and 35°C**

| Yeast isolate | Incubation at 30°C | | Incubation at 35°C | |
|---|---|---|---|---|
| | Ethanol concentration (% v/v at 20°C) | Ethanol yield ($Y_{ps}$, %) | Ethanol concentration (% v/v at 20°C) | Ethanol yield ($Y_{ps}$, %) |
| CL2.7A | $2.55^{c}$ | $52.13^{bc}$ | $1.86^{ef}$ | $41.33^{c}$ |
| CT1.3B | $2.81^{c}$ | $59.83^{ab}$ | $2.71^{cd}$ | $49.81^{b}$ |
| CT2.5D | $7.36^{a}$ | $64.76^{a}$ | $7.20^{a}$ | $64.68^{a}$ |
| PD1.1D | $2.14^{d}$ | $43.48^{cd}$ | $2.14^{de}$ | $43.48^{bc}$ |
| PD1.3B | $3.08^{b}$ | $56.41^{ab}$ | $2.36^{cde}$ | $48.83^{b}$ |
| PD1.5D | $1.30^{e}$ | $33.66^{d}$ | $1.23^{f}$ | $31.97^{d}$ |
| PD1.6B | $3.15^{b}$ | $61.77^{ab}$ | $2.87^{c}$ | $44.19^{bc}$ |
| PD1.6H | $7.40^{a}$ | $66.41^{a}$ | $6.37^{b}$ | $63.25^{a}$ |

**Note: Values in the table were the average values of triplication. The average values with the same letter were not significantly different at the 95% confidence level*

The ethanol concentrations produced by some isolates were not significantly different to another with 95% of confidence level such as CT2.5D and PD1.6H (7.36% and 7.40% v/v), PD1.3B and PD1.6B (3.08% and 3.15% v/v), CL2.7A and CT1.3B (2.55% and 2.81% v/v). According to research of Ueno *et al*. (2002), thermotolerant yeasts were capable of generating maximal ethanol con-

centration about 7.00-7.20% (w/v) at 30°C with nutritional substrate containing 15% (w/v) glucose. On the other hand, in the study of ethanol fermentation with molasses conducted by Lin *et al*. (2012), the maximal ethanol yield of *S. cerevesiae* BY4742 was about 61.93% at 30°C. Compared with the results obtained in the present study, all selected yeast strains could display well their ability of ethanol fermentation from molasses as a substrate, especially two isolates CT2.5D and PD1.6H.

At 35°C, there was a clearly difference in ethanol concentration among yeast isolates, ethanol concentrations and ethanol yields of all isolates were also lower. Growth of yeast cells also went up when temperature increased at a certain level in tolerant threshold of yeast but at that time the amount of ethanol production would be almost reduced. Moreover, the activity of intracellular enzyme in yeast cells was diminished at higher temperature leading to low ethanol production. CT2.5D and PD1.6H still displayed good fermentation ability when reaching 64.68% and 63.25% in ethanol yield at 35°C, respectively. Results of Periyasamy *et al*. (2009) showed that the maximal ethanol yield produced by *S. cerevisiae* with molasses at 35°C was about 53%. CT2.5D and PD1.6H isolates were selected for testing of ethanol production at higher temperatures (37°C, 40°C and 42°C) and the results were presented in Table 3.

**Table 3: Ethanol production of CT2.5 and PD1.6H isolates at different temperatures**

| Isolate | Ethanol | Temperature of incubation | | | | |
|---|---|---|---|---|---|---|
| | | 30°C | 35°C | 37°C | 40°C | 42°C |
| CT2.5D | Ethanol concentration (% v/v at 20°C) | 7.36[a] | 7.20[a] | 6.31[b] | 4.15[c] | 1.45[d] |
| | Ethanol yield ($Y_{ps}$, %) | 64.76[a] | 64.68[a] | 60.33[a] | 60.26[a] | 39.62[b] |
| PD1.6H | Ethanol concentration (% v/v at 20°C) | 7.40[a] | 6.37[b] | 6.26[b] | 3.92[c] | 1.43[d] |
| | Ethanol yield ($Y_{ps}$, %) | 66.41[a] | 63.25[a] | 62.51[a] | 60.06[a] | 41.99[b] |

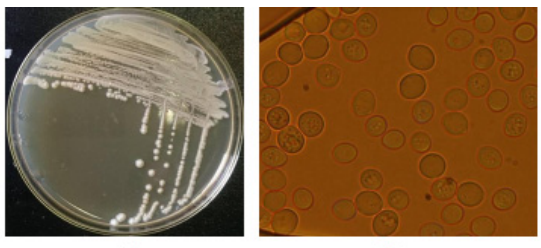
**Note: Values in the table were the average values of triplication. The average values with the same letter in the same row were not significantly different at the 95% confidence level*

Generally, both PD1.6H and CT2.5D were able to ferment molasses as substrate at high temperatures and had almost the same trend in fermentation, in which ethanol concentrations were decreased when temperatures were increased. Furthermore, no difference in ethanol yield with increase of temperature from 30-40°C was observed because both two isolates could withstand well up to 40°C. The produced ethanol concentrations were lower at 40°C (3.92-4.15% v/v) in comparison with the results obtained at 37°C (6.26-6.31% v/v) whereas the ethanol yields were not significantly decreased. This performance was due to the effect of temperature on the ethanol productivity, indicating the ethanol production by yeast was somehow limited at the certain high temperatures. At 42°C, the ethanol yields and concentrations produced by both isolates were significantly reduced, 1.45% (v/v) of ethanol concentration with 39.62% of ethanol yield created by CT2.5D and 1.43% (v/v) of ethanol concentration with 41.99% of ethanol yield produced by PD1.6H. At high temperature, the accumulation of intracellular ethanol of yeast cells was rose and stalled yeast growth so fermentation activity of yeast was also inhibited leading to lower ethanol produced (D'Amore *et al*., 1990).

### 3.5 Identification of selected yeast isolates

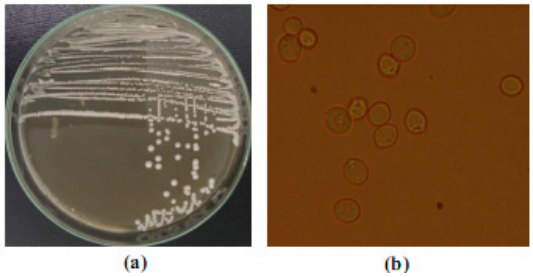
Yeast isolate CT2.5D (Figure 2) and isolate PD1.6H (Figure 3) were almost the same in term of colony morphology and cell characteristic. Colony morphology were 2-3 mm in diameter, 0.1 mm in height, convex growing onto the surface of culture medium, smooth surface, entire edge and cream color. Cell characteristics were in size of 5x5 µm, spherical shape, budding from multiple directions and forming 1-4 endospores in nutritional deficiency condition.

The results of sequencing and comparing the D1/D2 domain of the LSU rRNA gene to gene database on NCBI website showed that CT2.5D and PD1.6H were *Saccharomyces cerevisiae* with 100% of similarity.



(a) (b)

**Fig. 2: Colony (a) and cell (b) morphology of yeast isolate CT2.5D**



(a) (b)

**Fig. 3: Colony (a) and cell (b) morphology of yeast isolate PD1.6H**

*Saccharomyces cerevisiae* has been widely used as the most effective starter in alcoholic fermenting in industrial manufacture. *S. cerevisiae* was able to yield the ethanol concentration reaching 7.4% to 7.7% (w/v) in fermenting with molasses at room temperature. Also, this species was likely to grow in the range of high temperatures from 40-44°C (Sree *et al*., 1999; Addel-Fattah *et al*., 2000).

## 4 CONCLUSIONS

The diversity of yeast isolates purified from cocoa samples was examined, in which a number of ethanol- and thermo-tolerant ethanologenic yeasts was found. The feasibility of ethanol fermentation from molasses by the selected yeast isolates at high temperature was noticed, indicating the promising application of such isolates for the controlled ethanol production at high temperature.

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