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# Screening of thermotolerant yeasts for bioethanol production

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**Abstract** : The interest in bioethanol is increasing rapidly due to its several advantages as bio-fuel. The ethanol production at high temperature is very impressive since the process could reduce the cost of cooling system and contaminations during fermentation. The aims of this study are to screen thermotolerant yeasts that could produce ethanol at high temperature and study some important characteristics for ethanol production of isolated yeast strains. Fifty-five thermotolerant yeasts were isolated from 15samples of fermented fruit juice, fruits, soil, loog-pang and organic waste at 40˚C using YM broth supplemented with 0.01% chloramphenicol, 0.2% sodium propionate and 4% ethanol as enrichment media. All of the isolates were tested for their ability to produce ethanol by using Ebulliometer at 40˚C and 12 isolates were selected to study further with the highest ethanol produced at 4.8-5.2% from 12% glucose in the media after 48 hours of incubation. Spot test on YPD agar indicated that most of the isolates could grow at 42˚C and tolerate up to 30% of glucose when incubated at 40˚C. Moreover, all 12 isolates were studied for the suitable characteristics for ethanol production; foam formation, flocculation and dry film produced on the surface of liquid broth. The result showed that most of the isolates produced high amount of foam at 40˚C in both shaking and static condition. Almost all of the isolates also produced dry film on the surface of the media when incubated in static condition at 40˚C. Only one isolate showed flocculation when incubated at both 40 and 42˚C. This isolate will be initiated to pilot scale alcoholic fermentation for the next step.

**Keywords :** thermotolerant yeasts, ethanol production, bio-fuel

## Introduction

Recently, the use of bioethanol as alternative biofuel is increasing rapidly around the world due to the shortage of petroleum fuel, pollution and the accumulation of Carbon dioxide ($CO_2$) in the atmosphere (Badger, 2002). Therefore, bioethanol is one of the promising alternative energy that releases low amount of greenhouse gas (GHG) in combustion process (Prasad et al., 2007).

*Saccharomyces cerevisiae* is commonly used in ethanol production, especially in industrial scale. This yeast strain has the optimal temperature of growth at 25-30˚C and can also produce high percent of ethanol (Lin and Tanaka, 2006). However, the temperature in the fermenter will increase instantly in the fermentation process. Moreover, in tropical countries including Thailand, the average temperature can be up to 40-60˚C during the day (Hacking et al., 1984) which results in the cost of cooling system to control the fermenter temperature into the range of 30-35˚C for the whole fermentation process. Therefore, yeast strain used in ethanol production should possess the ability to grow and produce ethanol at high temperature (Limtonget al., 2007). Screening of the thermotolerant yeasts for ethanol production which has drawn a lot of attention recently can result in the effective ethanol production in which including reducing of contamination of other microbes and increasing the rate of fermentation.

There are only few yeast species which can produce ethanol and grow at the temperature above 40°C, however, it has been reported that *Kluyveromyces marxianus* IMB1, IMB2, IMB3, IMB4 and IMB5 could grow at 45-52°C and could produce ethanol at 45-50°C (Banat et al., 1992).

This research focused on screening for thermotolerant yeasts that show the ability to grow and produce ethanol at 40°C and above, especially for ethanol production in tropical countries.

## Methodology

**1. Samples**

Samples were collected from different locations indicated in table 1.

**2. Screening of thermotolerant yeasts**

Five grams of samples were enriched in 100-ml YM broth (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% glucose) supplemented with 0.01% chloramphenicol, 0.2% sodium propionate and 4% ethanol. The samples were incubated at 40°C in shaking condition 150 rpm for 48 hours before 10-fold serial dilution and spread plate at $10^{-2}$–$10^{-6}$were used to isolate yeast colonies on YM agar (YM and 1.5% agar). After that, the yeast cells were confirmed under microscopic observation and colony morphology was also studied. All isolates were tested for the thermotolerant growth by streaking on the YPD agar before incubating at 40 and 45°C for 48 hours.

**3. Ethanol production**

All yeast isolates were cultivated in 10-ml YPD broth (1% yeast extract, 2% peptone, 2% glucose) at 40°C for 18 hours as starter. The cells were transferred to 100-ml YP broth (1% yeast extract, 2% peptone) with 12% glucose in Erlenmeyer flasks and initial OD 600 nm was adjusted to 0.1. All flasks were incubated at 40°C in shaking condition 150 rpm for 9 hours before moved to static condition until 48 hours of incubation. OD 600 nm and ethanol for each sample were measured using Spectrophotometer and Ebulliometer, respectively. The yeast isolates that showed high ethanol production were selected to study for some important characteristics.

**4. Characteristics study**

Spot test was used to study cell tolerance to elevated temperature at 40 and 42°Candhigh glucose concentration; 10%, 20% and 30% incubated at 40°C.Other desirable characteristics; foam formation, flocculation and dry film on the surface were also observed in YPD broth.

## Results and Discussion

**1. Screening of thermotolerant yeasts**

Fifty-five isolates were screened from all 15 samples (Table 2). The morphology of the yeast colonies was also observed (Table 3). Some of the colonies isolated from the same samples showed the same morphology, however, there are a vast majority of differences in colonies and they were all collected for future studies. A lot of isolates showed rough morphology which is normally associated with pseudohyphal growth and sedimentation of cells during fermentation. Mostly, the yeasts with such morphology are non-Saccharomyces strains and the prevalence of these yeasts could be affected by oxygen (Basso et al., 2008).

Moreover, after incubating all yeast isolates at 40 and 45°C for 48 hours and observed the ability to grow, the result showed that all the isolates could grow very well at 40°C but only 6 isolates grew rapidly at 45°C which are PA1, PA2, PA3, PA4, PPA1 and PPA2 from pineapple samples. In 2007, *K. marxianus* DMKU 3-1042 which is the yeast strain that grow rapidly at 45°Cwas isolated from soil and water samples from sugar cane plantations and

sugar factoriesin Thailand and showed the ability to produce high ethanol from sugar cane juice media at 37°C (Limtonget al., 2007).

**2. Ethanol production**

The ethanol produced from each isolate in 12%glucose YPD broth was measured by Ebulliometer (Table4). The result showed that the highest ethanol produced is 5.2% from 7 isolates which are OW6, OW2/1, LC2, LC4, LC5, LG1 and DC6/2.There were also 10 isolates that showed high ethanol production at 5% which are HK1/1, HK2/5, OW1, OW2, MT1/1, MT1/2, G1, LP3, CORN1 and MY1.Nevertheless, the isolates from pineapple which could grow well at 45°C were able to produce only 0.8-3% of ethanol.

**3. Characteristics study**

All 12 selected isolates were studied some characteristics compared with Ethanol Red which is the commercial thermotolerant yeast used in Europe and North America that shows the ability to produce high ethanol at 30-40°C (Felixet al., 2014). The spot test showed that all of the isolates could grow well up to 42°C. Furthermore, the result showed that they could grow in the media contained 30% glucose which indicated the osmotic tolerance, however, CORN1 which was one of the isolates from corn sample could not grow well when compared to the others (Figure 1). The yeasts that can tolerate to high osmotic pressure are valuable in the ethanol industries because sugar concentration significantly changes during fermentation process and the cells are affected by this stress (Silva et al., 2013).

All the isolates showed to produce high amount of foam. Dry film on the surface of media were also observed in most of the isolates except LP3 (Table 5) which is the only one isolate that flocculated when incubated at 40 and 42°C. The Ethanol Red as control showed the same properties as LP3. Both yeast floatation and flocculation often occur together and reduce the contact of cell and substrate which will prolong the fermentation time and result in high sugar residual left in bioreactor However, it can benefit the continuous fermentation of ethanol in cell recycling process (Basso et al., 2008).

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**Table 1.** List of samples, number of samples and locations

| Types of Samples | Number | Locations |
|---|---|---|
| Fruits | 6 | -Muang, Chiang Mai |
| Soil | 4 | -Muang, Chaing Mai |
| Organic waste | 1 | -Chaing Mai University, Chiang Mai |
| Loog-pang | 2 | -Denchai, Phrae and Muang, Lumpang |
| Fermented fruit juice | 2 | -Muang, Chaing Mai |

**Table2.** List of isolates from different samples

| Samples | Number of isolates | Isolates |
|---|---|---|
| **Fruits** | | |
| -Mangosteen | 2 | MGT1, MGT2 |
| -Grape | 1 | G1 |
| -Pineapple | 6 | PA1, PA2, PA3, PA4, PPA1, PPA2 |
| -Lychee | 5 | LC1, LC2, LC3, LC4, LC5 |
| -Corn | 2 | CORN1, CORN2 |
| -Long-gong | 2 | LG1, LG2 |
| **Soil** | | |
| -Soil from HuayKaew waterfall | 8 | HK1/1, HK1/2, HK1/3, HK2/1, HK2/2, HK2/3, HK2/4, HK2/5 |
| -Soil from Montatarn waterfall | 4 | MT1/1, MT1/2, MT2/2, MT2/4 |
| **Organic waste** | | |
| -Organic waste | 13 | OW1, OW2, OW3, OW4, OW5, OW6, OW7, OW1/1, OW1/2, OW1/3, OW2/1, OW2/2, OW2/3 |
| **Loog-pang** | | |
| -Loog-pang from Lumpang | 5 | LP2, LP3, LP4, LP5, LP6 |
| -Loog-pang from Phrae | 5 | DC6/1, DC6/2, DC6/3, DC6/4, DC6/5 |
| **Fermented fruit juice** | | |
| -Gooseberry fermented fruit juice | 1 | MY |
| -Roselle fermented fruit juice | 1 | KJ |

**Table 3.** Colony morphology

| Isolates | Surface | Form | Margin | Elevation |
|---|---|---|---|---|
| MGT1 | smooth | circular | entire | convex |
| MGT2 | rough, rugose in the middle | irregular | undulate | umbonate |
| G1 | rough, rugose in the middle | irregular | undulate | umbonate |
| PA1 | smooth | circular | entire | convex |
| PA2 | smooth | circular | entire | convex |
| PA3 | smooth | circular | entire | convex |
| PA4 | smooth | circular | entire | convex |
| PPA1 | smooth | circular | entire | convex |
| PPA2 | smooth | circular | entire | convex |

**Table 3.** Colony morphology (continue)

| Isolates | Surface | Form | Margin | Elevation |
|---|---|---|---|---|
| LC1 | rough | irregular | undulate | umbonate |
| LC2 | rough, rugose in the middle | irregular | undulate | umbonate |
| LC3 | rough | irregular | undulate | umbonate |
| LC4 | rough | irregular | undulate | umbonate |
| LC5 | smooth | irregular | undulate | flat |
| CORN1 | rough | irregular | undulate | umbonate |
| CORN2 | smooth | circular | entire | convex |
| LG1 | rough, rugose in the middle | irregular | undulate | umbonate |
| LG2 | rough | irregular | undulate | flat |
| HK1/1 | rough | irregular | undulate | flat |
| HK1/2 | rough, rugose in the middle | irregular | undulate | umbonate |
| HK1/3 | rough, rugose in the middle | irregular | undulate | umbonate |
| HK2/1 | smooth | circular | entire | convex |
| HK2/2 | concentrically ringed | irregular | undulate | flat |
| HK2/3 | rough | irregular | undulate | flat |
| HK2/4 | concentrically ringed | irregular | undulate | flat |
| HK2/5 | rough, rugose in the middle | irregular | undulate | umbonate |
| MT1/1 | rough | irregular | undulate | flat |
| MT1/2 | rough, rugose in the middle | irregular | undulate | umbonate |
| MT2/2 | smooth | circular | entire | convex |
| MT2/4 | smooth | circular | entire | convex |
| OW1 | rough | irregular | undulate | raise |
| OW2 | rough, rugose in the middle | irregular | undulate | umbonate |
| OW3 | rough | irregular | undulate | raise |
| OW4 | rough, rugose in the middle | irregular | undulate | flat |
| OW5 | rough | irregular | undulate | raise |
| OW6 | rough | irregular | undulate | umbonate |
| OW7 | smooth | circular | entire | convex |
| OW1/1 | rough | irregular | undulate | flat |
| OW1/2 | concentrically ringed | irregular | undulate | flat |
| OW1/3 | rough, rugose in the middle | irregular | undulate | umbonate |
| OW2/1 | rough, rugose in the middle | irregular | undulate | umbonate |
| OW2/2 | rough, spot in the middle | irregular | undulate | umbonate |
| OW2/3 | rough, rugose in the middle | irregular | undulate | umbonate |
| LP2 | smooth | circular | entire | convex |
| LP3 | smooth | circular | entire | convex |
| LP4 | smooth | circular | entire | convex |
| LP5 | smooth | circular | entire | convex |

**Table 3.** Colony morphology (continue)

| Isolates | Surface | Form | Margin | Elevation |
|---|---|---|---|---|
| LP6 | smooth | circular | entire | convex |
| DC6/1 | concentrically ringed | irregular | undulate | flat |
| DC6/2 | concentrically ringed | irregular | undulate | flat |
| DC6/3 | rough, rugose in the middle | irregular | undulate | umbonate |
| DC6/4 | rough | circular | undulate | flat |
| DC6/5 | rough | irregular | undulate | raise |
| MY1 | rough | irregular | undulate | raise |
| KJ | smooth | circular | entire | convex |

**Table 4.** Ethanol production measured by Ebulliometer

| Isolates | Ethanol (%) | Isolates | Ethanol (%) | Isolates | Ethanol (%) |
|---|---|---|---|---|---|
| MGT1 | 2.4 | HK1/2 | 4.8 | OW1/2 | 4.8 |
| MGT2 | 4.8 | HK1/3 | 4.6 | OW1/3 | 4.8 |
| G1 | 5 | HK2/1 | 4.5 | OW2/1 | 5.2 |
| PA1 | 3 | HK2/2 | 4.6 | OW2/2 | 4.8 |
| PA2 | 1.7 | HK2/3 | 5 | OW2/3 | 4.6 |
| PA3 | 1.8 | HK2/4 | 4.6 | LP2 | 2.6 |
| PA4 | 1.9 | HK2/5 | 4.8 | LP3 | 5 |
| PPA1 | 2.6 | MT1/1 | 5 | LP4 | 0.8 |
| PPA2 | 0.8 | MT1/2 | 5 | LP5 | 3 |
| LC1 | 0 | MT2/2 | 4.8 | LP6 | 0.4 |
| LC2 | 5.2 | MT2/4 | 4.8 | DC6/1 | 4.8 |
| LC3 | 4.6 | OW1 | 5 | DC6/2 | 5.2 |
| LC4 | 5.2 | OW2 | 5 | DC6/3 | 4.8 |
| LC5 | 5.2 | OW3 | 4.3 | DC6/4 | 4.8 |
| CORN1 | 5 | OW4 | 4.6 | DC6/5 | 4.6 |
| CORN2 | 2.4 | OW5 | 3.8 | MY1 | 5 |
| LG1 | 5.2 | OW6 | 5.2 | KJ | 2.6 |
| LG2 | 3.8 | OW7 | 1.4 | | |
| HK1/1 | 5 | OW1/1 | 5.2 | | |

**Table 5.** Characteristics observed in YPD broth

| Isolates | Foam formation[a,b] | Dry film on surface[a] | Flocculation at40°C [b] | Flocculation at 42°C |
|---|---|---|---|---|
| HK1/1 | ++++ | + | - | - |
| HK2/5 | ++++ | + | - | - |
| OW6 | +++ | + | - | - |
| OW1/1 | ++++ | + | - | - |
| MT1/1 | +++ | + | - | - |
| G1 | ++++ | + | - | - |
| LP3 | ++ | - | + | ++ |
| CORN1 | ++++ | + | - | - |
| LC2 | ++ | + | - | - |
| LG1 | ++++ | + | - | - |
| MY1 | +++ | + | - | - |
| DC6/2 | +++ | + | - | - |
| Ethanol Red | + | - | + | + |

[a] Static condition

[b] Shaking condition

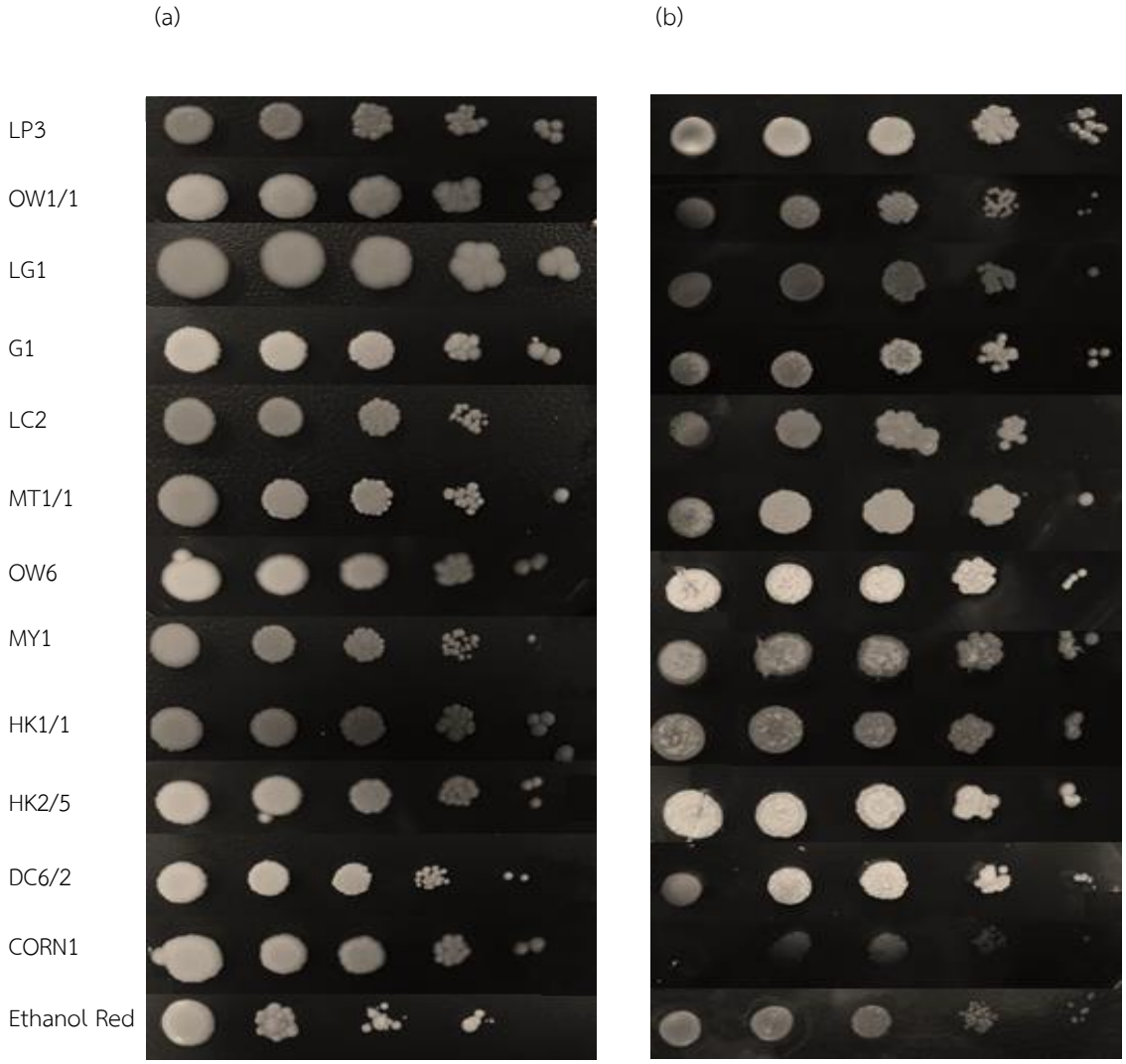


**Figure 1.** Spot test result (a) YPD agar at 42˚C for 48 hours (b) YPD agar with 30% glucose at 40˚C for 48 hours