Screening and Isolation of Thermotolerant Yeast for Ethanol Fermentation

Kannikar Charoensuk[1,*], Supranee Lauhkitikul[1], Narusan Wasikadilok[1], Boondarika Sumana[1], Pornthap Thanonkeo[2] and Mamoru Yamada[3, 4]

[1]Faculty of Agro-Industrial Technology, Rajamangala Univ.of Technology Tawan-ok,Chanthaburi campus, and [2]Faculty of Technology, Khon Kaen Univ., Thailand.

[3]Faculty of Agriculture, Yamaguchi Univ., and [4]Graduate School of Medicine, Yamaguchi Univ., Japan.

Abstract : The research attempted to screen and isolate yeast strains from soil in Chanthaburi, Thailand and Yamaguchi, Japan, which are able to grow under stressed conditions: i.e. mainly high temperature and high ethanol content, to support high temperature ethanol fermentation. A total of 12 isolated yeasts, designated as CK1-8, YUS5, YUS49, YUB34 and YUB49 which could grow in YP medium containing 15% w/v glucose under 37 °C were obtained and identified by 26S rRNA gene sequencing. Results obtained from this study revealed that CK1 was classified as Williopsis saturnus, CK3 as Candida sp., CK2 and CK4 as Zygosaccharomyces fermentati, and CK5-8, YUS5, YUS49, YUB34 and YUB49 as Pichia kudriavzevii. Interestingly, the first 8 strains isolated from Chanthaburi, Thailand and Yamaguchi, Japan, were able to grow with high glucose consumption rate at high temperature up to 40°C. Moreover, they could survive in YP medium containing 15% w/v glucose and 15 % v/v ethanol, and may be the potential candidates for high temperature ethanol fermentation.

Keywords : Thermotolerant yeast, ethanologenic microorganism, ethanol fermentation

*Corresponding Author : cc_kannikar@hotmail.com, Fax: (6639)307-270 (to) 25.

1. Introduction

Nowadays crude oil prices behave much as any other commodity with wide price swings in times of shortage or oversupply which have negative implications for the global economy (Mathew, [online]). The alternative energy comes to substitute for oil. In 2006, about 18 % of global final energy consumption came from renewable source such as ethanol or ethyl alcohol (C_2H_5OH); it is biodegradable, low in toxicity and causes little environmental pollution if spilt. However, the World Bank reported cause of increased ethanol production has contributed to the rise in food price, therefore, likely to be more difficult because of a complex of energy crisis and social factors. Thereby, increase the efficiency of bioethanol production processes by reduce the cost of ethanol production and/or increase the conversion rate of carbon sources to produce ethanol by use of high temperature fermentation process may be useful.

Since the ethanol fermentation process is exothermic, ethanologenic microorganisms seem to be exposed to heat stress in addition to other stresses such as low pH, high ethanol content, osmolarity, nutrient supply and etc., which may impact on cell growth, viability and also may cause stuck fermentation. We attempted to achieve high temperature ethanol fermentation with the advantages of the ethanologenic thermotolerant microorganisms, which are able to grow and produce high ethanol content at high temperature (Charoensuk *et al*., 2011) and decreased risk of contamination and also reduction in cooling costs (Babiker *et al*., 2010).

2. Materials and Methods

Materials: DNA sequencing kit (ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit) was purchased from Applied Bio Systems Japan (Kyoto, Japan). Oligonucleotide primers were synthesized by Proligo Japan (Tokyo). The other chemical were all of analytical grades.

Table 1 Primers used in this study

Name	Sequences
NL1	5′-GCATATCAATAAGCGGAGGAAAAG-3′
NL4	5′-GGTCCGTGTTTCAAGACGG-3′

2.1 Methods

Screening method; thermotolerant yeasts were isolated from soil in Chanthaburi, Thailand and Yamaguchi, Japan in September – December, 2010-2012. These isolates were screened on YP containing 15 % glucose, supplement with 0.2 % v/v acetic acid and 25 μg/ml Kanamycin to avoid bacterial growth, and incubated at 37 °C. All strains were compared their growth on YP containing 15 % glucose, supplement with 15 % v/v ethanol at 37 and 40 °C by replica plate technique. ***Isolation method***; the yeast strains were identified by morphological characterization and analysis of D1/D2 domain of 26S rDNA sequencing (Limtong *et al*., 2007). Briefly, whole genome from each isolate, which was grown in YPD medium for 24 h at 30 °C, was isolated by the hot phenol method (Sambrook *et al*., 2001) without RNAase treated, and *Lambda* DNA *HindIII* Marker was recommended

for sizing of their genomes. The D1/D2 domain was amplified by PCR from each whole genome with the universal primers NL1 and NL4 (Table 1) as follows: initial denaturation at 98°C for 10 sec. and 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 5 min, with a final extension at 72 °C for 5 min. The amplified DNA was concentrated, cleaned and sequenced in ABI sequencer at Yamaguchi University, Yamaguchi, Japan. The sequences were assembled and aligned with the blastn algorithm using the tool available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

Characterization of Isolated yeast strains; the isolated yeast strains were further characterized which their growth property in YP medium containing 2% glucose, 2% sucrose or 2% xylose and incubated under static condition at 37 °C for 48 h., and on YP agar medium containing 15 %v/v glucose and ethanol at 40 °C.

3. Results and Discussion

Screening of thermotolerant yeast from soil

In order to screening for thermotolerant yeasts, soil samples were taken from the yard of RMUTTO, Chanthaburi campus and from Yamaguchi Univ., Yoshida campus. Among about 150 isolates that could grow on YP containing 15 % w/v glucose supplement with 0.2 % v/v acetic acid and 25 μg/ml Kanamycin at 37 °C were observed their morphology and designed base on budding characteristic of yeast. All isolated strains were compared their thermo- and ethanol tolerant characteristics on YP containing 15 % w/v glucose supplement with 15 % v/v ethanol at 40 and 37 °C. As the result in figure 1, all isolated yeast could grow on 20 % v/v ethanol supplemented in YPD media at 37 but not at 40 °C, then 12 isolated strains were selected for future experiments

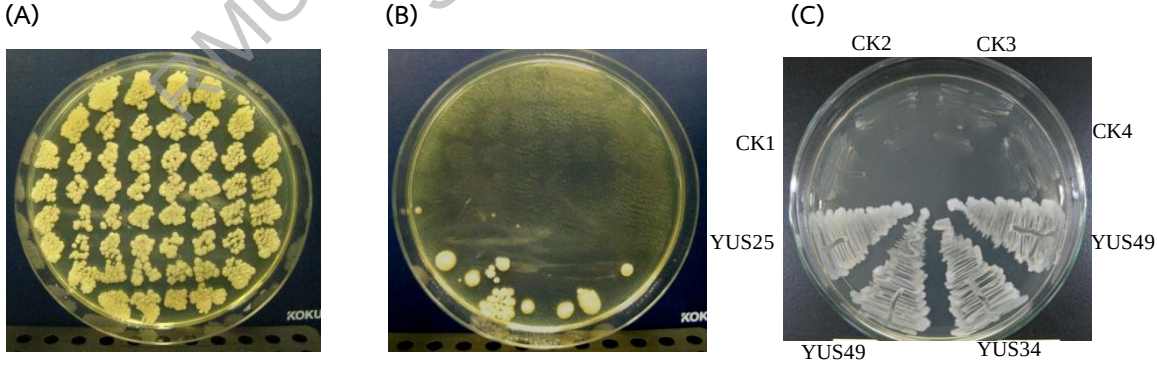


Fig. 1 Screening of thermotolerant yeasts (A) replica plate technique (B) growth of thermotolerant colonies after replica plate on YP containing 15 %v/v glucose at 40 °C (C) Growth characteristic of thermotolerant isolates on YP containing 15 %v/v glucose and ethanol at 40 °C.

Isolation and characterization of thermotolerant yeast

Among 12 isolates of thermo- and ethanol tolerant yeasts were isolated their whole genomes which their size may appear at 23.1 bp. (fig. 2A), are used as the templates for identified their species by analyzing the partial 26S rRNA gene nucleotide sequence with universal primers NL1 and NL4 (fig. 2B). As a result in table 2, 12 isolates were classified into 3 species in four genera; the strain was designated as CK1 was classified as *Williopsis saturnus*; the marine-derived yeast which well known as killer toxin producer (Wang *et al*., 2008), and was found to be capable of producing plant-growth-promoting; indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA) (Nassar *et al*., 2005), CK3 as *Candida sp*., CK2 and CK4 as *Zygosaccharomyces fermentati*; their ability to utilize cellobiose and produce ethanol, as well as their thermotolerance was reported by Pilna *et al*., 1986, and CK5-8, YUS5, YUS49, YUB34 and YUB49 as *Pichia kudriavzevii*; a newly isolated thermotolerant ethanol producer yeast (Oberoi *et al*., 2012).

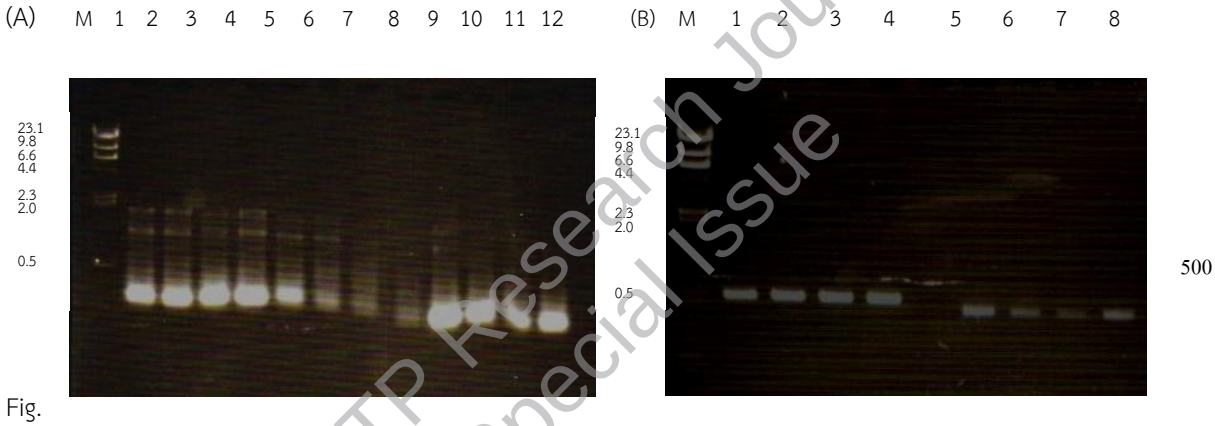
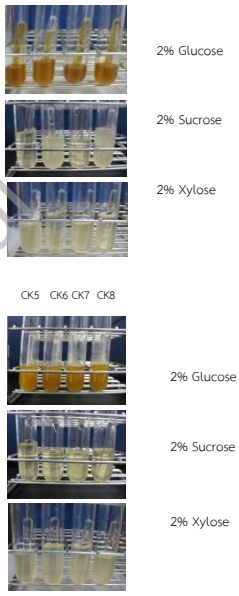


Fig. 2 Isolation of thermotolerant yeasts (A) whole genome extracted from 24 h culture at 30 °C of the isolated thermotolerant yeasts. Lane: 1, CK1; 2, CK2; 3, CK3; 4, CK4; 5, CK5; 6, CK6; 7, Ck7; 8, CK8; 9, YUS25; 10, YUS49; 11, YUB34; 12, YUB49. (B) PCR analysis of amplified 26S rDNA with universal primer NL1 and NL4 from representative of the isolated thermotolerant yeasts. Lane: 1, CK1; 2, CK2; 3, CK3; 4, CK4; 5, YUS25; 6, YUS49; 7, YUB34; 8, YUB49.

Table 2 Identification, based on BLAST comparison in NCBI, of the sequences obtained from ABI sequencer by using universal primers NL1 and NL4

Thermotolerant strains	Identification	Homology (%)	Growth under static condition			
			Glucose (2%)	Sucrose (2%)	Xylose (2%)	Growth characteristics
CK1	*Williopsis saturnus*	99	+++	-	++	
CK2	*Zygosaccharomyces fermentati*	97	+++	+++	-	
CK3	*Candida sp.*	98	+++	-	+	
CK4	*Zygosaccharomyces fermentati*	98	+++	+++	-	
CK5	*Pichia kudriavzevii*	98	++++	-	w	
CK6	*Pichia kudriavzevii*	98	++++	-	+	
CK7	*Pichia kudriavzevii*	99	++++	-	+	
CK8	*Pichia kudriavzevii*	98	++++	-	+	
YUS25	*Pichia kudriavzevii*	98	++++	-	w	
YUS49	*Pichia kudriavzevii*	99	++++	-	w	
YUB34	*Pichia kudriavzevii*	98	++++	-	+	
YUB49	*Pichia kudriavzevii*	99	++++	-	w	



Scored for response to tests: -, negative growth; w, weakly positive growth; +, positive growth

All of the isolated yeast strains could grow in YP medium containing 2% glucose under tested condition, and only *Z. fermentati* could utilize sucrose and produced gas. Xylose utilization was also investigated in this experiment. As the result, *W. saturnus* gave highest growth without gas in YP medium containing 2% xylose.

4. Conclusion

According to the fermentation is exothermic therefore the ethanologenic yeast may have defense mechanisms such as thermotolerance (Singer and Lindquist, 1998), ethanol tolerance (Amore and Stewart, 1987), multi-drug resistance (Nourani *et al*., 1997) and weak acid resistance (Mira *et al*., 2010) and to against many stresses. It is demonstrated herein the screening and isolation method of yeast with its stresses resistance property that are high acidity and antibiotic resistances, by replica plating on the selective medium and incubated under tested conditions, and fellow by the simple molecular technique. Among about 150 strains obtained, 12 isolated strains which could grow at high temperature up to 40 $^{\circ}$C were classified into 3 species in four genera; that are *W. saturnus*, *Candida sp*., *Z. fermentati* and *P. kudriavzevii*. And interestingly, the former strain which were isolated from Chanthaburi, Thailand and Yamaguchi, Japan, could survive on YP medium containing 15% w/v glucose and 15 % v/v ethanol at high temperature, which may be the potential candidate for use as the ethanologenic yeast for high temperature ethanol fermentation, which use of glucose but not sucrose or xylose, as there substrate.

5. Acknowledgements

This work was supported by the international academic cooperation project between the Faculty of Agro-Industrial Technology, Rajamangala University of Technology Tawan-ok, Thailand and the Faculty of Agriculture, Yamaguchi University, Japan.

6. References

Amore T. D. and Stewart G. G.: Review; Ethanol tolerance of yeast, *Enzyme and Microbial Technology*, Vol. 1987 No., 9(6): pp. 322–330.

Babiker M.A., Banat A., Hoshida H., Ano, A., Nonklang, S. and Akada, R.: High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast?, *Appl Microbiol Biotechnol*, Vol. 2010 No. 85, pp. 861–867.

Charoensuk, K., Irie, A., Lertwattanasakul, N., Sootsuwan, K., Thanonkao, P. and Yamada, M.: Physiological importance of cytochrome *c* peroxidase in ethanologenic thermotolerant *Z. mobilis*, *J Mol Microbiol Biotechnol*, Vol. 2011 No., 20 (2), pp. 70–82.

Limtong, S., Sringiew, C. and Yongmanitchai, W.: Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluveromyces marxianus, Bioresource Tehnology*, Vol. 2007 No., 98 (17), pp. 3367–3374.

Mathew, R.: Effect of declining oil prices on oil exporting countries. Available from: //www. Stanford.edu/class/e297c/trade_environment/energy/heffect.html Accessed: 2013 Mar 25.

Mira, N.P., Teixeira, M .C. and Correia, I. S.**:** Adaptive Response and Tolerance to Weak Acids in Saccharomyces cerevisiae: A Genome-Wide View, *Journal of Integrative Biology*, Vol**.** 2010 No., 14 (5), pp. 525-540.

Murata, M., Fujimoto, H., Nishimura, K., Charoensuk, K., Nagamitsu, H., Raina, S., Kosaka, T., Oshima, T., Ogasawara, N. and Yamada, M.: Molecular strategy for survival at a critical high temperature in *Eschierichia coli*, *PLoS ONE*, Vol. 2011 No., 6 (6), pp. 1- 9.

Nassar, A.H., El-Tarabily, K.A. and Sivasithamparam, K.: Promotion of plant growth by an auxin-producing isolate of the yeast *Williopsis saturnus* endophytic in maize (*Zea mays* L.) roots, *Biology and Fertility of Soils,* Vol. 2005 No., 42 (2), pp. 97-108.

Nourani A, Wesolowski-Louvel, M, Delaveau, T. and Delahodde, A.: Multiple-drug-resistance phenomenon in the yeast Saccharomyces cerevisiae: involvement of two hexose transporters, *Mol Cell Biol*. Vol. 1997 No., 17(9), pp. 5453–5460.

Oberoi H.S., Babar N., Sandhu S.K., Dhaliwal S.S., Kaur U., Chadha B.S. and Bhargav V.K.: Ethanol production from alkali-treated rice straw via simultaneous saccharification and fermentation using newly isolated thermotolerant *Pichia kudriavzevii* HOP-12012, *Journal of Industrial Microbiology & Biotechnology*, Vol. 2010 No., 39 (4), pp. 557 - 566.

Pilna, A., ISABEL, L., Calderon, I.L. and Benitez, T.: Intergeneric Hybrids of *Saccharomyces cerevisiae* and *Zygosaccharomyces fermentati* obtained by protoplast fusion, *Appl. Environ. Microbiol*, Vol. 1986 No., 51(5), pp. 995-1003.

Sambrook, J. and Russell, D.W.: Molecular cloning: A Laboratory Manual, ed 3. Cold Spring Harbour, Cold Spring Harbour Laboratory Press, 2001.

Singer, M.A. and Lindquist, S.: Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose, *TIBTECH*, Vol. 1998 No., 16, pp. 460-468.

Wang, L., Yue, L., Chi, Z. and Wang, X.: Marine killer yeasts active against a yeast strain pathogenic to crab *Portunus trituberculatus*, *Diseases of Aquatic Organisms*, Vol. 2008 No., 80, pp. 211–218.