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Alcohol Fermentation at High Temperature and the Strain-specific Characteristics Required to Endow the Thermotolerance of *Sacchromyces cerevisiae* KNU5377

Sangkyoo Paik[1], Insu Park[1], Ilsup Kim[1], Kyunghee Kang[1], Choonbal Yu[2], Inkoo Rhee[3], and Ingnyol Jin[1*]

[1] *Department of Microbiology, Kyungpook National University, Daegu 702-701, Korea,*

[2] *Department of Food Science and Technology, Daegu University, Gyeongsan 712-714, Korea,*

[3] *Department of Agricultural Chemistry, Kyungpook National University, Daegu 702-701, Korea*

*(*Corresponding author : Phone: +82-53-950-5377; Fax: +82-53-955-5522; E-mail: jinin@knu.ac.kr)*

Abstract

Saccharomyces cerevisiae KNU5377 is a thermotolerant strain, which can ferment ethanol from wasted papers and starch at 40℃ with the almost same rate as at 30℃. This strain showed alcohol fermentation ability to convert wasted papers 200 g (w/v) to ethanol 8.4% (v/v) at 40°C, meaning that 8.4% ethanol is acceptable enough to ferment in the industrial economy. As well, all kinds of starch that are using in the industry were converted into ethanol at 40℃ with the almost same rate as at 30℃.

Hyperthermic cell killing kinetics and differential scanning calorimetry (DSC) revealed that exponentially growing cells of this yeast strain KNU5377 were more thermotolerant than those of *S. cerevisiae* ATCC24858 used as a control. This intrinsic thermotolerance did not result from the stability of entire cellular components but possibly from that of a particular target. Heat shock induced similar results in whole cell DSC profiles of both strains and the accumulation of trehalose in the cells of both strains, but the trehalose contents in the strain KNU5377 were 2.6 fold higher than that in the control strain. On the contrary to the trehalose level, the neutral trehalase activity in the KNU5377 cells was not changed after the heat shock. This result made a conclusion that though the trehalose may stabilize cellular components, the surplus of trehalose in KNU5377 strain was not essential for stabilization of whole cellular components.

A constitutively thermotolerant yeast, *S. cerevisiae* KNU5377, was compared with a relatively thermosensitive control, *S. cerevisiae* ATCC24858, by assaying the fluidity and proton ATPase on the plasma membrane. Anisotropic values (r) of both strains were slightly increased by elevating the incubation temperatures from 25℃ to 37℃ when they were aerobically cultured for 12 hours in the YPD media, implying the membrane fluidity was decreased. While the temperature was elevated up to 40℃, the fluidity was not changed in the KNU5377 cell, but rather increased in the control. This result implies that the

plasma membrane of the KNU5377 cell can be characterized into the more stabilized state than the control. Besides, heat shock decreased the fluidity in the control strain, but not in the KNU5377 strain. This means also there's a stabilization of the plasma membrane in the KNU5377 cell. Furthermore, the proton ATPase assay indicated the KNU5377 cell kept a relatively more stabilized glucose metabolism at high temperature than the control cell. Therefore, the results were concluded that the stabilization of plasma membrane and the relatively higher glucose metabolism were related to the higher thermotolerance and the capacity for growth at high temperature for the KNU5377 cell.

Genome wide transcription analysis showed that the heat shock responses were very complex and combinatory in the KNU5377 cell. Induced by the heat shock, a number of genes were related with the ubiquitin mediated proteolysis, metallothionein (prevent ROS production from copper), hsp27 (88-fold induced remarkably, preventing the protein aggregation and denaturation), oxidative stress response (to remove the hydrogen peroxide), and etc.

Introduction

Saccharomyces cerevisiae KNU5377 is a natural isolate with strong thermotolerance that is one of the most important factors to produce alcohol at high temperature. This was used to produce ethanol at high temperature by using substrates such starch saccharified with amylases and waste papers saccharified with cellulases. Alcohol fermentation at high temperature gives us many advantages including reduction of the prime cost.

This yeast strain was used to assay its ability to ferment alcohol at such high temperature as 40℃, and to know the cellular and molecular phenomena of how it copes with extreme environment like this temperature.

Materials and Methods

1. Yeast strains and cultures

Saccharomycs cerevisiae KNU5377(abbreviated as KNU5377 afterward) was isolated from a sewage soil as a thermotolerant wild type strain. *Saccharomyces cerevisiae* ATCC24858 (abbreviated as ATCC24858 afterward) was used as a reference strain in this work, which was reported as an ethanol tolerant one in American Type Culture Collection (ATCC) and revealed into a moderately thermotolerant strain in this experiment. YEPD medium was mainly used to assay some characters of yeast.

2. Thermotolerance assay

1) Viability assay in liquid media

Cell were treated at different stress conditions, and the survived cells were serially diluted with sterile water to make the cell numbers of control into 100 - 200 colonies per a plate. The plates were incubated at

30℃ for overnight. Resulted colonies were counted and calculated as % survivors.

survivors(%) = (CFUs of post-heat treatment/CFUs of pre-heat treatments)×100

2) Viability assay on the agar plates

Overnight cultured cells as a streaked form on the YPD agar plate were replicated into daughter plates and then treated them thermally at different temperatures (55-65℃) during each given period. The plates were incubated at 30℃ for overnight and detected the viabilities or growth capacities. Plate replica was performed by overlaying the fresh plate (daughter plate) on velvet stained with mother plate cells.

3. DSC(Differential Scanning Calorimetry) assay

Cultures were harvested and washed twice with distilled water. After the harvesting step, the cell concentration was adjusted as 10^9 cells/㎖. 1.2 ㎖ of the cell suspension was injected into the sample cell of an MC-2 micro-calorimeter (Microcal Inc., Northampton, MA, USA) with degas process and subjected to DSC scans from 5 to 100℃ at a rate of 1 ℃/min. Distilled water was used as a reference. Both the sample and the reference were equilibrated at 5℃ and heated to 100℃ (1st scan). When the temperature was elevated to 100℃, they were rapidly cooled, equilibrated and then heated again from 5 to 100℃ (2nd scan). Measurements and data processing were conducted as previously described [Obuchi et al., 2000].

4. Membrane fluidity assay

The pobe Tma-DPH (*Molecular probes # T-204, Eugene, Oregon*) was prepared in tetrahydrofuran to give a 100 μM stock solution and added 10 ㎕ of the stock solution into the cell suspension. After the incubation at room temperature for 30 minutes, then it was washed twice and resuspended in 3 ㎖ of distilled water. Fluorophotometry was conducted with RF-1500 Spectroflurophotometer (Shimatsu co., Kyoto, Japan) with stirring the cell suspension and measured at 30℃ (Ex: 360 nm, Em: 445 nm). The anisotropic value (*Y*) was given as:

Y^* = IVV - GIVH / IVV+2GIVH

* *Y* = Anisotropic value(=1/membrane fluidity)

G = IHV/IHH , H = Horizontal , V = Vertical

Assay order : HH→HV→VH→VV

5. Assaying the plasma membrane H+-ATPase activity

The changed level of intracellular pH (pHi) was estimated by using a fluorescent pH indicator, 5-(and-6)-carboxy SNARF-1 acetoxymethylester acetate (C.SNARF-1- AM, Molecular Probes Inc. # C-1271, Eugene, Oregon, USA). Cells were washed and resuspended with 2 ㎖ of distilled water (ca. 105 cells/㎖), and added the dyes into the cell suspension to be 10 μM of final concentration. After the incubation at 30℃ for 40 minutes with shaking (100 rpm), Cells were washed twice and resuspended with 2 ㎖ of distilled water to remove the extracellular dyes. Fluorescence intensities (Ex: 514 nm, Em: 580 and

610 nm) of cell suspension were assayed for 1,000 seconds or more and recorded the data per a second. The stimulator or activator of H+-ATPase, 100㎕ of glucose (0.1 M), was added into the cell suspension as it was passing by 100 seconds. Intracellular pH was calibrated by using a fluorescence ratio (580/610 nm) vs. pH profile of C.SNARF-1-AM. H+-ATPase activity was measured with changes of intracellular pH (Δ pH for 200 seconds) after the activation by glucose.

6. FAMEs (Fatty Acid Methyl Esters) assay

Cells were aerobically cultured at 30, 37, and 40℃ for 12 hours in YPD media, and heat-shocked cells at 43℃ for 60 minutes were prepared from the exponentially grown cell cultured at 30℃. Cells were then washed twice with distilled water and then transferred to teflon-capped tube. Fatty acids are extracted from 40 ㎎ of cell wet weight per each sample and performed fatty acid extraction according to the Miller method [Miller et al., 1982], and analyzed the compositions by the MIDI system (Microbial ID, Inc., Sherlock version 3.0). In this fatty acid methyl esters (FAMEs) analysis, Hewlett Packard series II gas chromatography model 5890A (Microbial ID, Inc) was used, and the separation of fatty acids was performed with the methyl phenyl silicone fused silica capillary column (size: 250×220×33 mm, HP19091B-102).

7. Trehalose content analysis

Exponentially growing cells were transferred to fresh YPD media, then heat-shocked at (30, 37, 40, 43, 48℃) for 90 minutes. After the heat shock treatment, the cultures were immediately transferred to 30℃ and incubated further for 60 minutes. During the heat shock and recovery period, aliquots were sampled and resuspended with distilled water at 30 minutes intervals. The cell suspensions (1 ㎖) were boiled for 60 minutes and cytosol was extracted and treated with trehalase (α , α -trehalose glucohydrolase, EC 3.2.1.28., Sigma, USA) as already reported (Nwaka et al., 1998) to hydrolyze intracellular trehalose stoichiometrically. Thereafter, the liberated glucose was determined enzymatically using a glucose assay kit (Boeringer Mannheim Co. #716251, Mannheim, Germany). The trehalose content (ug trehalose synthesized by heat shock/mg of DCW) was defined as the ([㎍ trehalose of sample]-[㎍ trehalose of control]) per ㎎ of dried cell weight. The control illustrated in this equation denotes a cell that was not treated by the trehalase.

Trehalose content (ug trehalose synthesized by heat shock/mg of DCW)
= [ug trehalose of sample]-[ug trehalose of control]/mg of DCW

*DCW = Dry Cell Weight
** control = trehalase not treated, whole glucose conc. in the cell

8. Assaying the neutral trehalase activity

The prepared cells as described in the preceding section (trehalose accumulation) were disrupted with glass beads (0.5 ㎜ diameter) at 4℃ in the imidazole buffer [50 mM, pH 7.0 containing 10 mM PMSF

(phenyl methyl sulfonyl fluoride)], and then crude neutral trehalase fraction was extracted by removing cell debris with centrifugation (10,000 rpm for 2 minutes). Total protein concentration of the fraction was determined with protein assay kit (Bio-Rad DC # 500-0116, CA, USA). The reaction mixtures consisted of 0.1 M of trehalose and crude trehalase in the imidazole buffer. After allowing the enzymatic reaction at 37℃ for 20min, the reaction was terminated by boiling for 5 minutes, and the liberated glucose concentration was measured using the glucose assay kit mentioned above. Specific activity unit (U) was defined as the degraded trehalose concentration (㎍/㎖) per total protein concentration (㎍/㎖) over 20 minutes. Here, the control denotes the glucose concentration in a cell without the above enzymatic reaction.

1 unit = [ug/ml trehalose(sample-control)] / total protein(ug/ml)

9. Microarray analysis

Exponentially growing cells before and after heat shock at 43℃ for 2 hours were harvested and washed twice with distilled water, and it was frozen prior to the mRNA extraction. All processes for microarray analysis including mRNA isolation, probe preparation with fluorescence dye, hybridization, scanning of the hybridized array, and data processing were performed as described previously [Holstege *et al., 1998]*. Total RNA was firstly extracted from frozen cell pellets by a hot phenol method [Schmitt et al., 1990]. The mRNA was purified from the total RNA with an oligo(dT) selection step (Oligotex QIAGEN, Chatsworth, CA). Then double-stranded cDNA labeled by Cy5-dCTP (for pre-heat shocked cells) and Cy3-dCTP (for post-heat shocked cells) was prepared from the mRNA and applied it to Affymatrix chips (Affymetrix, Santa Clara, CA) according to the manufacturer's protocols. Resulted data were processed by the 'GeneSpring' program.

Results and Discussion

Saccharomyces cerevisiae KNU5377 is a thermotolerant strain, which can ferment ethanol from wasted papers and starch at 40℃ with the rate comparable to 30℃. This strain is a natural isolate, and was smartly

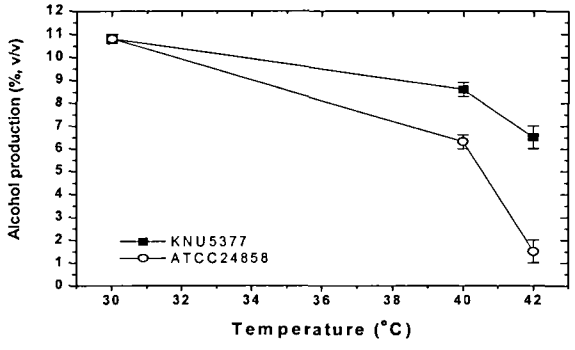

Fig. 1 Lab-scale fermentation with a mixed industrial substrate comprised of tapioca and corn meal (1:1). Fermentation was anaerobically performed for 72 hours at each temperature, including 30℃, 37℃, and 40℃. After completed the fermentation, produced alcohol was distilled and then measured the alcohol content (%, v/v) with alcohol hydrometer.

identified by ITS sequencing as shown in Fig 4. This strain showed alcohol fermentation ability to convert saccharified solution of 200 g (w/v) wasted papers to 8.4%(v/v) ethanol at 40°C with the fermentation rate of over 80 % like as shown in Fig 2, meaning that 8.4% ethanol and the fermentation rate are acceptable enough to ferment in the industrial economy. And in Fig 1 when using this strain, all kinds of starch(20% v/v) that are using in the industry could be converted into 10% ethanol with the fermentation rate of 80% or more at 40℃.

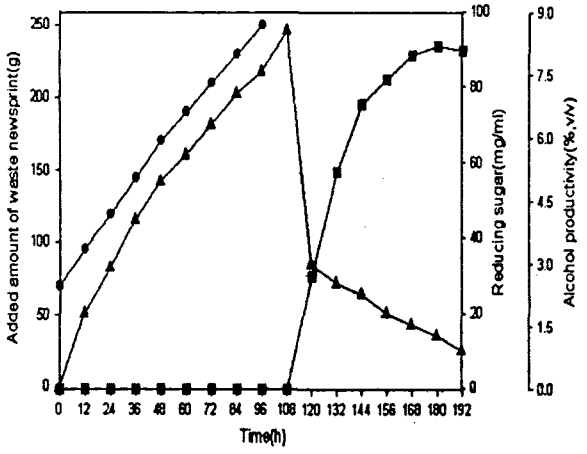

Fig. 2 Effect of inoculum amount on SSF by *S. cerevisiae* KNU5377 in 2L jar fermentor at 40℃ for 60h after fed-batch presacchari-fication at 50℃ for 110h.
Initial fermentation broth containing 650ml 0.1M sodium citrate buffer(pH4.8), 140 FPU XCL and 70 g of dry-defibrated waste newsprint.
● - ● added amounts of dry defibrated waste newsprint
▲- ▲ reducing sugar
■- ■ alcohol productivity

To investigate cellular and molecular mechanism of thermotolerance in this yeast, hyperthermic cell killing kinetics, differential scanning calorimetry (DSC) and others were measured. This KNU5377 strain showed much better survival ability than the reference strain, *S. cerevisiae* ATCC24858. As shown in Fig 3, preliminarily heat shocked to 43℃ for 30min, the KNU5377 cells exposed to each high temperature for given times could grow better on the agar plate, than the control cells. Interestingly, this strain showed two phased response to heat at 43℃ treatment, but its reason is not known yet. Both the test and reference strains did not have any difference in heat stability for all of cellular components like as shown in Fig 5. As shown in Fig 5, there is no change in the cells of two strains at the temperature range which most of cellular components would be denatured. However, the cells of two strains were observed to be stabilized by heat shock induction of cellular molecules under 50℃, where two seemed to be unstable to heat. This means that the intrinsic thermotolerance of the KNU5377 is not acquired by the heat stability of all cellular components. As well, observation of the stability of cellular components by the acquired thermotolerance indicates that there should be few relationship between thermotolernce of the yeast cells and the stability of cellular components.

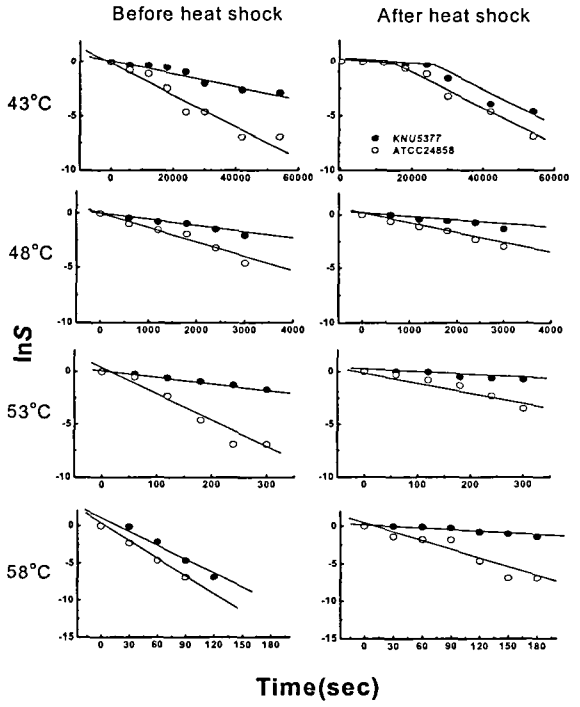

Fig. 3 Cell-killing profiles at pre- and post-heat shock at 43℃ for 90 min.

(A) ITS 1 sequence

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aagaaatttaataattttgaaaatggatttttttgttttggcaagagcatg     50
agagcttttactgggcaagaagacaagagatggagagtccagccgggcct     100
gcgcttaagtgcgcggtcttgctaggcttgtaagtttctttcttgctatt     150
ccaaactgtgagagatttctgtgcttttgttataggacaattaaaaccgt     200
ttcaatacaacacactgtggagttttcatatctttgcaacttttttctttg    250
ggcattcgagcaatcggggcccagaggtaacaaacacaaacaattttatt     300
tattcattaaatttttgtcaaaaacaagaattttcgtaactggaaatttt     350
aaaatattaa  360
```

Blast search result : *S. cerevisiae* DNA for internal transcribed spacer 1 [Identities = 319/321 (99%)]

(B) ITS 2 sequence

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ccttctcaaacattctgtttggtagtgagtgatactctttggagttaact     50
tgaaattgctggccttttcattggatgttttttttttccaaagagaggttt    100
ctctgcgtgcttgaggtataatgcaagtacggtcgttttaggttttacca     150
actgcggctaatctttttttatactgagcgtattggaacgttatcgataag    200
aagagagcgtctaggcgaacaatgttcttaaagt
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Blast search result : *S. cerevisiae* DNA for internal transcribed spacer 2 [Identities = 225/235 (95%)]

Fig. 4 Identification by ITS sequencing

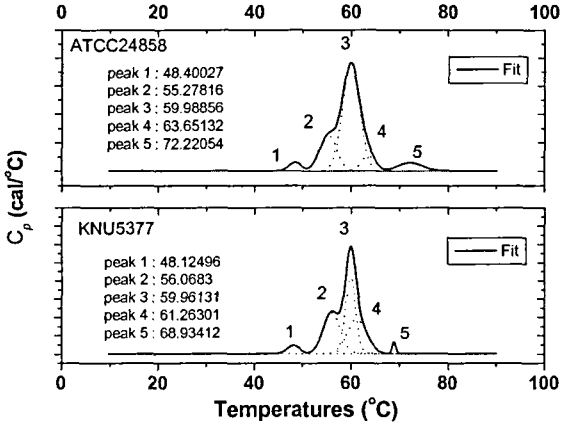

Fig. 5 Peak-fitted DSC profiles of exponentially growing cells.

In the KNU5377 cells exposed to heat shock, trehalose, a kind of disaccharide related to intracellular protection, was assayed in the 2.6 times more amounts than that in the reference cells, as shown in Fig 6. On the other side, the activity of neutral trehalase 1(Nth1p) in the KNU cells was lower relatively to the control cells. This result was that exposed to heat shock, the KNU 5377 cells could keep the more amounts than the control cells and this characteristics would give a positive effect to stabilize the KNU5377 cells. However, as resulted in the DSC file of the Fig 5, many amounts of synthesized trehalose would not be

directly propotional to the stabilization of cellular components, one of its representative functions. This suggests its other function should give an important effect to endow thermotolerance to the KNU5377 cells.

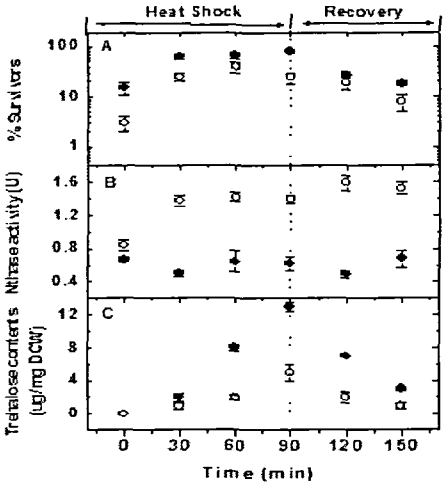

Fig. 6 Alterations of trehalose, neutral trehalase activity and thermotolerance of *S. cerevisiae* KNU5377 and ATCC 24858 during the heat shock and recovery period.

The KNU5377 cell kept relatively stabilized in the membrane even at the high temperature, compared with the control cell, as shown in Fig 8. Moreover, the H^{+-} ATPase, one of membranous enzymes, kept being in the high activity as well(Fig 9). This result says the membrane stability certainly played a very important role to endow thermotolerance to this yeast cell.

Responding to heat shock, the control cells was much changed to increase the unsaturated fatty acids, while the KNU5377 cells showed few change in those of the membrane, except of increased, but relatively less changed palmitoleic acid than the control cells, as shown in Fig 7. This means that this thermotolerant yeast strain manages to keep the homeostasis in the composition of membranous fatty acids. This suggests

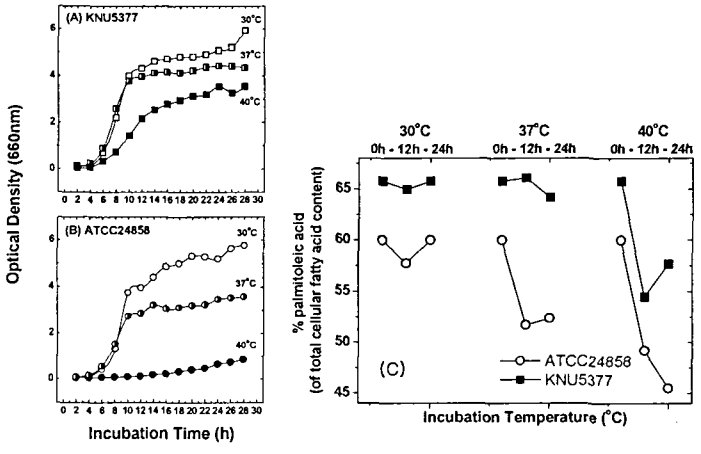

Fig. 7 Possible relationship between growth capacity and the alterations of palmitoleic acid.

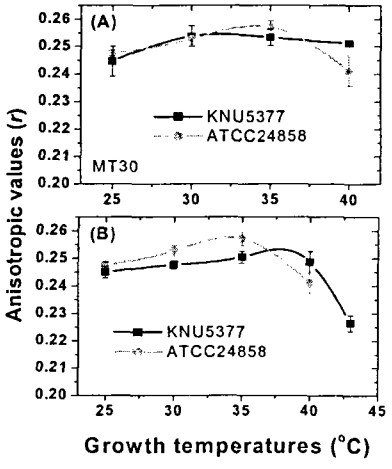

Fig. 8 Plasma membrane fluidities depending on the incubation temperatures.

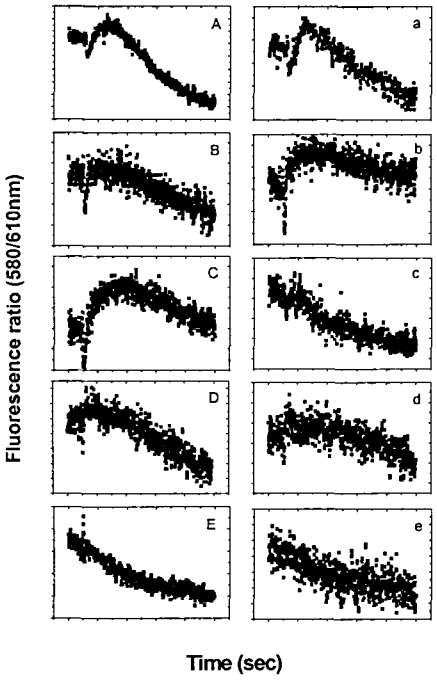

Fig. 9 Whole diagrams at different conditions including incubation temperatures and heat shock in order to detect the ratio jumping-up event after glucose addition.
Left panels illustrated capital letters, A to E, are all for the KNU5377, and right ones are for the ATCC 24858 illustrated as small letter, a to e. Panels A(a) to C(c) are indicating the incubation for 12hours at different temperatures.
Panels D(d) to E(e) are indicating the heat shock conditions. A(a), incubated at 30℃ B(b), incubated at 37℃(c), incubated at 40℃ D(d), heat-shocked at 40℃ for 40 min; E(e), heat-shocked at 43℃ for 60 min.

this KNU5377 cell has a possibility to have some stabilization factors to keep the composition of the membranous fatty acids. It is certain this strain should have an excellent capability to keep homeostatis in the structure and character of its membrane, while its structure and components are almost the same as the other yeast.

In a while, the KNU5377 cells have been grown at 40℃ with the almost similar pattern of growth as shown at the optimum temperature, when intracellular Hsp104 was remarkably more expressed than the control, suggesting that this Hsp104 has to play an important role for active growth of this strain at high temperature(not shown data).

In assaying the genetic background of this KNU5377 cell by microarray analysis as shown in Table 1, this cell has shown no remarkable difference from the reference cell. However, genes coding unclassified proteins were occupied to the most expressed group among total heat shocked ORFs, corresponding to 40%, and then the next were genes of cell organization, corresponded to 30%. It is very particular this heat shocked KNU5377 cells have expressed 80 times higher Hsp 27 than in its constitutive expression, but it is not yet known how it exactly functions in relation to thermotolerance of this cell at high temperature.

Considering all the above results shown in here, it is certain to say *S. cerevisiae* KNU5377 should be the very strain with the most excellent capabilities to produce ethanol under various extreme environments.

Table. 1 Heat-shock induced ORFs restricted by the functional category of stress responses

Heat Shock (43?)			Fluorescence ratio* > 5.0	
	Avg†	Stdev‡	Common	Description

YBR072W	83.86	6.34	HSP26	heat shock protein 26
YKL163W	14.09	0.22	PIR3	Protein containing tandem internal repeats
YDR059C	9.41	3.55	UBC5	ubiquitin-conjugating enzyme
YFR052W	8.89	1.38	RPN12	cytoplasmic 32 - 34 kDa protein
YOR208W	8.45	2.10	PTP2	protein tyrosine phosphatase
YFL053W	7.91	-	DAK2	dihydroxyacetone kinase
YER167W	7.40	2.13	BCK2	Serine\/threonine protein kinase of the protein kinase C pathway
YCR083W	7.37	0.15		
YCL035C	7.36	0.24		
YDR227W	6.24	-	SIR4	regulator of silent mating loci
YCR021C	6.08	1.10	HSP30	Protein induced by heat shock, ethanol treatment, and entry into stationary phase\; located in plasma membrane
YEL039C	5.72	2.95	CYC7	iso-2-cytochrome c
YHR057C	5.68	1.35	CYP2	Peptidylprolyl isomerase (cyclophilin) ER or secreted
YBR067C	5.66	0.82	TIP1	cell wall mannoprotein
YDL006W	5.52	0.73	PTC1	serine-threonine protein phosphatase
YLL060C	5.02	-		

* Fluorescence ratio 5.0 means the mRNA expression level of post-heat shock is 5 times higher than that of pre-heat shock or control.

† Avg ; Average ; ‡ Stdev ; Standard deviation

References

1. Attfield PV (1987). Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response. FEBS Lett 225:257-263.
2. Gross C, Watson K (1996). Heat shock protein synthesis and trehalose accumulation are not required for induced thermotolerance in depressed *Saccharomyces cerevisiae*. Biochem Biophys Res Comm 220:766-772.
3. Gross C, Watson K (1998). Application of mRNA differential display to investigate gene expression in themotolerant cells of *Saccharomyces cerevisiae*. Yeast 14:431-432.
4. Hohmann S, Mager WH (1997). Yeast stress responses. Springer-Verlag, Heidelberg
5. Hottiger T, Schmutz P, Wiemken A (1987). Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. J Bacteriol 169:5518-5522.
6. Iwahashi H, Obuchi K, Fujii S, Komatsu Y (1995). The correlative evidence suggesting that trehalose stabilizes membrane structure in the yeast *Saccharomyces cerevisiae*. Cell Mol Biol 41(6):763-769.
7. Kim JW, Jin IN, Seu JH (1995a). Isolation of *Saccharomyces cerevisiae* F 38-1, a thermotolerant yeast

for fuel alcohol production at higher temperature. Kor J Appl Microbiol Biotechnol 23:617-623.

8. Kim JW, Kim SH, Jin IN (1995b). The fermentation characteristics of *Saccharomyces cerevisiae* F 38-1, a thermotolerant yeast isolated for fuel alcohol production at elevated temperature. Kor J Appl Microbiol Biotechnol 23: 624-631.
9. Kirk N, Piper PW (1991). The determinants of heat-shock element-directed lacZ expression in *Saccharomyces cerevisiae*. Yeast 7:539-546.
10. Lepock JR, Frey HEA,Rodhal M, Kruuv J (1988) Thermal analysis of CHL V79 cells using differential scanning calorimetry: implication for hyperthermic cell killing and the heat shock response. J Cell Physiol 137:14-24.
11. Lepock JR, Kruuv J (1992). Mechanisms of thermal cytotoxicity. In Hyperthermic Oncology. Vol. 2, Gerner EW, Cetas CT (Eds.), Arizona Board of Regents, Tuscon, Arizona.
12. Lindquist S (1986). The Heat-shock response. Ann Rev Biochem 55:1151-1191.
13. Singer MA and Lindquist S (1998). Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. Trends in Biotechnol., 16, 460-468.
14. Miles DA, Knott L, Sumner IG, Bailey A (1998). Differences between the thermal stabilities of the three triple-helicle domains of type IX collagen. J Mol Biol 27:135-144.
15. Nwaka S, Holzer H (1998). Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. Progress in Nucleic Acid Research and Molecular Biology 58: 197-237.
16. Obuchi K, Iwahashi H, Lepock JR, Komatsu Y (1998). Stabilization of two families of critical targets for hyperthermic cell killing and acquired thermotolerance of yeast cells. Yeast 14:1249-1255.
17. Obuchi K, Iwahashi H, Lepock JR, Komatsu Y (2000). Calorimetric characterization of critical targets for killing and acquired thermotolerance in yeast. Yeast 16:111-119.
18. Ribeiro MJS, Silva JT, Panek AD (1994). Trehalose metabolism in *Saccharomyces cerevisiae* during heat shock. Biochem Biophys Acta 1200:139-147.
19. Wiemken A (1990). Trehalose in Yeast, stress protectant rather than reserve carbohydrate. Antonie Van Leeuwenhoek 58:209-217.