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Screening and Characterization of Thermotolerant Alcohol-producing Yeast

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Two strains of yeast (RA-74-2 and RA-912) showing superior fermenting ability at a high temperature were isolated from soils and wastewaters by an enrichment culture method. Based on the morphological and physiological charateristics, the two strains were identified as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, respectively. RA-74-2 was able to grow upto 43°C and sustain similar fermenting ability in the temperatures range from 30 to 40°C. In addition, the sugar- and ethanol-tolerance of RA-74-2 were 30% (w/v) glucose and 10% (v/v) ethanol, which appeared to be higher than those of nine other industrial yeast strains currently being used in the alcohol factories. The thermotolerant ethanol fermenting yeast RA-912 showed identical growth in the temperatures range from 35 to 45°C and was resistant to various heavy metals. The quality and quantity of byproducts of the isolated yeast strains in fermentation broth after fermentation at 40°C and 45°C were similiar with those obtained at 30°C. These results show that RA-74-2 can be adopted for the ethanol fermentation process where the expenses for cooling system is significant, and suggest that RA-912 may be applied in either SSF(simultaneous saccharification and fermentation) or Flash-fermentation process and RA-912 may be used as a gene donor for the development of thermotolerant ethanol-fermenting yeasts.

The research for alternative fuel has been carried out from the past and has accelerated in regard to the increase in air pollution and shortage of fossil resources in recent years (1). One of the possible alternatives is the use of fuel alcohols from renewable biomass. Attempts to produce fuel alcohol was actively carried out in the USA, Japan, France, Philipine and Brazil. However, the development of ethanol fermentation process employing thermotolerant alcohol-producing yeast and high-temperature fermentation is in a preliminary stage (1, 2). So far, there has been no economical advantage to be considered in that the fermentation must take place at a temperature above 40°C. The conventional process of alcohol-fermentation requires cooling systems due to the increase of external temperature and internal exothermal reaction, which suppress an economical production. Thermotolerant yeast could minimize the amount of cooling water required not only during the fermentation period but also after the sterilization. Considering the optimum temperature (1, 2, 5, 12, 13) of the majority of saccharifying enzyme (above 45°C),the thermostability of ethanol-fermenting yeast is essential in SSF (simultaneous saccharification and fermentation) and Flash fermentation (14, 15). The continuous process of ethanol fermentation also needs thermotolerant yeasts. In addition, we could expect other good results from using thermotolerant alcohol-producing yeasts, such as simplified operation, prevention of contamination during the fermentation period, less use of the equipment and labor, and increase of productivity along with highly metabolic ratio. However, there are also disadvantages in high-temperature fermentation, for example: sharp decrease of alcohol and/or sugar tolerance; decrease of theoretical yields by bypassways; requirement of additives; waste of substrates by increasing cell maintenance energy (2, 16, 17). In consequence, industrial application of high-temperature fermentation is not practical nowadays. In this study, we isolated and identified thermotolerant alcohol-fermenting yeasts from the soils and wastewaters in an attempt to develop hyperproductive alcohol-producing yeast. We further investigated the characteritics of isolated yeasts and compared them with those of the industrial strains.

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Key words: thermotolerant ethanol fermenting yeast, SSF, Flash fermentation

MATERIALS AND METHODS

Isolation of Strains and Media

S. cerevisiae RA-74-2 and *K. marxianus* RA-912 were isolated from the soils and wastewaters at high-temperature environments by an enrichment culture method and the subsequent three selective steps. Enrichment culture was carried out in a YPD medium containing glucose 10 g/L, polypeptone 5 g/L, and yeast extract 5 g/L at 45°C for 24 hrs. The three selective steps were done in sequence using a 20% glucose fermentation medium in Durham test tube scale, air-restricted flask scale, and jar fermentor scale, respectively. The selected strains were identified according to Lodder and Barnett classification methods (3).

The media for alcohol fermentation was composed of glucose 200 g/L, yeast extract 2 g/L, polypeptone 2 g/L, $(NH_4)_2SO_4$ 3g/L, KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 2 g/L. A potato dextrose agar (PDA) medium was used for preserving strain at 4°C. The nine industrial strains used as control were kindly supplied by the Research and Development Center for Energy and Resources of Korea.

Analytical Methods

Ethanol and byproduct content in the fermentation broth was determined by GC (GC 370.Gasukuro kogyo : FID : Supelco glass column, 6.6% carbowax 20 M, carbopack B 80~120 mesh: column 75°C, Injector 200°C, Detector 200°C: N_2 gas). For the quantitation of ethanol, alcohol hydrometer was used to determine the alcohol concentration after single distillation (4). The residual sugar in fermentation broth was determined by Somogyi-Nelson methods (5) using glucose as the standard. Cell growth was measured by Spectrophotometer (Beckman DU-40) at 660nm. The viable cell was counted by a haematometer after the methylene-blue staining (8).

Ethanol Fermentation Tests

Ethanol fermentation was carried out in a 250 m*l* flask with 50 m*l* fermentation medium. The flask was equipped with an air restrictor containing sulfuric acids (6). To quantitate the ethanol fermentation, the flask was inoculated with one loopful of cells and incubated at various temperatures ranging from 30°C to 45°C for 3~5 days. The loss in weight resulting from carbon dioxide was measured and was converted to fermentation ratio. For the final fermentation test of the yeast strains selected, jar fermentor test was performed with a working volume of 3 liters. After sterilzation of the fermentation medium, the inoculation was made at 3% (v/v) level and the culture was stirred at 200 rpm. The aeration was stopped immediately after inoculation. The fermentation temperature was maintained at 30°C, 40 °C or 45°C. The broth samples were obtained aseptically through the sample port and were tested for sugar content, ethanol content, cell growth and pH.

Measurements of Ethanol Tolerance.

In order to investigate how much the growth is affected by ethanol, the isolated strains were cultured for 40 hr in a YPD medium containing various concentrations of ethanol, and the growth O.D was measured at 660 nm (8). To examine ethanol tolerance of the fermentation, the cells were suspended in a YPD medium containing 15% ethanol for 48 hr and then the ethanol-treated seeds were inoculated into the fermentation media. Fermentation was carried out at 30°C, 40°C and 45°C. Industrial strain, *S.cerevisiae* B, was compared with the isolated strains.

Measurements of Sugar Tolerance.

To investigate how much the growth is affected by sugar,the isolated strains were cultured for 40 hr in a basal fermentation medium containing various concentrations of glucose. The growth O.D was measured at 660 nm after cultivation was carried out for 40 hr at 30°C and 40°C. As a control, industrial strain, *S.cerevisiae* B was employed.

Measurements of Resistance to Heavy Metals

In order to investigate the resistance to heavy metals, one loopful of the inoculum solution equilibrating 10^7 cells/m*l* was streaked on YPD agar media containing various heavy metals, and the growths were examined after culturing them for 4 days at 30°C. The concentration of heavy metals were in the range of 0~1200 ppm.

RESULTS

Isolation and Selection of Thermotolerant Yeast

About five hundred samples collected from the soils and wastewaters in high temperature environments were put into the YPD medium and cultured for 24 hr at 45°C in order to enrich the thermotolerant yeasts. Twenty strains of yeast, which were able to produce

Table 1. Fermentation test with air restricted fermentation-bung at various temperatures.

Strains	30°C	37°C	41°C	43°C	45°C
P-8	89	68	ND*	31	20
P-9	60	60	ND	39	27
R-15	51	39	ND	30	−
R-71	49	27	ND	21	−
R-74	ND	80	ND	9	−
R-77	ND	37	ND	9	−
RA-74-2	93	87	53	38	−
RA-422	42	43	40	40	24
RA-912	55	60	57	45	40

Fermentations were carried out in 20% glucose medium for 72 hours at various temperatures. The loss in weight resulting from carbon dioxide was measured and the result was expressed the relative fermentation ratio (%).
ND*: not determined, −: not fermented.

ethanol at temperature above 40°C, were isolated from the enriched culture broths through the Durham tube test. To further quantitate the ethanol concentration, air restricted flask with sulfuric acid was used, and 9 strains were selected (Table 1). By the final selection test performed in a mini-jar fermentor scale, two strains designated as RA-74-2 and RA-912 were obtained as the most promising strains based on the fermentation ratio and the amount of residual sugar (Fig. 1, Fig. 2).

Identification of Isolated Yeasts

Based on morphological and physiological charateristics examined according to the Lodder and Barnett classification methods (3), RA-74-2 and RA-912 were identified as *S. cerevisiae* and *K. marxianus*, respectively (Table 2). These two thermotolerant strains isolated were characterized by their small size in comparison to industrial strains. Particularly, *S. cerevisiae* RA-74-2 was able to grow at 43°C in 20% glucose medium.

Screened about 500 samples of high temperature environments
↓
Enriched culture at 43~45°C for 24hr in YPD medium.
↓
1st Durham tube test: Seed was used enriched culture broth.
↓
Single colony isolation from 1st Durham tube test broth.
↓
2nd Durham tube test to single colony for selection: 20 strains.
↓
Fermentation test in flask scale with air restricted fermentation-bung: 9 strains
↓
Fermentation test in jar-fermentor scale: 2 strains (RA-912, RA-74-2)

Fig. 1. The procedure of selective isolation for thermotolerant alcohol fermenting yeasts.

Characterization of the Isolated Strains

Growth at High Temperature: To examine growing ability at a high temperature, YPD (5% glucose,1% polypeptone, 0.5% yeast extract) media was inoculated with 0.1 ml of the culture fluid precultured at 30°C for 24 hr and the cultivation was carried at 20~55°C for 72 hours by Temperature-gradient incubator (Toyo Kagaku Sanyo Co LTD.Model TN-3). Since the yeast strain of B Company appeared to have the best heat-stability and fermentabilty among the industrial strains obtained from 9 different Alcohol Co., the strain was used as a control to evaluate if the isolants have enough potential to be applicable industrially. As shown in Fig. 3, RA-912 could grow at 48°C and RA-74-2 showed higher growth in comparison to the industrial strain of B company at 40°C.

Sugar Tolerance: As the results shown in Fig. 4, RA-74-2 showed satisfactory growth at 30°C in the various concentrations of glucose ranging from 5% to 30% whereas B company strain could grow in upto 25% glucose. The growth of RA-74-2 declined a little at a cultivation at 40°C. But growth was satisfactory in 20% glucose media by the prolonged culture. Also the isolated strains grew well at 40°C, 30% glucose condition but the 9 industrial strains failed.

Ethanol Tolerance: By measuring the growth inhibition of ethanol, absolute ethanol (99.9% v/v) was added immediately after inoculation (8). The results are shown in Fig. 5. The RA-74-2 and strain of B company showed growth inhibition at above 6% (v/v) ethanol concentration, but the growth of RA-74-2 was better than that of the strain of B company at 10% (v/v) concentration.

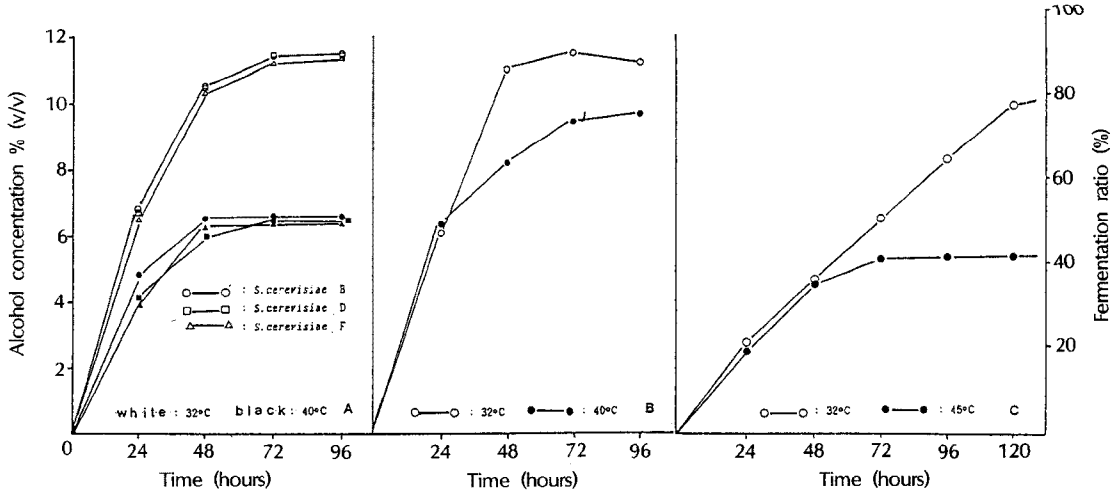

Fig. 2. Effect of temperature on ethanol fermentation in industrial strains (A), RA-74-2 (B) and RA-912 (C).
The fermentation was conducted in a jar fermentor with 3 liters of fermentation broth containing glucose 200 g/L, yeast extract 2 g/L, poly peptone 2 g/L, $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 2 g/L. The seed volume was 3%,and aeration was stopped immediately after inoculation. The pH was not controlled. Used industrial strains, *S. cerevisiae* B, D, F Strain, were kindly supplied by RaCER.

Table 2. Morphological and physiological characteristics of the isolated strains and industrial strain.

Morphological characteristics

RA-912: White or cream colony, multilateral budding, Simple and elaborate pseudohyphae, evanescent asci containing 1 to 4 smooth oval or round ascospore

RA-74-2: White or cream colony, multilateral budding, persistent asci containing 1 to 4 smooth, oval or round ascospore

	RA-912	RA-74-2	*S. cerevisiae* of *B and D ffactory*
Fermentation			
Glucose	+	+	+
Lactose	+	−	−
Sucrose	+	+	+
Galactose	+	+	+
Maltose	−	+	+
Raffinose	+	+	+
Inulin	+	−	−
Starch	−	−	−
Carbon assimilation			
Glucose	+	+	+
Mannose	+	+	+
Xylose	+	−	−
Galactose	+	+	+
Sucrose	+	+	+
Lactose	+	−	−
Salicin	+	−	−
Maltose	+	+	+
Inositol	−	−	−
Raffinose	+	+	+
Mannitol	−	−	−
Arabinose	−	−	−
Succinate	−	−	−
Citrate	−	−	−
Gluconate	−	−	−
Glycerol	+	−	−
Methanol	−	−	−
Ethanol	+	+	+
Inulin	+	−	−
Starch	−	−	−
Nitrogen assimilation			
NH_4^+	+	+	+
NO_3^-	−	−	−
NO_2^-	−	−	−
Additional characteristics			
0.01% cycloheximide	+	−	−
0.1% cycloheximide	+	−	−
Growth at 37°C	+	+	+
at 42°C	+	+	+
at 45°C	+	−	−
Urea hydrolysis	−	−	−
D.B.B reaction	−	−	−

While,the growth of RA-912 was inhibited from 4% (v/v) concentration at 30°C and fail to grow at 8% (v/v) concentration. The fermentation inhibition of ethanol was measured with ethanol-treated seeds (8, 9). Ethanol treatments were carried out by soaking the seeds in 15% ethanol for 2 days. The results are shown in table

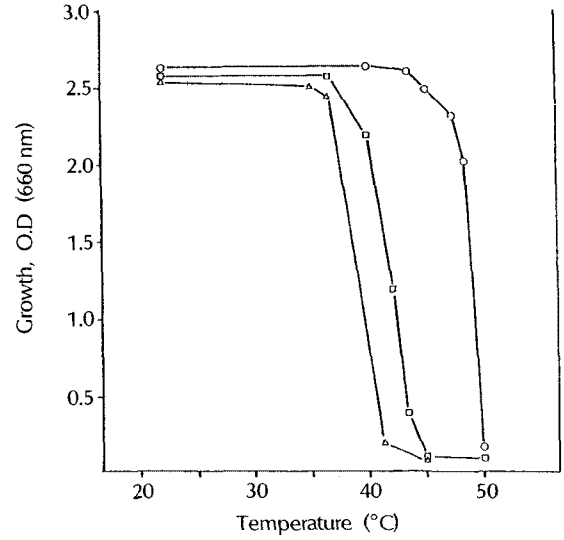

Fig. 3. Effect of temperature on the cell growth of industrial strain and isolated strains.
Cells were cultured on various temperatures for 3 days. Culture medium composition was glucose (5%),polypeptone (1%) and yeast extract (0.5%). △−△: *S. cerevisiae* B (Industrial strain), □−□: *S. cerevisiae* RA-74-2, ○−○: *K. marxianus* RA-912.

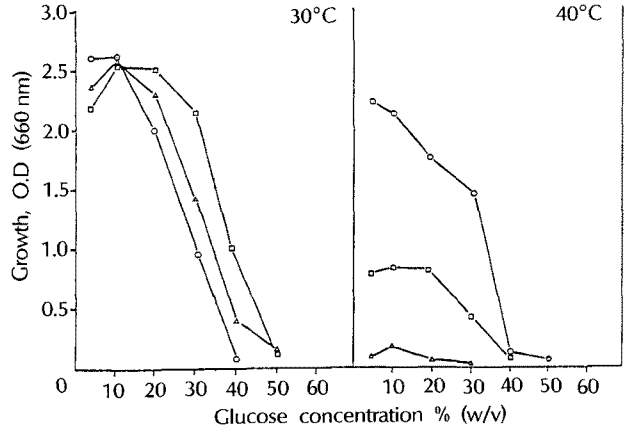

Fig. 4. Effect of glucose concentration on the cell growth at various temperatures of industrial strain and isolated strains.
Basal medium composition was polypeptone (1%) and yeast extract (0.5%). △−△: *S. cerevisiae* B (Industrial strain), □−□: *S. cerevisiae* RA-74-2, ○−○: *K. marxianus* RA-912.

3.

Identification of Byproducts in High Temperature Fermentation: In general, fermentation product was changed in quantity and quality according to metabolic change as temperature shift (10). The byproducts in high temperature or vacuum fermentation effect on cell viability and overall fermentation efficiency. Therefore we analyzed the byproducts from jar-fermentor broth by G.C. The results are shown in Table 4. In case of RA-74-2 ,there is no particular change detected in byproducts at 30°C and 40°C. However, isopropanol, not

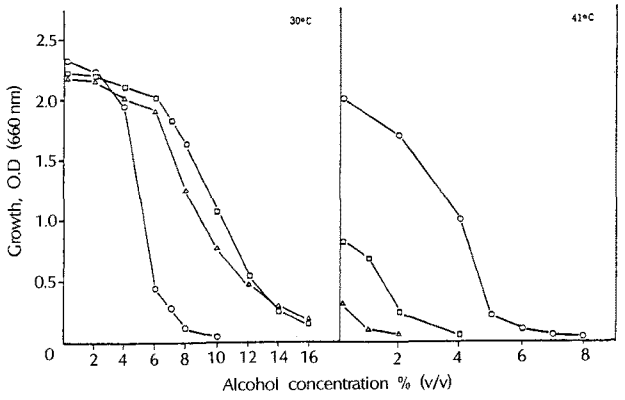

Fig. 5. Effect of ethanol concentration on the cell growth at various temperatures of industrial strain and isolated strains. Basal medium composition was polypeptone (1%), yeast extract (0.5%). and glucose (2%). △−△: *S. cerevisiae* B (Industrial strain), □−□: *S. cerevisiae* RA-74-2, ○−○: *K. marxianus* RA-912.

Table 3. Comparision of tolerance of the ethanol on the fermentation of among the S.cerevisiae B and isolated strains according to alcohol treatments.

Temperature	30°C		40°C		45°C	
Treated ethanol Concentration.% (v/v)	0	15%	0	15%	0	15%
S. cerevisiae B	93	93	57	33		
RA-74- 2	90	90	91	60		
RA-912	76	31			45	47

*The seeds used in this test were prepared to in YPD liquid medium with 15% (v/v) ethanol. After incubate for 2 days at 30°C in that media,the yeast cells were collected and used the fermentation inoculum.The fermentation was carried out at 30°C and 40°C to RA-74-2 and *S. cerevisiae* B for 72 hours. In case of RA-912,the fermentation was carried out at 30°C, 45°C for 72 hours. Agitation speed was adjusted 100 rpm.
**Units: Fermentation ratio (%).

produced at 30°C, was detected at 0.0267% (v/v) at 40°C and acetaldehyde concentratrion was increased 2.57 folds at 40°C in the strain of B company. RA-912 showed a pattern similar to that of strain of B company in that isopropanol was detected at 0.0134% (v/v),and acetaldehyde concentration was increased 1.68 folds at 45°C. But fermentation byproducts above 40°C in isolated strains were similar to those found in 30°C in this experiment.

Resistance of heavy metals: The result of the isolated strains' resistance to the heavy metal are shown in Table 5. The RA-912 has extensive tolerance in used heavy metals except for Co^{2+} and Hg^{2+}. The RA-74-2 and industrial strains appeared to have same heavy-metals tolerances but showed somewhat different resistance fashions in Hg^{2+}, Ni^{2+}. This chacteristic of various heavy-metal resistances are some major advantage for use of raw material substrate and applied strain developments.

Table 4. Comparision of fermentation byproducts of *S. cerevisiae* B and isolated strains by gas chromatography.

	S. cerevisiae B		RA-74-2		RA-912	
F.T[1]	30°C	40°C	30°C	40°C	30°C	45°C
1. Acetaldehyde	1.735	4.47	2.90	1.99	2.16	3.63
2. Methanol						
3. Isopropanol		0.0267				0.0134
4. Ethylacetate						
5. n-Propanol	0.0021	0.0031				
6. Isobutanol	0.0008	0.0008	0.0029	0.0011	0.0039	0.0017
7. n-Butanol		0.0008	0.0003	0.0006	0.0003	0.0005
8. Isoamylalcohol	0.0035	0.0015	0.0054	0.0049	0.0045	0.0008
9. n-Amylalcohol	0.0085	0.0030	0.0096	0.0117	0.0061	0.0043

*The data were mean values from duplicate experiments F.T[1]: Fermentation temperature.
**Industrial Units 1.: mg/100 ml, 2.: mg/ml, 3. 4. 5. 6. 7. 8. 9.: % (v/v).

Table 5. Comparison of various heavy metal resistances about the isolated strains and industrial strains.

		RA-912	RA-74-2	Industrial Strain B	Strain D
$PbCl_2$	400 ppm	+	+	+	+
	800 ppm	+	+	+	+
	1200 ppm	+	+	+	+
$CoCl_2$	400 ppm	−	−	−	−
	800 ppm	−	−	−	−
	1200 ppm	−	−	−	−
$CuSO_4$	400 ppm	+	−	−	−
	800 ppm	−	−	−	−
	1200 ppm	−	−	−	−
$HgCl_2$	400 ppm	+	+	+	−
	800 ppm	−	−	−	−
	1200 ppm	−	−	−	−
$AgNO_3$	400 ppm	+	+	+	−
	800 ppm	+	+	+	−
	1200 ppm	+	+	+	−
$SnCl_2$	400 ppm	+	+	+	+
	800 ppm	+	+	+	+
	1200 ppm	+	+	+	+
H_2WO_4	400 ppm	+	+	+	+
	800 ppm	+	+	+	+
	1200 ppm	+	+	+	+
$Ni(NO_3)_2$	400 ppm	+	−	+	−
	800 ppm	−	−	−	−
	1200 ppm	−	−	−	−

DISCUSSION

In order to use thermotolerant ethanol fermentation yeast for its high productivity and economical process, we screened thermotolelant yeast in soils and wastewa-

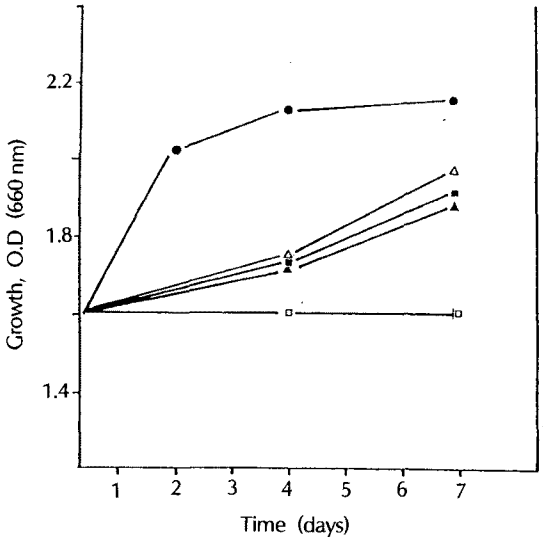

Fig. 6. Effect of the alcohol resistance in RA-74-2 according to the temporary heat-treatment.
The cultivation was carried out in YPD media at 30°C. ●−●: 30°C, alcohol free medium, □−□: 30°C, 15% (v/v) alcohol media, △−△: 55°C, 5 minutes (Heat shock) → 37°C, 1 hours preculture in alcohol free medium → 30°C, 15% (v/v) alcohol media, ▲−▲: 55°C, 5 minutes → 30°C, 15% (v/v) alcohol media, ■−■: 37°C, 1 hours (Heat stress) *k* 30°C, 15% (v/v) alcohol media.

ters in high-temperature environments. Among the isolants,two strains were selected through the enrichment culture method and the subsequent three selective steps. Particularly, in the process of strain selection, we used a jar-fermentor in order to investigate for the possibility of scale-up.

Fermentation in jar-fermentor scale, in general, was better than that in flask scale. In the scope of increased fermentability, there is a little difference in each strains. The strains for industrial utilization showed the stable fermentability in the process of scale-up. Also, in case of the final two selected strains, fermentation using the jar-fermentor was better than that using the flask scale. With these results in mind, isolants of this study were used in a large-scale fermentation (Fig. 2). The finally selected strains, RA-74-2 and RA-912, were identified as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* respectively. The isolated strains were excellent thermotolerant yeasts which grow at 40°C and 30% glucose concentrations. At the fermentation at 40°C for 96 hours, *S. cerevisiae* RA-74-2 was able to produce 86.6 g/L ethanol from 200 g/L glucose fermentation medium (85% of theoretical yields). The *K. marxianus* RA-912 that grows at 48°C, was able to produce 42.3 g/L ethanol from the 200 g/L glucose fermentation medium at 45°C for 72 hours (41.5% of theoretical yields). In a way, we know that the temporary heat-treated *S. cerevisiae* RA-74-2 increased ethanol resistance(Fig. 6). This effect could be explained that temporary-heat treatments induce cellular physiological condition that could grow in the presence of high-concentration of ethanol. Especially, the survival ratio of precultured yeast (37°C for 60 minutes) was higher than that without any preculture after temporary-heat treatments in 30°C, 15% alcohol media.This suggested that heat shock proteins, which were produced by heat-stress,are related to viability and to a survival program in high concentration of ethanol (7, 9, 11). On the contrary, the growth temperature of *S. cerevisiae* RA-74-2 was not increased by temporary treatments of 15% ethanol (data not shown). The facts could be applied not only to the research of ethanol resistance at high temperature but also to the development of ethanol-tolerant fermenting yeasts.

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