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The Fermentation Characteristics of Newly Selected Thermotolerant Yeasts at High Temperature

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In order to develop a method of economical production and to reduce energy-consumption in fuel alcohol production, we investigated the fermentation characters of two newly selected thermotolerant yeasts. The RA-74-2 showed stable and superior fermentability between 30 and 40°C in 20% glucose media in comparison to the industrial strains. The optimum concentration of glucose for economical fermentation at 40°C was 15~18%, and organic nitrogen was necessary for a satisfactory fermentation. The optimum pH was 4.0 and aeration was adversed for high temperature fermentation. Agitation was an important factor at 40°C and the addition of magnesium ion 0.2% was required in this experiment. When the inoculum was increased, ethanol productivity as well as the speed of fermentation increased. On the other hand RA-912, which can grow at 48°C, showed similar fermentability between 30~45°C in 20% glucose media. As the concentration of substrate decreased, fermentation ratio increased at 45°C (45%, 65%, 95% fermentation ratio in 20%, 15%, 10% glucose media, respectively). Also, requirement of organic nitrogen and magnesium ion in RA-912 was similar in RA-74-2. The optimum pH for fermentation was 5.0, and the effects of agitation were enhanced at 37°C than at 45°C. As the inoculum was increased, fermentation speed became more enhanced but the ethanol productivity was less affected. RA-912 showed fermentability with various substrates. Among the substrates used, inulin was the most promising substrate for the high-temperature fermentation. When 14.5% inulin was used as the substrate, 93% and 55% fermentation ratios were shown at 37°C and 45°C, respectively.

The research for alternative clean energy has been actively carried out because of increase in air pollution and the shortage of fossil resources (1. 2). Ethanol,up to 20%, can be blended with gasoline to achieve octane enhancement and be used without engine modification. In practice, some countries have used it as fuel for a long time (3). Also researches of fuel alcohol have been continuously carried out domestically and many technical problems in alcohol fermentation have been mostly solved. However, considering its economical importance,there are surprisingly few publications on the high-temperature fermentation by yeasts. Fermentation at 37~40°C or above has advantages such as recovery of ethanol and significant saving on operational costs of refrigeration controls in distilleries for fuel alcohol production (4). In addition, prevent of contamination, more simple operation, increasement of productivity along with highly metabolic ratio can be expected from thermotolerant yeast in high temperature fermentation. But,thermotolerant fermentation yeasts reported in *Saccharomyces cerevisiae, S. fermentati, S. thermoantitonum, S. anomaluus, Kluyveromyces marxianus, Hansenula* sp., *Brettanomyces* sp., have not been applied in industrially (5, 6). We previously reported that thermotolerant fermentation yeasts, *S. cerevisiae* RA-74-2 and *K. marxianus* RA-912, were the most promising strains for industrial use in high-temperature fermentation (7). Therefore, in this research, we studied the effects of chemical and physical factors on the high-temperature fermentation using the two selected strains of thermotolelant yeasts isolated in our laboratory.

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Key words: fuel alcohol, agitation effect, themotolerant ethanol producing yeast

MATERIALS AND METHODS

Strains

The used strains were *S. cerevisiae* RA-74-2 and *K. marxianus* RA-912; the most promising strains at high temperature fermentation, isolated in our laboratory (7). The strain of B company, which has the best thermostability and fermentability among the strains from 9 different alcohol industry Co., was used compared with the

isolated strains.

Media and Substrates

For seed culture, yeast strains were grown in a YPD medium containing 0.5% yeast extract, 0.5% polypeptone and 1% glucose (pH 6.0). Media for the fermentation consisted of various concentrations of glucose,yeast extract 5 g/L, $(NH_4)_2SO_4$ 3 g/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. Glucose was purchased from Sun-il Co. Lactose, xylose, and inulin were purchased from Sigma Co. The starting pH of the fermentation media was 5.2~5.4 after sterilization.

Ethanol Fermentation.

Ethanol fermentation was carried out in a 250 ml flask with 50 ml fermentation medium. The flask was equipped with an air restrictor containing sulfuric acids. To quantitate the ethanol fermentation, the flask was inoculated with one loopful of cells and incubated at 30~45°C waterbath for 3~5 days. The loss in weight resulting from carbon dioxide was measured and the result was expressed fermentation ratio (7). In case of the jar fermentor tests with a working volume of 3 liters, inoculation was made at 3% (v/v) level. The cultures were stirred at 200 rpm and the pH was not controlled. The cooling water from Handy cooler was used to entrap the volatiles for high temperature and to evolve carbon dioxide. The broth samples were obtained aseptically through the sample port and each sample was tested for sugar contents, ethanol contents, cell growth and pH. The fermentation temperature was controlled at 30~45°C by a fermentation controller (Mituwa Co.).

Analytical Methods

Ethanol in the fermentation broth was determined after single distillation by alcohol hydrometer (8). The residual sugar was measured by Somogyi-Nelson methods using glucose as the standard (9). Cell growth was measured either by Spectrophotometer (Beckman DU-40) at 660 nm or by the D.C.W after washing it with sterilized water three times.

RESULTS

In order to investigate the fermentation characteristics at high temperature, 20% glucose was used as the substrate in flask scale.

Effects of Temperature

The fermentability of nine industrial strains was tested at various temperatures for 96 hours in a 20% glucose fermentation medium. As the result, strains of A, B ,D companies showed superior fermentation at 30°C and the strain of B company was more actively fermented than any other strains at above 37°C (Fig. 1A). Therefore the effect of fermentation temperature on the isolated

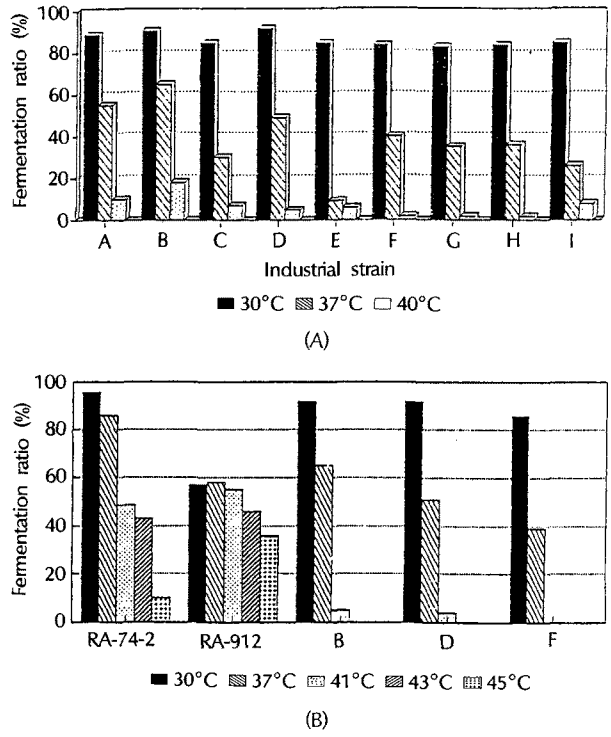

Fig. 1. (A) Effect of temperature on fermentation of industrial strains.

The fermentation was conducted in 250 ml flask with 50 ml of fermentation broth containing Glucose 200 g/L,Yeast extract 2 g/L, Polypeptone 2 g/L, $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 2 g/L. To quantitate the ethanol fermentation, the flask was inoculated with one loopful of cells and incubated at various temperatures for 96 hours. The loss in weight resulting from carbon dioxide production was measured and the result was expressed the relative fermentation ratio (%). Used industrial strains, *S. cerevisiae* A-I strains, were kindly supplied by RaCER.

(B) Comparision of fermentation ability at various temperatures of industrial and isolated strains.

The fermentation condition and alcohol quantitative analysis methods were the same as Fig. 1A.

strains, *S. cerevisiae* RA-74-2 and *K. marxianus* RA-912, were investigated in comparison to that on the strain of B company. The RA-74-2 showed 93%, 50% fermentation ratio at 30°C, 41°C respectively. The RA-912 showed stable fermentability at a high temperature within the limits of 37~45°C (Fig. 1B).

Effects of Initial pH

Cell growth and fermentation were affected by the initial pH (10), and so we investigated the optimum initial pH for high temperature ethanol fermentation (40 or 45°C). As the result is shown in Table 1, the optimum initial pH was 6.0. However, more efficient fermentation was carried out at pH 4.0 and pH 5.0 for RA-74-2 and RA-912 respectively. The initial pH was controlled by 2N HCl.

Effects of Agitation

To examine the effects of agitation,standing and sha-

Table 1. Effect of initial pH on alcohol fermentation of RA-74-2 and RA-912.

pH	Total sugar (mg/ml)	Alc concentration % (v/v)	Fermentation ratio (%)	Remaining Sugars (mg/ml)	$Y_{p/s}$*
RA-74-2					
3.0	200	2.5	19.5	140	0.33
4.0	200	5.1	39.7	112	0.46
5.0	200	5.9	45.9	94	0.44
6.0	200	6.7	52.2	76	0.43
RA-912					
3.0	200	2.1	16.3	148	0.32
4.0	200	5.5	42.8	91	0.40
5.0	200	5.9	46.0	89	0.422
6.0	200	6.1	47.5	80	0.403

$Y_{p/s}$*: g Ethanol/Consumed g Glucose. The fermentation was conducted in 250 ml flask with 50 ml of fermentation broth containing Glucose 200 g/L,Yeast extract 2 g/L, Polypeptone 2 g/L, $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 2 g/L . Fermentation was carried out in standing waterbath for 72 hours at 40°C for RA-74-2 and 45°C for RA-912.

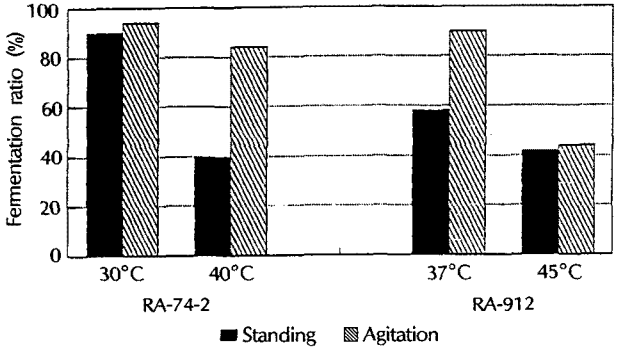

Fig. 2. Effect of agitation on the ethanol fermentation of RA-74-2 and RA-912.
Cells were cultured at various temperatures for 96 hours in 20% glucose medium. Agitation speed was adjusted to 100 rpm.

king cultures were carried out for 96 hours. As the result is shown in Fig. 2, effects of agitation on RA-74-2 increased more at 40°C than at 30°C, and that on RA-912 increased more at 37°C than at 45°C. This suggested that agitation is a key factor in the high-temperature fermentation of isolated strains.

Effects of Substrate Concentration

In order to achieve the optimum concentration of sugar for high- temperature fermentation (40 or 45°C), 10~20% glucose media was used. As the result is shown in Fig. 3, the strains used decreased the fermentation ratio as the sugar concentration increased. These results show that the fermentation ability was affected by complex surrounding factors such as temperature, sugar concentration,ethanol concentration etc (10~12). The optimum sugar concentration .for economical fermentation at above 40°C were 15~18%, 10~15% for

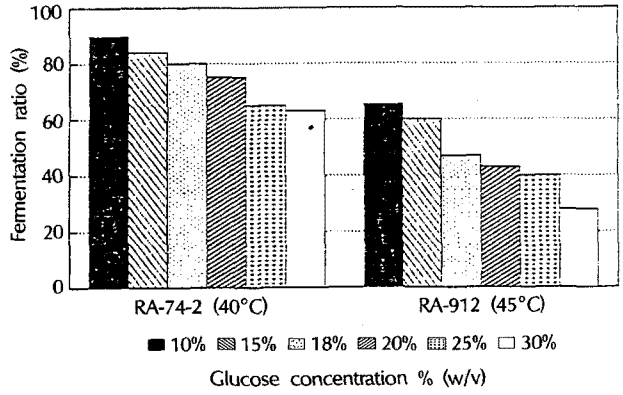

Fig. 3. Effect of initial glucose concentration on the ethanol fermentation of RA-74-2 and RA-912.
The fermentation was conducted at 40°C to RA-74-2 and at 45°C to RA-912 for 96 hours in 20% glucose medium. Agitation speed was adjusted to 100 rpm and the initial pH was 5.2 after sterilization.

RA-74-2, RA-912, respectively.

Effects of Nitrogen Sources

Since growth and ethanol productivity of yeast can be affected by the nitrogen sources in fermentation broth, the effects of nitrogen sources on fermentation were investigated. As the result show in Fig. 4A, organic nitrogens,such as yeast extract or com steep liquor (C.S. L), were better than inorganic nitrogens in high temperature fermentation. As the amount of organic nitrogens were increased in the culture broth, fermentability was enhanced (Fig. 4B). This fact with coincide the previous reports (13). In our study, a substrate was used to purify glucose, and possibly because of for that reason, fermentation may have been affected by vitamin, trace elements and nitrogen sources.

Effects of Metal Ions

Since the glycolytic enzymes requires the metal ions for activity, effect of metal ions on high temperature fermentation were examined. Concentrations of metal ion above 2% enhanced ethanol productivity and growth rate in the bacteria (14). Among the metal ions used,magnesium was required in the high-temperature fermentation and only 0.2% magnesium was sufficient in this experiment (Fig. 5). According to other previous reports (15), ethanol productivity increased when calcium or alumina was added. The effect of magnesium ion on RA-912 was replaced by that of calcium ion, and the addition of both metal ions did not increased ethanol productivity. The effect of magnesium ion on RA-74-2 was not replaced by that of calcium ion, but the ethanol fermentation increased a little when those ions were added (data not shown).

Effects of Antifoam

To investigate effect of antifoam on the high-temperature fermentation, various antifoam agents were added

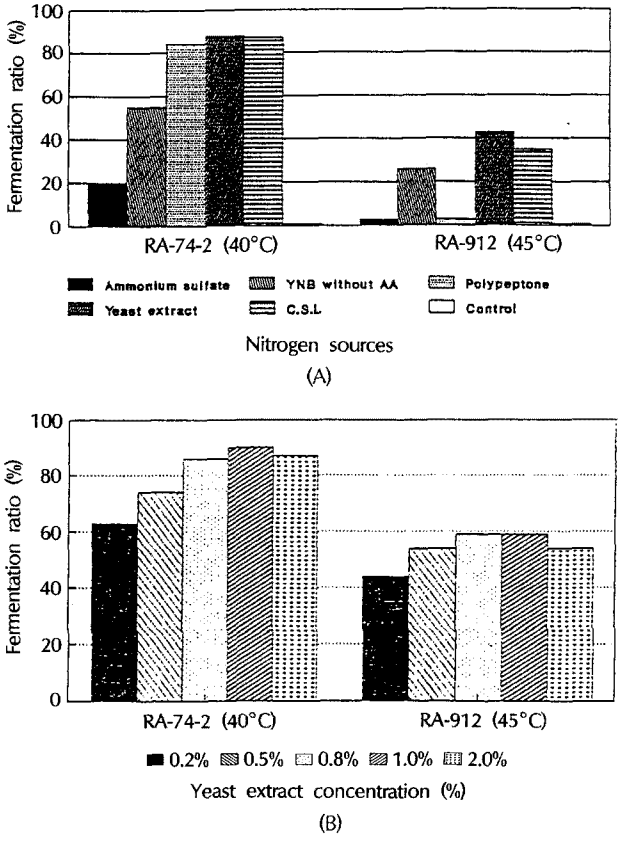

Fig. 4. Effect of nitrogen sources (A) and yeast extract (B) on ethanol fermentation of RA-74-2 and RA-912.
The fermentation was conducted at 40°C to RA-74-2 and at 45°C to RA-912 for 96 hours in 20% glucose medium. Agitation speed was adjusted to 100 rpm and the initial pH was 5 after sterilization.

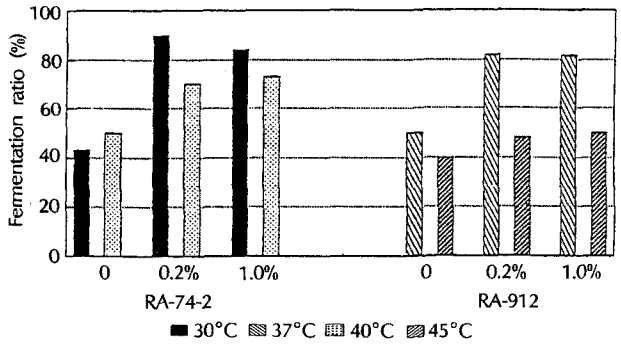

Fig. 5. Effect of magnesium ion on ethanol fermentation of RA-74-2 and RA-912.
The fermentation was conducted at 30, 40°C to RA-74-2 and at 37, 45°C to RA-912 for 96 hours in 20% glucose medium. Agitation speed was adjusted to 100 rpm and the initial pH was 5 after sterilization.

Table 2. Effect of antifoam agents on ethanol fermentation of RA-74-2 and RA-912.

	Antifoams	0	0.1%	0.2%	0.5%
RA-74-2	Dawcoming DB-110A	70.0	70.0	69.5	64.5
	FL-70	70.0	67.5	67.0	65.3
	Tween-20	70.0	64.1	62.5	58.7
	Silicagate-glycerin ester	70.0	66.5	65.7	58.5
RA-912	Dawcoming DB-110A	39.0	41.1	45.0	34.4
	FL-70	39.0	38.0	35.4	35.6
	Tween-20	39.0	36.9	39.0	40.2
	Silicagate-glycerin ester	39.0	30.3	29.3	22.5

The fermentation was conducted at 40°C to RA-74-2 and at 45°C to RA-912 for 72 hours in 20% glucose medium. Agitation speed was adjusted to 100 rpm and the initial pH was 5 as not adjusted. Units: Theoretical fermentation ratio (%).

to the fermentation media.In general, ethanol resistance was enhanced by the addition of antifoam agent (16, 17). The antifoams used were Dowcoming DB-110A (Lucky Silicon Co.), silicagate glycerin ester, Tween-20, FL-70 (Fisher scientific Co.). But the yeasts used in high-temperature fermentation were not affected by the addition of antifoams in regard to the fermentation yields and fermentation speed (Table 2).

Effects of Inoculum

The seed volume in the conventional process of ethanol fermentation is 10% (v/v) to fresh broth regardless of the substrate for the purpose of less contamination and rapid fermentation. Therefore, we examined the effect of seed volume on the high-temperature ethanol fermentation of 20% glucose media. The culture conditions were maintained at 40°C, 100 rpm for RA-74-2 and 45°C, 100 rpm for RA-912. As shown in Fig. 6, when the seed volume was 10% (v/v), the ethanol productivity was enhanced and the period of fermentation was shortened compared with that of 1% (v/v) (18). As seed volume was 10% (v/v), the RA-74-2 showed 88% fermentation ratio during the 24 hours at 40°C and the RA-912 showed 53% fermentation ratio for 48 hours at 45°C.

Fermentability with Various Substrates of RA-912

Since the thermotolerant yeast RA-912 has fermentability with various substrate, we used lactose, xylose and inulin as substrate. Inulin and xylose in hemicellulose is attracting attention as a possible carbohydrate resources (20, 21), and lactose is the major carbohydrate of whey (22). Therefore these substrates could contribute to economic fermentation by cutting down the substrates costs. As shown in Table 3, when 14.5% inulin was used as the substrate, 93% and 55% fermentation ratios were marked at 37°C and 45°C respectively.

In order to investigate the fermentation characteristics at high temperature, 20% glucose was used the substrate in jar-fermentor scale.

Effects of Aeration

To investigate the effect of aeration on high-temperature fermentation,thermotolerant yeast RA-74-2 was cul-

tured in the jar fermentor scale with an air compressor (Iwaki AP-115N). In general, the dissolved oxygen is required for yeast cell growth and maintainance, and oxygen solubility to water is limited at atmospheric pressure, and more restricted at high temperature (17, 19). Therefore we aerated 16 hours at 0.5 vvm after inoculation in this experiment in comparison to the 8~10 hours aeration in the industrial scale. Fermentation temperature was maintained at 40°C. As shown in Fig. 7, ethanol productivity and cell growth with aeration was

Table 3. Comparision of ethanol fermentation with various substrates of RA-912.

RA-912		Fermentation ratio (%) 37°C	45°C
Xylose	(6%)	10	32
Lactose	(11%)	38	30
	(15.6%)	22	13
	(19.2%)	17	8
Inulin	(14.5%)	93	55

The fermentation was conducted at 37 or 45°C to RA-912 for 48 hours in various substrates. Agitation speed was adjusted to 100 rpm.

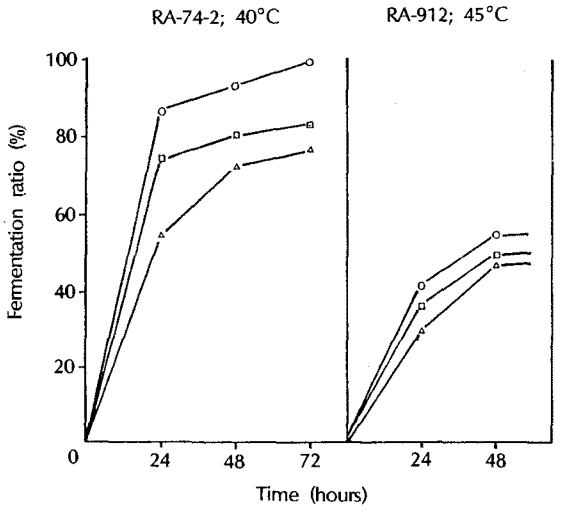

Fig. 6. Effect of amount of inoculum on ethanol fermentation of RA-74-2 and RA-912 .
The fermentation was conducted at 40°C to RA-74-2 and at 45°C to RA-912 in 20% glucose medium. Agitation speed was adjusted to 100 rpm and the initial pH was 5 after sterilization △−△: 1%, □−□: 5%, ○−○: 10%.

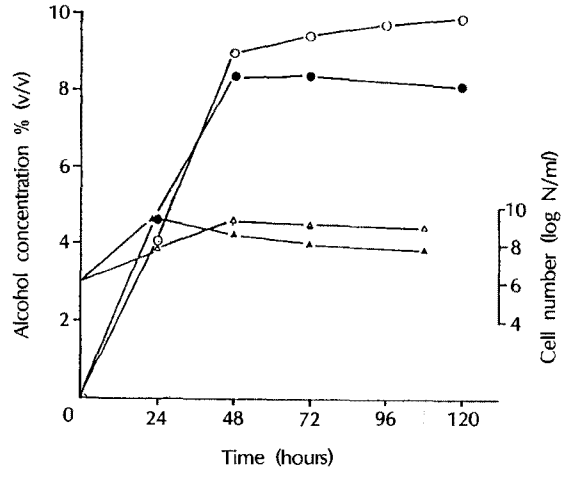

Fig. 7. Effect of aeration on ethanol fermentation of RA-74-2.
The fermentation was conducted in a jar-fermentor with 3 liters of fermentation broth containing Glucose 200 g/L, Yeast extract 5 g/L, $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 2 g/L. The seed volume was 2% and pH was not controlled. The fermentation was carried out at 40°C, 200 rpm.
Black: 16 hours aeration, White: No aeration, ○−○: Alcohol concentration, △−△: cell number.

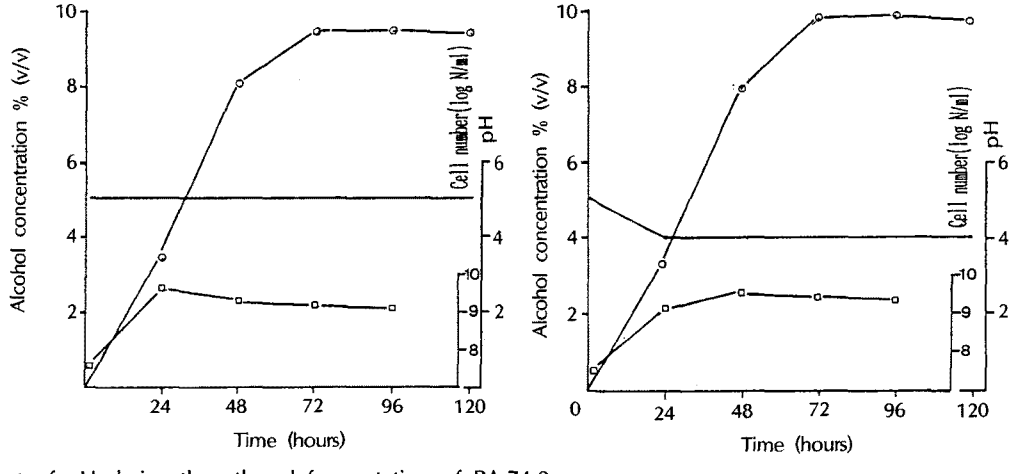

Fig. 8. Effect of pH during the ethanol fermentation of RA-74-2.
The fermentation was conducted in a jar-fermentor with 3 liters of fermentation broth containing Glucose 200 g/L, Yeast extract 5 g/L, $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 2 g/L. The seed volume was 2% and pH was controlled to 5.0 and 4.0 with 2N NaOH. In case of pH 4.0, pH was controlled after 24 hours. The fermentation was carried out at 40°C, 200 rpm. ○−○: Cell number, −: pH.

better than those of not-aerated fermentation during the 24 hours. However, after 48 hours, aerated fermentation was inferior to not-aerated fermentation in ethanol productivity and cell growth. Also, final fermentation ratios and Yp/s (g ethanol/g utilized sugar) were 77%, 88% and 0.4765, 0.4876 in aerated and not-aerated fermentation, respectively.

Effects of Fermentation pH

To investigate the effect of fermentation pH on the high-temperature fermentation, RA-74-2 was cultured in jar fermentor scale with a pH controller (Mituwa Co.). The pH 4.0 was maintained with 2N NaOH after 24 hours. The culture conditions were maintained at 40°C, 200 rpm, not-aerated. As shown in Fig. 8, 9.4% and 9.74% (v/v) alcohol was produced at pH 5.0 and pH 4.0 during the 72 hours, respectively (while, the fermentation pH was maintained to 3.95~4.05 with 5N NaOH for 0~72 hours, 11.2% (v/v) alcohol was produced; data not shown). In pH 5.0, cell number was sharply increasd to 4.2×10^9 cell/ml at 24 hours and slowly decreasd as time passed. In pH 4.0, cell number slowly increased to 3×10^9 cell/ml during the 72 hours.

Effects of Agitation Speed

To investigate the effect of agitation speed on the high-temperature fermentation, RA-74-2 was cultured in a jar fermentor with 2L fermentation broth. The impeller was an open turbine type (fermentor diameter, 15 cm; impeller diameter, 5 cm; impeller height, 0.5 cm; liquid height, 14 cm) and the agitation speed was adjusted to 50, 100, 200, 300 rpm respectively. The fermentation was performed for 96 hours at 40°C. As shown in Fig. 9, efficient agitation speed was 100~200 rpm. The fermentation was inhibited at below 100 rpm and above 200 rpm.

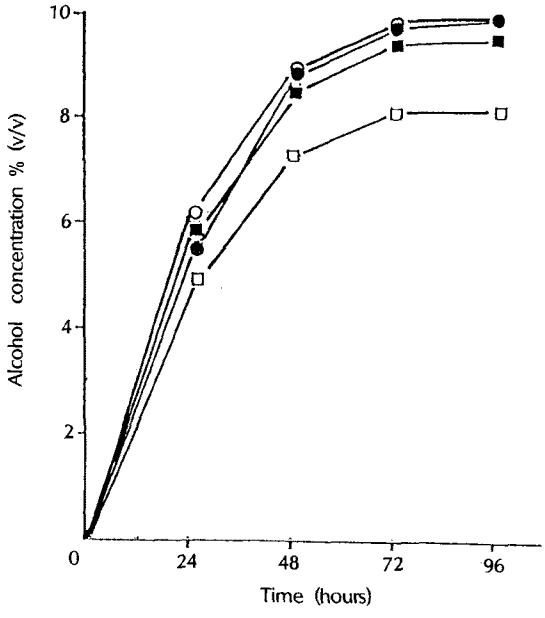

Fig. 9. Effect of agitation speed on ethanol fermentation of RA-74-2.

The fermentation was conducted in a jar-fermentor with 2 liters of fermentation broth. The impeller was an open turbine type (fermentor diameter, 15 cm; impeller diameter, 5 cm; impeller height, 0.5 cm; liquid height, 14 cm). The fermentation was carried out at 40°C and pH was not controlled.

□−□: 50 rpm, ●−●: 100 rpm, ○−○: 200 rpm, ■−■: 300 rpm.

DISCUSSION

We investigated the fermentation characteristics of newly selected thermotolelant yeasts (RA-912, RA-74-2) for hyperproductivity and economical process. In case of RA-74-2, it showed stable fermentability between 30 and 40°C, in comparison to the industrial strains (Fig. 1B). Agitation was an important fermentation factor at 40°C but it was less affective at 30°C. On the other hand, RA-912 showed similar fermentability between 30~45°C and the effects of agitation were more affected at 37°C than at 45°C (Fig. 2). Therefore it is suggested that agitation affected thermophilic yeast more affected at a low temperature than at a high temperature, and that agitation affected thermotolerant yeast more at a high temperature than at a low temperature. Among the nitrogen sources, CSL (corn steep liquor), which is inferior to the yeast extract at 30°C, was an excellent source at a 42°C-fermentation with RA-912, RA-74-2. The strain of B company was not affected by CSL. Polypeptone,in particular, was of no use at a 45°C fermentation for RA-912 (Fig. 4A, Fig. 10). This result suggested that amino acids were not essential molecule on high temperature fermentation. The optimum substrate concentration was 15~18% for RA-74-2 and 10~15% glucose for RA-912 and 0.2% $MgSO_4$ was sufficient for a satisfactory fermentation. In RA-74-2 with 16-hour aeration (The reason for 16-hour aeration was to prevent alcohol loss by aeration at 40°C) after inoculation, cell growth and ethanol productivity was better than those of not-aerated fermentation at 40°C during the initial 24 hours. However,the fermentation stopped and cell growth decreased after 48 hours of aerated fermentation (Fig. 7). The results were relative to the byproducts from aerated high-temperature fermentation (data not shown). Then inulin, xylose and lactose were ready to be as used substrate for RA-912. Especially inulin was the most promising one among the substrates used in the high-temperature fermentation. When 14.5% inulin was used, 93% and 55% fermentation ratios were marked at 37°C and 45°C respectively. Considering the inulinase (optimum pH 4.5~5.0, optimum temperature 45~50°C: data not shown) secreated by RA-912, these

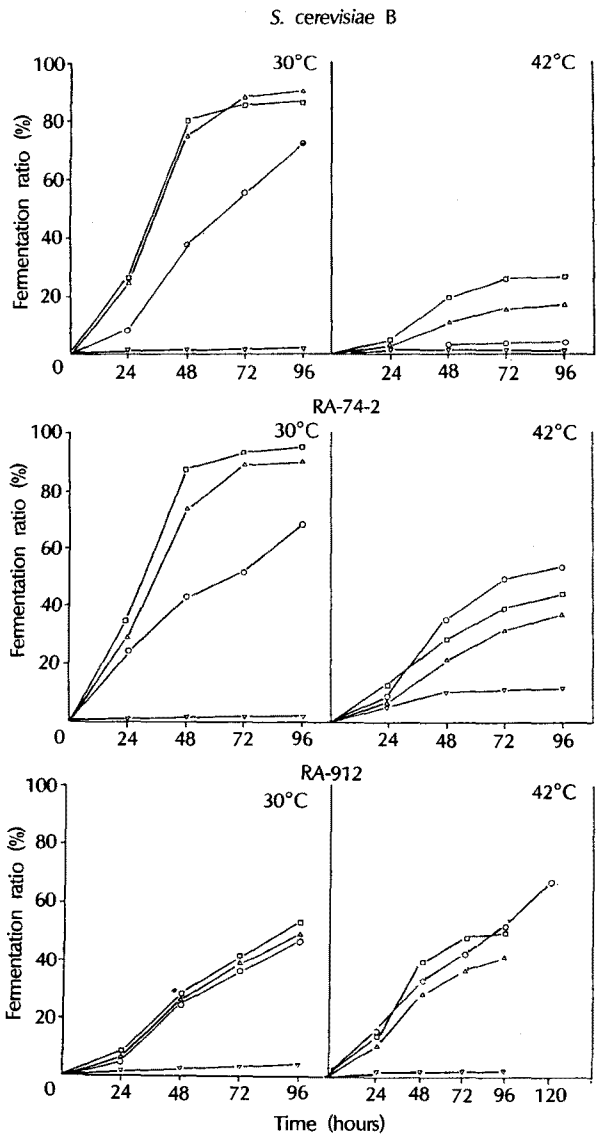

Fig. 10. Comparision of nitrogen sources on the ethanol fermentation at various temperatures of *S. cerevisiae* B (Industrial strain), RA-74-2 and RA-912.

The fermentation was conducted at 30 or 42°C for strain of B alcohol factory. Agitation speed was adjusted to 100 rpm and the initial pH was 5 after sterilization. ▽−▽: 1% $(NH_4)_2SO_4$, □−□: 1% Yeast extract, ○−○: 1% C.S.L. △−△: 0.3% Yeast extract, 0.3% Polypeptone, 0.4% $(NH_4)_2SO_4$.

results suggested that RA-912 could be applied to SSF with inulin at above 37°C and that an economical production of the fuel ethanol was possible.

Acknowledgement

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REFERENCES

1. Park, Jang-Ho, Bun-Sam Lim and Moon-Jin Chun. 1989. Continuous production of ethanol using the immobilized and flocculating cells of *Saccharomyces cerevisiae*. *J. Inst. Biotechnol. Korea Univ.* **1**: 27-31.
2. Fiechter, A., Advances in Biochemical Engineering. 1981. Springer-Verlag Berlin Heidelberg New York. 1-14.
3. Thomas, J., Mullins. 1985. Enzymatic hydrolysis and fermentation of com for fuel alcohol. *Biotech. & Bioeng.* **27**: 321-326.
4. Ryu, Beung-Ho, Ki-Du Nam, Hae-Sung Kim, Dong-Seuk Kim, Young-Ae Ji, and Soo-Ja Jung. 1988. Screening of thermotolerant yeast strain for ethanol fermentation. *Kor. J. Appl. Microbiol. Bioeng.* **16**: 265-269.
5. Pratima Bajpai and Argyrios Margaritis. 1987. The effect of temperature and pH on ethanol production by free and immobilized cells of *Kluyveromyces marxianus* grown on Jerusalem Artichoke extract. *Biotech. and Bioeng.* **30**: 306-313.
6. Cheol Seung Shin and Yoon-Joong Park. 1984. Studies on the Thermophiic yeast for ethanol fermentation. *Res. Rep. Agri. Sci. Tech. Chungnam Natl. Univ. Korea* **11**: 25-33.
7. Sohn, Ho-Yong and Jung-Hwn Seu, Screening and characterization of thermotolerant alcohol-producing yeast. 1994. *J. Microbiol. Biotechnol* (in press).
8. Seu, J.H. and Y.H. Kim. 1989. Ethanol fermentation of fusant between heterologous transformant of *Saccharomyces cerevisiae* and *Candida tropialis* in mini-jarfermentor scale. *Kor. J. Appl. Microbiol. Bioeng.* **17**: 8-13.
9. Kim, Y.H., D.Y. Jun, and J.H. Seu. 1988. Heterologous transformation of *Saccharomyces cerevisiae* by glucoamylase gene of *Saccharomyces diastaticus*. *Kor. J. Appl. Microbiol. Bioeng.* **16**: 489-493.
10. Buzas, Zs., K. Dalimann, and B. Szajani. 1989. Influence of pH and growth and ethanol production of free and immobilized *Saccharomyces cerevisiae* cells. *Biotech and Bioeng.* **34**: 882-884.
11. Laudrinand, I., G. goma. 1982. Ethanol production by *Zymomonas moblis*: Effect of temperature on the cell growth, ethanol production and Intracellular ethanol accumulation. *Biotechnology Letters.* **4**: 537-542.
12. Byung Ki Hur, Hyung Chul Kim, Ji won Yang, and Young Il Mok. 1991. Characteristic of alcohol fermentation by the culture of high cell density (Functional relationship among specific growth rate, sugar concentration, cell concentration and alcohol concentration). *Korean J. Biotechnol. Bioeng.* **6**: 85-90.
13. Seu, J.H., Y.H. Kim, S.M. Lee, and B.H. Bang. 1989. Ethanol fermentation of fusant between heterologous transfomant of *Saccharomyces cerevisiae* and *Candida tropicalis* in Pilot scale. *Kor. J. Appl. Microbiol. Bioeng.* **17**: 14-18.
14. Pramod, K., Bajpai and Argyris Magaritis. 1984. Effect of calcium choride on the growth and ethanol production by free cells of *Zymomonas mobilis*. *Biotechnol. letters.* **6**: 673-676.
15. Kanellaki, M., A.A. Koutinas, K. Kana, M. Nicolopoulou, A. Papadimitriou, and A. Lycourghitis. 1989. Ethanol producton by *Saccharomyces cerevisiae* promoted by gamma-

Alumina. *Biotech. and Bioeng.* **34**: 121-125.
16. Tony, D'Amore and Grahamm G. Stewart. 1987. Ethanol tolerance of yeast. *Enzyme Microbe. Technol.* **9**: 321-330.
17. Ryu, Yeon Woo and Heang-Wook Jang. 1991. Effect of oxygen and Unsaturated fatty acids on the ethanol tolerance of yeast strains. *J. Microbiol and Biotech.* **1**: 6-11.
18. Strehaiano, P., M. Mota, G. Goma. 1983. Effects of inoculum level on kinetics of alcohol fermentation. *Biotech. letters.* **5**: 135-140.
19. Kim, H.J., H.W. Jang, and Y.W. Ryu. 1989. The conditions affecting ethanol tolerance of yeast strains in alcohol fermentation—Study on the aeration and lipid addition. *Korean J. Biotechnol. Bioeng.* **4**: 172-176.
20. Ansa Tolvola, D. Yarrow, E. Bosch, J. Duken, and W.A. Scheffers. 1984. Alcoholic fermentation of D-Xylose by yeasts. *Appl. Environ. Microbiol.* **47**: 1221-1223.
21. Chae, Eun-Mi and Eon-Ho Chol. 1991. Optimization for alcohol fermentation by *Kluyveromyces marxianus* using Jerusalem articoke powder. *Kor. J. Appl Microbiol. Biotechnol.* **19**: 265-271.
22. Lee, C.H., J.H. Bang, and Doo Hyun Nam. 1992. Ethanol production from lactose by immobilized reactor system using a fusant yeast strain of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*. *Kor. Jour. Microbiol.* **30**: 355-359.

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