Journal of Microbiology and Biotechnology
Vol. 4, No. 4, 316-321, 1994

Application of Thermotolerant Yeast at High Temperature in Jar-fermentor Scale.

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We investigated the possibility of industrial application and economic process of high temperature fermentation by thermotolerant alcohol producing yeasts as previously reported. From the 20% glucose media, the RA-74-2 produced 11.8% (v/v) ethanol at 32°C (0.5% inoculum) and 10.6% (v/v) ethanol at 40°C (3% inoculum), respectively. Also, 11.3% (v/v) ethanol was produced for 96 hours in the temperature-gradient fermentation. These results suggest that the RA-74-2 could successfully be applied to save the cooling water and energy in industrial scale without re-investment or modification of established fermentation systems. When potato starch was used as the substrate for the RA-74-2, high temperature fermentation above 40°C was more appropriate for industrial utilization because organic nitrogen was not necessary to economical fermentation. As the naked barley media just prior to industrial inoculation, taken from the Poongkuk alcohol industry Co., were used, 9.6% (v/v) ethanol was produced at 40°C for 48 hours in jar-fermentor scale (actually, 9.5- 9.8% (v/v) ethanol was produced at 30~32°C for 100 hours in industrial scale). The ethanol productivity was increased by the high glucoamylase activity as well as the high metabolic ratio at 40°C. Therefore, if the thermotolerant yeast RA-74-2 would be used in industrial scale, we could obtain a high productivity and saving of the cooling water and energy. Meanwhile, the RA-912 produced 6%(v/v) ethanol in 10% glucose media at 45°C and showed the less ethanol-tolerance compared with industrial strains. As the produced alcohol was recovered by the vacuum evaporator at 45°C in 15% glucose media, the final fermentation ratio was enhanced (76% of theoretical yields). This suggest that a hyperproductive process could be achieved by a continuous input of the substrate and continuous recovery of the product under vacuum in high cell-density culture.

The energy consumption has been sharply increased in domestic and the energy resources, especially fossil resources, are mostly imported. Since the fossil resources have energy related problems (for example, air toxic waste and energy crisis), exploitation of the alternative clean energy would be important project which could determine the future of the nation. Among the candidates for the alternative energy resource, the fuel alcohol produced from renewable biomass, was the most prominent because of the accumulated technique in alcohol industry and tremendous deposits of biomass in worldwide (10, 11). As the fuel alcohol may be used in motors without engine modification or change and has various environmental merits (8), research of the fuel alcohol in pilot plant scale has been carried out by the Ministry of Energy and Resources in domestic (9). Research of the fuel alcohol fermentation was divided into the process development and strain improvement, which could bring about an economical mass production and reduction of energy use in fermentation. Among the many trivial problems in the strain improvement, development of the thermotolerant alcohol producing yeast is the most urgent matters. If the thermotolerant yeast should be applied in industrial scale, we could reduce the cooling cost (the fermentation may be started with high temperature) and expect other economic gains, such as volumetric reduction of the effluent, increased productivity along with highly metabolic ratio and low contamination risk (12, 15). Therefore in this study, we investigated the possibility of an industrial application and economical process on high temperature fermentation by the thermotolerant alcohol producing yeasts as previously reported (16).

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Key words: high temperature fermentation, temperature-gradient fermentation, vacuum fermentation, cooling water and energy, naked barley

MATERRIALS AND METHODS

Strains

The strains used were *S. cerevisiae* RA-74-2 and *K.*

marxianus RA-912, the most promising strains on high temperature fermentation, isolated in our laboratory (16). Industrial strain of B company, which has the best thermostability and fermentability among the strains of 9 alcohol industry Co., was used to be compared with the isolated strains.

Media

The media composition for seed culture and fermentation broth was the same as those previously reported. When starch hydrolysate was used as the substrate, no nutrient was added into the fermentation media. Potato dextrose agar (PDA) medium was used for strain preservation at 4°C.

Substrates and Starch Pretreatment.

Glucose was purchased from Sun-il Co. and potato starch was purchased from Kanto chemical Co.. Potato starch for the fermentation was pretreated as follows. The various concentrations of potato starch were prepared in tap water. 0.8% (v/w, to starch content) of Thermamyl (α-amylase, Novo co). was added at room temperature and allowed to liquify the potato starch for 30 minutes at 95°C. After cooling to 60°C of the liquified broth, 1% (v/w) glucoamylase (25 units/g-starch) was added and left alone at room temperature. Glucoamylase was kindly supplied by Poonguk alcohol factory Co.. Liquified and saccharified naked barley broth in industrial scale was used for a practical application. The starting pH of the fermentation media was 5.2-5.4 after sterilization.

Ethanol Fermentation.

Ethanol fermentation was carried out by the same methods as previously reported (16). The cultures were stirred at 200 rpm and the pH was not controlled. In case of temperature-gradient fermentation, cooling water from Handy cooler HC-10 was used for temperature shift. Broth samples were obtained aseptically through the sample port and each sample was tested for sugar contents, ethanol contents, cell growth and pH. Temperature accuracy was better than 0.2°C in this experiment.

Vacuum Fermentation.

Since alcohol was easily vaporized in high temperature or low pressure, product recovery was attempted by vacuum evaporator (Tokyo Rikakikai Co.LTD) and aspirator A-3S (Eyela:Tokyo Rikakikai Co.). The RA-912 was cultivated in 15% glucose media at 45°C for 48 hours and alcohol produced was removed by vacuum for 10-15 minutes (Fig.1). Subsequently, cultivation was carried out for more ethanol production.

Analytical Methods

Ethanol in the fermentation broth was determined by alcohol hydrometer after single distillation (16). The residual sugar was measured by Somogyi -Nelson method using glucose as the standard. Cell growth was measured either by spectrophotometer (Beckman DU-40) at 660 nm or the D.C.W (dry cell weight) after washing it three times with sterilized water. In case of the starch fermentation broth, cell number was directly counted by haematometer.

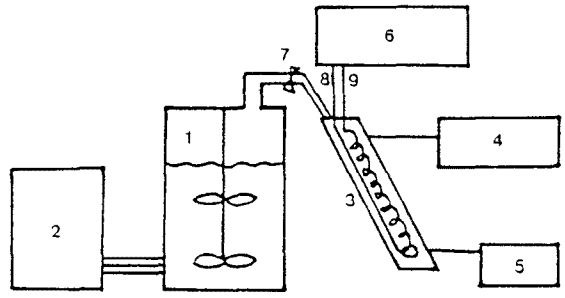

Fig. 1. Schematic diagram for vacuum fermentor with aspirator.

1. Main fermentor(Mituwa KMJ-5A)
2. Fermentor controller(Mituwa)
3. Cooling duct(Tokyo Rikakikai Co.)
4. Aspirator(Eyela A-3S)
5. Product reservoir
6. Cooler(Handy cooler HC-10)
7. Stop valve.
8. Inlet of cooling water
9. Outlet of cooling water.

RESULTS

Ethanol Productivities from Naked Barley of Industrial-pretreated Substrate at Various Temperatures

To investigate the ethanol productivity at various temperatures, 20% glucose media was used for RA-74-2 and RA-912 in jar-fermentor scale. The result is shown in Table 1, Fig. 2. In case of the RA-74-2, 11.8% (v/v), 11.5% (v/v) and 10.6% (v/v) ethanol was produced at 32°C, 38°C and 40°C, respectively. As concentration of yeast extract in fermentation broth was increased to 0.8%, 11.2% (v/v) ethanol was produced at 40°C during the 72 hours (14). However, the industrial strains failed ethanol production above 8.9% (v/v) at 40°C during the 120 hours. The RA-912 was produced 6.3% (v/v) and 5.3% (v/v) ethanol at 45°C in 10% and 20% glucose media (Table 1).

Temperature-gradient Fermentation

In general, commercial alcohol production was divided into 3 parts; preparation of the fermentation media, main fermentation and product recovery (13). When starchy material was used as the substrate, a pretreatment process was necessary. After starch was liquified at 90~95 °C, cooling the media to 30°C is prerequisite to inoculation. Cooling water and immense energy was consumed for optimum fermentor temperature during the fermentation as well as preparation of the media. Thus, we investigated the temperature-gradient system of RA-74-2 to accomodate this problem. The RA-74-2 was inoculated at 40.5°C and fermented for initial 24 hours. Thereafter it was cooled at 37°C by the Handy cooler

Table 1. Jar-fermentor test with 20% glucose fermentation media at various temperatures of industrial strains (B,D company), RA-912 and RA-74-2.

Strains	Temp (°C)	Substrate (%)	Time (hrs)	Alcohol concen -tration % (v/v)	Fermentation ratio (%)	Reference
Industrial strain B	32	20	120	11.8	92.0	Glucose
	40	20	120	8.9	69.3	Glucose
Industrial strain D	32	20	120	11.8	91.9	Glucose
	38	20	50	9.4	73.0	Glucose
	32	20	120	11.8	91.9	PP[2] (-)
	32	20	120	11.0	85.6	Glucose
	38	20	90	11.5	90.4	Glucose
	38	18	96	10.4	90.1	Glucose
RA-74-2	39	20	72	11.5	89.6	Glucose
	40	20	84	10.6	82.5	PP[2] (-)
		20	72	11.2	87.2	PP[2](-), YE[3]0.8%
	32	18% P.S[1]	72	10.6	82.6	N sourcs(-)
	40	15% P.S[1]	96	8.1	76.9	N sourcs(-)
	45	10	48	6.3	98.1	Glucose
	45	10	48	6.2	96.5	Glucose
	45	15	60	6.1	64.1	Glucose
RA-912	45	20	96	5.3	41.3	Glucose
	43	20	96	ND*	55.2	CO_2 decrease**
	42	20	96	ND*	55.2	CO_2 decrease

*N. D: not determined, P.S[1]: Potato starch, PP[2]: Polypeptone, YE[3]: Yeast extract

** CO_2 decrease: The fermentations were conducted in 1 liter suction flask with air restricted fermentation-bung containing 500 ml fermentation broth. To quantitate the ethanol fermentation, the flask was inoculated with one loopful of cells and incubated at various temperatures for 9 6hours. The loss in weight resulting from carbon dioxide production was measured and the result was expressed as the relative fermentation ratio (%). In case of glucose fermentation media, yeast extract was added only 0.2% concentration for industrial application.

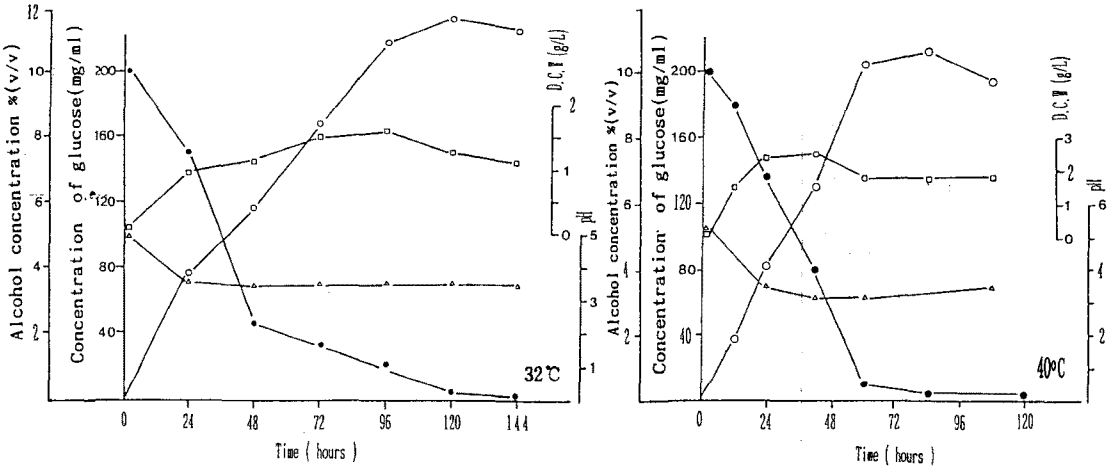

Fig. 2. Ethanol fermentation in 20% glucose at 32°C and 40°C of RA-74-2.
The fermentation broth containing glucose 200g/L, yeast extract 5g/L, $(NH_4)_2SO_4$ 3g/L, KH_2PO_4 1g/L, $MgSO_4$ $7H_2O$ 2g/L. The seed volume was 0.5% and 3% at 32°C and 40°C respectively. The pH was not controlled and agitation speed was maintained o 200 rpm. ○—○: Alcohol concentration, □—□: D.C.W, ●—●: Glucose concentration, △—△: pH

HC-10 and fermented for the next 24 hours. We subsequently fermented at 35°C and 33°C for 24 hours, respectively. This model devised was assuming that the cooling system of the plant scale would not work. That is, temperature is increased to 40°C or above at the initial stage of the fermentation (initial 30-40 hours after inoculation) and decreased to 30°C or below at the final stage of the fermentation. As shown in Fig. 3, RA-74-2 produced 11.3% (v/v) for 96 hours. When RA-74-2 was fermented at 40.5°C for 24 hours, rapid metabolic ratio

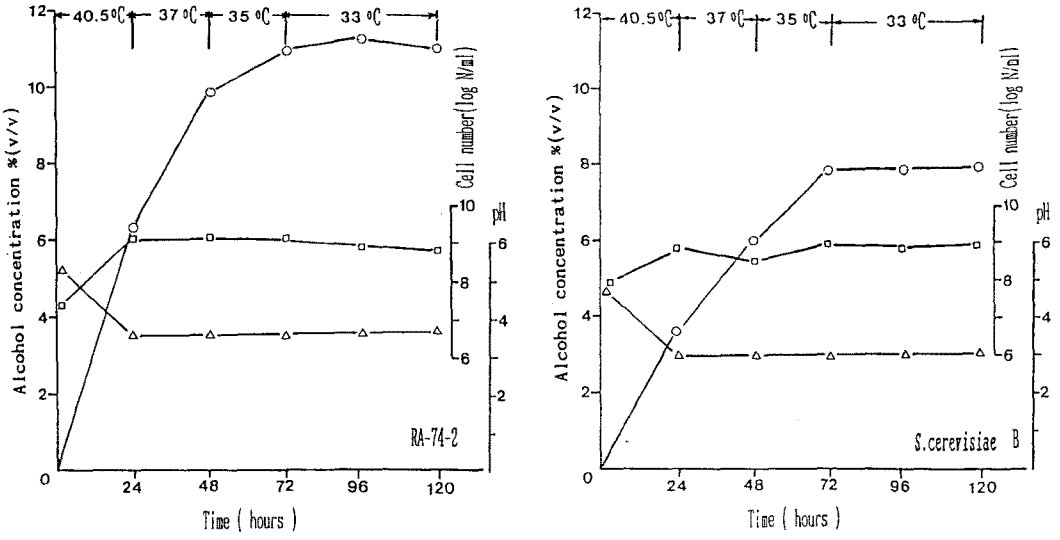

Fig. 3. Temperature-gradient fermentation of RA-74-2 and *S. cerevisiae* B (Industrial strain)
The fermentation was conducted in jar-fermentor with 3 liters of fermentation broth containing glucose 200g/L,yeast extract 5g/L, $(NH_4)_2SO_4$ 3g/L, KH_2PO_4 1g/L, $MgSO_4$ $7H_2O$ 2g/L. The seed volume was 2.5% and the pH was not controlled. O—O: Alcohol concentration, □—□: Cell number, △—△: pH

by high temperature was showed that 6.3% (v/v) ethanol was produced. However, the strain of B company produced 8% (v/v) during the 120 hours under the same condition. These results suggest that RA-74-2 could be sucessfully applied to an industrial fermentation scale without the re-investment or modification of the equipments.

Vacuum Fermentation of RA-912

The alcohol recovery process till present has involved distillation, which consume a tremendous amount of energy above 30% of the total energy-consumation (3, 5). The distillation process was improved to lower energy-consuming process for fuel alcohol in industrial scale (1, 2, 4, 7). In this study, we used vacuum evaporator with aspirator for product recovery, because of the high temperature fermentation. As the alcohol concentration for an economical recovery was 6% (v/v) at 45°C with 50-150 torr as previously reported (1-3, 7), RA-912 was applied in high temperature- vacuum fermentation. The cultivation was carried out in 15% glucose medium at 45°C for 48 hours. Thereafter, alcohol produced was recovered by a vacuum evaporator and subsequently fermented at 45°C. As shown in Fig. 4, RA-912 was shown to have 76% fermentation ratio for 144 hours.

Ethanol Productivities from Potato Starch at Various Temperatures

To investigate the industrial application, we used saccharified potato starch as the substrate. The cultivation was carried out at various temperatures in 10~18% potato starch without any additives. As shown in Fig. 5, RA-74-2 produced 10.6% (v/v) ethanol in 18% potato

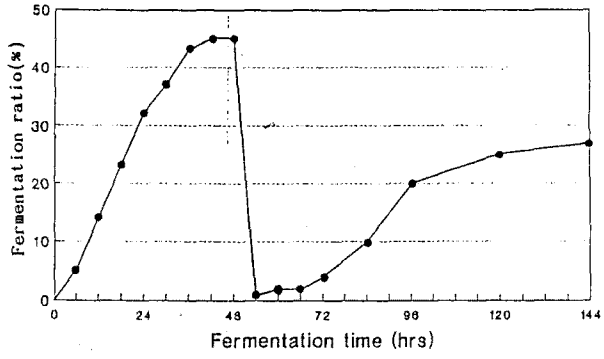

Fig. 4. Vacuum fermentation of RA-912.
The fermentation was conducted in jar-fermentor with 3 liters of fermentation broth containing glucose 150g/L, yeast extract 5g/L, $(NH_4)_2SO_4$ 3g/L, KH_2PO_4 1g/L, $MgSO_4$ $7H_2O$ 2g/L. The seed volume was 3% and the pH was not controlled. The fermentation conditions were maintained at 45-50°C, 200 rpm. The removal of produced ethanol at 48 hours was carried out by aspirator A-3S and vacuum evaporator. The total fermentation ratio was 76% of theoretical yield.

starch at 32°C (83% of theoretical yield) and 8.1% (v/v) ethanol in 15% potato starch at 40°C (77% theoretical yield), respectively. The results suggest that the organic nitrogen sources were less required in starch media than in glucose media at high temperature (Fig. 5). Consequently we used naked barley, which has more nitrogen sources than potato starch, in the next experiments.

Ethanol Productivities from Glucose Media at Various Temperatures

The media just prior to industrial inoculation were received in Poongkuk alcohol industy Co.. About 18% (v/v) total sugar was contained in saccharified media of

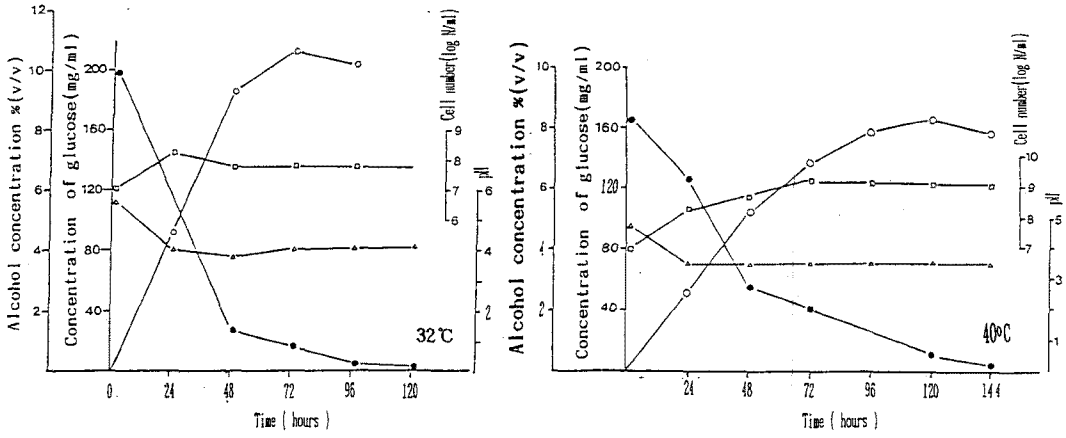

Fig. 5. Ethanol fermentation in 18% potato starch at 32°C and 15% potato starch at 40°C of RA-74-2.
In case of potato starch broth, no nutrients was added and seed volume was 10%. The pH was not controlled. ○—○: Alcohol concentration, □—□: Cell nmber, ●—●: Glucose concentration △—△: pH

Table 2. The ethanol fermentation from naked barley of industrial-pretreated substrate at various temperatures.

Culture temperature (°C)	Concentration of alcohol, % (v/v) 24hrs	48hrs	72hrs	96hrs	Final reducing sugar (mg/ml)
RA-74-2					
30°C	ND	9.1	9.4	9.4	0.5
40°C	6.1	9.6	9.4	9.0	0.5
RA-912					
37°C	3.8	5.8	5.6	5.6	N.D
45°C	4.0	5.8	5.6	5.4	N.D

*N.D: not determined.

naked barley. In industrial scale, 9.5~9.8% (v/v) ethanol was produced at 30-32°C for 100 hours in that media. However, RA-74-2 produced 9.4, 9.6% (v/v) alcohol at 30, 40°C and RA-912 produced 5.8% (v/v) alcohol at 37~45°C, respectively (Table 2).

DISCUSSION

We investigated the possibility of industrial application and economic process on high temperature fermentation by thermotolerant alcohol-producing yeast as previously reported. In 20% glucose media, we obtained 11.8% (v/v) ethanol at 32°C with 0.5% seed (92% of theoretical yields) and 10.6% (v/v) ethanol at 40°C with 3% seed (83% of theoretical yields), respectively. In temperature-gradient fermentation, which assuming the cooling system would not work in industrial scale (actually, cooling system in industrial scale was operated during the initial 30-40 hours in ethanol fermentation) 11.3% (v/v) ethanol was produced for 96 hours by the RA-74-2. However, the strain of B company was failed to 8% (v/v) ethanol production during the 120 hours with ten-folds seed concentration as RA-74-2 did (Fig. 3). This result suggest that the RA-74-2 could be sucessfully applied to save the cooling water and energy in industrial fermentation scale without re-investment or modification of the established systems. Since the RA-912 produced 6% (v/v) ethanol in 10% glucose media at 45°C and showed the less ethanol tolerance as compared with industrial strains, vacuum fermentation was conducted at 45°C in 15% glucose media. As a result, RA-912 showed the enhanced fermentation ratio (76% of theoretical yields). However, cell damage was observed by the ethanol produced in fermentation broth above 6% (v/v) ethanol. It suggests that hyperproductive process could enlight by a continuous input of the substrate and continuous recovery of the product in high cell-density culture (6). When potato starch is used as the substrate, high temperature fermentation was more readily put into an industrial application because no organic nitrogen was necessary. As the naked barley media, taken from the Poongkuk alcohol industy Co., were used on a high temperature fermentation, higher ethanol productivity was shown in jar-fermentor scale at 40°C than in industrial scale at 30-32°C. Since this media did not contain thermotolerant alcohol fermentating microorganisms and the fermentation ratio was decreased in the sterillized naked-barley media at 121°C for 20 minutes (data not shown), in-

creased ethanol productivity was caused by higer glucoamylase activity as well as higher metabolic ratio at 40°C. Therefore, if the thermotolerant yeast RA-74-2 were used in industrial scale, we would obtain the hyperproductivity due to higher glucoamylase activity and metabolic ratio and save the cooling water and energy. The strain improvement, however, remained in the more efficient fuel alcohol production.

Acknowledgements

This work was supported by a research grant from Research and Development Center for Energy and Resources (1993).

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(Received July 18, 1994)