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Ethanol Fermentation in Lactose Medium Using a Fusant Strain of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*

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The fermentative characteristics in ethanol production from lactose, with increased ethanol tolerance, of a fusant yeast strain constructed by protoplast fusion of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* were studied. The ethanol tolerance of this strain was increased to 8.0%, compared with the parent *K. fragilis*. During batch ethanol fermentation the optimal cultivation conditions for this fusant yeast were an initial pH of 4.5, a culture temperature 30°C, stirring at 100 rpm without aeration in 10% lactose medium (supplied with 1.0% yeast extract). Using this fusant strain in whey fermentation to ethanol, maximum ethanol production reached 3.41% (w/v) (theoretical yield; 66.7%) after a 48 hour cultivation period.

Whey is the liquid effluent generated by the cheese and casein manufacturing industries. Annual production of this material is estimated at 74×10^6 tons (6). Whey has a BOD_5 value of 60 to 70×10^3 mg/*l*, which makes disposal a serious environmental problem. Efforts to use whey in the dairy industry as a renewable resource have centered on fermenting lactose in whey to produce ethanol.

There have been two difficulties preventing large scale whey use for alcohol production. Yeast strains able to convert whey to ethanol cannot tolerate high levels of alcohol, whereas yeast strains having high ethanol tolerance cannot use the lactose in whey. Recently, several research groups (5,10,12) reported new hybrid strains of *Saccharomyces cerevisiae* and *Kluyveromyces* sp., constructed by protoplast fusion techniques. These fusant strains are able to use the lactose in whey efficiently, with a higher alcohol tolerance.

We investigated the fermentative characteristics of one of these fusant yeast strains in the production of ethanol from lactose. The fusant strain studied was developed by Farahnak *et al.* (5) from the parental strains, *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*.

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MATERIALS AND METHODS

Microorganisms and Cultivation

Saccharomyces cerevisiae STX23-5B (Ade^- Trp^-), *Kluyveromyces fragilis* 55-55 (Met^-), and their fusant yeast strain (Fus7, Ade^- Trp^- Met^-) were kindly supplied by Dewey D.Y. Ryu, Department of Chemical Engineering, University of California, Davis, USA. These yeast strains were maintained in a YPD medium of 1% yeast extract, 2% peptone, and 2% glucose.

In flask-fermentation study, inoculum of 10% stock culture broth, cultured in YPD medium at 30℃ for 30 hours, were inoculated into Erlenmeyer flasks of new culture media having different nutrients adjusted to appropriate pH values. The inoculated broths were fermented at 30℃ for 60 to 72 hours with agitating at 100 rpm on a rotary shaker.

A 2.5 *l* jar fermentor (Korea Fermentor Co., Ltd, Model SY) was employed in testing the effects of aeration rate, agitation speed, and culture temperature, and in examining the overall fermentation profiles. Whey was purchased from Sigma Chemical Company, MO, USA, which contained 13% protein, 65% lactose, 8% ash, and 2% lactic acid.

Comparison of Ethanol Tolerance

In order to determine the ethanol tolerance of the yeast strains, 10% inoculums of the yeast culture were

transferred into new YPD media, exogenously supplied with different concentrations of ethanol (5~15%), and cultivated at 30℃ for 7 days with shaking at 150 rpm (3, 11). Viable yeast cells in the final culture broth, were counted on YPD agar plates using the serial dilution method. The maximum ethanol tolerant concentration was estimated as that concentration which caused a drastic decrease in number of viable yeast cells.

Analysis of Fermentation Broth

The biomass of the yeast cells was measured as dry cell weight. 10 *ml* samples of fermentation broth were centrifuged and precipitated cells were washed with distilled water, then dried *in vacuo* overnight at 80℃.

Several components in the supernatants, obtained by centrifugation of the culture broth, were analyzed. The concentrations of the produced ethanol were determined by the gas chromatograhic method. The Gas Chromatograph (Model 5890A) of Hewlett Packard S.A. (Meyrin, Switzerland) was used with a Porapak Q column (80~100 mesh) and a flame ionization detector. Culture samples were mixed with equal volumes of 2% propanol for use as an internal standard, then injected into the injection port at 200℃. The samples were passed through the column in 150℃ oven.

The amount of residual lactose in the culture broth was assayed by the method of Nickerson *et al.* (9). The change in total reducing sugar concentration during fermentation was analyzed by the Somogyi-Nelson method (8).

RESULTS AND DISCUSSION

Comparison of the Ethanol Tolerance of the Fusant Strain with Its Parental Strains

Since one of the aims in constructing a fusant yeast strain is to increase the ethanol tolerance during the ethanol fermentation, the ethanol tolerances of the fusant yeast strain and its parental strains, *S. cerevisiae* and *K. fragilis*, were investigated by cultivation in YPD media for 7 days with exogenously added ethanol (5~15%). As illustrated in Fig. 1, the maximum ethanol tolerant concentration of the fusant strain was 8.0%, which was slightly higher than that of *K. fragilis* (7.0%). Surprisingly, this ethanol tolerance was much lower than the tolerance of *S. cerevisiae*(13.0%).

Under microscopic examination, *K. fragilis* has a long cylindrical shape, whereas *S. cerevisiae* is nearly round (Fig. 2). The fusant yeast strain showed an intermediate morphology, having a short cylindrical form. Even though the fusant strain has intermediate shape and auxotrophic nutrient requirements, this result shows that the ethanol tolerance of the fusant strain comes mostly from the genes of *K. fragilis* rather than the genes of *S. cerevisiae*.

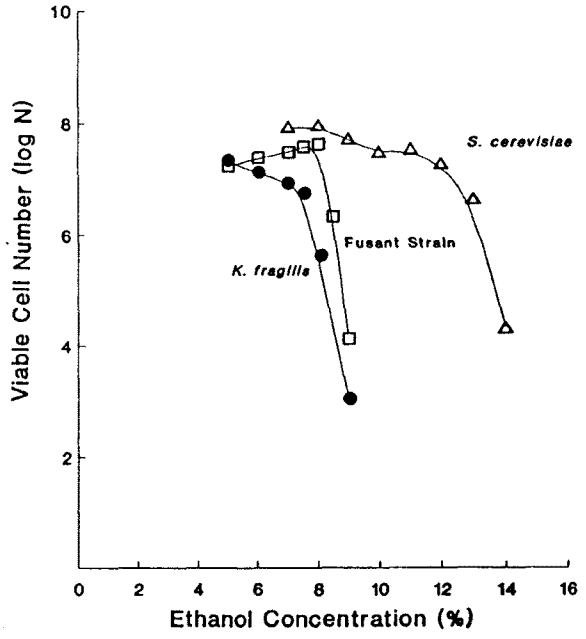

Fig. 1. Ethanol tolerances of the fusant yeast strain and its parental strains, *Kluyveromyces fragilis* and *Saccharomyces cerevisiae*.
Yeasts were cultivated in new YPD media exogenously supplied with different concentrations of ethanol (5~15%) at 30℃ for 7 days with shaking at 150 rpm, and viable yeast cells were counted on YPD agar plates by the serial dilution method.

Fermentative Characteristics of the Fusant Strain in a Shaken Flask

The ability of the fusant yeast strain to ferment sugars in a shaken flask (100 rpm) was compared with the abilities of the parental yeast strains. In glucose media, the three yeast strains showed similar fermenting abilities (Table 1), even though ethanol productivities were low due to aeration by shaking. The fusant strain, like *K. fragilis*, did not efficiently use glucose in a culture medium at a high glucose concentration (20%). This suggests that the osmotic tolerance of the fusant strain may be lower than the osmotic tolerance of *S. cerevisiae*. In lactose media, the ethanol productivities of the fusant strain and one of the parent strain, *K. fragilis*, were gradually increased as the concentration of the supplied carbon source was increased up to 10%(Table 2). The other parental strain, *S. cerevisiae*, could not ferment ethanol due to its lack of a lactose-utilizing system.

In addition to a carbon source, the other nutrients, including a nitrogen source and other growth factor (mineral ions or vitamins) are required to support minimal

Table 1. Ethanol fermentation in glucose media by the fusant strain and its parental strains in a shaken flask.

Yeast Strain	Concentration of Glucose (g/l)	Remaining Glucose (g/l)	Dry Cell Weight (g/l)	$Y_{X/S}$ (g/g)	Produced Ethanol (g/l)	$Y_{P/S}$ (g/g)	Final pH
Kluyveromyces	100	0.4	3.90	0.039	15.86	0.159	4.58
fragilis	150	0.6	3.95	0.024	17.28	0.116	4.56
55-55	200	27.7	4.17	0.024	17.99	0.104	4.53
Saccharomyces	100	0.2	3.16	0.032	14.83	0.149	4.64
cerevisiae	150	0.3	3.98	0.027	17.28	0.115	4.52
STX23-5B	200	1.5	4.09	0.021	18.86	0.095	4.48
Fusant (Fus 7)	100	0.4	4.17	0.042	15.78	0.158	4.58
	150	0.6	5.13	0.034	17.52	0.117	4.50
	200	13.1	4.95	0.026	19.01	0.101	4.47

Microorganisms were cultivated in glucose media with added 1% yeast extract and 2% peptone (pH 5.0) at 30℃ for 72 hours with shaking at 100 rpm.

Table 2. Ethanol fermentation in lactose media by the fusant strain and its parental strains in a shaken flask.

Yeast Strain	Concentration of Lactose (g/l)	Remaining Glucose (g/l)	Dry Cell Weight (g/l)	$Y_{X/S}$ (g/g)	Produced Ethanol (g/l)	$Y_{P/S}$ (g/g)	Final pH
	20	0.3	4.20	0.213	3.79	0.192	6.05
Kluyveromyces	40	0.3	4.28	0.108	12.07	0.304	4.08
fragilis	60	0.3	4.23	0.071	12.55	0.210	4.06
55-55	80	0.3	4.71	0.053	14.12	0.177	4.09
	100	0.4	4.10	0.041	15.15	0.152	4.77
	20	12.0	1.52		0.016		6.29
Saccharomyces	40	35.0	1.32		0.079		5.70
cerevisiae	60	58.0	1.53		0.063		6.01
STX23-5B	80	74.0	1.63		0.095		6.03
	100	85.0	1.56		0.158		5.52
	20	0.3	4.27	0.216	3.31	0.168	6.02
	40	0.3	4.70	0.118	12.07	0.304	4.21
Fusant (Fus 7)	60	0.3	5.08	0.085	12.55	0.210	4.28
	80	0.4	5.49	0.068	14.44	0.181	4.09
	100	0.4	5.54	0.055	15.39	0.154	4.14

Microorganisms were cultivated in lactose media with added 1% yeast extract and 2% peptone (pH 5.0) at 30℃ for 72 hours with shaking at 100 rpm.

cell growth during ethanol fermentation. However, Moulin *et al.* (7) reported that no significant effect of nitrogen source supplementation was observed, and Castillo (1) reported that the addition of salts and other growth factors produced negative effects on ethanol production. On the other hand, Chen *et al.* (2) emphasized the importance of yeast extract (0.7%) supplementation on ethanol yield from lactose by *Kluyveromyces*. Using the fusant strain, the effect of nitrogen source supplementation of the fermentation broth was investigated (Table 3). No remarkable improvement of ethanol yield by the fusant strain was observed even though yeast cell masses were somewhat increased. With the fusant strain and *K. fragilis*, however, ammonium phosphate and urea supplementation produced an increase in ethanol productivity.

Lactose fermentation to ethanol in a shaken flask at different initial pH values, was also investigated (Table

4). Even though the optimum pH for *Kluyveromyces* β-galactosidase is around pH 7.25 (4), the fusant yeast strain showed a broad optimum pH range in the acidic range (pH 3.0~6.0) for ethanol production from lactose. However, the best value for ethanol production by the fusant yeast strain was a pH of 4.5. This value is consistent with the result of Vienne *et al.* (14) who showed that the energetic metabolism of yeast shifts toward the production of glycerol concomitant with a decrease in ethanol yield at higher pH.

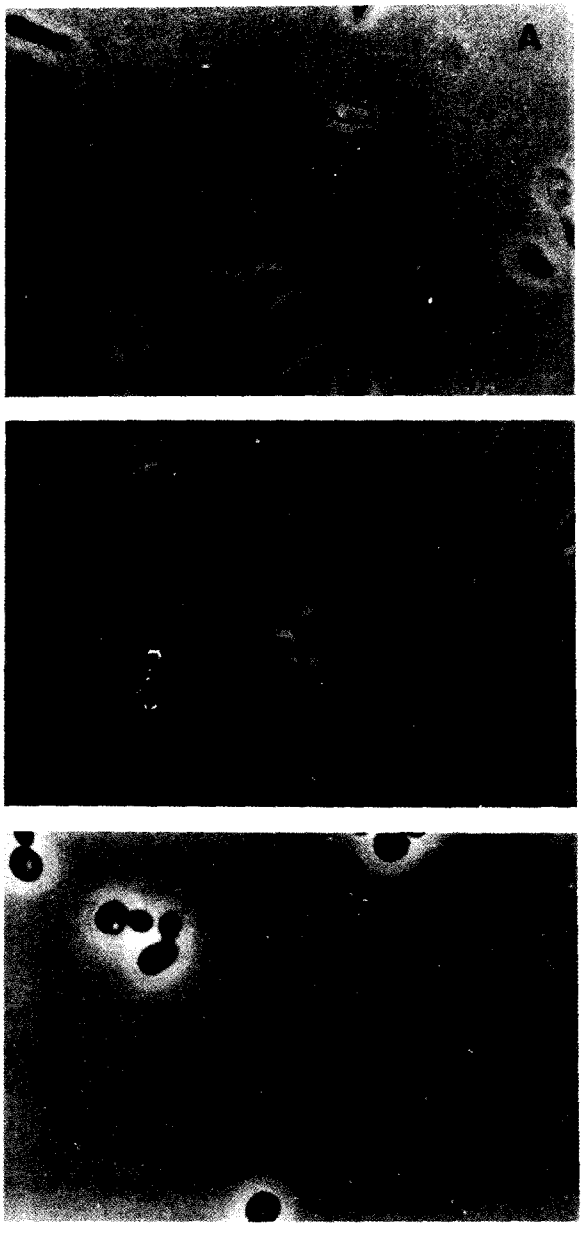

Fig. 2. Microscopic features of the fusant yeast strain and its parental strains, *Kluyveromyces fragilis* and *Saccharomyces cerevisiae*.
A: *Kluyveromyces fragilis*; B: fusant yeast; C: *Saccharomyces cerevisiae*.

Fermentative Characteristics of the Fusant Strain in a Jar Fermentor

Based on the fermentation results in the shaken flask, batch ethanol fermentation in a 2.5 *l* jar fermentor was conducted using the fusant strain. Ethanol production in a lactose medium was carried out at different agitation speeds and aeration rates. As shown in Fig. 3, the ethanol yields of the fusant strain at high agitation speeds, or with aeration, were greatly reduced, which reflects the fact that ethanol formation in yeast cells takes place in a strict anaerobic condition. However, exceedingly low agitation speed without aeration also inhibited ethanol production, presumably due to the retardation of fusant cell growth.

Microbial systems show a high sensitivity to the temperature of their environment. The optimum temperature range from 32℃ to 37℃ for *K. fragilis* strain was reported by Chen *et al.* (2), and 38℃, by Vienne *et al.*(13). In our experiments using the fusant strain, ethanol production in a jar fermentor at 30℃ was greater than ethanol production at 35℃, even though the initial cell growth rate and ethanol yield at 35℃ were slightly greater than that at 30℃ (Fig. 4). The growth of the fusant strain at 35℃ stopped suddenly after 24 hours, probably due to the depletion of the lactose carbon source.

In the jar fermentor experiments the effect of the amount of lactose in the culture broth was also examined (Fig. 5). The same result was obtained as in the shaken flask fermentation. However, the ethanol productivity was decreased when more than 10% lactose was supplied. This suggests that the osmotic tolerance, in addition

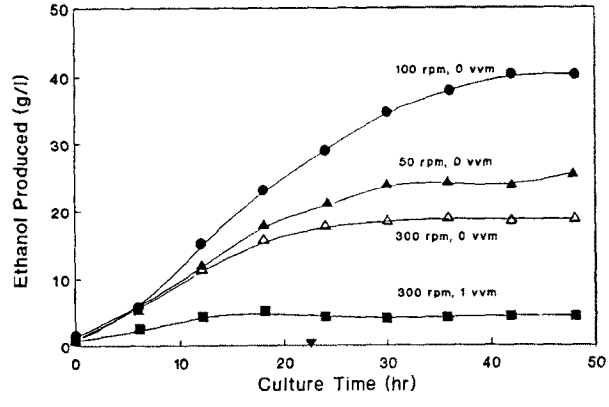

Fig. 3. The effect of agitation speed and aeration rate on ethanol production from lactose by the fusant strain in a 2.5*l* jar fermentor.
The fusant strain was cultivated in 10% lactose media with added 1% yeast extract at 30℃ with a controlling pH of 4.5.

Table 3. Ethanol fermentation by *Kluyveromyces fragilis* and the fusant strain in lactose media supplemented with different nitrogen sources.

Yeast Strain	Nitrogen Sources	Remaining Lactose (g/l)	Dry Cell Weight (g/l)	$Y_{X/S}$ (g/g)	Produced Ethanol (g/l)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	Final pH
Kluyveromyces fragilis 55-55	None	2.0	5.76	0.059	17.44	0.178	3.03	4.24
	Casamino Acid	2.0	5.82	0.059	17.20	0.176	2.96	4.30
	Peptone	3.0	6.88	0.071	17.20	0.177	2.50	4.19
	Tryptone	3.0	6.52	0.067	17.20	0.177	2.64	4.22
	Tryptic Soy Broth	3.0	7.38	0.076	17.44	0.180	2.36	4.07
	Sodium Nitrate	3.0	5.56	0.057	16.25	0.168	2.92	3.98
	Ammonium Phosphate	2.0	4.74	0.048	18.46	0.188	3.89	4.14
	Urea	6.0	4.28	0.046	17.52	0.186	4.09	4.22
Fusant (Fus 7)	None	2.0	5.46	0.056	18.23	0.185	3.33	4.14
	Casamino Acid	2.0	7.11	0.073	16.81	0.171	2.36	4.01
	Peptone	2.0	7.48	0.076	17.28	0.176	2.31	3.94
	Tryptone	3.0	7.52	0.075	16.25	0.168	2.16	4.00
	Tryptic Soy Broth	4.0	6.00	0.063	17.52	0.182	2.92	4.30
	Sodium Nitrate	2.0	6.86	0.070	16.81	0.171	2.45	3.88
	Ammonium Phosphate	2.0	5.69	0.058	17.52	0.179	3.08	4.01
	Urea	4.0	5.16	0.054	16.57	0.173	3.21	4.12

Microorganisms were cultivated in 10% lactose media with added 1% yeast extract and 1% nitrogen sources (pH 5.0) at 30℃ for 72 hours with shaking at 100 rpm.

Table 4. The effect of initial culture pH on the production of ethanol from lactose by Kluyveromyces fragilis and the fusant strain.

Yeast Strain	Initial Culture pH	Remaining Lactose (g/l)	Dry Cell Weight (g/l)	$Y_{X/S}$ (g/g)	Produced Ethanol (g/l)	$Y_{P/S}$ (g/g)	Final pH
Kluyveromyces fragilis 55-55	3.0	0.5	3.04	0.031	13.41	0.134	3.30
	3.5	0.5	3.80	0.038	13.65	0.137	3.67
	4.0	0.7	4.74	0.048	14.20	0.143	3.95
	4.5	0.8	5.29	0.053	14.12	0.142	4.11
	5.0	1.1	5.52	0.056	13.89	0.140	4.20
	6.0	1.0	4.99	0.050	13.89	0.140	4.18
	7.0	1.0	5.02	0.051	12.78	0.129	4.21
Fusant (Fus 7)	3.0	0.5	4.47	0.044	13.65	0.137	3.24
	3.5	0.6	5.35	0.054	13.73	0.138	3.60
	4.0	0.8	5.96	0.060	13.65	0.138	3.87
	4.5	1.0	6.21	0.063	14.20	0.143	4.05
	5.0	1.1	6.31	0.063	13.89	0.140	4.13
	6.0	1.0	4.33	0.044	13.89	0.140	4.13
	7.0	1.1	4.28	0.043	12.31	0.124	4.14

Microorganisms were cultivated in 10% lactose media with added 1% yeast extract, which was initially adjusted to different pH values, at 30℃ for 72 hours with shaking at 100 rpm.

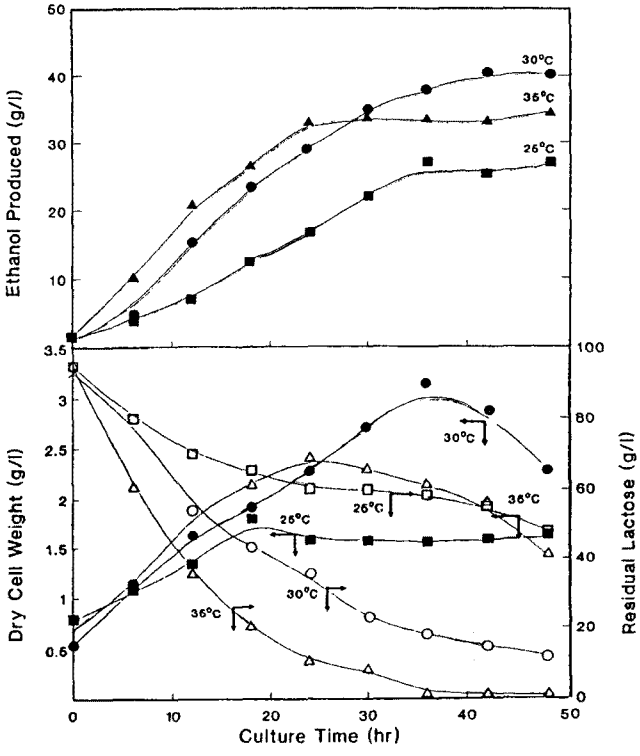

Fig. 4. The effect of culture temperature on ethanol production from lactose by the fusant strain in a 2.5*l* jar fermentor.
The fusant strain was cultivated in 10% lactose media with added 1% yeast extract at different temperatures with a controlling pH of 4.5, stirring at 100 rpm, and no aeration.

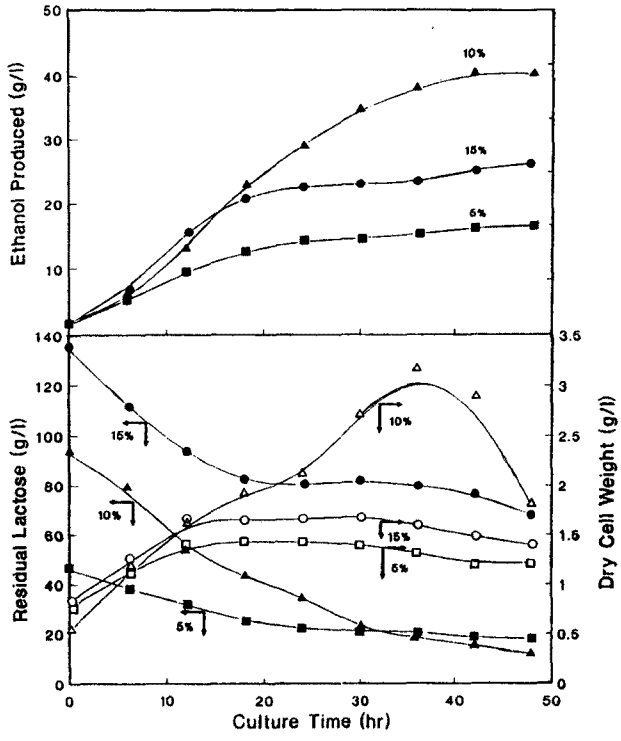

Fig. 5. Ethanol fermentation pattern of the fusant strain in media containing different concentrations of lactose in a 2.5*l* jar fermentor.
The fusant strain was cultivated at 30℃ in media having different concentrations of lactose and 1% yeast extract, with a controlling pH of 4.5, stirring at 100 rpm, and no aeration.

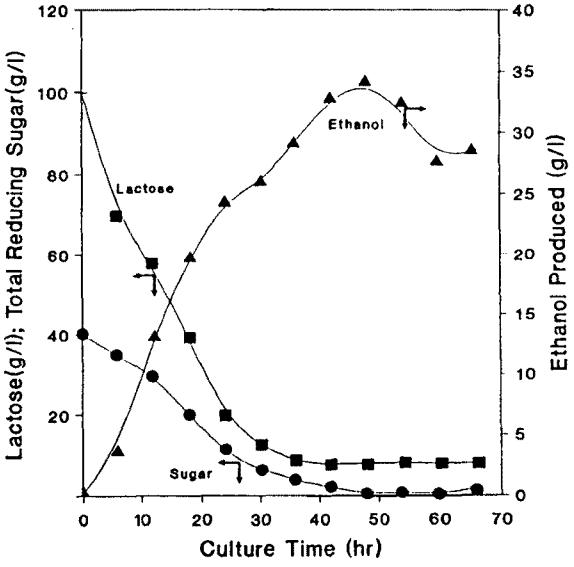

Fig. 6. Fermentation profile of ethanol from cheese whey (15% as dry weight) by the fusant strain.
The fusant strain was cultivated in 15% cheese whey (9.75% as lactose base) at 30℃ with a controlling pH of 4.5, stirring at 100 rpm, and no aeration.

to the ethanol tolerance, is important in ethanol production by the fusant strain.

The maximum ethanol production of the fusant strain in a 10% lactose medium was 4.05% (w/v) (theoretical yield; 79.3%) after 48 hours of fermentation at 30℃ with a controlling pH of 4.5, stirring at 100 rpm with no aeration.

Ethanol Fermentation from Cheese Whey Using the Fusant Strain

Based on the results obtained from the experiments using lactose, ethanol fermentation from cheese whey (15% as dry weight, 9.75% in lactose base) using the fusant strain was attempted at 30℃ with a controlling pH of 4.5 (Fig. 6). The highest ethanol concentration was 3.41% (w/v) (theoretical yield; 66.7%) after 48 hours of cultivation in a jar fermentor. After 48 hours the lactose in the culture broth was almost completely consumed.

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