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Biomass Production of *Saccharomyces cerevisiae* KFCC 10823 and Its Use in Prepararion of *Doenjang*

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An ethanolic fermentation process was developed for preparing *Doenjang* with high ethanol. Higher and efficient viable cell production of salt-tolerant ethanolic yeast is a prerequisite for the successful commercial-scale process of ethanol production during *Doenjang* fermentation. Culture conditions of salt-tolerant yeast, *S. cerevisiae* KFCC 10823, was studied in terms of the effect of several environmental and nutritional factors. Viable cell numbers were the highest in a medium containing the following components per liter of water: soysauce, 300 ml; dextrose, 50 g; beef extract, 5 g; yeast extract, 5 g; KH_2PO_4, 5 g; NaCl, 50 g. The optimal culture conditions of *S. cerevisiae* KFCC 10823 were pH 5.5, 25°C, 200 rpm and 0.5 vvm. Yeast viability during batch fermentation was gradually decreased to a level less than 90% after 35 hours. The maximum cell number was 2.2×10^7 cells/ml at the optimal condition. *Doenjang* prepared with ethanolic yeast was ripened after 45 days at 30°C. This *Doenjang* contains 470 mg% of amino nitrogen and 2.5% ethanol. The shelf-life at 30°C was theoretically estimated as 444 days.

Doenjang is an extremely popular fermented foods that are used as a seasoning stuff in cooking or as a dip for green pepper and onion in Korea. The *Doenjang* has been traditionally prepared by mixing and fermenting a moldy cooked soybean (*Meju*) and brine (18%) in a porcelain pot. However, it is now prepared commercially by local firms using a slightly different procedure. They are now preparing *Doenjang* by mixing and fermenting moldy wheat (*Koji*), cooked soybean and salt in a stainless steel or plastic tank. The reason for this is to meet the massive demand from apartment complexes in urban areas. Traditional *Doenjang* does not have spoilage problem due to its high salt concentration. However, consumers do not favor products with high salt content and thus manufacturers decreased the level of salt in commercial products to 8-10%. As they are marketed in bottles or plastic containers to consumers, this frequently resulted in large quantities of swollen products on the local market, despite the use of preservatives. They usually use chemical preservatives such as sorbic acid or its potasium salt to prevent microbial deterioration and gas production of final products. These preservatives are regarded undesirable additives, especially in a food that is often eaten daily, by a growing number of consumers as a result of increased awareness of health related issues. In a previous experiment, ethanol was found to be a good preservative and also verified for use in fermented soybean products (18). However, ethanol added in *Doenjang* preparation is another cost factor. Therefore, a highly salt-tolerant ethanolic yeast (KFCC 10823) was developed through an adaptation process as a candidate for a co-culture system for *in-situ* ethanol production and deposited with KFCC. Higher and efficient viable cell production of *Saccharomyces cerevisiae* KFCC 10823 is a prerequisite of business for the commercial-scale production of preservative-free *Doenjang*. In this paper, optimal culture conditions for *S. cerevisiae* KFCC 10823 and experimental data from a semi-pilot scale processing line are reported.

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MATERIALS AND METHODS

Microorganism and Cultivation

Ethanolic yeast, *S. cerevisiae* KFCC 10823, was used as a starter of *Doenjang* fermentation. This strain was derived from *S. cerevisiae* ATCC 42940 through a mutation and adaptation process as previously described (18). The strain was maintained on YM agar containing 10% NaCl at 30°C. Cultivation was performed in MULTIGEN™ jar fermentors (NBS, N.J., U.S.A., total volume 500 ml) or Bioengineering Fermentors (19 liter, Model NLF 22, Switzerland). The fermentation medium was for-

mulated using fresh soysauce (total nitrogen, 1.8%; amino N, 1.0%; NaCl, 19%; reducing sugar, 3.75%), glucose, yeast extract (0.5%), beef extract (0.5%), KH_2PO_4 (0.5%) and soybean oil (1%) as an antifoamer. The cultivation conditions were optimized in terms of nutrients and environmental factors such as aeration, agitation, temperature and pH.

Preparation of *Doenjang* and Shelf-life Dating

Doenjang was prepared by mixing cooked soybean with wheat and soybean koji cultured by *Aspergillus oryzae*. The mixture was pulverized and salted in such a way as to adjust the final concentration to 12%. Fresh culture of *S. cerevisiae* KFCC 10823 was added at the concentration of 10^5 cfu/g. The slurry was fermented and ripened for 45 days in an FRP tank. Total fermentation scale was 1,000 kg. The final product was crushed and packed in 500 g plastic pouches. The products were stored at 20 and 30°C. Samples at each interval were subjected to the determination of pack volume gain. Shelf-life was defined by the time taken to reach 100 ml of gas production. Those parameters were analyzed against storage time. From the rate constant of gas evolution, a theoretical shelf-life was derived (18).

Analysis

Cell concentration was determined using a Haemacytometer after methylene blue staining (4, 5). pH was measured with an Orion model 520 pH meter. Total sugar was measured spectrophotometrically, using the dinitrosalysilic acid method (13). Lipid was extracted from the yeast cell by the Bligher & Dyer method using chloroform and methanol (2/1) (2). Lipid fraction and fatty acid were determined by TLC-FID (Iatron Inc. Japan) and gas chromatograph with capillary column (supelcowax 10, 30 mm × 0.32 mm, 0.32 μm), respectively as previously described (20). Amino nitrogen was analyzed by the formalin titration method. Ethyl alcohol was determined by headspace analysis using GC with a 15% FFAP column (2 mm × 2 m) (7, 10) Pack volume of plastic pouches was obtained by measuring the overflowing water from a cylinder in which the pouch were soaked (10).

RESULT AND DISCUSSION

Development of Salt-tolerant Yeast and Characterization

In order to induce *in-situ* ethanol fermentation during *Doenjang* preparation, a co-culture system was designed. For this process, it is necessary to get salt-tolerlant, highly ethanolic yeast. *S. cerevisiae* ATCC 42940 was found to produce ethanol in high concentrations during wine-making (19). Mutations of the yeast were performed using ethylmethanesulfonate and a colony formed on the medium with 10% NaCl was adapted to higher salt (18%) concentration. Mutation frequency was 10^{-8}. *S. cerevisiae* KFCC 10823 was able to grow on the medium with 18% NaCl. The strain produced 4.2% ethanol in the medium with 10% NaCl after 4 days of fermentation, even though ethanol was not detectable in the medium with the parent strain. The morphology of yeast changed from ellipsoidal to spherical (data not shown). This is thought to be as a result of change in the biological matrix of the cell that is composed of globular integral protein dispersed in a lipid matrix (16). Membrane lipid is known to influence membrane fluidity, depending on the type of acyl chain esterified, amount of sterol and polar lipids (15). Cellular lipid was analyzed to compare any compositional difference. The sterol content of the mutant was lower by 6% that means higher fluidity of the lipid phase of cell membrane (6). Polar fraction of cellular lipid slightly increased from 95.3% to 97.01% (Table 1). Fatty acids of total lipid was analyzed (Table 2). They are in the range of C_{16}-C_{18} fatty acids as in other eukaryotic cells (14). The unsaturation index was increased from 0.65 to 0.97. This is not consistent with Hosono's data with *Zygosaccharomyces rouxii* grown at high salt concentrations (8).

Optimization of Growth Medium for *S. cerevisiae* KFCC 10823

As already mentioned, *S. cerevisiae* KFCC 10823 was developed for ethanol production during *Doenjang* fermentation. Therefore, the medium for high density

Table 1. Lipid class composition of cellular lipids of *S. cerevisiae* ATCC 42940 (parent strain) and KFCC 10823 (mutant).

Lipid class	ATCC 42940	KFCC 10823
Esterified sterol	0.17	0.11
Triglyceride	0.92	0.11
Diglyceride	0.22	0.28
Free fatty acid	0.96	1.15
Free sterol	1.45	1.36
Polar lipid	95.3	97.01

Table 2. Fatty acid composition of cellular lipids of *S. cerevisiae* ATCC 42940 (parent strain) and KFCC 10823 (mutant).

FFA	ATCC 42940	KFCC 10823
$C_{12:0}$	0.67	0.51
$C_{14:0}$	1.06	–
$C_{16:0}$	16.06	15.03
$C_{16:1}$	49.35	11.05
$C_{18:0}$	9.17	2.62
$C_{18:1}$	2.69	57.37
$C_{18:2}$	5.43	12.10
$C_{18:3}$	0.66	0.49
$C_{20:4(6)}$	–	0.17
$C_{20:4(3)}$	–	0.38
$C_{22:1}$	–	0.23
Unsaturation index	0.65	0.97

growing of the yeast must be of such a composition as to not adversely influnce the organolepic properties. The best carbon and nitrogen source could be fresh soysauce. Fig. 1 shows the effect of soysauce concentration on growth. The specific growth rate (μ) was not substantially affected by the concentration. The μ was in the range of 0.20-0.22 h^{-1}. The higher cell growth was obtained as the concentration reached 30% and then reduced at the concentration above this level. As the basal medium contained 5% glucose and NaCl, salt concentration will rise with the amount of soysauce added. 40% soysauce in the medium amounts to an 8% increase of total salt. That is the reason for lowered maximum cell production. Ammonium sulfate instead of yeast extract was not much more favorable for growth (Table 3). Peptone with a lowered amount of yeast extract was somewhat desirable as a nitrogen and vitamin source. Yeast extract at 0.5% level was enough to get 10^7 cell/ml of viability after 40 h of cultivation. Additional minerals such as Ca^{+2}, Mg^{+2}, Fe^{+3}, Mo^{+6} and Mn^{+2} in the 0.5% yeast extract medium did not help the growth but maintained similar level of biomass (data not shown).

Carbohydrate concentration is reported elsewhere as affecting biomass and metabolite production (9, 11). The effect of glucose concentration in soysauce medium was evaluated (Table 4). The cell number was high in a medium with 5% additional glucose, although the concentration effect was not so clear. This strain can grow well in a medium with 25% glucose as parent strain (17). The μs were 0.20-0.21 h^{-1} (Fig. 2). In general, the μ is decreasing to some extent with increased carbohydrate concentration (11). In this case, soysauce medium already contained 1.54% reducing sugar before adding glucose. This is thought be a reason for the constant μs at different concentrations. Aeration and agitation are indispensable environmental factors in biomass production by yeast (3). Optimal aeration rate and agitation speed for KFCC 10823 were 0.5 vvm and 200 rpm, respectively (Table 5). Higher aeration and agitation did not induce higher biomass production. Lee *et al.* (11) attained the highest cell production by *S. cerevisiae* CA-1 at 150 rpm. Choi *et al.* (3) reported that optimal aeration for *S. rouxii* was 2 vvm. Inoculum size is also a factor in an economic fermentation process. 0.1% inoculum was enough to get a fermentation broth with 10^7 cell/ml. But

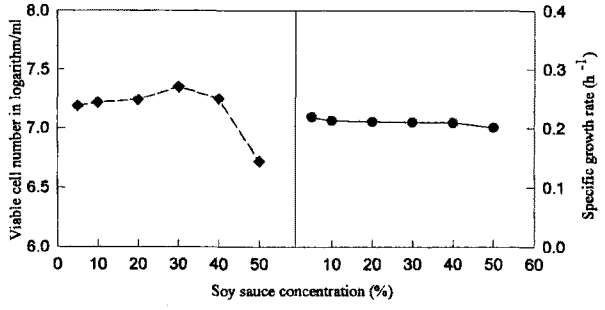

Fig. 1. Specific growth rate and cell growth of *S. cerevisiae* KFCC 10823 as affected by soy sauce concentration in the growth medium (glucose 50 g/l, 30°C, pH 5.0, 0.5 vvm, 100 rpm, 30 h).

Table 3. Effect of nitrogen sources on the production of viable cell number of *S. cerevisiae* KFCC 10823 in 30% soy sauce medium.

Nitrogen source	Concentration (%)	Total cell numbers/ml	Viable cell numbers/ml
Yeast extract	0.5	2.5×10^7	2.2×10^7
	0.8	2.3×10^7	2.1×10^7
	1.0	1.6×10^7	1.5×10^7
Amminoium sulfate	0.5	9.1×10^6	7.2×10^6
Yeast extract (0.3%) +Peptone(0.5%)		2.3×10^7	2.1×10^7

*The culture was performed in 350 ml jar fermentor at 30°C for 60 h and initial pH was 5.0.

Table 4. Cell growth of *S. cerevisiae* KFCC 10823 as affected by initial glucose concentration in medium at 30°C after 54 h.

Initial concentration of glucose (%)	Initial cell density (CFU/ml)	Maximum cell density (CFU/ml)	Specific growth rate (μ) (h^{-1})
1	2.8×10^4	6.9×10^6	0.20
5	9.6×10^3	4.3×10^7	0.21
15	3.6×10^4	3.1×10^7	0.21
25	2.4×10^4	2.5×10^7	0.21

The culture was performed in 350 ml jar fermentor at 30°C for 54 h and initial pH was 5.0.

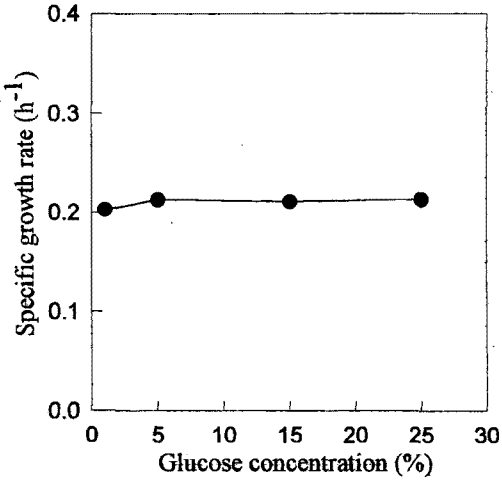

Fig. 2. Specific growth rate of *S. cerevisiae* KFCC as affected by glucose concentration in the soy sauce medium (soysauce 30%, 30°C, pH 5.0, 0.5 vvm, 100 rpm, 30 h).

larger inoculum sizes induced an earlier stationary phase. 45-50 h of fermentation was enough to get maximum level of viable cell production.

Effect of cultivation temperature was compared (Fig. 3) as various yeasts have their temperature optimum (12). Actual optimum growth temperatures of the parent strain was reported as 24°C (1). The mutant was able to grow well between 20-30°C. Viable cell production at 25°C was 2.3×10^7 cell/ml after 45 h. The cell production at above 25°C was low. The μ decreased as the cultivation temperature decreased. The activation energy for growth was 4.2 kcal/mole. Initial pH of yeast fermentation can affect the cell production. Lee *et al.* (19) reported the optimum initial pH for biomass production by *Torulopsis candida* as 4.0. Growth profile *vs.* pH was assymetric around pH optimum (Fig. 4). However, KFCC 10823 produced a similar amount of cell production below pH 5.5 which is optimum.

From these results, optimal condition for growing *S. cerevisiae* KFCC 10823 are to grow in a medium containing 5% NaCl, 5% glucose, 30% soysauce, 0.5% beef extract, 0.5% yeast extract, 0.5% KH_2PO_4 in the conditions of pH 5.5, 0.1% inoculum, 0.5 vvm and 200 rpm. The process was scaled up from 300 ml to 13 liters (Total volume : 19 l) to prepare a yeast culture for *Doenjang* production. The scaled-up process showed a typical fermentation profile (Fig. 5). Growth reached a stationary phase after 25 h of inoculation. Cell production after 38 h was 2.2×10^7 cell/ml. Yeast consumed carbohydrate slowly during the late logarithmic phase and used it up in the stationary phase.

Table 5. Effect of agitation, inoculum size and aeration on the growth of *S. cerevisiae* KFCC 10823 at 30°C after 50 h.

Fermentation condition		Final pH	Total cell numbers/ml	Viable cell numbers/ml
Aeration (vvm)	0.5	4.95	2.3×10^7	2.1×10^7
	1	5.10	1.8×10^7	1.5×10^7
	2	5.05	1.4×10^7	1.2×10^7
	2.5	5.01	1.4×10^7	1.1×10^7
Agitation (RPM)	100	5.09	1.4×10^7	1.1×10^7
	200	4.98	2.6×10^7	2.1×10^7
	300	5.08	1.4×10^7	1.0×10^7
Amount of inoculum (%)	0.1	4.94	2.9×10^7	2.3×10^7
	1	4.92	3.7×10^7	3.4×10^7
	10	4.93	3.5×10^7	3.2×10^7

*The culture was performed in 350 ml jar fermentor with 30% soy sauce medium. Initial pH was 5.0.

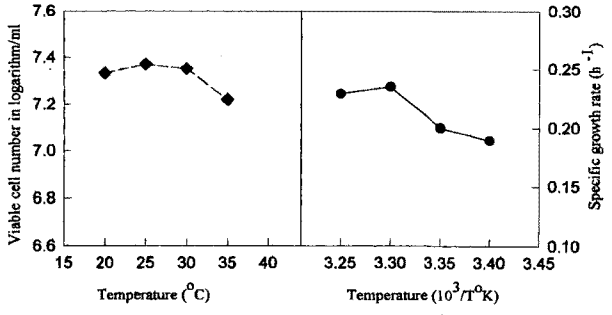

Fig. 3. Specific growth rate and cell growth of *S. cerevisiae* KFCC 10823 in soy sauce medium as affected by the growth temperature (glucose 50 g/l, soysauce 30%, 30°C, pH 5.0, 0.5 vvm, 100 rpm, 50 h).

Preparation of *Doenjang* and Shelf-life Dating

Doenjang was prepared on 1,000 kg scale. The mash was formulated with wheat *koji*, soybean, salt and water. Final moisture and NaCl were 50% and 12%. Fermenta-

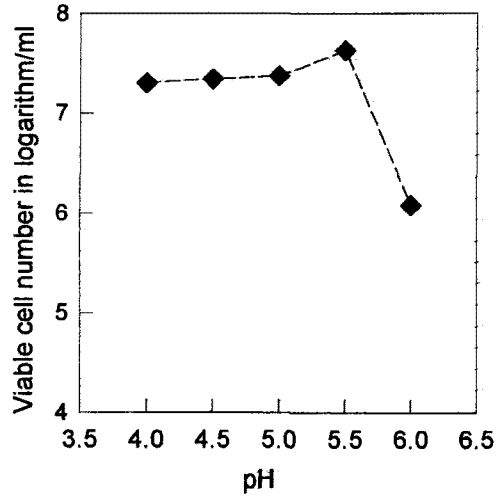

Fig. 4. Cell growth of *S. cerevisiae* KFCC as affected by pH of the soy sauce medium (glucose 50 g/l, soysauce 30%, 30°C, 0.5 vvm, 100 rpm, 50 h).

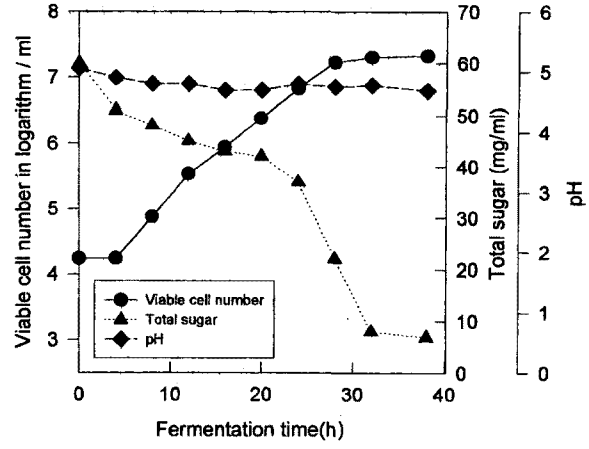

Fig. 5. Time course of growth of *S. cerevisiae* KFCC 10823 in the soy sauce medium (glucose 50 g/l, soysauce 30%, 30°C, pH 5.5, 0.5 vvm, 200 rpm).

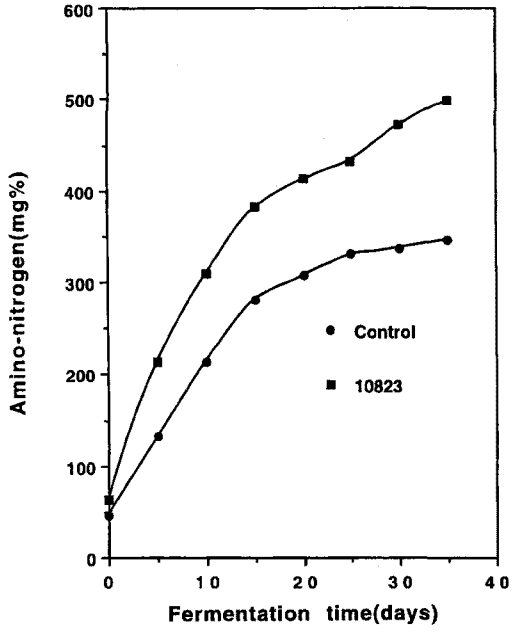

Fig. 6. Changes in amino nitrogen during *Doenjang* fermentation (30°C).

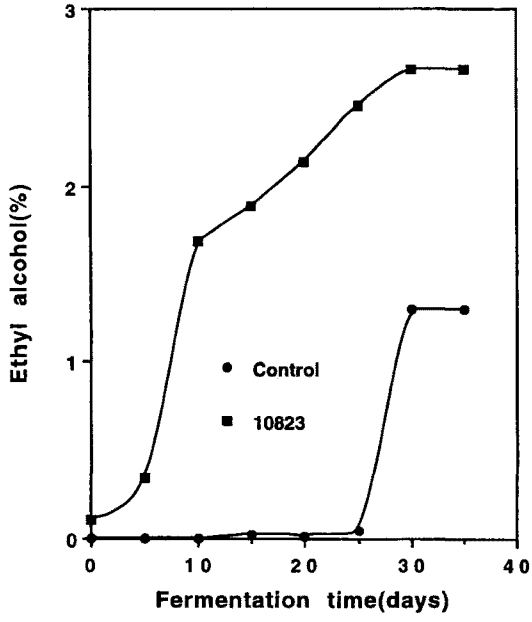

Fig. 7. Changes in ethyl alcohol during *Doenjang* fermentation (30°C).

tion was performed for 45 days at 30°C. 300 mg% amino nitrogen was liberated after 20 days of fermentation. Production was faster in a process with KFCC 10823 (Fig. 6). This indicated that use of *S. cerevisiae* helped ripening of *Doenjang*. Ethyl alcohol was produced faster

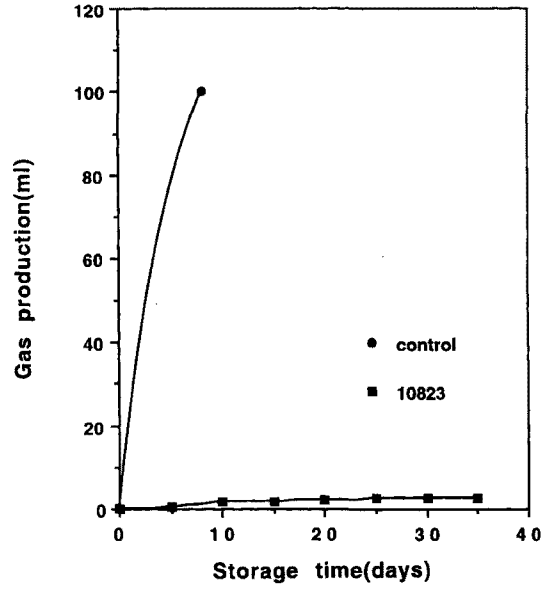

Fig. 8. Gas production in pouch during the storage of *Doenjang* at 30°C.

in the process with KFCC 10823 (Fig. 7). Final ethanol concentration was 2.5%, whereas 0.7% ethyl alcohol was produced in the control batch process without KFCC 10823. The *Doenjang* thus prepared after 45 days of fermentation was properly mixed and packaged in a plastic pouch. Those products were subjected to a storage test. Gas production in terms of pack volume gain was measured (Fig. 8). *Doenjang* with normal process started swelling and the pouch was destroyed after 3-4 days of incubation. However, *Doenjang* with KFCC 10823 produced less than 10 ml of gas even after 40 days at 30°C. Gas evolution rate constants at 20 and 30°C were calculated from the graphs. From the rate equation, shelf-life of the *Doenjang* was predicted to be 444 days at 30°C.

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