Journal of Microbiology and Biotechnology
Vol. 3, No. 1, 24-30, 1993

Development of a Novel Yeast Strain Which Ferments Soy Sauce by Protoplast Fusion

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In order to develop a novel yeast which produces the charateristic aroma of soy sauce, a protoplast fusion between *Zygosaccharomyces rouxii* WFS4 and *Torulopsis versatilis* IAM 4993 was carried out. Auxotrophic mutants as selective markers were obtained from *Zygosaccharomyces rouxii* and *Torulopsis versatilis* by treatment of N-methyl-N'-nitro-N-nitrosoguanidine. The conditions of the protoplast formation and the regeneration for fusion were examined. The protoplast fusion using polyethylene glycol 4000 led to the fusion frequency of $4\sim5\times10^{-7}$ cells/ml. Among fusants, a fusant ST723-F31 presented the best results in terms of the aromaticity of fragrance, the growth pattern, the resistance against salt and the degree of growth according to pH. It makes easy to control the production and the balance of aroma components so that it gives a good flavor, shortens the fermentation period and, simplifies the preparation process when using a bioreactor into which fusant is immobilized.

Traditional Korean soy sauce is used for flavoring food in many Korean dishes. It is prepared by adding saline to soybean malt and fermenting it. In this procedure, naturally occurring microorgaisms are involved (6). These microorganisms influence the taste and the flavor of traditional Korean soy sauce during fermentation (5).

During the fermentation process, bacteria of the *Bacillus* genus and the *Lactobacillus* genus are propagated, and the hydrolyzed proteins change into amino acids or peptides and produce various acids including lactic acis. Particularly, yeasts such as *Zygosaccharomyces rouxii* and *Torulopsis versatilis* grow and produce alcohols and esters in soy sauce, which enhance the flavor.

However, the conventional process for preparing traditional soy sauce has many problems, because naturally occurring microorganisms regulate the fermentation and the aging of soy sauce.

The naturally occurring microorganisms make it difficult to control the production or the balancing of aroma components and in result make it difficult to adjust the taste and the flavor of soy sauce. It also takes a long time, about 6 to 12 months, to ferment and age soy sauce, but this is a necessary step in obtaining good taste and aroma.

Zygosaccharomyces rouxii and *Torulopsis versatilis*, which serve as major yeasts in the fermentation and the aging of traditional Korean soy sauce and Japanese fermented soy sauce, determine the flavor of the soy sauce by producing the aroma components; 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone(HEMF), dihydro-5-methyl-2(3H)-furanone, 4-ethylguaiacol(4-EG) and alcohols (16, 17).

Zygosaccharomyces rouxii is involved in the initial stage of soy sauce fermentation whereas *Torulopsis versatilis* is involved in the latter stage. It takes a long time for fermentation and aging and, since the two strains are employed seperately, managing them can be complicated and cumbersome.

Recently, the method to ferment the soy sauce and age quickly is empolyed immobilization of *Zygosaccharomyces rouxii* and *Torulopsis versatilis* (9).

In this study, we carried out an intergeneric and intraspecific fusion between *Zygosaccaromyces rouxii* and *Torulopsis versatilis* to obtain a novel strain which has the characteristics of both strains, and investigated the conditions of a protoplast fusion.

The fusant makes it possible to shorten the period and to eliminate the cumbersome problem resulting from the seperate use of the two strains.

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Key words: fusant ST723-F31, soy sauce fermentation, protoplast fusion, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF), 4-ethylguaiacol(4-EG), *Zygosaccahromyces rouxii*, *Torulopsis versatilis*

MATERIALS AND METHODS

Strains

As genetic markers of protoplast fusion, the auxotrophic mutants of *Zygosaccharomyces rouxii* WFS4 and *Torulopsis versatilis* IAM 4993 were selected after treatment with N-methyl-N'-nitro-N-nitrosoguanidine(NTG) (2, 7). The selected auxotrophic mutants, *Zygosaccharomyces rouxii* SMn7 and *Torulopsis versatilis* TMn23, were an arginine-requiring mutant and a leucine-requiring mutant, respectively.

The mutants produced aromas of soy sauce which were better or similar to those of the wild types.

Media and Culture Conditions

The compositions of the propagation(PM) for both yeast strains were 4% glucose, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$. The complete medium (CM) used for fusion consisted of 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.2% $(NH_4)_2SO_4$, 0.1% polypeptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, and the minimal medium(MM) were composed of 2% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05 % KCl, 0.001% $FeSO_4 \cdot 7H_2O$. The temperature of cultivation and treatment was 30℃.

As regeneration medium, complete or minimum medium containing 4.5% of KCl was used. The pH of the media was 5.5.

Protoplast Formation and Regeneration

The protoplast formation of the yeast cells was carried out according to the modified method of Fournier (10).

The yeasts harvested in the exponential growth phase were washed with 99 mM Tris-HCl buffer (pH 8.0) containing 0.7 M KCl and 20 mM $CaCl_2$, and resuspended in 10 m*l* of PTP buffer (100 mM Tris, 860 μM EDTA and 20 mM β-mercaptoethanol, pH 8.0) and incubated at 30℃ for 10 minutes. The cells were collected by centrifugation at 3000 rpm for 10 minutes and suspended in 4 m*l* of the enzyme solution; 2 m*l* of 1.2 M KCl, 1 m*l* of 50 mM β-mercaptoethanol and 1 m*l* of Lyticase 100 unit/m*l* (Sigma Co.). The mixture was incubated at 30℃ for an appropriate period.

Protoplast Fusion and Fusant Selection

Protoplast fusion was induced under the action of polyethyleneglycol (PEG) 4000. The parental protoplasts (5×10^8 cells/m*l* of each auxotroph) were mixed in a 1 : 1 ratio and recollected by centrifugation. The mixed protoplasts were resuspended in 3 m*l* of 100 mM Tris-HCl buffer (pH 8.0) containing 40%(M/V) PEG 4000, 50 mM glycine and 20 mM $CaCl_2$, and incubated at 30℃ for an appropriate period. After the fusion reaction, the protoplasts were collected and washed with 0.7 M KCl solution. Serial dilutions of treated protoplast were mixed with 10 m*l* of MM or CM containing 4.5% KCl and 1% agar which was melted and maintained 42℃, and immediately poured onto agar plates of the same medium containing 4.5% KCl and incubated at 30℃ for 5~7 days.

The fusion frequency was calculated by dividing the number of colonies per milliter on hypertonic complete medium.

Aroma Production

To investigate the characteristics of the soy sauce aroma produced by the fusant, the fusant was inoculated in an aroma producing medium: glucose 2.0%, yeast extract 0.5%, polypeptone 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, soybean extract 10%, NaCl 10% and ferulic acid 0.1%, and stationary cultured at 30℃ for 26 days. The preparation of the soybean extract was as follows: smashed soybeans (100 g) were add to distilled water (400 m*l*) and boiled for 3 hours and filtered. The filtrate was used as the soybean extract.

The aromaticity of the fragrance produced in head space was organoletically tested by a trained pannel in accordance with the Korean Industrial Standard (3, 4) and the growth pattern of the fusant in broth was observed.

Extraction and Analysis of Volatile Aroma Components

The volatile aroma components were extracted from the broth of fusant cultured in an aroma producing medium with a simultanous steam distillation-extraction (SDE) apparatus (12), and their peaks were identified with preparative gas chromatography (8).

Each peak component was organoleptically tested and the conditions of preparative gas chromatography were as follows; Instrument: Shimadzu GC-8A gas chromatography, column: chemically bonded fused sillica, inj. and detec. temp.: 240℃, column temp.: 60~200℃ (10℃ /min), carrier gas: N_2 (8 m*l*/min), range: 10^3, attenuation: 1, detector: FID.

Identification of Volatile Aroma Components

Mass spectrum of fragrance materials were obtained by using GC-mass and the aroma components were identified by a computerized library search.

4-EG was identified with a standard reagent (TOKYO KASEI Co.) by preparative GC and GC-mass, and HEMF was identified by GC-mass.

Instrument and operating conditions of GC and GC-mass were as follows; Instrument: Finnigan MAT 4510B GC-mass spectrometer, column: Carbowax-20M-25M, splite ratio: 30 : 1, inj.temp.: 230℃, detect.temp.: 150℃, carrier gas: He (5 m*l*/min), temp. program: 45℃ for 2 min, 45~220℃(15℃/min) and 220℃ for 11.4 min, elec. volt: 70 eV, elec.muli.: 1100 V, ionizer temp.: 150 ℃.

Effect of Salt Concentration and pH on the Growth

The resistance of the fusant against salt was examined. The fusant was inoculated in a solid medium and liquid medium, which was prepared by using various concentrations of salt (15~27%), and the complete medium was cultivated at 30℃ for 8 days.

The degree of growth of the fusant in various pH was investiga-ted. The fusant was cultivated in a complete media with a pH range of 3 to 10 at 30℃ for 8 days, and the degree of growth and the pH after cultivation were observed.

RESULTS AND DISCUSSION

Conditions for Protoplast Formation and Regeneration

In order to investigate the optimum conditions for the preparation and the regeneration of yeast protoplasts in high yields, we examined lysis ratio(ratio of protoplast formation) and regeneration ratio of *Zygosaccharomyces rouxii* SMn7 and *Torulopsis versatilis* TMn23 depending on Lyticase treatment times.

As shown in Fig. 1, the lysis ratio of *Zygosaccharomyces rouxii* SMn7 was good during the treatment time from 30 minutes to 120 minutes with time being increased, and was maximum at 120 minutes as 83 percents, but the degree of regeneration was remarkably low.

We infered that the ratio of protoplast formation was better, and that the generation was worse because the degradation of the cell wall was relatively serious.

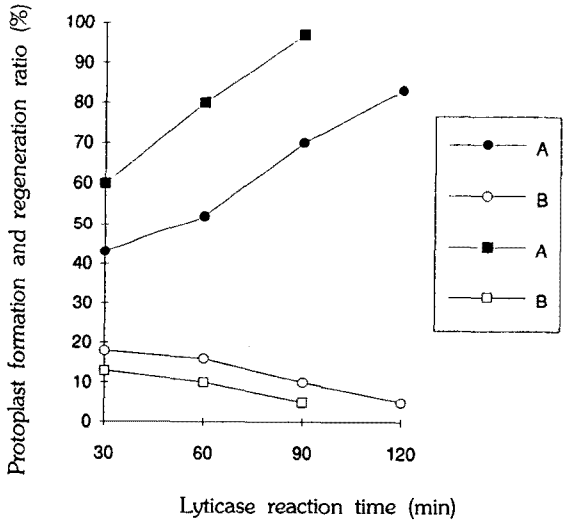

Fig. 1. Effect of Lyticase reaction time on the formation and regeneration of protoplast.
Zygosaccharomyces rouxii (●−●), *Torulopsis versatilis* (■−■)
A: Protoplast formation ratio, B: regeneration ratio

In the case of *Torulopsis versatilis* TMn23, the ratio of protoplast formation was better than that of *Zygosaccharomyces rouxii* SMn7 but the regeneration ratio was worse.

On the basis of the lysis ratio and the regeneration ratio, the treatment times of Lyticase for *Zygosaccharomyce rouxii* SMn7 and *Torulopsis versatilis* TMn23 were 90 and 60 minutes, respectively.

The protoplast fusion by 40% of PEG 4000 as fusogen was at maximum frequency when the reaction time was 10 minutes. The maximum frequency of intergeneric fusion between protoplasts of *Zygosaccharomyces rouxii* SMn7 and *Torulopsis versatilis* TMn23 was $4\sim5\times10^{-7}$ cells/ml.

This result showed that the frequency is lower than those from the other papers of intraspecific and intergenic fusion (1, 11, 13-15).

Selection of Fusant

Each parental strain carrying auxotrophic requirements as genetic markers, was fused by intraspecific and intergeneric combination complementarily, so the fusants grown in minimal medium were selected. They were apt to divide to parental strains, because intraspecific fusants were hereditarily unstable. To give hereditary stability, the fusants were passaged culture in a minimal medium several times at 7 days intervals.

To select the best fusant among the fusants isolated, the ability to produce of good soy sauce aroma and the degree of growth were investigated in aroma production medium at 30℃ for 26 days, and the volatile aroma components produced by fusants were analized with preparative gas chromatography.

Among the fusants, funsant ST723-F31 was selected as the best fusant. To identify chromosomal fusion of fusant ST723-F31, its chromosome was observed with a trasmission electron microscope.

Fig. 2 is a photograph of thin sectioned of fusant ST 723-F31 cell where one fused chromosome appeared. Fig. 3 is a scanning electron micrograph of fusant ST 723-F31.

Charateristics of Aroma Produced by Fusant ST 723-F31

After fusant ST723-F31 and the wild types were cultured in the aroma production medium at 30℃ for 26 days, the aromaticity and the growth pattern were investigated.

The aromaticity of the fragrance produced by wild types was that of sweet and sour soy sauce, but that produced by fusant ST723-F31 was that of good sweet soy sauce. The growth pattern of fusant ST723-F31 was observed. Fusant ST723-F31 showed the growth type of both *Zygosaccharomyces rouxii* (top yeast) and *Toru-*

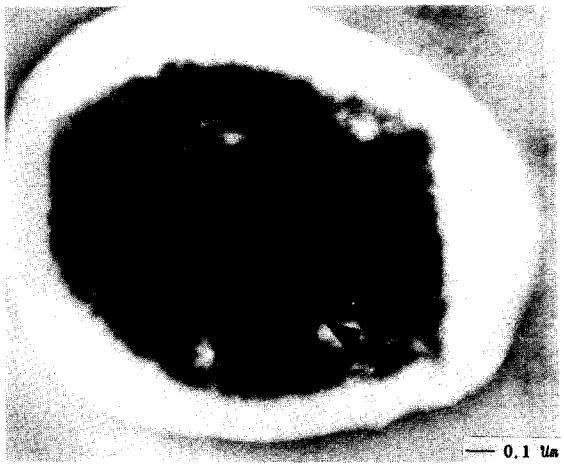

Fig. 2. Transmission electron micrograph of thin sectioned fusant ST723-F31 cell.
→: nucleus

Fig. 3. Scanning electron micrograph of fusant ST723-F31.

lopsis versatilis (bottom yeast).

The fact that fusant ST723-F31 has the growth habit of both *Zygosaccharomyces rouxii* and *Torulopsis versatilis* means that chromosomal fusion between the two strains had occured.

Analysis and Identification of the Volatile Aroma Components

The volatile aroma components produced by wild types and hybridoma ST723-F31 were extracted from culture with a SDE apparatus and analyzed by preparative gas chromatography.

Each analyzed peak component was organoleptically tested and the results are shown in Fig. 4~6. *Zygosaccharomyce rouxii* produce HEMF, the characteristic aroma component of Japanese soy sauce which has a particular sweet aroma similar to caramel. As appeared in the gas chromatogram (Fig. 4) of *Zygosaccharomyces*

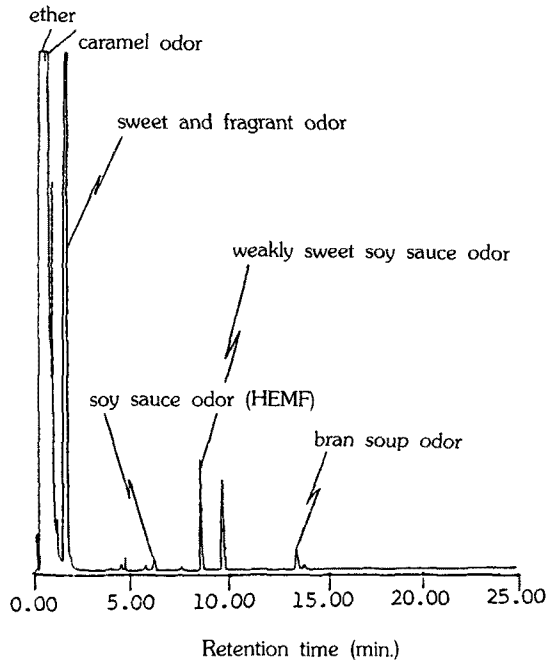

Fig. 4. Gas chromatogram and organoleptic characteristics of volitile components produced by *Zygosaccharomyces rouxii* SMn7.

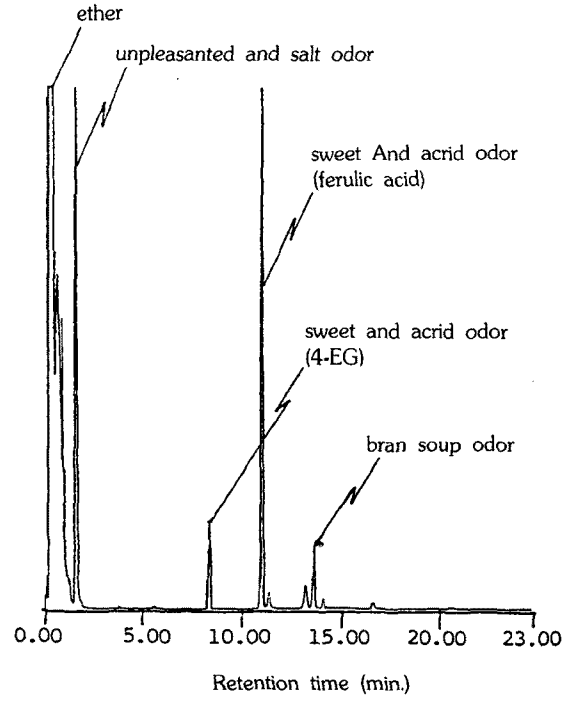

Fig. 5. Gas chromatogram and organoleptic characteristics of volitile components produced by *Torulopsis versatilis* TMn23.

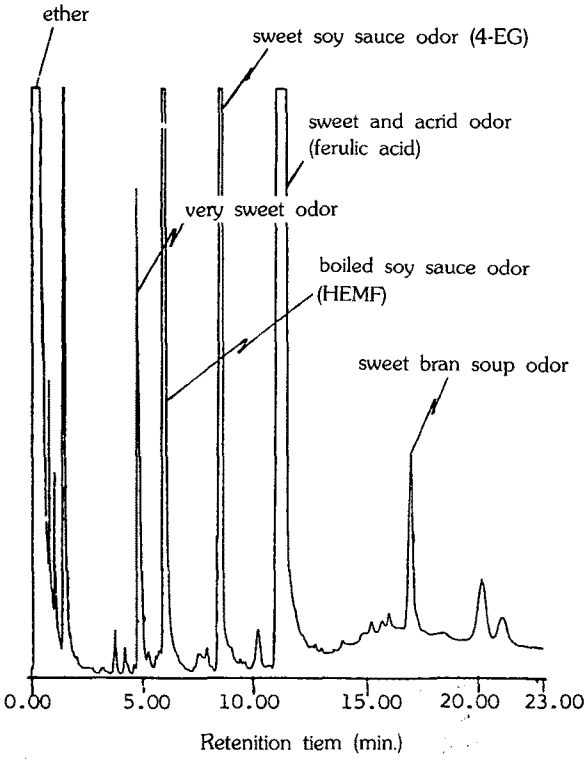

Fig. 6. Gas chromatogram and organoleptic characteristics of volitile components produced by fusant ST723-F31.

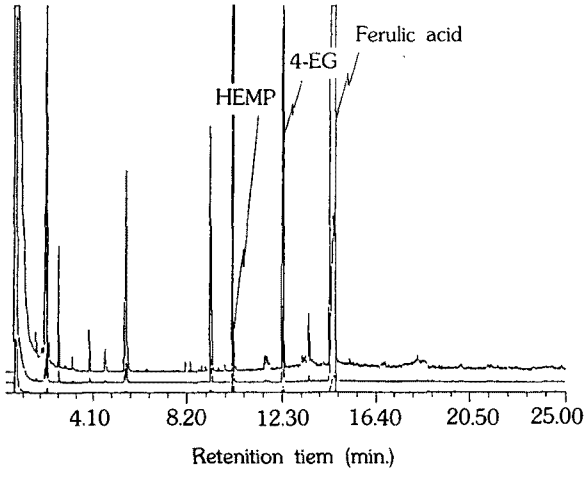

Fig. 7. Gas chromatogram of volatile components produced by fusant ST723-F31.

rouxii, the peak which presented a sweet soy sauce aroma at a retention time of 6.348 was HEMF.

Also 4-EG converted from ferulic acid by *Torulopsis versatilis* is a charateristic aroma component of Japanese soy sauce. In the gas chromatogram (Fig. 5) of *Torulopsis*

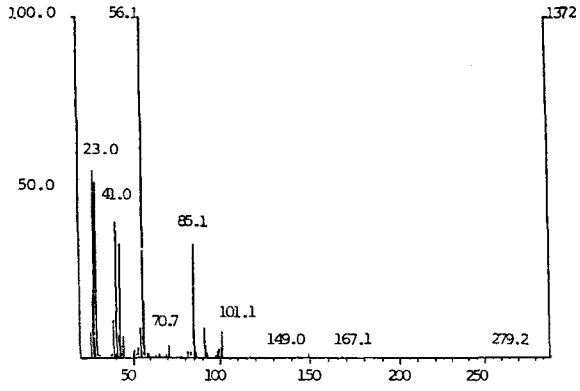

Fig. 8. Mass spectrum of the indicated peak of HEMF shown in Fig. 7.

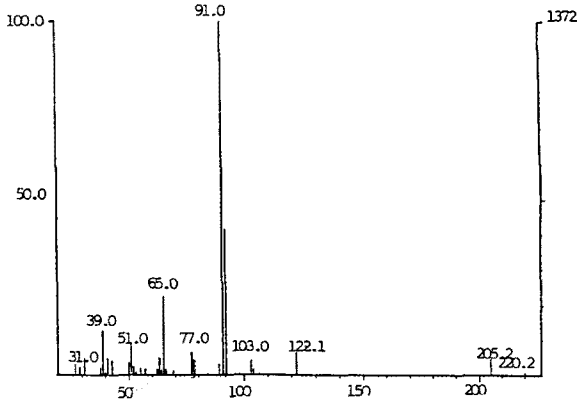

Fig. 9. Mass spectrum of the peak of 4-EG shown in Fig. 7.

versatilis, the retention time of 9.005 of the 4-EG peak and the retention time of 11.253 of the ferulic acid peak were identified to be comparable with the retention time of standard reagents.

As shown in the gas chromatogram of fusant ST723-F31 (Fig. 6), the appearance of a HEMF peak at retention time 6.308 and 4-EG peak at retention time 8.963 indicates that fusant ST723-F31 can produce two characteristic aroma components.

Mass spectrum of fragrance components produced by fusant ST723-F31 was obtained by using GC-mass, and the aroma components were identified by library search using computer. The peaks of HEMF and 4-EG in the gas chromatogram are shown in Fig. 7, and the mass spectrum of the investigated HEMF and 4-EG are shown in Fig. 8 and Fig. 9, respectively.

Effect of Salt Concentration and pH on Growth

To investigate the optimum condition of growth during

Table 1. Degree of growth of the wild type and fusant ST723-F31 in various salt concentrations

Strains	Salt Con.	15%		18%		20%		22%		25%		27%	
	Midium	S	L	S	L	S	L	S	L	S	L	S	L[a]
S. rouxii WFS4		+++	+	+	W	+	W	−	−	−	−	−	−[b]
T. versatilis IAM 4993		++	+	+	+	+	W	+	−	−	−	−	−
Fusant ST723-F31		+++	+	++	+	++	+	+	+	−	W	−	

[a]S; Agar-added solid medium, L; Liquid medium, [b]+++; Good growth (colony diameter 4~6 mm), ++; Fair growth (colony diameter 2~4 mm), +; Growth (colony diameter 0~2 mm), W; Weak growth, −; No growth

Table 2. Degree of growth of the wild type and fusant ST723-F31 in various pH and pH changes

Strains	pH	3			4			5			6		
	Medium	L[a]		C pH	S	L	C pH	S	L	C pH	S	L	C pH[b]
Z.rouxii		+[c]		2.81	+++	+	3.69	++	+	4.14	+++	+	4.39
T.versatilis		+		2.80	++	+	3.83	++	+	4.34	+	+	4.57
Fusant ST723-F31		+		2.85	+++ +	+	3.77	++	+	4.10	++	+	5.55

Strains	pH	7			8			9			10		
	Medium	S	L	C pH	S	L	C pH	S	L	C pH	S	L	C pH
Z.rouxii		+++	+	4.50	++	+	5.16	++++	+	5.25	++	+	5.50
T.versatilis		+	+	5.27	+	+	5.54	+	+	6.25	++	+	5.87
Fusant ST723-F31		+++	+	4.28	+++	+	5.20	++	+	5.42	++	+	5.63

[a]L; Liquid medium, S; solid medium, [b]pH after cultivation, [c]++++; Very good growth (colony diameter 6~8 mm), +++; Good growth (colony diamter 4~6 mm), ++; Fair growth (colony diameter 2~4 mm), +; Growth (colony diameter 0~2 mm)

fermentation of soy sauce, the resistance and the degree of growth according to the salt concentrations of wild type and fusant ST723-F31 were compared and examined. As shown in Table 1, fusant ST723-F31 can even grow in a 25% salt concentration indicating its strong resistance against salt, but the wild types cannot grow in salt concentrations higher than 20%.

The degree of growth of the wild type and fusant ST723-F31 in various pH was examined and the results are presented in Table 2.

The wild type and fusant ST723-F31 can grow in a wide pH range of 3 to 10. After culture, the pH become acidic. This result corresponded with the point that the pH of traditional Korean soy sauce is acidic.

When fusant ST723-F31 was used industrially, the following merits were identified: it is possible to do fermentation in high salt concentrations in which other microorganisms can't grow, and to produce industrially a soy sauce aroma without negative effects on the pH.

Also, the fusant ST723-F31 produces both of the charateristic aroma components produced by *Zygosaccharomyces rouxii* and *Torulopsis versatilis* simultaneously, so it eliminates the problems found in the conventional soy sauce preparation method; it makes it easy to control the production and the balance of aroma components so that it gives a good flavor, shortens the fermentation period and, in the case of using a bioreactor into which fusant is immobilized, simplifies the preparation process.

Acknowledgement

This sutdy was supported by grant from the Korea Science and Engineering Foundation.

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(Received 4 May, 1993)