Ethanol production from starch by protoplast fusion between *Aspergillus oryzae and Saccharomyces cerevisiae*

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사상균과 효모의 세포융합에 의한 녹말로부터의 에탄올 생산

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ABSTRACT : Amylolytic filamentous fungus, *Aspergillus oryzae* and nonamylolytic sugar fermentable yeast, *Saccharomyces cerevisiae* were fused by protoplast fusion in order to develope microorganisms having their integrated function. Amino acid auxotrophic properties were used as a genetic marker of protoplast fusion, and 35% PEG 4000 was used as a fusogenic agent.Complementation frequengy of fusion was 4.6×10^{-6} Obtained fusants showed the morphology of yeast strains, the amylase activity and the ethanol productivity. Among the properties of the fusants, morphology and prototrophic property were sustained stably but their ethanol productivity from starch was reduced. Although fusant strains had 0.5-fold ethanol productivity compared to that of *S. cerevisiae* in glucose medium, they produced ethanol from strach by direct fermentation.

KEY WORDS □ *Aspergillus oryzae*, *Saccharomyces cerevisiae*, fusion, alcohol production.

The production of biomass fuels such as ethanol has become important recently. Although the starchy biomass is a favorable source of ethanol, two energy consuming steps are involoved in converting it to ethanol, liquefaction of starch by endo-amylase, and then saccharification of the liquefaction products to produce fermentable sugars(Sakai *et al.*, 1985; Abouzied and Reddy, 1986). Commercial enzymes are used for liquefaction and saccharification of starch and it represent a significant expense in the production of fuel alcohol from starchy materials. Thus, the development of a new method is desirable to reduce the cost of ethanol production. Initial studies to devise a new method aimed at the elimination of the enzymatic liquefaction and saccharification step by using symbiotic culture of amylolytic and sugar-fermenting organisms(Abouzied and Reddy, 1986).

In recent years fungal amylase have gained more and more interest for carring out starch hydrolysis in brewing(Bhella and Altosaar, 1984), *Aspergillus oryzae* was well known as producer of amylase(Thorberk and Eplor, 1974). Also, *Saccharomyces cerevisiae* has been used very frequently for ethanol production(Rose and Beavan, 1982 ; Seki *et al.*, 1983)

In this paper, we describe the protoplast fusion of amylolytic fungus, *Aspergillus ouyzae* and sugar fermentable yeast, *Saccharomyces cerevisiae* which aimed to develope a microogarnisms being able to produce ethanol from starch by direct fermentation. We believe that this is to be the first original paper on the protoplast fusion of them.

Materials and Methods

Strains and media

The strains of *A. oryzae* and *S. cerevisiae* used in these experiments are listed in Table 1. Cultures of *A. oryzae* and of *S. cerevisiae* were grown on Malt extract medium(Peberdy, 1979) and YPD medium (Sherman et al., 1982), respectibely. SD medium was used as minimal medium(Serman et al., 1982) for

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selection of fusion products. In protoplast regeneration and fusion experiments both media were supplemented with osmotic stabilizer, 0.6M KCl, and designated RYPD and RSD restectively. The fermentation media used for ethanol production were YPD and SYP media. SYP medium was identical to growth medium, YPD except that glucose was replaced with soluble starch.

Table 1. *Strains of Aspergillus oryzae and Saccharomyces cerevisiae used in this study*

Stain	Genotype and Phenotype
A. oryzae ATCC 16507	requires lysine
S. cerevisiae KCTC 7073	MATA his4 leu2 met10 ural ade 2, 4

Protoplast formation, regeneration, and fusion

Protoplast of *A. oryzae* were prepared using the modified procedure described by Anné and Peberdy (1976), and separated from mycelial debris by filtration through a sintered glass filter(porosity 1). The filtered protoplasts suspension were twice washed with protoplast buffer(0.2M phosphate buffer pH 5.8 containing 0.6M KCl), and counted by haemocytometer. The suspension was prepared by a slight modification of the method of Soligen and Plaat (1977).

Protoplast fusion between *A. oryzae* and *S. cerevisiae* was performed by the standard protoplast fusion method of *S. cerevisiae* described by Farahnak et al.(1976). Produced protoplasts of *A. oryzae* and *S. cerevisiae* were fused using 35% PEG 4000 and 10mM $CaCl_2$ as fusogenic agents and regenerated on RYPD and RSD media. Fusion frequency was calculated as the ratio colonies growing on RSD and RYPD.

Halo formation

For the halo formation test, a small amount of cell were smeared on a YPSB plate. Then the plate was incubated at 28℃ for 4~6 days and at 4℃ for 2 days. Glucoamylase-producing cells formed holos around their colonies, while non-producing cells did not(Yamashita and Fukui, 1983).

Stabilityof fusant

Colonies selected as fusants by growing on minimal were inoculated separately into YPD broth, and cultured 24 hrs at 30℃ with shaking. One loop of this culture was streaked out on YPD plate and incubated for 2 days to isolate single colonies as subclones. The amino acid requiring property of these subclones were tested by incubating to selective media. Also, the ethanol productivity of these subclones was tested by gas chromatography as described below.

Ethanol fermentation test for starch

Ethand concentration was measured by gas chromatography on a column coated with polyethylene glycol with 2% butanol as internal standard ; column temperature was set at 50℃.

Results and Discussions

Isolation of fusion products between *A. oryzae* and *S. cerevisiae*

To improve the fermenting ability, amylolytic fungus, *Aspergillus oryzae* and sugar fermenting yeast, *Saccharomyces cerevisiae* were fused by protoplast fusion. Amino acid auxotrophic properties were used as a genetic marker of protoplast fusion. Colonies growing on the minimal medium were selected as fusants.

Complementation frequency of fusion was 4.6×10^{-6} (Table 2). Fusants of intraspecific fusion in *S. cerevisiae* were larger than their parents. In other hand, obtained fusants between *A. oryzae* and *S. cerevisiae* showed yeast form in their morphology but their size were very small compared to that of parent, *S. cerevisiae* KCTC 7073. And most of obtained fusants showed glucoamylase activity in halo formation test.

Ethanol productivity of fusants and parents in glucose and starch media

The ethanol productivities in glucose and starch media of various fusants having glucoamylase producing ability in halo formation test were listed in

Table 2. *Complementation frequency by the protoplast fusion of A. oryae and S. cerevisiae*

Strain	Reversion frequency[1] (less than)	Regeneration frequency (%)	Fusion frequency*
A. oryzae ATCC 16507	1.4×10^{-6}	9.8	
S. cerevisiae KCTC 7073	5.0×10^{-7}	1.1	4.6×10^{-6}

[1] Reversion frequency was defined as the ratio of the number of protoplasts regenerating on RSD(minimal, medium) and RYPD(complete medium). None colonies showed on RSD medium.

* Fusion frequency was shown as the ratio of colonies appearing on RSD and RYPD.

Table 3. The ethanol productivity changed with the carbon source used. All tested fusants had about 0.5-fold ethanol productivity in glucose medium compared to that of parental strain, *S. cerevisiae* KCTC 7073 but the ethanol producing ability in starch medium varied dupending on the fusants.

Table 3. *Ethand productivity of fusants and parents in glucose and starch medium*

Strain	Halo formation	Growth on starch medium	Ethanol productivity* (g/100m/day)	
			Glucose medium	Starch medium
Fusants :				
Fus 1	+	+	0.676	0.048
Fus 2	+	+	0.647	0.124
Fus 3	+	+	0.647	0.147
Fus 4	+	+	0.515	0.148
Parents :				
ATCC 16507	+	+	−	−
KCTC 7073	−	−	1.03	−

*The ethanol produced was measured after 24 hr fermentation (at 30℃)
Amylase activity of *A. oryzae* ATCC 16507 was determined by Ogawa's method (1988).

Although fusant strains showed relatively low starch-fermenting activity, they produced ethanol from starch by direct fermentation. These results allowed us to develope starch fermenting microorgarnisms by means of protoplast fusion of *A. oryzae* and *S. cerevisiae*. However, further experiment must be made to find out the reason why glucose and starch fermenting ability were low.

Stability of fusants during subculture

The stability of obtained fusant strains after propagation was examined as shown in method. The properties of obtained fusants were stable. Especially, the prototrophic property of the fusants was very stable and all tested fusant didn't segregate into parented auxotroph but sustained the prototrophic property(Table 4). We cannot decide that the fusants are diploids without their DNA content estimation and other experiments. However, above result suggest that the fusants are not heterokaryons. Also, ethanol productivities in glucose media were examined(Table 5). In glucose media, ethanol productivity of subclone was similar to that of original fusants. However, ethanol production from starch was reduced strikely. This suggest that amylase activity of fusants may be reduced during the subculture. Therefore, maintenance media and screening method are necessary to devised to select stable subclones.

Table 4. *The stability of prototrophic property of fusants.*

Protoplast pair	Segregation into auxotroph	Appereance frequency of prototroph
ATCC 16507	None	100%
KCTC 7073	None	

200 subclones from 23 different fusants by the procedure described in the method were all prototrophs, and they were not segregated into parental auxotroph.

Table 5. *Ethanol productivity of fusant F4 during on subcultures.*

	Ethanol productivity(g/l/day)	
	Glucose medium	Starch medium
0	5.15	1.48
8 weeks	6.62	0.20
9 weeks	5.39	0.21

적 요

녹말로부터 에탄올을 직접 생산하는 균주를 개발하고자 amylase 활성이 높은 *Aspergillus oryzae*와 알콜발효능이 있는 *Saccharomyces cerevisiae*의 원형질체를 융합하였다. 이들의 융합을 유도하기 위해서 fusogen으로 35% PEG 4000을 사용하였으며, 융합체의 선별을 위한 유전적 지표로는 아미노산 요구성을 사용하였다. 융합률은 4.6×10^{-6}이었다. 선별된 융합체들은 효모의 형태였으며 amylase활성을 나타내었고 에탄올을 생산하였다. 그리고 이들 성질 중에서 형태는 후세대에도 안정하게 유지되었으나 녹말로부터의 알콜 생성능은 현저히 감소하였다. 비록 포도당 배지에서의 융합체들의 알콜 생산은 *S. cerevisiae*의 0.5배에 지나지 않았으나, Prototroph인 *S. cerevisiae*와는 달리 이들은 녹말로부터 직접 에탄올을 생산할 수 있었다.

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