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Ethanol Fermentation of Fusant between Heterologous Transformant of *Saccharomyces cerevisiae* and *Candida tropicalis* in Mini-jar Fermentor Scale

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Mini-jar fermentor Scale에서의 Fusant의 Ethanol 발효

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The optimum conditions for ethanol fermentation and ethanol productivity of the fusant FSC-14-75 were examined in a mini-jar fermentor scale (working volume = 2.5 liters) to assess the possibility of practical application. Addition of yeast extract to fermentation broth greatly enhanced the ethanol productivity and shortened the period of fermentation. The pH 4.2 was more favorable than pH 5.5 with respect to ethanol productivity and fermentation speed. The optimum concentration of liquefied potato starch for ethanol fermentation of FSC-14-75 was 15%(*w/v*) and the corresponding productivity was 8.7%(*v/v*) of ethanol with an efficiency of 80.6% to the theoretical maximum. When the fresh fermentation broth containing 20% of liquefied potato starch was inoculated with 10%(*v/v*) of inoculum, the fusant FSC-14-75 produced 11.0%(*v/v*) of ethanol in 4 days, which is considered comparable to that from an industrial process. From the liquefied cassava starch or the equal mixture of liquefied barley and sweet potato starch prepared according to the same method as in the industrial process except saccharification step, the fusant FSC-14-75 produced 8.5%(*v/v*) or 7.6%(*v/v*) of ethanol in 4 days, respectively.

Starch, which consists of two high molecular weight components such as amylose and amylopectin, is the principal raw material for ethanol production. In conventional ethanol fermentations, an enzymatic step is required to obtain low molecular weight sugars which can be subsequently fermented to ethanol by *Saccharomyces* sp. Moreover the enzymatic pretreatment of starch is actually one of the main causes to increase the alcohol plant cost(1).

Thus in our previous work(2-4), we attempted transformation of the intact cells of *S. cerevisiae* by partially BamHI-digested chromosomal DNA of *S. diastaticus* and subsequently intergeneric protoplast fusion between the transformant and *Candida tropicalis* to develop a new brewing yeast. The successful fusion products were genetically very stable, and were capable of utilizing starch and producing ethanol from liquefied potato starch (DE = 3.0) with high efficiency.

In this study, we have focused on the investigation of the optimum condition for ethanol fermentation in a laboratory mani-jar fermentor scale to assess the possibility of practical application of the fusant FSC-14-75 which was the most favorable strain in terms of genetic stability, amylase activity, and ethanol productivity in flask experiments.

Key words: Fusant, mini-jar fermentor scale fermentation, ethanol productivity, liquefied raw starch
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Materials and Methods

Strains

The used strains were *Saccharomyces diastaticus* IFO 1046, and FSC-14-75, the most promising one among the fusants obtained from intergeneric protoplast fusion between TSD-14 and *Candida tropicalis* RCT-40(lys$^-$) in the previous work (4).

Media

For seed culture, yeast strains were grown in YPS medium containing 1% yeast extract, 2% peptone, and 3% starch hydrolysate (pH 6.0). Media for starch fermentation consisted of various concentrations of starch hydrolysate, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.2% yeast extract.

Substrates

The potato starch was purchased from Junsei Co., and crude tapioca meal, sweet potato meal, and barley meal were kindly supplied by Phung Gook Alcohol Industry Co.

Starch liquefaction

As the substrates for the ethanol fermentation, potato, tapioca, barley and sweet potato starch were liquefied by treatment of Thermamyl (alpha-amylase, Novo Co.). The various suspensions of starch were prepared in distilled water. When the temperature reached 50°C, 0.8%(v/w, to starch content) of Thermamyl was added and the temperature was raised to 90°C with continuous stirring for 20 min. After liquefaction, the liquefied starch was used for preparing the fermentation medium.

Fermentation conditions

Ethanol fermentation of various liquefied starch was performed in a mini-jar fermentor (Tokyo Rikakikai Co.) with a working volume of 2.5 liters, and the inoculation was made at 1%(v/v) level. The incubation temperature in the fermentor was maintained at 30°C and the cultures were stirred at 150-200 rpm with aeration at 0.5 vvm for 8 hours. The pH was controlled to 4.2 with 2N NaOH every 12 hours. Broth samples were obtained aseptically by removing 200 m*l* of sample through the sample port. Each sample was tested for sugar content, ethanol content, and pH.

Analytical methods

The residual sugar content was determined by the procedure of acid hydrolysis followed by Somogyi-Nelson method(5). The ethanol content was measured by alcohol hydrometer after distillation(5).

Results

Effect of yeast extract on ethanol productivity from liquefied potato starch

Since the ethanol productivity of *S. cerevisiae* can be enhanced by the addition of vitamines to fermentation broth, the effect of yeast extract on ethanol productivity of the fusant FSC-14-75 was investigated in the fermentation broth containing 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 15% or 20% liquefied potato starch. The seed volume was 1.0%, and culture condition was maintained at 30°C and pH 4.2. The agitation speed was 200 rpm and aeration was made at a rate of 0.5 vvm for 8 hours.

As showned in Fig. 1, the ethanol productivity of FSC-14-75 was preferable to that of the parental strain, *S. diastaticus* IFO 1046, regardless of the concentration of liquefied potato starch. It is also noted that the addition of 0.2% yeast extract further increased the ethanol production and shortened the period of fermentation.

Effect of pH on ethanol productivity from liquefied potato starch

To examine the optimum pH for ethanol fermentation of the fusant FSC-14-75, the ethanol productivity from 20% liquefied potato starch supplemented with 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.2% yeast extract was investigated at pH 4.2 and 5.5, respectively.

As shown in Fig. 2, pH 4.2 was more favorable than pH 5.5 in terms of the fermentation speed and ethanol productivity.

Ethanol productivities from various concentrations of liquefied potato starch

In order to investigate the optimum concentration of liquefied potato starch for ethanol fermentation of the fusant FSC-14-75, 15%, 20%, or 25% liquefied potato starch was directly fermented by the fusant at 30°C. As the results summarized in Table 1, the fusant FSC-14-75 showed consider-

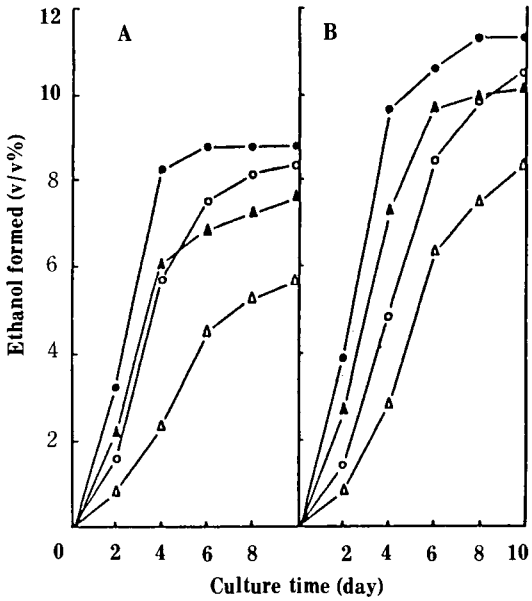

Fig. 1. Effect of yeast extract on ethanol fermentation in 15% liquefied potato starch (A), and 20% liquefied potato starch (B).

The fermentation was conducted in a mini-jar fermentor with 2.5 liters of fermentation broth containing 15% or 20% liquefied potato starch, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and if necessary, 0.2% yeast extract was added. The seed volume was 1%, and the pH was controlled to 4.2 with 2N NaOH. Symbols: △, *S. diastaticus* IFO 1046 (no yeast extract); ▲, *S. diastaticus* IFO 1046 (0.2% yeast extract); ○, FSC-14-75 (no yeast extract); ●, FSC-14-75 (0.2% yeast extract).

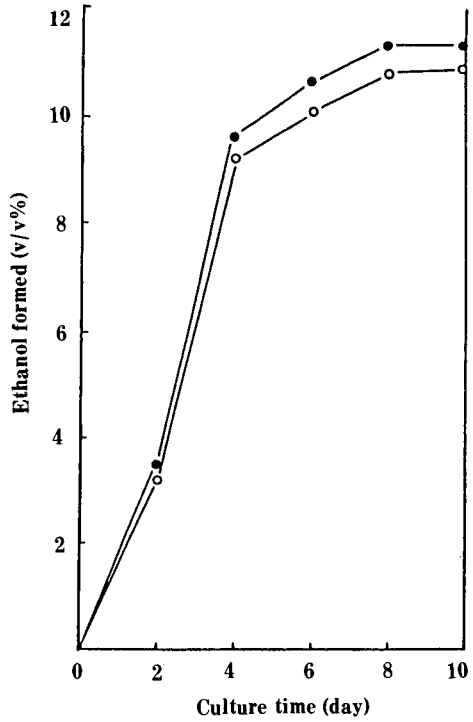

Fig. 2. Effect of pH on ethanol fermentation of FSC-14-75.

The fermentation was conducted in a mini-jar fermentor with 2.5 liters of fermentation broth containing 20% liquefied potato starch, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.2% yeast extract. The pH was controlled with 2N NaOH. Symbols: ●, pH 4.2; ○, pH 5.5.

abely improved ethanol productivity compared to the parental strain. Although the fermentation efficiency decreased, the ethanol production increased consistent with the increase of starch concentration. FSC-14-75 produced 13.7% (v/v) of ethanol from 25% liquefied potato starch in 10 days. Especially, FSC-14-75 produced 8.7% (v/v) of ethanol from 15% liquefied potato starch in 6 days by direct fermentation with an efficiency of 80.6% to total sugars.

Effect of amount of inoculum on ethanol fermentation

The seed volume used in this experiment was 1%. However, the seed volume in the conventional processes of ethanol fermentation is 10%(v/v) to the fresh broth regardless of the substrates for the purpose of preventing contamination and shortening the period of fermentation.

Table 1. Ethanol fermentation in various concentrations of liquefied potato starch

Strain	Starch concentration(%)	Ethanol productivity %(v/v) 4	6	8	10(day)	Yield (%)
S. diastaticus IFO 1046	15	6.0	6.8	7.2	7.6	70.7
	20	7.3	9.6	9.9	10.1	70.3
	25	7.1	8.6	8.8	9.6	53.5
FSC-14-75	15	8.2	8.7	8.7	8.7	80.6
	20	9.6	10.6	11.3	11.3	78.5
	25	10.1	12.0	13.3	13.7	76.3

The fermentation was conducted in a mini-jar fermentor with 2.5 liters of fermentation broth containing 15%, 20% or 25% potato starch (liquefied with Thermamyl) 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% yeast extract at 30 °C, and seed volume was 1%. The pH was controlled to 4.2 with 2N NaOH every 12 hours.

Table 2. Effect of seed volume on ethanol fermentation of FSC-14-75 in liquefied potato starch

Seed volume %(v/v)	Starch concentration(%)	Ethanol productivity %(v/v) 2	4	6	8	10(day)
1	20	3.8	9.4	10.6	11.2	11.2
10	20	6.6	11.0	11.3	11.6	11.6

The fermentation broth consisted of 20% liquefied potato starch, 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.2% yeast extract. Other fermentation conditions were as in Table 1.

Thus we examined the effect of seed volume on ethanol productivity from 20% of liquefied potato starch. The culture conditions were maintained at pH 4.2, 30 °C, 200 rpm with aeration at 0.5 vvm for 8 hr.

As shown in Table 2, when the seed volume was 10% (v/v), the ethanol productivity was enhanced as well as the period of fermentation was shortened as compared with 1%. The fusant FSC-14-75 produced 11.0% (v/v) of ethanol from 20% liquefied potato starch in 4 days in this case.

Ethanol productivity from liquefied cassava starch

As the result of ethanol fermentation of the fusant FSC-14-75 in liquefied potato starch, the productivity was considered high enough for practical application. Hence, in order to assess the possibility of industrial application of FSC-14-75 to direct fermentation of liquefied starch, ethanol productivity from liquefied cassava starch was investigated in a mini-jar fermentor scale with the same manner as a plant-scale process.

For the preparation of fermentation broth, a 25% (w/v) suspension of cassava starch (40 mesh) was liquefied by Thermamyl for 60 minutes at 80 °C, to which 0.3% $(NH_4)_2SO_4$ was added, and then sterilized for 30 minutes at 121 °C. The fresh broth was inoculated with 1% of inoculum and was maintained at 30 °C. The pH was not controlled during the period of fermentation contrary to the case of liquefied potato starch.

As shown in Table 3, the ethanol productivity of FSC-14-75 was superior to that of parental strain *S. diastaticus* IFO 1046. The FSC-14-75 produced 8.5% (v/v) of ethanol in 4 days, whereas the *S. diastaticus* produced only 3.4% (v/v) of ethanol. However, when the cassava starch was treated with 0.03% (v/w, to starch content) of Thermamyl, ethanol productivity considerably decreased.

Table 3. Ethanol fermentation in liquefied cassava starch

Starain	α-amylase %(v/w)	Nitrogen source	Ethanol productivity %(v/v) 4	6	8(day)
S. diastaticus IFO 1046	0.8	0.3%$(NH_4)_2SO_4$	3.4	3.8	3.8
FSC-14-75	0.8	0.3% $(NH_4)_2SO_4$	8.5	—	—
FSC-14-75	0.03	0.3% $(NH_4)_2SO_4$ 0.2% yeast extract	—	—	6.2

The concentration of cassava starch was 25g/100 m*l*, and the pH was not controlled during fermentation. Other fermentation conditions were as in Table 1.

Table 4. Ethanol fermentation in equal mixture of liquefied barley and sweet potato starch

strain	α-amylase %(v/w)	Nitrogen source	Ethanol productivity %(v/v) after 4 day
FSC-14-75	0.8	0.3%$(NH_4)_2SO_4$	7.6

The concentration of starch was 25g/100 m*l* and consisted of equal mixture of liquefied barley and sweet potato starch.

Ethanol productivity from equal mixture of liquefied barley and sweet potato starch

In an industrial process of ethanol fermentation, the barley and sweet potato starch also were used as substrates besides the cassava starch.

The result, appeared in Table 4, shows the ethanol productivity from the equal mixture of liquefied barley and sweet potato starch. The fusant FSC-14-75 produced 7.6% (v/v) of ethanol in 4 days, whereas the parental strain *S. diastaticus* produced only 3.0% (v/v).

Discussion

In other to assess the possibility of practical application of the most promising fusant FSC-14-75 to a new industrial process for ethanol fermentation employing starch-fermenting yeast, optimum conditions for ethanol fermentation of FSC-14-75 were investigated in a laboratory mini-jar fermentor scale.

It was observed that the addition of 0.2% yeast extract significantly improved the ethanol production and shortened the period of fermentation when liquefied potato starch was used as the substrate. On the other hand, when the raw starchy materials such as cassava meal, sweet potato meal, and barley meal were used as the substrates, the addition of yeast extract was not as much effective as the case of purified potato starch. Especially, the addition of yeast extract was more favorable in the early phase of fermentation than the late phase.

As the optimum pH of the amylases of FSC-14-75 originated from *S. diastaticus* and *C. tropicalis* lay in the range of 5.0 to 6.0 (6,7), ethanol productivity was examined at pH 4.2 and pH 5.5, respectively. As showed in Fig. 2, pH 4.2 was better than 5.5 in terms of ethanol productivity as well as fermentation speed.

Based on the above results, 15%, 20%, or 25% liquefied potato starch was directly fermented by FSC-14-75 at 30°C in order to examine the optimum concentration of liquefied potato starch for ethanol fermentation. FSC-14-75 showed considerably improved ethanol productivity compared to the parental strain *S. diastaticus*. This result suggested that the amylase originated from *C. tropicalis* also did play an important role in direct fermentation of liquefied potato starch. The ethanol productivity increased consistent with the increase of starch concentration. Especially, FSC-14-75 could produce 8.7% (v/v) of ethanol from 15% liquefied potato starch in 6 days, and 11.3% (v/v) of ethanol from 20% liquefied potato starch in 8 days with an efficiency of 78.5%, which is comparable to the level of industrial ethanol fermentation.

When the seed volume was 10%, the ethanol productivity was enhanced and the period of fermentation required to reach maximum level was considerably reduced. In fact, FSC-14-75 produced 11.6% (v/v) of ethanol from 20% liquefied potato starch and the corresponding efficiency was 81.0%.

Although FSC-14-75 produced 8.5% (v/v) of ethanol from liquefied cassava starch prepared with the same manner as the industrial process except saccharification step, *S. diastaticus* produced 3.4% (v/v) of ethanol. These results might be due to the characteristics of the glucoamylase produced from *S. diastaticus*, which did not possess debranching activity (8).

On the other hand, from the equal mixture of liquefied barley and sweet potato starch, FSC-14-75 produced 7.6% (v/v) of ethanol. This result showed that the barley and sweet potato starch were more resistant to the action of amylase than cassava starch because of the structural difference.

As the results shown above, it was thought that the fusant FSC-14-75 has sufficient potentiality to be used as a new brewing yeast capable of fermenting liquefied starch directly in the industrial processes.

요 약

Transformant TSD-14와 *C. tropicalis*간의 이속간 융합체인 FSC-14-75의 산업적 이용가능성을 검토하기 위하여 mini-jar fermentor(working volume=2.5 liters)를 사용하여 ethanol 발효의 최적조건 및 ethanol productivity를 조사하였다.

Thermamyl(α-amylase, Novo Co.)로서 액화시킨 liquefied potato starch를 15%, 20%, 25% 농도로서 발효를 한 경우 총당에 대해 약 80%의 발효율을 나타내었으며 이는 yeast extract의 첨가에 의해 향상되었다. Ethanol 발효조건으로는 pH 4.2가 pH 5.5에 비해 효과적이었으며, seed volume을 공업적 수준인 10%로 하면 4일만에 20%의 liquefied potato starch로부터 11.0%(v/v)의 ethanol을 생성하여 현재의 공업적 사용균주의 성적에 필적할만 하였다.

또한 본 균주는 현재의 공업적 ethanol 발효생산에서 발효기질로 실제 이용하고 있는 Cassava starch 혹은 barley 및 sweet potato starch 등을 원료로 하였을 때도 8.5%(v/v) 및 7.6%(v/v)의 ethanol을 직접적으로 발효 생산하였으며, 한편 이와 같은 raw starch를 원료로 할 경우는 yeast extract를 첨가하지 않아도 무방하여 이상의 결과로 본 효모균주는 당화, 발효를 동시에 할 수 있는 효모로서 공업적 이용이 가능할 것으로 기대되었다.

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