Alcohol Fermentation of Starch by a Genetic Recombinant Yeast Having Glucoamylase Activity

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Alcohol fermentation of starch was investigated using a direct starch fermenting yeast, *Saccharomyces cerevisiae* SR93, constructed by integrating a glucoamylase-producing gene (*STA1*) into the chromosome of *Saccharomyces cerevisiae* SH1089. The glucoamylase was constitutively produced by the recombinant yeast. The ethanol concentration produced by the recombinant yeast was 14.3 g/L which was about 1.5-fold higher than by the conventional mixed culture using an amylolytic microorganism and a fermenting microorganism. About 60% of the starch was converted into ethanol by the recombinant yeast, and the ethanol yield reached its maximum value of 0.48 at the initial starch concentration of 50 g/L. The fed-batch culture, which maintains the starch concentration in the range of 30 to 50 g/L, was used to produce a large amount of ethanol from starch. The amount of ethanol produced in the fed-batch culture increased about 20% compared to the batch culture.
Key words: starch fermentation • recombinant yeast • ethanol production • glucoamylase activity • fed-batch culture

INTRODUCTION

Starch is the most abundant utilizable resource in plant biomass and can be hydrolyzed enzymatically into fermentable sugar available to microorganisms. Traditional conversion of starch into alcohol requires a two-stage process: hydrolysis of starch by acid or amylolytic enzyme and fermentation by anaerobic bacterium or yeast. Research has been focused recently on the enzymatic hydrolysis by amylase or amylolytic microorganism, on the alcohol fermentation by immobilized yeast and on substrate-controlled fed-batch cultures using an automatic analyzer to increase the production rate and recover the cell mass in a continuous culture (Hoshino et al., 1990; Nakasaki et al., 1989; Park and Rivera, 1982; Shimizu et al., 1988; Wada et al., 1981).

Simultaneous saccharification and fermentation with mixed microorganisms such as an amylolytic microorganism and an ethanol-fermenting microorganism is an effective method for the direct fermentation of starch (Dostalek and Haggstrom, 1983; Han and Steinberg, 1987; Kurosawa et al., 1989; Lee et al., 1983; Tanaka et al., 1986). The advantages of the simultaneous saccharification and fermentation are that a two-stage process for the conversion of starch into ethanol is realized in one reactor and the glucose produced is rapidly converted into ethanol (Beschkov et al., 1984). However, in this system the ethanol yield decreased because much starch was consumed by the growth of amylolytic microorganisms. To increase the production of ethanol, it is necessary to breed a microorganism by a genetic manipulation, which can directly ferment starch into ethanol. Recently, there has been much interest in genetic engineering for expression of heterologous genes in a yeast as well as *Escherichia coli* (Park et al., 1993; Patkar and Seo, 1992; Silva and Bailey, 1991; Walls and Gainer, 1991).

The genetic manipulation required to insert a recombinant plasmid with a heterologous gene into a cell is a comparatively easy procedure. However, the loss of plasmid with a heterologous gene has been observed during longer incubation. Although Sardonini and DiBiasio (1987) attempted to avoid the loss of plasmids in *Saccharomyces cerevisiae* containing a recombinant plasmid, by using a selective media, some of the plasmids leaked from the cells. Therefore, to avoid the loss of the heterologous gene, it is necessary to insert a heterologous gene into the microbial chromosome (Mochizuki et al., 1994; Scherer and Davis, 1979).

In this work, the direct conversion of starch into ethanol by a genetic recombinant yeast having glucoamylase activity was investigated. The effect of initial starch concentration on the production of glucoamylase by the recombinant yeast was evaluated experimentally. The amount of ethanol produced by the recombinant yeast was compared to that produced by the conventional mixed culture using an amylolytic microorganism and a fermenting microorganism. The fed-batch culture using recombinant yeast was tested for the large-scale production of ethanol from starch.

MATERIALS AND METHODS

Host Strains and Plasmids

Saccharomyces cerevisiae SH1089 (ura-3 leu-2), kindly supplied by S. Harashima (Osaka University), and *Esch-*

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erichia coli JM109 were used as host strains. The glucoamylase gene, *STA1*, originating from *Saccharomyces diastaticus*, was obtained by digesting pSTA1-7-18 plasmid, kindly provided by I. Yamashita (Hiroshima University) (Yamashita et al., 1985). A yeast plasmid, YIP5, and an *Escherichia coli* plasmid, pUC19, were used for gene manipulation.

Construction of Recombinant Yeast

The *STA1* gene was integrated into the chromosome of *Saccharomyces cerevisiae* SH1089 by the homologous recombination method (Orr-Weaver et al., 1981). The first screening was done with minimal medium without uracil and the second screening was done with optimized carbon-limited minimal medium containing only starch as carbon source. One clone expressing a glucoamylase activity was obtained and named *Saccharomyces cerevisiae* SR93.

Culture Media and Cultivation

YPS medium was used for the incubation experiments of recombinant yeast and contained 5 g/L of yeast extract, 10 g/L of polypeptone and various concentrations of soluble starch. All biochemicals of the medium were from Wako Pure Chemical. *Saccharomyces cerevisiae* SR93 was precultured in the L-test tube containing 10 mL of YPD medium (5 g/L yeast extract, 10 g/L polypeptone, and 20 g/L glucose). When the cell concentration reached 1.0 g/L, 1 mL of the culture was poured into 100 mL of YPS medium and shaken. The incubation was carried out at pH 5 and 30°C for 200 h by rotary shaker with 100-rpm agitation (BR-300L; Taiyo Kagaku Kogyo).

Analysis

The cell concentration was estimated by measuring the dry cell weight. The glucose concentration was measured by the mutarotase GOD method (Glucose C-Test; Wako Pure Chemical). The starch concentration was determined by subtracting the amount of glucose from the total amount of sugar as measured by the phenol–sulfuric acid method (Dubois et al., 1956). The ethanol concentration was measured by gas chromatography (Shimadzu, GC-8APF). Glucoamylase activity was assayed by measuring the glucose released from the soluble starch when 800 μL of culture filtrate was mixed with 200 μL (final volume of 1000 μL) of 2% (w/v) soluble starch in 0.4 *M* acetate buffer at pH 5 and incubated at 50°C for 30 min (Lin et al., 1993; Yamashita and Fukui, 1984). One unit of glucoamylase activity is defined as the amount of enzyme required to produce 1 μmol of glucose/min at pH 5 and 50°C.

RESULTS AND DISCUSSION

Figure 1 shows the time courses of glucoamylase activity and ethanol concentration under various initial starch concentrations. The glucoamylase activity rose rapidly with the increase of initial starch concentration and reached its maximum value at a relatively short incubation time. This value at the initial starch concentration of 200 g/L was about 2.5-fold higher than that at the initial starch concentration of 10 g/L. The reason why the maximum glucoamylase activity increased with the increase of initial starch concentration is unknown in detail, but it seems that the secretion of glucoamylase was promoted by the high specific growth rate at the higher initial starch concentration. The ethanol concentration increased with the increase of incubation time and the maximum ethanol concentration increased with the increase of initial starch concentration. The glucoamylase activity decreased rapidly when ethanol concentration exceeded about 15 g/L. This suggests that an ethanol concentration above 15 g/L exhibits an inhibitory effect on glucoamylase activity. No loss of the *STA1* gene inserted into the chromosome was observed during an incubation time of 200 h.

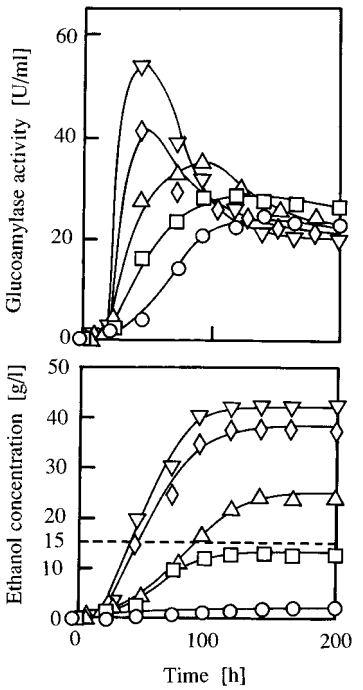

Figure 1. Time courses of glucoamylase activity and ethanol concentration under various initial starch concentrations. Symbols: (○) 10 g/L; (□) 50 g/L; (△) 100 g/L; (◇) 150 g/L; and (▽) 200 g/L.

The experimental results of the direct fermentation of starch using the recombinant yeast and of the simultaneous saccharification and fermentation of starch using mixed microorganisms, *Aspergillus awamori* and *Saccharomyces cerevisiae*, are shown in Figure 2a and b, respectively. As shown in Figure 2a, the recombinant yeast converted the glucose produced into ethanol by hydrolyzing the starch. The glucose concentration increased with the increase of incubation time reaching its maximum value at 48 h, after which it decreased.

The reason for the accumulation of glucose seemed to be

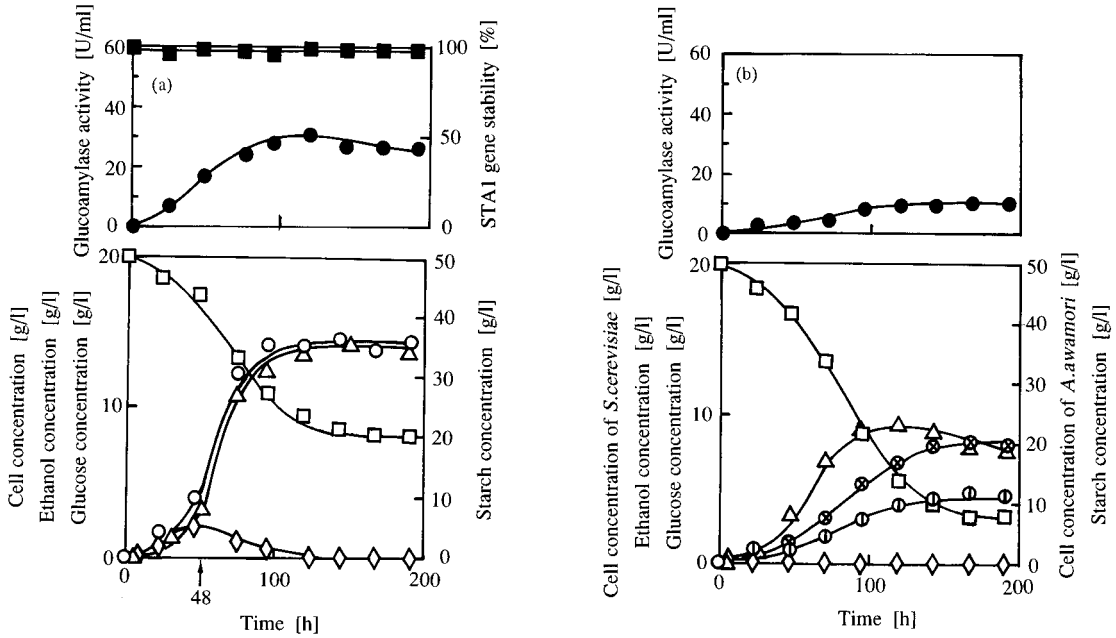

Figure 2. Starch fementation by: (a) recombinant yeast, *Saccharomyces cerevisiae* SR93; and (b) mixed microorganisms, *Aspergillus awamori* and *Saccharomyces cerevisiae.* Symbols: (○) cell concentration of *S. cerevisiae* SR93; (ⓘ) cell concentration of *S. cerevisiae;* (⊗) cell concentration of *A. awamori;* (□) starch concentration; (△) ethanol concentration; (◇) glucose concentration; (●) glucoamylase activity; and (■) *STA1* gene stability.

that the hydrolysis rate of soluble starch into glucose was larger than the conversion rate of glucose into ethanol. The cell concentration and the ethanol concentration increased rapidly with the hydrolysis of starch and reached their maximum values after an incubation time of 140 h. The maximum ethanol concentration was 14.3 g/L and the unhydrolyzed soluble starch was 20.1 g/L. Because the initial starch concentration was 50 g/L, only 60% of the starch could be hydrolyzed by the recombinant yeast. This suggests that the glucoamylase secreted by the recombinant yeast could not break the α-1,6-glucoside bond of amylopectin in the starch (Nikuni, 1961), and thus some parts of the starch remained undegraded. The comparison between the experimental results of starch degradation by recombinant yeast and by conventional mixed culture shows that the starch was degraded to only about 20 g/L by the recombinant yeast, as shown in Figure 2a, but the starch was efficiently degraded to about 8 g/L by the mixed culture, as shown in Figure 2b. It seems that the mixed culture could break the starch linkage, which could be degraded with difficulty by the recombinant yeast, because *A. awamori* produced an amylase for breaking the α-1,6-glucoside bond of amylopectin in the starch. However, because the starch was used mainly for the growth of *A. awamori* and not principally for the growth of the fermented yeast, *S. cerevisiae,* the amount of ethanol produced by the mixed culture was about 60% compared with that produced by the recombinant yeast. This result indicates that the direct fermentation of starch by the recombinant yeast obtained a comparatively smaller amount of degraded starch but a significantly larger amount of ethanol in comparison to the mixed culture.

Table I shows a comparison of the amount of ethanol produced and the production rate of ethanol from the soluble starch under several incubation systems. Dostalek and Haggstrom (1983) studied the conversion of starch into ethanol in a mixed culture of an amylolytic yeast, *Saccharomycopsis fibuliger,* and an anaerobic bacterium, *Zymomonas mobilis,* and they obtained a maximum ethanol concentration of 9.7 g/L and a production rate of ethanol of 0.54 g/L · h in the fermentation of 30 g/L of starch. Tanaka et al. (1986) investigated the production of ethanol from starch by a coimmobilized mixed culture system of aerobic and an-

Table I. Comparison of amount of ethanol produced and production rate of ethanol from soluble starch under several incubation systems.

Microorganism		Initial soluble starch concentration	Amount of ethanol produced	Production rate of ethanol	
Saccharification	Fermentation	(g/L)	(g/L)	(g/L · h)	References
S. fibuliger	*Z. mobilis*	30	9.7	0.54	Dostalek and Haggstrom (1983)
A. awamori	*Z. mobilis*	100	22.0	0.61	Tanaka et al. (1986)
S. cerevisiae SR93		100	24.9	0.66	This work

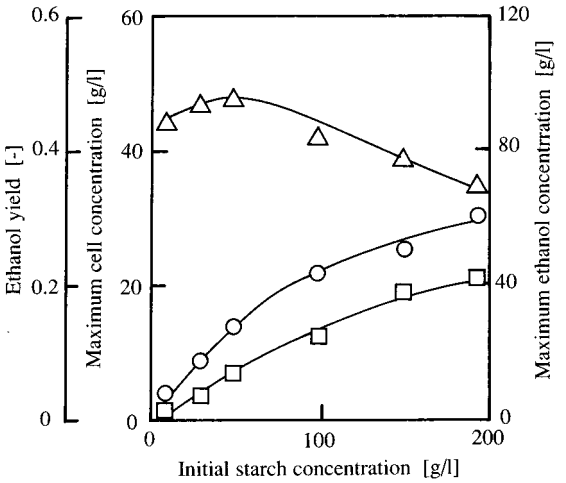

Figure 3. Relationship between maximum cell concentration, maximum ethanol concentration, ethanol yield, and initial starch concentration. Symbols: (○) maximum cell concentration; (□) maximum ethanol concentration; and (△) ethanol yield.

aerobic microorganisms, *Aspergillus awamori* and *Zymomonas mobilis*, in Ca-alginate gel beads and they obtained a maximum ethanol concentration of 22.0 g/L and a production rate of ethanol of 0.61 g/L · h in the fermentation of 100 g/L of starch. In our experiment using the recombinant yeast, *Saccharomyces cerevisiae* SR93, the maximum ethanol concentration was 24.9 g/L and the production rate of ethanol was 0.66 g/L · h in the fermentation of 100 g/L of starch. From this result, it was found that the direct fermentation of starch by the recombinant yeast was an effective method because it used only one microorganism and because it obtained a maximum ethanol concentration and a production rate of ethanol not less than those in mixed cultures.

Figure 3 shows the relationship between the maximum cell concentration, the maximum ethanol concentration, the ethanol yield and the initial starch concentration.

As the maximum ethanol concentration increased with the increase of the maximum cell concentration, it was found that the production of ethanol by the recombinant yeast was growth associated. The ethanol yield increased with the increase of the initial starch concentration, reaching the maximum value of 0.48 at the initial starch concentration of 50 g/L, and then decreased when the starch concentration exceeded 50 g/L. The reason why the ethanol yield was low at the lower initial starch concentration seems to be that the glucose produced by the glucoamylase was used for cell growth before it was converted into ethanol. A large amount of citric acid and isocitric acid were detected at the high initial soluble starch concentration (data not shown). Thus, the reason why the ethanol yield was low at the higher initial starch concentration seems to be that the metabolite pathway of glucose was changed by the glucose accumulated and a lot of organic acids such as citric acid were produced as byproducts (Tabuchi et al., 1968).

Next, an incubation method for producing large amounts of ethanol was examined. The incubation method to maintain the starch concentration in the liquid medium from 30 to 50 g/L seems to be effective because the ethanol yield reaches its maximum value at approx. 30 to 50 g/L, as shown in Figure 3. Figure 4 shows the result of fed-batch culture. The concentrated starch was intermittently added to increase the starch concentration from 30 to 50 g/L.

The cell concentration and the ethanol concentration increased with each increase of starch, but the cell growth rate and the ethanol production rate gradually decreased. This may depend on the fact that the ratio of undegradable starch concentration to starch concentration increases with each increase of starch. The cell concentration and the ethanol concentration at the final culture time were 25 and 28.2 g/L, respectively, and no loss of the *STA1* gene in the recombi-

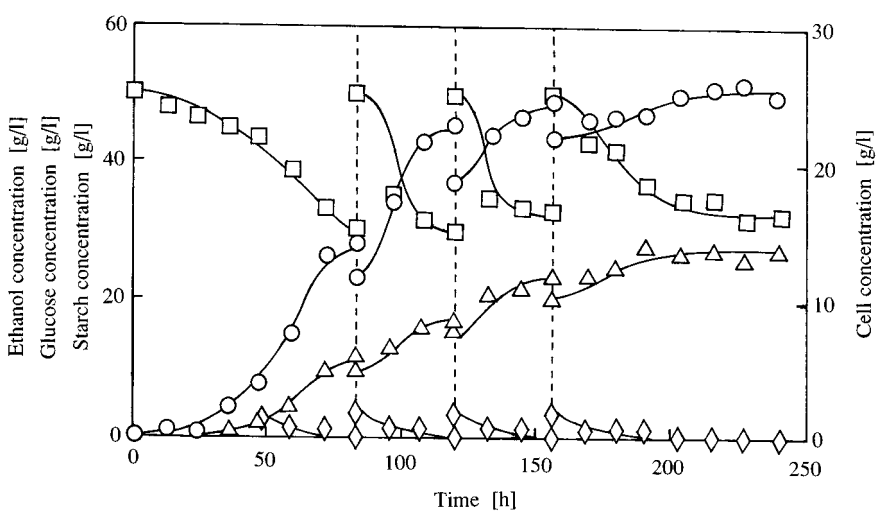

Figure 4. Fed-batch culture to maintain starch concentration in the range of 30 to 50 g/L. Symbols: (○) cell concentration; (□) starch concentration; (△) ethanol concentration; and (◇) glucose concentration.

nant yeast was observed. Because 24.9 g/L of ethanol was produced from 100 g/L of starch in the batch culture, as shown in Figure 1, and since 28.2 g/L of ethanol was produced from 94 g/L of starch in the fed-batch culture, as shown in Figure 4, the amount of ethanol produced in the fed-batch culture increased by about 20% compared with the batch culture. For the promotion of ethanol production, it is necessary to maintain the starch concentration at an optimal value, such as 50 g/L, with continuous addition of concentrated starch.

In conclusion, the following findings were obtained: (1) that the genetic recombinant yeast having glucoamylase activity could convert about 60% of the starch into ethanol directly and provide a high ethanol yield (0.48); (2) that the alcohol fermentation by the genetic recombinant yeast produced ethanol from the starch more rapidly and efficiently than a conventional mixed culture using an amylolytic microorganism and a fermenting microorganism; and (3) that the recombinant yeast fed-batch culture enhanced ethanol production. From these results, it is suggested that the genetic recombinant yeast can be effectively applied to the conversion of pretreated raw starch into ethanol. Future study will be focused on constructing a mathematical model of alcohol fermentation using genetic recombinant yeast and on increasing the efficiency of ethanol production using the fed-batch culture system based on a process analysis of this model.

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