Production of Ethanol from Distilled Shochu Mash through Saccharification by a Cellulase and Fermentation by a Saccharomyces cerevisiae : study for Basic Experimental Conditions of Saccharification

Production of Ethanol from Distilled Shochu Mash through Saccharification by a Cellulase and Fermentation by a *Saccharomyces cerevisiae*

—— Study for Basic Experimental Conditions of Saccharification ——

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ABSTRACT

Distilled shochu mash, a waste product from the shochu industry, has mainly been dumped into the sea as a means of disposal. However, this practise is due to be banned within a number of years. We proposed utilizing the distilled shochu mash as a biomass, which will convert to ethanol *via* saccharides, e.g. glucose, using cellulases. Basic experimental conditions of saccharification, such as temperature, pH, enzyme concentration and mash pretreatment, were investigated empirically.

1. INTRODUCTION

Shochu is one of the authentic Japanese alcoholic beverages. It is made by distilling the mash from sweet potatoes, rice or other grains, and molasses. In Kagoshima, sweet potatoes shochu has been produced and cherished. For a long time, the distilled shochu mash has been dumped into the sea and its amount is quite large. In Kyushu only, the annual amount of the distilled mash to be dumped into the sea is some 220,000 tons. The situation is changing for the shochu producers because of the possible banning of the dumping. The forecast is not bright for the time being, even though many alternatives have been examined. Incineration after dehydration is the perfect way to eradicate that in a sense. The countermeasure, however, costs more than sea-dumping. From not only the economical reason but also a recent industrial viewpoint (emissionless), it is required to utilize or recycle the distilled shochu mash as a biomass. Many alternatives have been attempted, including domesticated animal feed, mushroom culture, compost and so forth.

The authors started to contribute to this field by trying to utilize the distilled mash as a raw material of ethanol fermentation *via* saccharification using cellulases. One of the features of this method is that the product ethanol is a very versatile raw material which can be incorporated into current industrial processes, while the amounts of the products from most of other countermeasures to utilize the distilled mash are too huge to be consumed properly. There are some reports in which cellulose biomass, such as pulp[1] and orange peel[2], was saccharified enzymatically, but none of them has tried to use distilled shochu or other liquor mash as a cellulose source.

In this study, saccharification of distilled rice and sweet potato shochu mash by cellulases was carried out to investigate the effects of basic conditions (temperature, pH, enzyme concentration and mash pretreatment) on glucose yield. Also, using a supernatant of saccharified mash, alcohol fermentation was performed by a *Saccharomyces cerevisiae*.

2. MATERIALS AND METHODS

Substrates, Enzymes and Reagents

Distilled rice shochu mash (Komasa Jozoh Co., Ltd.) and distilled sweet potato shochu mash (Satsuma Shuzoh Co., Ltd.) were used as substrates. After autoclaving at 150℃ for 15 min, these were stored frozen. The solid contents of the distilled rice and sweet potato shochu mash were 8.6 and 5.7 wt%, respectively. Cellulases from a *Trichoderma viride* (Wako Pure Chemical Industries, Ltd.) was used for saccharification of distilled rice shochu mash. For saccharification of distilled sweet potato shochu mash, Meicelase (Meiji Seika Co., Ltd.) which consist of 40% cellulases was used. To keep the pH of reaction

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mixture constant, 0.1 M sodium acetate buffer was used. Sodium acetate and acetic acid were of reagent grade, and were purchased from Wako Pure Chemical Industries, Ltd.

Microorganism and Media

Saccharomyces cerevisiae K_0 was used for fermentation. The pure culture for inocula was stored on yeast extract-peptone-glucose (Difco Laboratories, Detroit, Michigan, USA) agar kept at 4℃. The composition of preculture media was (grams per 100 ml of distilled water) : yeast extract, 1; tryptone, 2; glucose, 2.

Saccharification

The mash was mixed with the buffer solution in an Erlenmeyer flask in the ratio of 1:1. The enzyme was added into this solution to a desired level. The reaction mixture were then shaken in a reciprocal shaker operating at 150 rpm.

During the course of saccharification, samples were taken at certain times and analyzed for glucose as follows. Enzymatic saccharification was terminated by putting the tube containing each sample in boiling water for 5 min. The supernatant of the sample was collected by centrifugation at 100,000 rpm for 5 min, and was filtered through a 0.2㎛ filter. Glucose in supernatant was determined enzymatically by means of Iatro-Chrom GLU-Lq reagent (Iatron Laboratories, Inc., Tokyo).

Fermentation

Saccharomyces cerevisiae K_0, so-called Kagoshima Kohbo, used for shochu production was inoculated into 5 ml of preculture medium in a test tube of 15 mm in an internal diameter and was shaken in a reciprocal shaker at 30℃ and 150 rpm for 6 h. After collecting them by centrifugation at 50,000 rpm for 10 min, the cells were transferred to an Erlenmeyer flask containing 100 ml of supernatant of a saccharified mixture, and then incubated under static condition at 30℃.

The course of fermentation was followed by measuring the concentration of glucose and ethanol. Ethanol concentration was determined by a gas chromatograph (Shimadzu GC-9AIF with G-205 capillary column).

3. RESULTS AND DISCUSSION

3.1. Rice Shochu Mash

At the first study, basic conditions of saccharification of distilled rice shochu mash using Cellulases from a *Trichoderma viride* were investigated. It was followed by fermentation of the resulting glucose. The results are as follows.

3.1.1. Effect of temperature

The mash was saccharified with 0.01g-Cellulases/g-mash at various temperatures. The result is shown in Figure 1. Reaction was most effective at 40℃. Thus, all reaction experiments were carried out at this temperature thereafter.

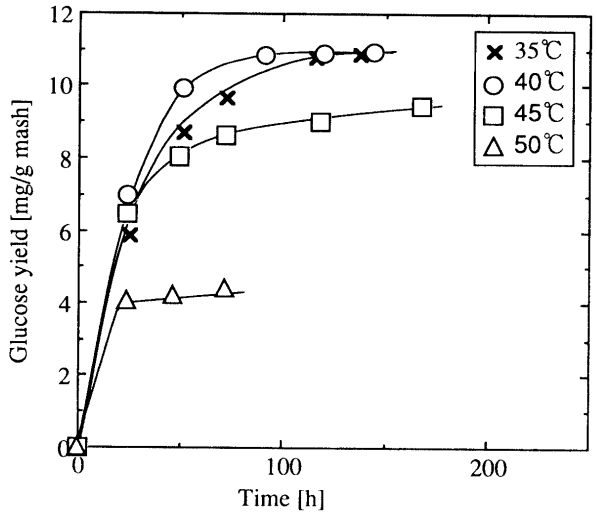

Figure 1. Effect of temperature on glucose yield. The mash and 0.1 M sodium acetate buffer (pH 4.0) were mixed in a ratio of 1:1. Enzyme concentration was 0.01 g /g mash.

It is obvious that at temperatures of 45 and 50℃ the enzyme underwent more serious deactivation than at lower temperatures (35 and 40℃). The question left is why the reaction stopped when the glucose yield became 11 mg/g-mash at 35 and 40℃ after 100 h. To get more information about this, the following experiment was carried out. For a reaction run at 40℃, another 0.01 g/g-mash of enzyme was added to the reaction mixture after 160 h. Just after the addition, glucose concentration started to rise again, though its slope against process time was smaller than the initial slope (about one tenth). It is likely that deactivation

of the enzyme caused the termination even at 40℃ shown in Figure 1. What we are not aware of is if there are substantial decrease in substrate or product inhibition.[1]

3.1.2. Effects of enzyme concentration and mash pretreatment with ball-milling

Because of cellulose being insoluble in water, size reduction of fibers in distilled shochu mash will be effective to enhance the saccharification rate and yield. Thus, we tried to give a pretreatment to the mash before saccharification process. The mash was milled using glass balls (d=5 mm) in a rotation ball mill at 250 rpm for 1 hour, and was saccharified at various concentrations of the enzyme for 22 h. The glass balls used were 130 g per 100 g of the mash. The result is shown in Figure 2. This indicates that glucose formation rate increased with the amount of enzyme added, and by the mash pretreatment carried out.

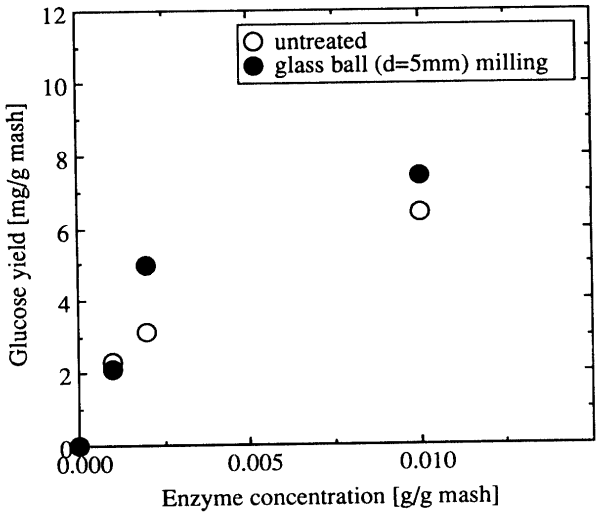

Figure 2. Effects of enzyme concentration and mash pretreatment by glass ball milling. Milling time, 1 h; the mash and buffer solution were mixed in a ratio of 1:1; temperature, 40℃; reaction time, 22 h.

Expecting more milling effect, stainless steel balls, which have a higher density than glass ones, were employed. They were of 4.8 and 20 mm in diameter. The stainless steel balls used were 350 g per 100 g of the mash. After milling in a rotation ball mill at 250 rpm for 16 h, the mash was then saccharified at various concentrations of enzyme until the reaction came to an end (162 h). Glucose yields after 22 and 162 hour reaction are shown in Figure 3. The glucose yield of 22 h (Figure 3(a)) means a glucose formation rate, and that of 162 h (Figure 3(b)) gives the maximum yield (see Figure 1). Glucose formation rate and maximum glucose yield increased with the amount of enzyme added, and when the mash pretreatment was carried out. As shown in Figure 3(a), small sized stainless steel ball milling pretreatment exhibited the highest rate of glucose formation among the three groups including untreated, 20 mm stainless steel balls treated and 4.8 mm stainless balls treated. As shown in Figure 3(b), however, milling pretreatment gave only a little effect on the maximum glucose yield.

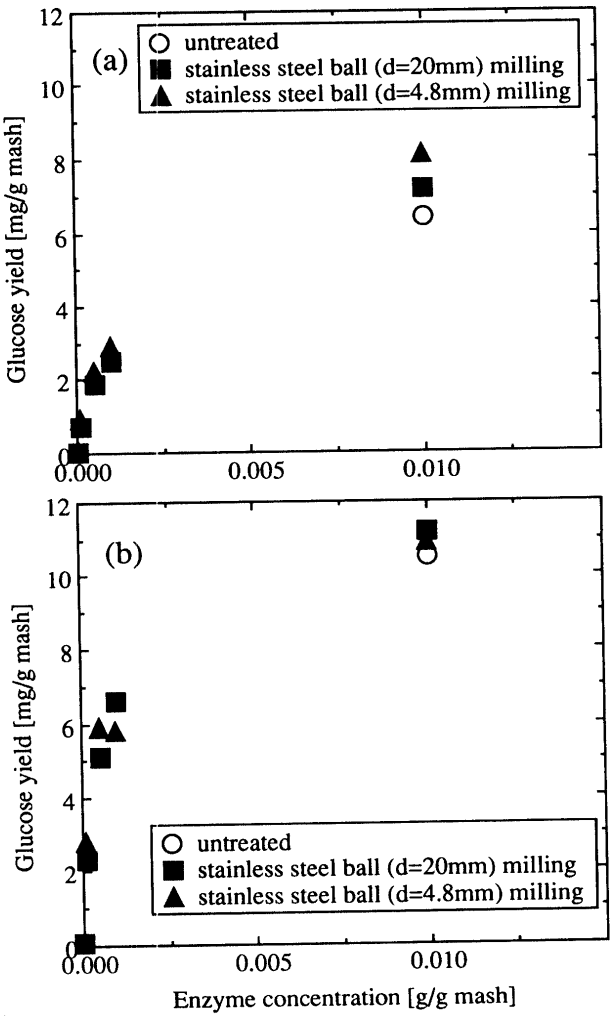

Figure 3. Effects of enzyme concentration and mash pretreatment by stainless steel ball milling. Milling time, 16 h; the mash and buffer solution were mixed in a ratio of 1:1; temperature, 40℃; reaction time, (a), 22h, (b) 162h.

3.1.3. Fermentation of supernatant of saccharified mash

The result is shown in Figure 4. This result indicates that fermentation was completed in one day, and the glucose formed in saccharification process was completely converted to ethanol. From the result, our primary apprehension, in which shochu mash might contain substances suppressing alcohol fermentation by *S. cerevisiae*, has eased off substantially.

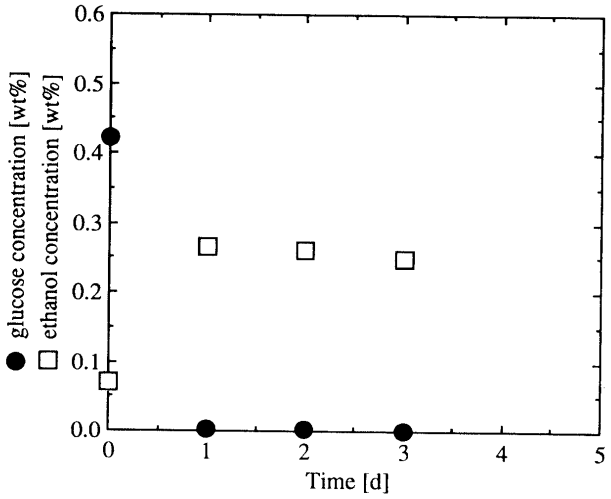

Figure 4. Time courses of glucose and ethanol concentrations during fermentation of supernatant of saccharified mash with *S. cerevisiae* Ko at 30℃. (●), glucose concentration; (□), ethanol concentration.

The ethanol yield obtained from these experiments was too low, it was only 0.5% from the mash. Financially, it would not be profitably because of the high price of Cellulales (5800 yen per 5 grams). Therefore, it was required to substitute the enzymes used to less expensive ones.

3.2. Sweet Potato Shochu Mash

Saccharification of distilled sweet potato shochu mash was then performed by Meicelase (the price is 5000 yen per kg). Effects of temperature, pH and mash pretreatment by centrifugation were investigated. The results are as follows.

3.2.1. Effect of temperature

The mash was saccharified at temperatures of 40, 45 and 50℃. The amount of cellulases used was adjusted so that the mass of enzyme per mass of solid in mash is the same as experiments using rice shochu mash. Thus, 0.017 g of Meicelase was added to 1 g of mash. The result is shown in Figure 5. At the end of the experiment, the highest glucose yield was obtained when reaction was carried out at 50℃, but the reaction time needed was too long (170 h). However, reaction at 45℃ gave higher glucose yield than at 50℃ during the initial time period. Thus, we decided to carry out saccharification at 45℃ thereafter.

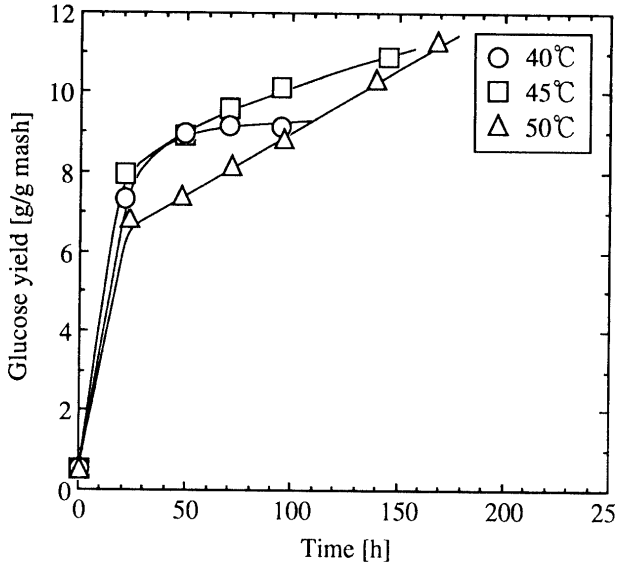

Figure 5. Effect of temperature on saccharification of distilled sweet potato shochu mash. The mash: buffer solution (pH 4.5) = 1:1; Meicelase conc., 0.017 g/g mash.

3.2.2. Effect of pH

The saccharification of the mash with 0.017 g-Meicelase/g-mash was carried out at various pH of reaction mixtures as shown in Figure 6. Reaction was most effective at pH 4.5. Thus, all reaction experiments were carried out at 45℃ and pH 4.5 in the following experiments. At both of pH 3.5 and 4.5, glucose formation rate decreased rapidly after 25 h, while at pH 5.5 the amount of glucose itself decreased. At first we suspected this phenomenon at pH 5.5 could be from contamination. The reproducibility was however excellent. There is still a possibility that this is from contamination. Another possible explanation is losing the reducing group in glucose. Degradation of glucose is very unlikely because of its chemical stability. Anyway, as we chose pH 4.5. The problem will be scrutinized later.

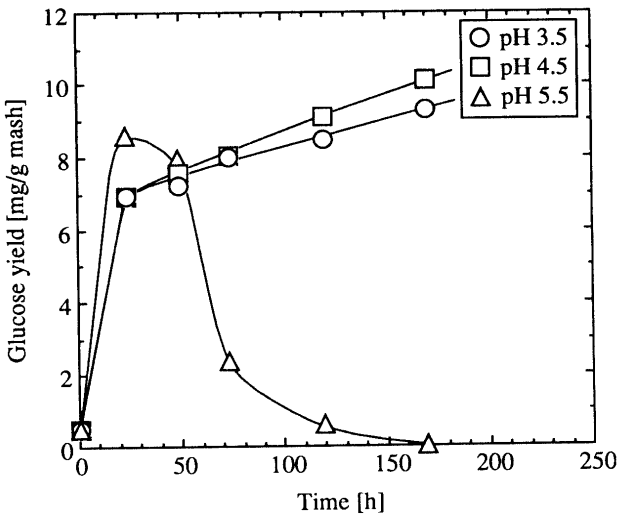

Figure 6. Effect of pH on saccharication of distilled sweet potato shochu mash. The mash: buffer solution=1:1; Meicelase conc., 0.017 g/g mash; temperature, 45℃.

3.2.3. Effect of glucose addition

The glucose formation suppression after 50 h shown in Figures 1, 5 and 6 could be caused by glucose inhibition. In order to clarify this question, the following experiment was carried out. 10.8 mg/g mash, maximum glucose yield we have had, of glucose was added into the reaction mixture initially. The reaction mixture was then saccharified with 0.017 g-Meicelase /g-mash at 45℃ and pH 4.5. The result shown in Figure 7 exhibits that the decreasing of glucose formation rate is not caused by inhibition of glucose which formed in saccharification process.

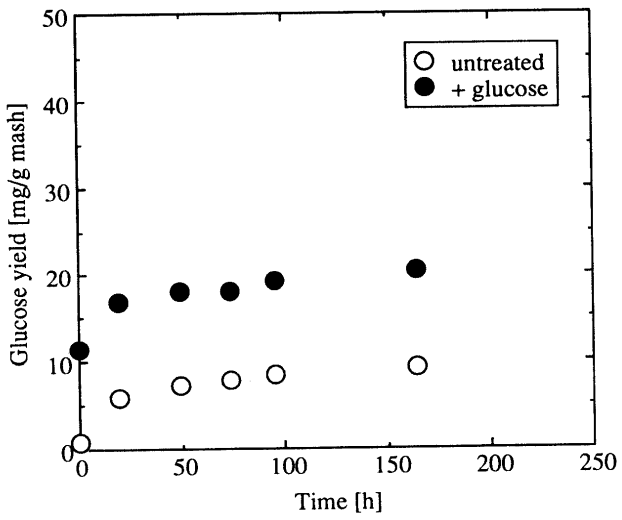

Figure 7. Effect of addition of glucose into the reaction mixture. The mash: buffer solution (pH 4.5) =1:1; Meicelase conc., 0.017 g/g mash; addition of glucose, 10 mg/g mash; temperature, 45℃.

3.2.4. Effect of solid loading in reaction mixture

Distilled shochu mash contains more than 90% of water. We tried to reduce this water content by centrifugation before the saccharification was carried out. After centrifugation at 50,000 rpm for 10 min, supernatant was removed as much as possible. Thus, the mash solid content increased from 5.8 to 12.8wt%. The mash was then saccharified with two kinds of enzyme concentrations (0.017 and 0.037 g/g-mash). As shown in Figure 8, glucose yield increased about three times when solid loading was increased by applying centrifugation, even though the enzyme concentration used was the same. On the other hand, from the experiment with 0.037 g-enzyme/g-mash, it is found that there is no significant effect if enzyme amount is increased.

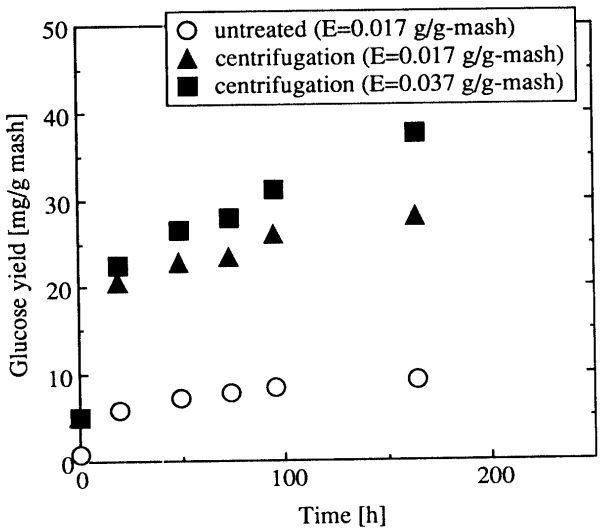

Figure 8. Effect of pretreatment by centrifugation (at 50,000 rpm, 10 min). The mash: buffer solution (pH 4.5)=1:1; Meicelase conc., 0.017 g/g mash; temperature, 45℃.

4. CONCLUSION

Basic conditions of saccharification (temperature, enzyme loading and mash pretreatment) of distilled rice shochu mash by cellulases from a *Trichoderma viride* were investigated. It was followed by ethanol fermentation of the glucose obtained using

Saccharomyces cerevisiae K_0. For the saccharification experiments of temperature effect, the result of 40℃ gave the best rate and yield. For the saccharification experiments of pretreatment effect, ball-milling enhanced the rate while a marginal improvement was observed for the maximum yield. In the fermentation test, all glucose in a supernatant from a saccharified mash was converted to ethanol stoichiometrically even though the yield was not high (0.5% from the mash). Financially it would not be profitably because the cellulase cost is rather expensive. Therefore, it was required to substitute the enzymes used to less expensive ones.

The saccharification of distilled sweet potato shochu mash by Meicelase was then performed. Effects of temperature, pH and pretreatment by centrifugation were investigated. This enzyme was most effective when the reaction was carried out at 45℃ and pH 4.5. We are also convinced that the decreasing of glucose formation rate after 25 h reaction time was not caused by product inhibition. For the experiment of centrifugation pretreatment effect, higher solid content in mash could enhance the glucose yield about three times using the same amount of enzyme. However, when the enzyme amount was increased that the enzyme amounts are the same in solid basis, no significant effect for enhancing glucose yield was observed. It is necessary to investigate if higher solid content than those tried is more efficient or not.

ACKNOWLEDGEMENT

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