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Bioconversion of Soy Sauce Residue Treated with Steam Explosion into Ethanol by Meicelase and *Mucor indicus*

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Abstract: Steam explosion was attempted as a pretreatment for the efficient conversion of soy sauce residue into ethanol. The Simultaneous Saccharification and Fermentation (SSF) of steam-exploded soy sauce residue at a steam pressure of 10 atm and a steaming time of 5 min was carried out by using Meicelase and halotolerant *Mucor indicus* ATCC24905 and gave a maximum ethanol yield, i.e., 0.057 g-ethanol g^{-1} dry soy sauce residue, at a substrate concentration of 100 g L^{-1}. This value corresponds to 1.9 times higher than that of untreated soy sauce residue, i.e., 0.030 g-ethanol g^{-1} dry soy sauce residue. At 200 g L^{-1} of steam-exploded soy sauce residue, though the ethanol yield was 0.052 g-ethanol g^{-1} dry soy sauce residue, the maximum ethanol productivity, i.e., 0.43 g-ethanol/L/h was obtained. Furthermore, the same ethanol yield and ethanol productivity were obtained at a substrate concentration of 100 g L^{-1} even if using the steam-exploded soy sauce residue without nutrient medium ingredients such as nitrogen sources and mineral salts.

Key words: Soy sauce residue, ethanol fermentation, enzymatic saccharification, steam explosion, productivity, mineral salt

INTRODUCTION

In late years biomass that is a renewable energy source derived from living organisms has attentions to be an alternative resource of fossil fuels (Klass, 1989). Soy sauce residue, a byproduct of soy sauce manufacturing process is one of the biomass and discharges 80,000 ton a year in Japan. Though some parts are used as animal feed almost parts are disposed by processing suppliers as an industrial waste. In addition, the use as the animal feed is a tendency to decrease because the soy sauce residue contains a large amount of salt. Therefore, it is desired to develop an efficient conversion method of soy sauce residue into useful materials and fuels in view points of both environmental protection and effective utilization of unutilized biomass.

Steam explosion that consists of steam hydrolysis with a high temperature and a high pressure and the mechanical treatment of the resulting hydrolyzed product with a sudden decrease in pressure has been attractive as an effective pretreatment for degrading and depolymelizing plant biomass (Asada *et al*., 2005; Li *et al*., 2009; Chen and Qiu, 2010). It seems that the steam explosion is effective as a pretreatment of soy sauce residue for alcohol fermentation. In this research the ethanol production from soy sauce residue was carried out by using steam explosion, Meicelase and halotolerant *Mucor indicus* ATCC24905. The effects of steam explosion, substrate concentrations in SSF and nutrient ingredients on the ethanol yield and the ethanol productivity were evaluated.

MATERIALS AND METHODS

Samples: Soy sauce residue was provided by Marukin Chyuyu Co., Ltd. and used as a sample throughout this investigation.

Steam explosion pretreatment: Steam explosion pretreatment which was used as a physicochemical pretreatment was carried out in a batch pilot unit equipped with a 1 L reaction vessel (Yamashita *et al*., 2010). About 100 g of soy sauce residue was introduced into the reaction vessel and exposed to saturated steam with 10 atm (181°C) for 5 min. After exposure to the saturated steam, a ball valve at the bottom of the reactor was suddenly opened to bring the reactor rapidly to atmospheric pressure. A product containing liquid and solid fractions was obtained as the steam exploded samples.

Microorganism and inoculum medium: *Mucor indicus* ATCC24905 was maintained on agar medium and stored at 4°C then transferred to fresh plate to be used within 24 h of incubation at 30°C. Cells were pre-grown in 10 mL

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L-tube containing 5 mL fermentation medium of 20 g L^{-1} glucose, 5 g L^{-1} yeast extract, 7.5 g L^{-1} $(NH_4)_2SO_4$, 3.5 g L^{-1} K_2HPO_4, 0.75 g L^{-1} $MgSO_4$•$7H_2O$ and 1 g L^{-1} $CaCl_2$•$2H_2O$ in 0.05 M acetate buffer at 30°C.

Simultaneous Saccharification and Fermentation (SSF): Simultaneous saccharification and fermentation was performed in 200 mL Erlenmeyer flasks containing 50 mL fermentation medium with various concentrations of steam-exploded soy sauce residue, 5 g L^{-1} yeast extract, 7.5 g L^{-1} $(NH_4)_2SO_4$, 3.5 g L^{-1} K_2HPO_4, 0.75 g L^{-1} $MgSO_4$•$7H_2O$ and 1 g L^{-1} $CaCl_2$•$2H_2O$ in 0.05 M acetate buffer at 37°C. For saccharification and fermentation, appropriate amounts of Meicellase (20 FPU g^{-1} substrate) from Meiji Seika Co., Ltd. and *M. indicus* cells separated from 5 mL pre-cultured solution were added, respectively. Samples were taken at 12, 18, 24, 36, 48 h and the amount of ethanol produced was analyzed by High Performance Liquid Chromatography (HPLC). All determinations were performed in triplicate biological replications. The ethanol yield was defined as the percentage ratio of ethanol produced to dry weight of untreated or steam-exploded soy sauce residue.

Analytical methods: The amount of chlorine in sample was determined by the method reported by Sisido and Yagi (1948). The amounts of protein and lipid in sample were measured by Kjeldahl method and the diethyl ether method, respectively (Kagawa, 2003). The ash content without chlorine was calculated by subtracting the chlorine content from the residual amount of sample after heating in an electric muffle furnace at 550°C for 20 h. Furthermore, the sample was hydrolyzed with 10 mL of 72% (w/w) H_2SO_4 at 30°C for 60 min. after then the reaction mixture was diluted to 4% (w/w) H_2SO_4 and autoclaved at 121°C for 60 min. This hydrolyzed solution was measured by HPLC as above condition. Holocellulose (cellulose and hemicellulose) content was established based on monomer content in acid extraction. After this procedure the insoluble matter was washed with hot water dried at about 105°C to constant weight and weighed. This substance was lignin. For the simultaneous saccharification and fermentation, the glucose concentration was measured by the mutarotase GOD method (Glucose C-Test; Wako Pure Chemical, Osaka, Japan) and the ethanol concentration was determined was determined by HPLC using an Aminex column HPX-87H (300×7.8 mm) (Bio-rad, Richmond, CA).

RESULTS AND DISCUSSION

Effect of steam explosion on composition of soy sauce residue: Table 1 shows the composition of components in untreated and steam-exploded soy sauce residue. The soy sauce residue contains salt, ash, protein, lipid, lignin and holocellulose (cellulose). The steam-exploded soy sauce residue was very finely fibrillized, giving a muddy appearance. The lignin content of steam-exploded soy sauce residue was higher than that of untreated soy sauce residue. This increase of lignin content is attributed to the formation of lignin by condensation reactions of the products of decomposition. On the other hand, the amount of cellulose, a substrate for ethanol fermentation, did not decrease at all and it was 0.152 g g^{-1} sample of steam-exploded soy sauce residue; this corresponds to 0.167 g-glucose g^{-1} sample if hydrolyzed. Since the theoretical ethanol yield is 0.51, 0.085 g-ethanol is obtained from 1 g sample of steam-exploded soy sauce residue theoretically. Furthermore, since the soy sauce residue has a large amount of salt, it is necessary to use halotorelant fermenting microorganism for the alcohol production from soy sauce residue. Therefore, in this research halotolerant *Mucor indicus* ATCC24905 was used as a fermenting microorganism.

Simultaneous saccharification and fermentation of soy sauce residue: Figure 1 shows the time course of ethanol concentration in the simultaneous saccharification and fermentation of untreated and steam-exploded soy sauce residue. Since the glucose concentration was maintained at a very low level due to the rapid assimilation by *M. indicus*, no accumulation of glucose was observed. The ethanol concentration increased rapidly with the increase of incubation time reaching its maximum value and then decreased slightly. This decrease seems to be that the cells consumed the ethanol produced. The ethanol concentrations obtained from the steam-exploded soy sauce residue were about 1.6-2.0 times higher than

Table 1: Composition of untreated and steam-exploded soy sauce residue without moisture

	Component (%)					
Samples	Salt	Ash without salt	Protein	Lipid	Lignin	Holocellulos (cellulose)
Untreated soy sauce residue	8.36	11.6	26.0	9.3	2.7	35.5 (15.6)
Steam-exploded soy sauce residue	8.79	10.8	25.3	10.7	8.7	33.3 (15.2)

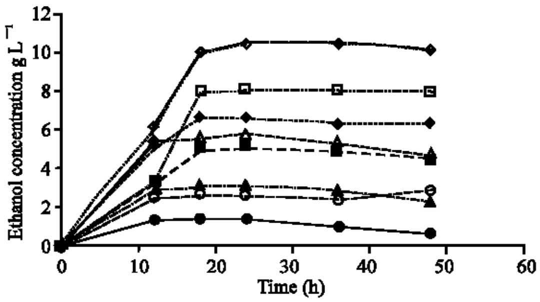

Fig. 1: Simultaneous saccharification and fermentation of untreated and steam-exploded soy sauce residue. Symbols: (●), 50 g L^{-1}; (▲), 100 g L^{-1}; (■), 150 g L^{-1}; (♦), 200 g L^{-1} of untreated soy sauce residue. (○), 50 g L^{-1}; (Δ), 100 g L^{-1}; (□), 150 g L^{-1}; (◊), 200 g L^{-1} of steam-exploded soy sauce residue

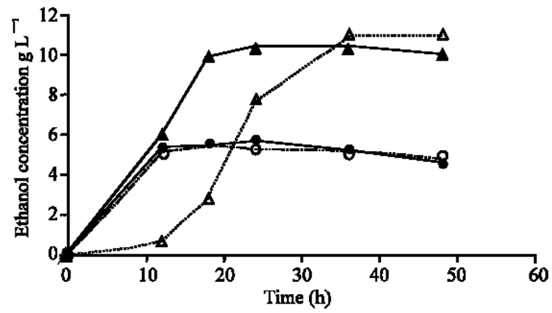

Fig. 2: Simultaneous saccharification and fermentation of steam-exploded soy sauce residue with or without nutrient medium ingredients. Symbols: (●), 100 g L^{-1}; (▲), 200 g L^{-1} with nutrient medium ingredients (○), 100 g L^{-1}; (Δ), 200 g L^{-1} without nutrient medium ingredients

those from the untreated soy sauce residue. The reason why the higher ethanol concentrations were obtained from the steam-exploded soy sauce residue seems to be that the soy sauce residue was hydrolyzed and fibrillized by the steam explosion and the cellulose component became more susceptible to the enzyme and cells (Kurosumi *et al*., 2006; Take *et al*., 2006). The maximum ethanol yield, i.e., 0.057 g-ethanol g^{-1} dry soy sauce residue (this value correspond to 67% of the theoretical ethanol yield) was obtained at a substrate concentration of 100 g L^{-1}.

This value corresponds to 1.9 times higher than that of untreated soy sauce residue, i.e., 0.030 g-ethanol g^{-1} dry soy sauce residue. At 200 g L^{-1} of steam-exploded soy sauce residue, though the ethanol yield was 0.052 g-ethanol g^{-1} dry soy sauce residue, the maximum ethanol productivity, i.e., 0.43 g-ethanol L h^{-1} was obtained. As a result, it was found that the steam explosion at a steam pressure of 10 min and a steaming time of 5 min is effective as a pretreatment for the alcohol production from the soy sauce residue.

Effect of fermentation medium on alcohol production: Figure 2 shows the comparison of alcohol production in the fermentation medium with nutrient medium ingredients to that without nutrient medium ingredients. At 100 g L^{-1} of steam-exploded soy sauce residue, the production profile of ethanol without the ingredients was almost same as that with the ingredients and the maximum ethanol concentration, i.e., about 5.7 g-ethanol L^{-1} was obtained at an incubation time of 24 h. On the other hand, at 200 g L^{-1} of steam-exploded soy sauce residue, though the ethanol production rate without ingredients was very slow compared to that with ingredients, the almost maximum ethanol concentration, i.e., about 10.5 g L^{-1} was obtained in both mediums. Since the soy sauce residue contains not only polysaccharides but also other components, i.e., protein, lipid and ash, etc., these components seems to be converted into nitrogen sources and mineral salts necessary to alcohol production of cells by the steam explosion.

This result demonstrates that the steam-exploded soy sauce residue can be converted into ethanol without nutrient medium ingredients such as nitrogen sources and mineral salts.

CONCLUSION

The effect of steam explosion method was investigated for the effective production of ethanol from the soy sauce residue. A large amount of ethanol obtained from the steam-exploded soy sauce residue and its value corresponds to about nearly 2.0 times higher than that from the untreated soy sauce residue. Furthermore, even if without nutrient medium ingredients the same ethanol yield was obtained from the steam-exploded soy sauce residue. Future study will be focused on the determination of optimal steam explosion conditions such as steam pressure and steaming time for the enhancement of ethanol yield from the steam-exploded soy sauce residue.

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