ORIGINAL ARTICLE

Ethanol production from sugi pulp under simultaneous saccharification and fermentation using a cocktail enzyme of *T. reesei* and *A. tubingensis* produced by solid-state fermentation

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Abstract The process of solid-state fermentation was used to produce a cocktail enzyme of *Trichoderma reesei* ATCC 66587 and *Aspergillus tubingensis* KRCF 700-33. Wheat bran, corncob, and sugi pulp were supplemented with ammonium sulfate as an enzyme-producing medium using *T. reesei* and *A. tubingensis*. The corncob blend ratio, duration of incubation, and ammonium sulfate concentration were optimized for enhancing cellulase production from *T. reesei* using a Box-Behnken design. Filter paper degrading activity more than tripled when *T. reesei* was grown in the optimized medium, as compared with the initial medium. The highest activity of 4.03 FPU/ml (about 29 FPU/g of dry material) was obtained with a cocktail enzyme having a 25 % content of *A. tubingensis* and 75 % of *T. reesei*. The sugi pulp was then fermented to ethanol with the cocktail enzyme and thermotolerant yeast (*Saccharomyces cerevisiae* BA-11) under simultaneous saccharification and fermentation (SSF) at 40 °C. An ethanol concentration of 4.48 % (w/v) was achieved using the cocktail enzyme (4 FPU/g-pulp) that was produced on-site with a substrate loading level of 12.5 wt %, which achieved an ethanol yield of 76 % after 72 h.

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Introduction

Woody biomass is widespread renewable resource, and quite a large volume has been accumulated. A growing focus of research concerns the production of bioethanol from woody biomass, due to its immense potential as an alternative fuel that does not compete with food. Given Japan's large forest area and the ratio of sugi (*Cryptomeria japonica*) in artificial planted forests, promoting the effective utilization of sugi should be a weighty subject [1]. A large portion of forests planted mainly during the 1950s and 1960s have reached the age of maturity [2]. And the conversion process of lignocellulosic materials via enzymatic saccharification generally needs some kind of pretreatment to increase the saccharification yield for a more effective production of fermentable sugars [3, 4]. Alkaline pretreatment causes hemicellulose and some lignin to solubilize, and the removal of both has a positive effect on the degradability of residual pulp. Oxygen delignification in alkaline solutions has also been reported as an effective technique of further removing residual lignin from pulp and thereby improving the saccharification yield [5, 6].

Cellulolytic enzymes are responsible for the hydrolysis of lignocelluloses and the production thereof is among the most important factors in the economical production of bioethanol. The supply of highly active, inexpensive cellulolytic enzymes is indispensable in developing bioethanol as a viable energy pathway. In the face of enzyme production costs, such low-cost materials as agro-industrial residues should be utilized as media for enzyme production. The process of

solid-state fermentation is a promising method of cultivation in terms of using agricultural and forest products, as well as waste. Microorganisms grow on the surface of solid or semisolid media consisting of the low-cost materials used in solid-state fermentation. This fermentation process is considered to yield more natural conditions than submerged fermentation for filamentous fungi and could induce the potential cellulolytic activity of microorganisms [7, 8].

In our previous research, alkaline-treated sugi chip (sugi pulp) was effectively saccharified when the culture extract of *Aspergillus tubingensis* (prepared by solid-state fermentation) was mixed with a commercial cellulase preparation obtained from *Trichoderma reesei*. *A. tubingensis* produced remarkable amounts of lignocellulose-degrading enzymes including endoglucanase, β-glucosidase, mannanase, xylanase, and β-xylosidase, as well as filter paper-degrading activity. The enzyme system from *A. tubingensis* was excellent for creating an enzyme cocktail with that of *T. reesei* to achieve the efficient cooperative hydrolysis of sugi pulp. [9] Given the ability of *T. reesei* to produce an intense cellulase system under solid-state fermentation, whole crushed media produced from *T. reesei* and *A. tubingensis* could thus be made available for producing bioethanol from sugi pulp under simultaneous saccharification and fermentation[10] (SSF). When considering the powerful cellulase system represented by cellobiohydrolase, the cellulase productivity of *T. reesei* is apparently a quality necessary for efficient, low-cost bioethanol production. In the study presented here, an attempt was made to employ response surface methodology to identify the optimum media conditions for producing cellulase from *T. reesei* with agro-industrial residues. Response surface methodology is a combination of mathematical and statistical techniques that are useful for modeling and analyzing problems, where several variables affect the response of interest. And statistical designs for optimization have recently been successfully employed in enzyme production [11, 12]. Because Box-Behnken designs typically have fewer design points, such designs are often less expensive to run than central composite designs with the same number of factors, particularly when all factors are not set simultaneously to the most extreme (low or high) settings [13]. The combined whole crushed media produced from *T. reesei* and *A. tubingensis* (as grown in an optimized medium) were mixed with sugi pulp and thermotolerant yeast (*Saccharomyces cerevisiae* BA-11 [14]), to obtain a higher concentration of ethanol under SSF.

Materials and method

Reagent and raw material

Sugi pulp was prepared by soda-anthraquinone cooking at 170 °C for 30 min followed by oxygen bleaching (once for
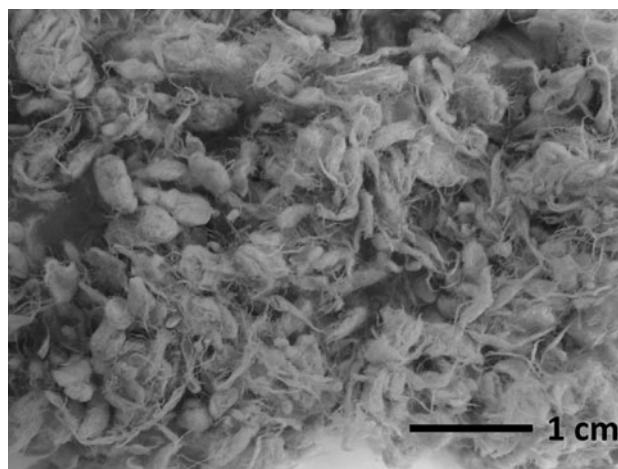

Fig. 1 Shape of wet sugi pulp

30 min followed by 3 times for 15 min). After cooking, the pulp was passed through a flat screen (0.125 mm). Wet sugi pulp had varying sizes as the wet squeezed pulp (having moisture content of 66.7 %) was used through the study (Fig. 1). To determine the relative sugar composition of the pulp, corresponding neutral monosaccharides in the acid hydrolyzate were analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) equipped with a CarboPac PA1 column (Dionex). The monosaccharides were eluted with 1 mM NaOH containing 0.3 mM acetic acid at 30 °C at a flow rate of 1 ml/min. The relative sugar composition analysis of sugi pulp showed 82 % glucose, 7.3 % mannose, 9.3 % Xylose, 0.9 % galactose, and 1.0 % arabinose (mol %). The Klason lignin content of the pulp was 8.8 %. *A. tubingensi* KRCF 700-33 was generated from *A. tubingensis* KRCF 700 by UV irradiation at FFPRI. The mutated strain, KRCF 700-33, produced filter paper-degrading activity (FPase activity) about 20 % higher than the original strain under solid-state fermentation with a medium composed of wheat bran and rice bran (data not show). The thermotolerant yeast, S*accharomyces cerevisiae* BA-11 (BioAcademia), was employed for ethanol fermentation. Mannnobiose, mannnotriose, and isoprimeverose were obtained from Megazyme.

Enzyme production under solid-state fermentation

T. reesei ATCC 66587 was grown on PDA plates at 28 °C for 10 days before use in a dark place. Under this growth condition, *T. reesei* ATCC 66587 did not form definite spores on the PDA plates. Mycerial plugs (6 mm in diameter) were prepared with a sterile cork borer from each PDA plate and then crushed by a multi-bead shocker (Yasui Kikai, Japan) in 1 ml of sterilized water. The moisture content of the solid culture medium was adjusted

to 70 %, and then 10 g of the medium composed of wheat bran, corn cob (all granules passed through a 6-mm sieve), and 5 % sugi pulp supplemented with ammonium sulfate (with a dry material content of 3 g) was autoclaved (at 121 °C for 20 min) in a 100-ml Erlenmeyer flask. The crushed mycerial solution was then inoculated into the flask under sterile conditions. *A. tubingensis* KRCF 700-33 was grown on a PDA plate for 2 weeks, with the mycerial plugs being crushed with spores. Both of *T. reesei* and *A. tubingensis* were grown using the medium optimized to *T. reesei* to facilitate the enzyme preparation step. The inoculated culture flasks were placed in an incubator in the dark, at a temperature of 28 °C and relative humidity of 60 %. After incubation, 15 ml of 50 mM sodium citrate buffer (pH 4.8) was added to the flask, and then the medium was homogenized using a handy homogenizer (with generator probe G10-195ST being inserted into Omni homogenizer TH-01). The supernatant after centrifugation was used as a crude enzyme solution for the determination of FPase activity and filter paper cellulase unit (FPU). The whole crushed medium was used for SSF. Approximately 22 g of whole crushed medium was obtained from 3 g of dry culture material.

Analytical procedures

FPase activity was determined by measuring the increase in reducing sugars from the hydrolysis of filter paper No. 1 (Whatman 1001-813, 2.6 × 3.1 cm, ca. 70 mg) using the 3,5-dinitro-salicilic acid (DNS) method with glucose as standard. The reaction mixture consisting of 40 μl of enzyme extract, 910 μl of 50 mM sodium citrate buffer (pH 4.8), 550 μl of distilled water, and one piece of curled filter paper was incubated at 50 °C for 1 h under continuous shaking (BioShaker M·BR-022UP, TAITEC). The reaction was stopped by heating the reaction mixture at 100 °C for 5 min. One unit (U) of FPase activity was defined as the amount of enzymes required to liberate an equivalent of 1 μmol of reducing sugars per min. FPU was determined according to IUPAC guidelines [15]. β-Glucosidase activity was assayed by measuring the amount of glucose liberated from cellobiose. Biosensor BF-5D (Oji Keisoku) was used to measure the concentration of glucose. One IU of β-Glucosidase activity was defined as the amount of enzymes required to degrade 1 μmol of cellobiose per min. Protein was determined by Bradford method (Bio-Rad) with bovine β-globulins as standard. Some sugars in the fermented reactants after SSF were analyzed by HPAEC-PAD. The reactants were centrifuged at 15,000 g for 3 min, and the aliquots (25 μl) of the diluted supernatants were analyzed using the CarboPac PA1 column after filtration at 30 °C at a flow rate of 1 ml/min. A linear gradient elution within 30 min from 20 mM to 100 mM NaOH was applied after injection.

Table 1 The variables and their levels for the Box-Behnken design

Factor code	Factor	Levels		
		−1	0	+1
X_1	$(NH_4)_2SO_4$ (%)	0	2	4
X_2	Corn cob (%)	0	25	50
X_3	Culture period (d)	3	5	7

Experimental design

MINITAB Release 14 statistical software (MINITAB Inc., PA, USA) was used in the generation of experimental designs. Response surface methodology using the Box-Behnken design of experiments was employed to develop a statistical correlation between three variables on the production of FPase. The three independent variables were the ammonium sulfate level (X_1), corncob level (X_2), and culture period (X_3). The low, medium, and high content levels of each were designated as −1, 0 and +1, respectively, as listed in Table 1. The system behavior is explained by the following quadratic model equation:

$$G = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3, \quad (1)$$

where G denotes the predicted response, β_0 the intercept, $\beta_1 \beta_2$ and β_3 the linear coefficients, $\beta_{11} \beta_{22}$ and β_{33} the squared coefficients, and $\beta_{12} \beta_{13}$ and β_{23} the interaction coefficients. The same software was used to generate the analysis of variance (ANOVA), contour plots, and optimized values of the variables.

The three variables and related levels for the Box-Behnken design were determined based on some preliminary experiments. The basic medium was composed of 95 % wheat bran and 5 % sugi pulp. The higher ratio of corncob in the medium lowered the ratio of wheat bran. On the other hand, the level of ammonium sulfate was expressed in proportion to the dry matter content in the medium. In other words, 0.06 g of ammonium sulfate was added to the medium when the level of X_1 was 2 %, since a dry matter content of 3 g was set as a default in this study.

Simultaneous saccharification and fermentation

The yeast was precultured in 100 ml of YM broth (Difco) for 48 h at 30 °C in a 300-ml flask placed on an orbital shaker operating at 100 rpm. The culture broth was then centrifuged and the yeast pellets were collected. The SSF experiments were conducted using 50-ml flasks with a working weight of 15 g. The mixture contained 12.5 wt % sugi pulp, 25 mM sodium citrate buffer (pH 4.8), crushed enzyme medium, and the yeast pellets (wet weight 20 g/kg). The reaction mixture

was stirred at 40 °C for 4 days. The mixture was difficult to stir during the early phase of the reaction. The concentrations of ethanol and glucose were determined using the supernatant of the reactant, along with biosensor BF-5D.

The ethanol yield from the concentration (EY) was calculated as

$$EY = 100 \frac{[ethanol]}{0.51(0.83[pulp]1.11}, \qquad (2)$$

where [ethanol] refers to the ethanol concentration (w/v %) of the supernatant at the end of fermentation, 0.51 is the conversion factor for hexose to ethanol based on the stoichiometric biochemistry of yeast, 0.83 the hexose-related polysaccharide fraction of dry sugi pulp in this study (g/g), [pulp] the dry pulp concentration at the beginning of fermentation (wt %), and 1.11 the conversion of hexose-related polysaccharide to equivalent hexose.

Results

Optimization of FPase production by experimental design

In our previous study, *T. reesei* NBRC 31329 (QM 9414) effectively induced FPase activity under solid-state fermentation with a medium composed of 10 % rice bran and 90 % wheat bran [9]. Three mutant strains (ATCC 66587, ATCC 66588, and ATCC 66589 [16]) showed higher FPase productivity than NBRC 31129 in the same culture medium. Among the strains, ATCC 66587 showed the highest FPase activity after 3 days of cultivation (data not shown). The culture variables were thus optimized for further FPase production with ATCC 66587.

Preliminary experiments conducted on one factor at a time revealed that sugi pulp, corncob, and ammonium sulfate were also effective additives to raise FPase activity under solid-state fermentation in a basal wheat bran medium. Ammonium sulfate (0–4 %), corncob (0–50 %), and culture period (3–5 days) were selected as the three variables in a wheat bran medium containing 5 % sugi pulp to study the related interactions and effects on FPase production using the Box-Behnken design of experiments (Table 1). Table 2 depicts FPase activity as a response to 15 runs of the three variables. The initial results of response surface regression for FPase production as analyzed using coded units showed that all linear and squared model terms were significant ($p < 0.05$) for FPase production. The interactions of X_1 with X_3 and X_2 with X_3 were also significant. Conversely, the interaction of X_1 with X_2 was not significant ($p = 0.341$). After removing the nonsignificant interaction term (X_1X_2), the final model in uncoded units was

Table 2 Experimental design and results of the Box-Behnken design of response surface methodology for the optimization of filter paper-degrading activity from *T. reesei* ATCC 66587

Run no.	$(NH_4)_2SO_4$ (%)	Corn cob (%)	Culture period (days)	FP ase (U/ml)	
				Experimental	Predicted
1	−1	−1	0	2.21	2.03
2	1	−1	0	4.57	4.40
3	−1	1	0	4.41	3.73
4	1	1	0	5.09	6.09
5	−1	0	−1	2.02	2.70
6	1	0	−1	2.53	2.36
7	−1	0	1	1.85	2.00
8	1	0	1	7.75	7.06
9	0	−1	−1	4.26	4.15
10	0	1	−1	2.61	2.17
11	0	−1	1	2.05	2.47
12	0	1	1	7.75	7.84
13	0	0	0	8.42	8.57
14	0	0	0	9.35	8.57
15	0	0	0	7.97	8.57

$$\begin{aligned} FPase(U/ml) = &-7.952 + 1.476X_1 + 0.005X_2 \\ &+ 5.088X_3 - 0.643X_1^2 - 0.003X_2^2 \\ &- 0.618X_3^2 + 0.337X_1X_3 + 0.037X_2X_3 \quad (3) \end{aligned}$$

The coefficient of determination (R^2) for the final model is 96.2 %. The analysis of variance (ANOVA) showed that the lack of fit is not significant in the final model ($p = 0.444$). According to Eq. 3, Table 2 lists the results for predicted FPase activity, along with the experimental responses. As listed in Table 2, higher FPase production was observed at run Nos. 13–15. The results of run Nos. 13–15 had a mean value of 8.58 ± 0.70, or values 3.9 times higher than that of run No.1 (the basic medium containing 95 % wheat bran and 5 % sugi pulp).

In order to determine the optimal settings of variance for FPase production, contour plots were generated to indicate the regions of variance settings (data not shown). Response Optimizer in the software was also used to pinpoint the optimum settings. As a result, the optimized medium was composed of 5 % sugi pulp, 33 % corncob, 62 % wheat bran, and 2.5 % ammonium sulfate. The duration of incubation was set at a point of 5.7 days according to Eq. 3. At this optimized point, FPase activity of the supernatant from *T. reesei* ATCC 66587 was 8.41 U/ml, or equivalent to 3.25 FPU/ml. The concentration of protein in the optimized *T. reesei* crude enzyme was 6.04 mg/ml. The actual experimental value at the optimum settings was slightly lower than the activity of 9.15 U/ml as predicted by the model (in Eq. 3), but still located at the high FPase

activity level (run Nos. 13–15 in Table 2) in this study. The measured β-glucosidase activity of the enzyme supernatant of ATCC 66587 was 0.39 IU/ml.

The effect of adding *A. tubingensis* enzyme on filter paper activity

The enzyme supernatants of *T. reesei* ATCC 66587 were mixed with those of *A. tubingensis* KRCF 700-33 at different ratios in enzyme volume to investigate the cocktail effect of the enzymes on filter paper-degrading unit (FPU). As shown in Fig. 2, the highest activity of 4.03 FPU/ml was obtained with a 25 % mixture of KRCF 700-33 and ATCC 66587 (1:3). The 25 % cocktail enzyme had a higher FPU level than that of the crude extract from ATCC 66587 alone. Increasing the ratio to more than 25 % of KRCF 700-33 against ATCC 66587 lowered the resultant mixture's FPU. The β-glucosidase activity of the 25 % cocktail enzyme was 1.17 IU/ml.

Producing ethanol from sugi pulp under SSF

In addition to enzyme production, the distillation of fermented broth is also a costly process. One of the most energy-intensive steps in the wood-to-ethanol process is the recovery of ethanol by distillation. The concentration of ethanol reportedly has a major effect on energy demand, especially at concentrations less than 4 % (w/v) [17]. Therefore, the goal was set to obtain a concentration greater than 4 % (w/v). At a water-insoluble solids concentration of 10 wt % and 12.5 wt % sugi pulp, the theoretical ethanol concentrations based on hexose conversion were 4.72 and 5.89 % (w/v), respectively. Previous research confirmed that higher dry matter content tends to lower the ethanol yield under SSF [18, 19]. The ethanol concentration will not reach 4 % (w/v) at an assumed ethanol yield of 80 % with 10 wt % sugi pulp. A substrate
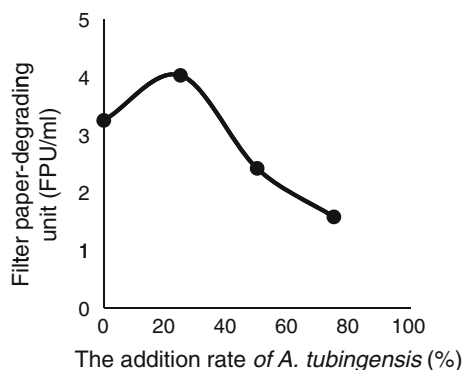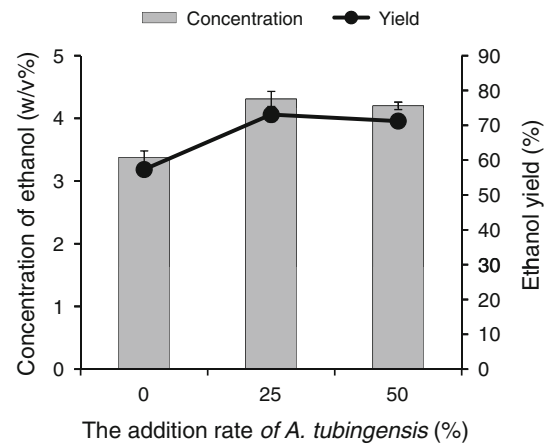
Fig. 3 Cocktail effect of *A. tubingensis* and *T. reesei* enzymes on ethanol production under SSF with 12.5 wt % sugi pulp. The dosage of whole crushed enzyme was 0.8 g (4–6 mg protein per gram of dry sugi pulp). Mean values ± SD (*n* = 3) are given

concentration of 12.5 wt % will make it possible to produce an ethanol concentration of 4.71 % (w/v) even under the same condition. Given the fact that SSF took 3–6 days to achieve the best yield in many cases, the fermentation time of SSF was fixed at 4 days.

SSF was run with the whole crushed media as enzyme source. The crushed enzyme dosage level was 0.8 g (crude enzyme) per gram of dry sugi pulp. The cocktail effects of crushed enzymes from *T. reesei* and *A. tubingensis* for ethanol conversion under SSF was investigated using 0, 25, and 50 % (w/w) cocktail enzymes (Fig. 3). The reaction mixtures were difficult to stir during the early period of reaction since the reaction mixtures were set at a high dry matter content of 12.5 wt % to obtain a higher concentration of ethanol. Regarding the *T. reesei* enzyme alone (0 % cocktail enzyme), the mixture did not turn into fluid slurry even after 4 days of SSF. Conversely, the reaction mixture with the cocktail enzyme fractions gained fluidity as the reaction progressed. The fluidity of the reaction mixtures permitted constant stirring of the reactants. An ethanol concentration of 4.31 % (w/v) was achieved with the 25 % (w/w) cocktail enzyme (Fig. 3), thus accounting for 73 % of the theoretical yield as based on hexose conversion (Eq. 2).

Figure 4 shows the analyses of residual sugars in the supernatant of the reactant after 4 days of SSF with 100 % *T. reesei* enzyme (B) and with a cocktail enzyme having a 25 % content of *A. tubigensis* and 75 % of *T. reesei*. Both of the crushed enzyme dosage levels were 0.8 g per gram of dry sugi pulp. Apparent peak, which showed the same retention time with that of authentic cellobiose was detected in the reactant with 100 % *T. reesei* enzyme and was decreased using the 25 % cocktail enzyme. Authentic

Fig. 2 Cocktail effect of *A. tubingensis* and *T. reesei* enzymes on filter paper-degrading unit (FPU). Results are expressed as the means of two independent experiments
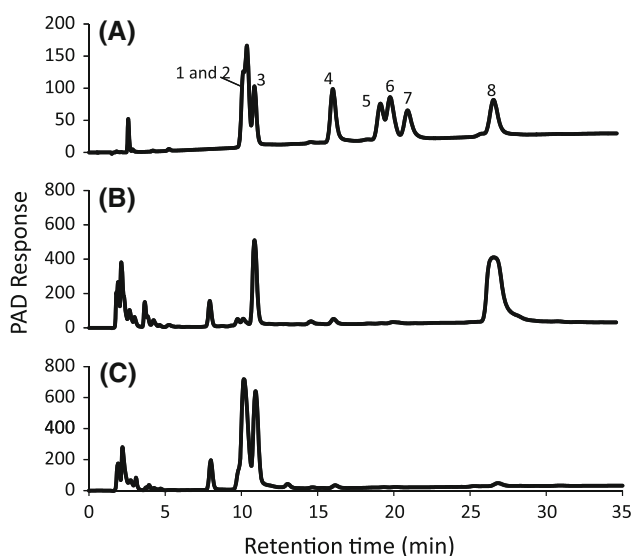
Fig. 4 HPAEC chromatograms of the fermented reactants after SSF. **a** Standard sugars: *1* and *2* glucose and mannose, *3* xylose, *4* mannnobiose, *5* xylobiose, *6* mannnotriose, *7* isoprimeverose, *8* cellobiose. **b** Reactant with *T. reesei* enzyme, **c** reactant with a cocktail enzyme having a 25 % content of *A. tubingensis* and 75 % of *T. reesei*. The supernatants of the reactants were diluted 100 times before analyses
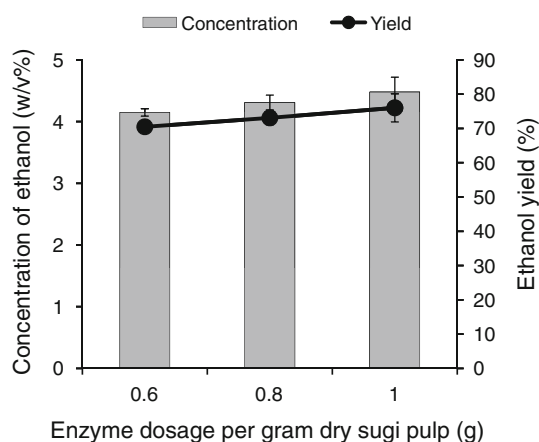

Fig. 5 Effect of enzyme dosage on ethanol production under SSF with 12.5 wt % sugi pulp. The amount of protein contained in 0.6, 0.8, and 1.0 g of the 25 % cocktail enzyme was approx. 3, 4, and 5 mg, respectively. Mean values ± SD (*n* = 3) are given

cellotriose and mannnotriose were not eluted from the column under the experimental condition.

Figure 5 shows the effect of enzyme dosage on ethanol yield with the 25 % cocktail enzyme. The highest ethanol concentration of 4.48 % (w/v) was obtained when sugi pulp was fermented with the highest enzyme dosage of 1.0 g per gram of dry sugi pulp or equivalent to about 5 mg of protein and 4.0 FPU per gram of dry pulp. Even when the least amount of 0.6 g of enzyme (about 3 mg of protein

and 2.4 FPU per pulp) was applied, the target ethanol concentration of 4 % (w/v) was still achieved (4.15 %) with an ethanol yield of 70 %. Regardless of the increased enzyme dosage, the ethanol concentration seemed to have reached its maximum. The increase in ethanol yield was not in proportion to the enzyme dosage.

Discussion

The enzymatic hydrolysis of cellulose and hemicellulose is conducted by cellulases and hemicellulases, and the production economics of bioethanol largely depend on the cost of enzymes. There are two possible ways of obtaining economically viable enzymes: one is reducing the enzyme dosage, and the other is lowering the enzyme unit price [20]. Extensive research has demonstrated that the presence of yeast along with a cellulolytic enzyme complex under SSF reduces the accumulation of inhibiting sugars, thereby increasing the yield. The SSF process using a low-cost enzyme might yield the best from both sides. Wheat bran and corncob used in the enzyme-producing media in this study are agricultural byproducts commonly used in the edible mushroom industry. Byproducts are attractive materials for low-cost enzyme media, though their prices vary depending on many factors. At the same time, agricultural byproducts naturally contain variations in quality from lot to lot that might affect the enzyme yield. Box-Behnken design can be a useful method of finely adjusting the mixing ratio of ingredients to obtain the maximal enzyme yield.

In this study, the proportion of sugi pulp in the medium was set as 5 %. The increase in cellulase productivity was not in direct proportion to the amount of sugi pulp. The cellulase activity was not doubled even if the content of the sugi pulp was doubled in the medium. Because 80 % of sugi pulp used in this study consisted of cellulose (glucan), cellulase, the filter paper-degrading activity was supposed to play an important role for sugi pulp saccharification. In fact, the saccharification yields of sugi pulp improved by increasing the amount of filter paper unit (FPU) per gram of dry sugi pulp (data not shown).

The low estimated costs of solid-state fermentation are due to the use of complex and heterogeneous agricultural wastes as substrates, low-cost technology regarding sterility, and regulatory demands. The estimated production cost of cellulases for in situ solid-state fermentation was very economical, in contrast to that in a stirred tank reactor [21, 22]. Mekala et al. used sugar cane bagasse as the substrate for cellulase production using *T. reesei* RUT C30 with a cellulase inducer and basal mineral salts in the medium. They produced 25.6 FPAase units per gram of dry substrate [11]. Oberoi et al. [23] achieved the highest filter paper

cellulase (FPase) activity of 13.4 IU per gram of dry substrate using *T. reesei* Rut C-30 with wheat bran to supplement the kinnow pulp. Zhai et al. [24] used water hyacinth as the substrate and produced filter paper enzyme activity (FPIU) of 13.4 per gram of dry solid using *T. reesei* SEMCC-3.217, supplemented with corn steep liquor, soybean meal, and some salts. In this study we achieved 8.41 FPase U/ml (3.25 FPU/ml). A clear difference in FPase activity was not observed in the supernatant and whole crushed media (data not shown), thereby resulting in productivity of about 29 FPU per gram of dry substrate. Kovacs et al. [25] reported the superiority of whole enzyme broth using *T. reesei* in the hydrolysis of pretreated spruce as compared with that obtained from using supernatant. In the case of a whole crushed enzyme, the broth contained both extracellular enzymes and enzymes bound to the cell wall. The superiority of the whole crushed crude enzyme could be explained in part by β-glucosidase obtained from *T. reesei* that has been reported as being tightly bound to the cell wall of fungus [26].

The cocktail effect of β-glucosidase obtained from *Aspergillus niger* for the cellulase system obtained from *T. reesei* is widely known to enhance the saccharification yield of biomass resources [27]. *A. tubingensis* is also an excellent β-glucosidase producer [9, 28]. The crude enzyme solution from *A. tubingensis* KRCF 700-33 showed a cocktail effect along with that of *T. reesei*, and the highest cellulase activity (FPU) was obtained with a 25 % cocktail enzyme using optimized culture medium (Fig. 2). Although it is difficult to figure out all enzymes that contribute to the saccharification yield of sugi pulp at present, supplementing β-glucosidase certainly increases the yield.

The cocktail effect was also shown under the SSF process. SSF originally offers the advantage of avoiding end-product inhibition that affects the hydrolysis rate of cellulose, as glucose is consumed by the yeast as soon as it forms [3, 29]. The enzyme system from *A. tubingensis* contributed to decrease the remaining sugars in the reactant after 4 days of fermentation (Fig. 4). An enhanced hydrolysis yield due to supplementing with lignocellulose-degrading enzymes, including β-glucosidase from *A. tubingensis*, resulted in favorable ethanol conversion. Moreover, supplementing with the *A. tubingensis* enzyme system apparently promoted the liquefaction of sugi pulp as related to advanced substrate degradation. Under a high substrate concentration, a complete mixing of the content is difficult, particularly in the initial stage of the fermentation process. Regional accumulation of the end product might delay the total conversion process, thereby making efficient mixing of the reactant is very important to the conversion process. The boosted liquefaction of the reactant by the enzyme system from *A. tubingensis* must be one of the reasons for the effective fermentation of sugi pulp. The crude enzyme of *A. tubingensis* was produced using the medium optimized to *T. reesei* to facilitate the enzyme preparation step in this study. Further optimization of the culture conditions of *A. tubingensis* and a detailed investigation of the cocktail effect of these fungi will lead to an improved ethanol yield of sugi pulp.

The rise of reaction temperature due to the use of thermotolerant yeast also allowed effective conversion under SSF. The main disadvantage of SSF with conventional *S. cerevisiae* strain lies in the difference of optimal temperatures for saccharification by cellulase (around 50 °C) and ethanol fermentation by yeast (around 33 °C). The ethanol yield of sugi pulp under SSF with a conventional *S. cerevisiae* NBRC 2347 at 33 °C remained lower compared with the result obtained with a thermotolerant yeast BA-11 at 40 °C (data not shown). As one example, Stenberg et al. [18] reported the importance of the role played by yeast during SSF *S. cerevisiae* BA-11 was capable of efficiently fermenting the waste molasses with a high salt concentration (6 %) at 40 °C [11]. Effective ethanol conversion can be attributed to the improvement of cellulolytic activity of the cocktail enzyme by prompt removal of the end product, glucose, by thermotolerant yeast BA-11 under SSF. No special nutrients were supplied in the SSF experiments, suggesting that the yeast may take nutrients derived from the reaction mixture during the SSF process.

From 10 % impregnated spruce, Hoyer et al. produced ethanol of more than 4 % (w/v) with an ethanol yield of 95.8 % under SSF [18]. In this study, an ethanol concentration of 4.48 % (w/v) was produced using a cocktail enzyme produced on-site with an ethanol yield of 76 %. The ethanol yield in this study was calculated by Eq. 2, and when the moisture content of the reactant was taken into consideration, the actual ethanol yield should become a little lower. The final ethanol yield from sugi pulp might be slightly increased by increasing the enzyme dosage under SSF. However, the higher yield must be limited as shown in Fig. 5. Rather than simply increasing the amount of enzyme added, the ethanol yield from sugi pulp will be enhanced through the accumulation of steady efforts, such as an improved pretreatment method and enzyme productivity, optimized stirring speed, optimum pH, and the proper amount of yeast—all factors that might affect the final yield.

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