

短報 (Note)

Screening of lignocellulolytic enzyme producers: enzyme system from *Aspergillus tubingensis* for hydrolysis of sugi pulp

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Abstract

Lignocellulolytic enzyme activities from some wood decay fungi were screened for the effective hydrolysis of sugi (*Cryptomeria japonica*) pulp. The cellulolytic fungi were first screened using agar plates containing AZCL-HE-cellulose. In addition to the *Trichoderma* species, *Grammothele fuligo*, *Aspergillus tubingensis*, and *Pycnoporus coccineus* showed greater filter paper-degrading activity under subsequent screening under solid-state fermentation. *A. tubingensis* KRCF 700 produced remarkable amounts of lignocellulolytic enzymes that include endoglucanase, β -glucosidase, mannanase, xylanase, β -xylosidase, and filter paper-degrading activities. Among the tested strains, the highest synergy between cellulase preparation and the crude extract against sugi pulp hydrolysis was achieved when the extract from *A. tubingensis* KRCF 700 was used, followed by *Aspergillus niger* NBRC 31125. The diversity of the lignocellulolytic enzyme system from *A. tubingensis* might lead to the effective synergy with cellulase system from *T. reesei*.

Key words : screening, cellulase, saccharification, solid-state fermentation, *Aspergillus tubingensis*

Introduction

Sugi (*Cryptomeria japonica*) is widespread bioresource that need further promotion for effective use. In our previous research, though sugi wood has higher lignin content and less carbohydrate content compared to eucalyptus wood, both had almost the same hexose content (about 60%). The hexose content of sugi pulp was around 90% after alkaline soda cooking (Ikeda et al., 2007). Alkaline pulping is a widely used pretreatment method to achieve increased enzymatic accessibility (Hendriks • Zeeman, 2009). Since the enzymatic hydrolysis of woody lignocelluloses is gaining increased research attention due to its immense potential for transformation into fermentable sugars, the supply of highly active, inexpensive cellulolytic enzymes is indispensable in developing an economically feasible biotechnical process (Jorgensen et al., 2007; Wyman, 2007).

The well-known cellulolytic fungus *Trichoderma reesei* (*Hypocrea jecorina*) produces a powerful cellulase system mainly composed of cellobiohydrolase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and β -glucosidase (EC 3.2.1.21) with synergism (Woodward, 1991; Beguin

• Aubert, 1994; Baldrian • Valaskova, 2008). However, the amount of β -glucosidase in the *Trichoderma* cellulase system is reported to be lower than that needed for the efficient saccharification of lignocelluloses (Holtzapfel et al., 1990; Xiao et al., 2004). Genome sequencing of *H. jecorina* (*T. reesei*) has revealed that it contains less cell wall-degrading enzymes when compared to some fungal genes (Martinez et al., 2008). Since the saccharification process of lignocelluloses is too complicated to overcome the recalcitrance of substrates, using a cocktail of different cellulolytic enzymes might be an effective way for complete biomass saccharification with reduced amounts of enzyme preparation (Buaban et al., 2010; Andrić et al., 2010). The screening of cellulolytic microorganisms existing in nature remains important from the standpoint of industrial development and biodiversity surveys.

In this study, cell wall-degrading enzyme activities from some wood decay fungi were analyzed for the effective hydrolysis of sugi pulp. The cellulolytic microorganisms were first screened and isolated using agar plates containing AZCL-HE-cellulose. The strains gave larger halos on the plates were further screened under solid state fermentation

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(SSF) for the ability of enzymatic saccharification of alkaline-treated pulp from sugi

Materials and methods

AZCL-HE-cellulose and reduced 1,4- β -D-mannan were obtained from Megazyme. Carboxymethyl cellulose (CMC) sodium salt (low viscosity type) was obtained from Sigma-Aldrich. Soluble xylan was prepared from birchwood xylan (Fluka, Buchs, Switzerland). Sugi pulp was prepared by soda-anthraquinone cooking followed by oxygen bleaching (Ikedad et al., 2007). To determine the relative sugar composition of the pulp, corresponding neutral monosaccharides in the acid hydrolyzates were analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD; Dionex) equipped with a CarboPac PA1 column. 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 sugi pulp was 8.18%. The cellulase preparation Accellerase 1500 was provided by Genencore Co., Ltd. (42 FPU/ml; Filter-paper degrading activity (FPase), 55 U/ml; β -glucosidase activity, 480 U/ml).

Soil samples were collected from different locations in Japan for the isolation of cellulolytic microorganisms. The isolated microorganisms were maintained on PDA plates. From the destructive fungi collection in Japanese edible mushroom industry, maintained in the Kyusyu Research Center of FFPRI (KRCF strains), 26 strains were selected for the screening of cellulolytic activity. From the collection of wood decay fungi, maintained in the microbial ecology laboratory in our institute (WD strains), 79 strains obtained from Japanese forests were selected for the screening. Some isolated fungal strains with high cellulase activity were identified by BLAST (28S rDNA-D1/D2) by Techno Suruga Lab. Co., Ltd. (Shizuoka, Japan).

The plate with the AZCL-HE cellulose consisted of 1.0% CMC, 0.1% polypeptone, 0.3% yeast extract, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% AZCL-HE-cellulose, and 2.0% Agar. Kanamycin 25 mg/L and chloramphenicol 34 mg/L were added to inhibit bacterial growth. Serial dilutions of each soil sample were prepared with sterilized distilled water, and 0.1 ml of diluted solution was spread on the AZCL-HE-cellulose plate, then incubated at 28°C. The isolated colonies were maintained on the PDA plates. As for maintained strains (NBRC, etc.), a 6-mm diameter mycelial mat of the strains grown on the PDA plate was inoculated on the AZCL-HE-cellulose plate to discern cellulolytic

activity.

The solid medium for SSF was composed of 10% rice bran and 90% wheat bran. The moisture content of the medium was adjusted to 70%, and then 10 g of the moisture-adjusted substrate was autoclaved (121°C, 20 min) in a 100 ml Erlenmeyer flask. A 1-cm² mycelial mat grown on a PDA plate for 2-3 weeks was crushed in 1 ml of sterilized water using a multi-beads shocker (Yasui Kikai, Japan). The crushed solution was inoculated in the flask under sterile conditions. The culture flask was placed in the incubator in the dark, at a temperature of 28°C, and at a humidity of 60% for three days. 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. The supernatant after centrifugation was used as a crude enzyme extract.

FPase activity was determined by measuring the increase of reducing sugars from the hydrolysis of filter paper No. 1 (Whatman 1001-813, 2.6×3.1 cm, about 70 mg) using the 3,5-dinitro-salicylic acid (DNS) method (Miller 1959) with glucose as the standard. The reaction mixture consisting of 40 μ l of enzyme extract, 910 μ l of 50 mM sodium citrate buffer (pH 4.8), 550 μ l of distilled water, and one piece of curled filter paper was incubated in a test tube at 50°C for 1 h under continuous shaking. One unit (U) of enzyme activity was defined as the amount of enzymes required to liberate the equivalent of 1 μ mol of reducing sugar per min. Endoglucanase (CMCase), xylanase, and mannanase activity was assayed using CMC, soluble birchwood xylan, and reduced 1,4- β -D-mannan as substrates, respectively. β -Glucosidase and β -xylosidase activity was assayed by measuring the amounts of *p*-nitrophenol liberated from *p*-nitrophenyl- β -D-glucopyranoside and β -D-xylopyranoside, respectively, as described previously (Shimokawa et al., 2007).

Wet sugi pulp was used to measure saccharification yields. The moisture content of the pulp was 66.72%. The reaction mixture consisted of 25 mg (dry weight) of pulp and 100 μ l of the crude extract for sugi pulp hydrolysis in a 1 ml 50 mM sodium citrate buffer pH 4.8. The mixture was incubated at 50°C for 24 h with continuous shaking, and the reaction was stopped by heating the mixture at 100°C for 5 min. The amounts of liberated reducing sugars were measured using the DNS method with glucose as the standard. The saccharification yield was calculated from the weight of polysaccharides in the substrates. The results were expressed as the means of three experiments. In additional experiments with sugi pulp saccharification, cellulase preparation (Accellerase 1500, 10 FPU/g) was added to the reaction mixtures. Degree of synergies were

evaluated by the determining the ratio of reducing sugar levels produced by the combination of the enzymes (crude enzymes containing Accellerase 1500) to that produced by the separate reactions.

Results and discussion

CMC was used as a carbon source for the AZCL-HE plate because it dissolved readily in water and the solution was transparent, which was helpful for the visual assessment of the halo zones. More than 90 fungal or bacterial strains were isolated from the Japanese soil samples using AZCL-HE plates. All the isolated strains were tested for their ability to produce FPase as an index of cellulolytic enzyme activity under SSF. The species of the 12 strains exhibited greater activity were identified by BLAST. They were assigned to be *Hypocrea* (*Trichoderma*), *Aspergillus*, and *Penicillium* species. These species have been recognized as excellent cellulolytic enzyme producers, and many commercial cellulase preparations are made from *T. reesei* and *A. niger* (Cherry • Fidantsef, 2003).

All the 26 strains picked up from the destructive fungi collection in Japanese edible mushroom industry were tested for their FPase activity under SSF. The 17 of the 26 strains belonged to the *Hypocrea* (*Trichoderma*) species: *T. harzianum* (11 strains), *T. atroviride* (2), *T. cf. stramineum*, *T. pseudokoningii*, *T. longibranchiatum*, and *T. virens*. The rest belonged to *P. brebicompectum*, *P. fellutanum*, *P. cf. paneum*, *P. expansum*, *Spicellum roseum*, *Cladobotryum varium*, *Gliocladium viride*, *A. fumigatus*, and *A. tubingensis*. Among the wood-decaying

fungi (WD), 79 different strains were screened on the AZCL-HE plates. The tested WD strains, selected for their rapid growth and potential for wood deterioration, were: *Abortiporus biennis*, *Antrodia multipapillata*, *Antrodia multipapillata*, *Antrodiella semisupina*, *Bjerkandera adusta*, *Bondarzewia montana*, *Castanoporus* (*Cystidiophorus*) *castaneus*, *Ceriporia viridians*, *Ceriporiopsis aurantitinges*, *Cerrena unicolor*, *Climacocystis borealis*, *Climacodon septentrionalis*, *Coniophora puteana*, *Coriolopsis glabrorigens*, *Cyclomyces fusca*, *Daedalea aurora*, *Daedaleopsis conchiformis*, *Datronia stereoides*, *Dichomitus squalens*, *Diplomitoporus lindbladii*, *Echinochaete ruficeps*, *Echinodontium japonicum*, *Fistulina hepatica*, *Fomes fomentarius*, *Fomitopsis palustris*, *F. rosea*, *F. spraguei*, *Ganoderma lucidum*, *Gloeophyllum odorum*, *Gloeoporus dichrous*, *Gloeostereum incarnatum*, *Grammothele fuligo*, *Grifora frondosa*, *Hapalopilus croceus*, *Hericium erinaceum*, *Hexagonia tenuis*, *Hydnochaete tabacinoides*, *Irpex lacteus*, *Ischnoderma resinousum*, *Junghuhnia nitida*, *Laccocephalum hartmanii*, *Lenzites betulina*, *Melanoporia castanea*, *Meripilus giganteus*, *Merulius tremellosus*, *Microporellus nanus*, *Mycocleptodonoides aitchisonii*, *Nigroporus vinosus*, *Oxyporus cuneatus*, *Paratrachaptum acculatum*, *Perenniporia fraxinea*, *Phaeolus schweirii*, *Phanerochaete chrysorhiza*, *Phanerochaete chrysosporium*, *Phellinus igniarius*, *Phlebia strigoso-zonata*, *Physisporinus vitreus*, *Piptoporus betulinus*, *Polyporus arcularius*, *Porodisculus pendulus*, *Protomerulius caryae*, *Pulcherricium caeruleum*, *Pycnoporellus fulgens*, *Pycnoporus cinnabarinus*, *P. coccineus*, *Rigidoporus*

Table 1. Lignocellulolytic enzyme activities in the crude extracts from screened and controlstrains. Mean values \pm SE (n=3) are given.

	Activity (U/ml)						
	FPase		CMCase	β -Gluco sidase	Mannanase	Xylanase	β -Xylo sidase
	3 days	6 days	3days				
<i>Aspergillus tubingensis</i> KRCF 700	0.80 \pm 0.08	0.96 \pm 0.07	1.06 \pm 0.24	1.60 \pm 0.45	4.81 \pm 0.50	5.85 \pm 0.52	3.06 \pm 0.35
<i>Grammothele fuligo</i> WD 844	1.31 \pm 0.28	0.20 \pm 0.03	0.68 \pm 0.07	0.07 \pm 0.03	1.34 \pm 0.84	0.87 \pm 0.46	0
<i>Perenniporia fraxinea</i> WD 1518	0.42 \pm 0.19	0.71 \pm 0.03	1.42 \pm 0.12	0.62 \pm 0.15	0.70 \pm 0.21	5.58 \pm 0.18	0.11 \pm 0.05
<i>Phanerochaete chrysosporium</i> WD 1416	0.67 \pm 0.10	0.86 \pm 0.10	1.24 \pm 0.17	0.76 \pm 0.16	1.15 \pm 0.36	3.89 \pm 1.05 \pm	0.14 \pm 0.02
<i>Pycnoporus coccineus</i> WD 2263	0.93 \pm 0.08	0.27 \pm 0.05	0.95 \pm 0.27	0.79 \pm 0.16	1.18 \pm 0.36	1.64 \pm 0.37	0.01 \pm 0.05
<i>Trichoderma harzianum</i> KRCF 131	0.49 \pm 0.08	0.35 \pm 0.09	1.48 \pm 0.19	0.88 \pm 0.45	2.87 \pm 0.35	5.76 \pm 0.84	0.50 \pm 0.06
<i>Trichoderma</i> sp. Oga-16	1.12 \pm 0.13	1.19 \pm 0.17	1.34 \pm 0.40	1.11 \pm 0.28	2.86 \pm 0.42	7.00 \pm 0.40	0.29 \pm 0.11
<i>Trichoderma</i> sp. Sh-3	0.90 \pm 0.09	0.89 \pm 0.13	1.43 \pm 0.13	0.80 \pm 0.20	2.80 \pm 0.35	5.72 \pm 0.12	0.65 \pm 0.18
<i>Trichoderma</i> sp. Sh-8	0.98 \pm 0.08	0.90 \pm 0.19	1.47 \pm 0.25	1.04 \pm 0.39	2.52 \pm 0.11	5.29 \pm 0.11	0.72 \pm 0.20
<i>Trichoderma</i> sp. Sh-23	0.54 \pm 0.13	0.01 \pm 0.02	0.70 \pm 0.27	0.11 \pm 0.03	1.02 \pm 0.29	3.00 \pm 0.75	0.12 \pm 0.04
<i>Trichoderma reesei</i> NBRC 31329	1.34 \pm 0.15	0.62 \pm 0.13	1.03 \pm 0.03	0.21 \pm 0.12	1.27 \pm 0.22	6.06 \pm 0.50	0.51 \pm 0.14
<i>Aspergillus niger</i> NBRC 31125	0.68 \pm 0.07	0.76 \pm 0.11	0.77 \pm 0.21	2.85 \pm 0.71	2.66 \pm 0.75	5.23 \pm 0.35	1.47 \pm 0.39

cinereus, *Skeletocutis nivea*, *Spongipellis delectans*, *Steccherinum ochraceum*, *Stereum hirsutum*, *Theleporus calcicolor*, *Tinctoporellus epimiltinus*, *Trametes conchifer*, *T. elegans*, *T. versicolor*, *Trechispora mollusca*, *Trichaptum bifforme*, *Tyromyces chioneus*, *Wolfiporia cocos*, and *Xylobolus annosus*. After three days of cultivation at 28°C, 23 strains did not produce visible halos on the plates. Most of the strains did not show good growth on the plates at 28°C, suggesting mismatched growth temperature or a lack of CMC utilization ability as a carbon source. Fifteen strains that showed large halos with vigorous growth on the plates were further screened under SSF. The first-screened WD strains belonged to *A. biennis*, *B. adusta*, *C. aurantingens*, *G. fuligo*, *I. lacteus*, *M. tremellosus*, *M. nanus*, *P. fraxinea*, *P. chrysosporium*, *T. epimiltinus*, *T. conchifer*, and *T. mollusca*. There are few reports about cellulase production by *C. aurantingens*, *G. fuligo*, *T. epimiltinus*, *T. conchifer*, *T. mollusca*, and *M. nanus*.

Among the approximately 200 isolated strains, 10 strains that showed higher FPase activity under SSF were selected for further research (Table 1). The strains named Oga-16, Sh-3, Sh-8, and Sh-23 were screened from soil samples. The amounts of FPase activity after three and six days of cultivation were shown with *T. reesei* NBRC 31329 and *A. niger* NBRC 31125 (Hong et al., 2001) as control strains. *T. reesei* showed the highest FPase activity and *G. fuligo* WD 844 was comparable with that of *T. reesei* NBRC 31329 after three days of cultivation. However, the FPase activity from *G. fuligo* decreased more rapidly during the three to six days. The FPase activity from *P. coccineus* also decreased during the three to six days of cultivation. *A. tubingensis* KRCF 700 showed higher FPase activity than that of *A. niger* NBRC 31125. Although many lignocellulolytic enzymes, including CMCase (Seo et al., 2003) have been reported from *A. tubingensis*, very few studies have reported their filter paper-degrading activity. When KRCF 700 was cultured in a liquid medium containing 0.5% α -cellulose as a carbon source, no remarkable FPase activity was detected though the control strain *T. reesei* NBRC 31329 showed apparent FPase activity during two weeks of cultivation (data not shown). Endoglucanase (CMCase), β -glucosidase, and hemicellulase activities from the selected strains are also summarized in Table 1. Two *Aspergillus* species (NBRC 31125 and KRCF 700) exhibited high β -glucosidase, β -xylosidase, mannanase, and xylanase activity. In particular, KRCF 700 was an excellent mannanase and β -xylosidase producer. Since *G. fuligo*, *P. flaxinea*, and *P. coccineus* are not well-characterized cellulolytic fungi, this study offers considerable information about their cellulolytic enzymes.

Among the tested strains listed in Table 1, both KRCF 700 and NBRC 31125 showed the highest saccharification yields of 12.2%, followed by NBRC 31329 (11.0%), when sugi pulp was hydrolyzed with only the crude extracts (Fig. 1). Hydrolysis of the sugi pulp with commercial cellulase preparation Accellerase 1500 (10 FPU/g) alone resulted in a saccharification yield of 36.5%. The highest synergy between cellulase preparation and the crude extracts on the pulp hydrolyses was achieved when the extract from KRCF 700 were used (degree of synergy was 1.21), followed by NBRC 31125 (degree of synergy was 1.10). The crude extract from *T. reesei* NBRC 31329 could not show the apparent synergy effect with cellulase preparation from *T. reesei* (degree of synergy was 0.98). The enzymatic reaction with Accellerase 1500 and the crude extract from KRCF 700 resulted in a saccharification yield of 58.7% on the hydrolysis of sugi pulp. A number of studies revealed that the enzyme system of *Aspergillus* sp. is excellent for an enzyme cocktail for the efficient cooperative hydrolysis of lignocellulosic biomass, in most cases to offset a shortage of β -glucosidase (Tengborg et al., 2001; Xiao et al., 2004).

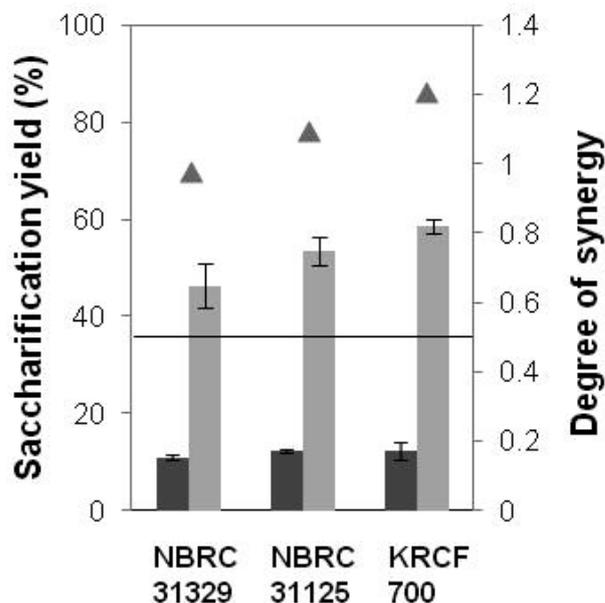


Fig. 1. Hydrolyses of sugi pulp by crude extracts and cocktail enzymes. Saccharification yields of sugi pulp hydrolyzed with the crude extracts alone (black bar) and that hydrolyzed with the cocktail enzymes consisting of crude enzymes and cellulase preparation (gray bar) are shown. Line in the figure represents saccharification yield gained by cellulase preparation alone. Degree of synergies (\blacktriangle) were evaluated by determining the ratio of reducing sugar level produced by the combination of the enzymes to that produced by separate enzymes.

However, the amount of β -glucosidase activity was the highest in the extract from NBRC 31125 in this study. When compared with NBRC 31125, KRCF 700 produced much higher levels of the lignocellulolytic enzymes: FPase, CMCase, mannanase, xylanase, and β -xylosidase. The diversity of the lignocellulose-degrading enzyme system from KRCF 700 might lead to the most effective synergy with cellulase preparation. In addition, Decker et al. (2001) have reported the multiplicity of β -glucosidase from *A. tubingensis* with respect to substrate specificity, glucose inhibition, and acid tolerance, which broaden out a variety of substrates by taking advantage of the synergistic action of the distinct enzymes. Moreover, an on-site-produced crude enzyme from KRCF 700 in this study showed considerable amounts of FPase activity, and to our knowledge this is the first report to show FPase activity from *A. tubingensis*. These findings reinforce the superiority of the enzyme system from *A. tubingensis*. The strain KRCF 700 was a good producer of helpful lignocellulolytic enzymes under SSF and may be used for possible biotechnological applications.

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リグノセルロース分解酵素生産菌のスクリーニング： *Aspergillus tubingensis* の生産する酵素によるスギパルプの分解

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要旨

数種の木材劣化菌について、スギパルプを効率的に分解することを目的としてリグノセルロース分解酵素活性のスクリーニングを行った。1次スクリーニングは、AZCL-HE-セルロースを使用した平板培地で行った。引き続き固体培地においてスクリーニングを行うことでトリコデルマ種、アイアナタケ、アスペルギルス種、ヒイロタケに高い紙分解活性を確認した。*Aspergillus tubingensis* KRCF 700 株は、エンドグルカナーゼ、 β -グルコシダーゼ、マンナーゼ、キシラナーゼ、 β -キシロシダーゼ及びろ紙分解活性の高生産株であった。試験した菌株のうち、市販のトリコデルマ由来セルラーゼ製剤と併用した場合に相乗効果を示すことで最も効率的なスギパルプ分解作用を示したのは *A. tubingensis* KRCF 700 株であり、次が *Aspergillus niger* NBRC 31125 株であった。*A. tubingensis* は多様なリグノセルロース分解酵素を生産しており、その多様性がトリコデルマ由来の酵素製剤との効率的な相乗作用につながったと考えられる。

キーワード：スクリーニング、セルラーゼ、固体培養、アスペルギルス・ツビゲンシス

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