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論　文（Original article）

Effect of gamma-ray irradiation on enzymatic hydrolysis of spent corncob substrates from edible mushroom, enokitake (*Flammulina velutipes*) cultivation

SHIMOKAWA Tomoko[1)*], NAKAMURA Masaya[1)], NAGASAWA Naotsugu[2)], TAMADA Masao[2)] and ISHIHARA Mitsuro[1)]

Abstract

The effect of gamma-ray irradiation on enzymatic hydrolysis of spent enokitake mushroom substrate containing corncob meal and rice bran as major components was studied. Almost all the lignin component remained in the bottle though 34% of the original substrate was consumed. Polysaccharides consisting of glucose and xylose residues were the major components of the spent substrate. The saccharification rate of the spent substrate doubled with irradiation at a dose of 500 kGy, and the irradiated sample formed finer particles than the untreated sample after the same period of grinding. The holocellulose was apparently depolymerized by irradiation, resulted in the fragility of the substrate. An appreciable amount of xylan-derived sugars were extracted with water from the irradiated sample. The enzymatic saccharification rate of the irradiated spent enokitake substrate increased to over 80% with Cellulosin TP 25 from Trichoderma viride, which possesses endo-β-D-xylanase and β-D-xylosidase activities as well as cellulase activities.

Key words : Gamma irradiation, Spent mushroom substrate, *Flammulina velutipes*, Enzymatic hydrolysis, Xylanase, Cellulase

Introduction

Enokitake (*Flammulina velutipes*), a white-rot fungus, is one of the most popular edible mushrooms in Japan. Enokitake mushrooms on the market are generally produced on spawned supplemented substrates in plastic bottle, and in 2003, production reached over 110,000 tons in Japan (Forestry Agency, 2004). The spent substrates used in cultivation amount to several times more than the enokitake products and although they are regarded as lingocellulosic biomass resources, the majority is discharged from mushroom culture facilities instead of being profitably utilized. The development of a technique to hydrolyze lignocellulosic materials into sugars, which are then convertible into bioethanol by fermentation has attracted a great deal of attention from the view point replacing fossil fuels with biomass fuels to preserve the global environment.

Enzymatic hydrolysis has many advantages over acid hydrolysis although it needs physical or chemical pretreatments to destroy the cell wall structure of lignocelluloses where lignin prevents enzymatic accessibility and hydrolysis (Fan et al., 1980; Bisaria and Ghose, 1981). Numerous efforts to develop pretreatment processes to enhance the rates of enzymatic saccharification have been proposed. Of these, mechanical milling has been reported to be an effective pretreatment causing structural modification of cell wall, and consequently increasing enzymatic hydrolysis rates of the substrates (Mandels et al., 1974; Matsumura et al., 1977). However, mechanical milling requires a long operation time and large energy inputs, and is therefore, a costly option (Lynd et al., 1996). Earlier works have reported that gamma-ray irradiation on various lignocellulosic biomasses such as bagasse (Kumakura and Kaetsu, 1983), chaff (Kumakura et al., 1986), rice straw and sawdust (Kumakura and Kaetsu, 1978; Begum et al., 1988; Khan et al., 1986; Bhatt et al., 1992) is effective to improve enzymatic saccharification rate. Gamma-ray irradiation induces cellulose depolymerization (Charlesby, 1955; Lowton et al. 1951) and irradiated materials are ground more easily than untreated samples (Kumakura et al., 1986).

In this study, the effect of gamma-ray irradiation on rates of enzymatic saccharification of the spent corncob enokitake substrates was studied. The irradiated spent substrates were ground and hydrolyzed with commercial enzyme preparations from *Trichoderma viride* and *Aspergillus niger*.

Materials and Methods

Materials and irradiation: The pre-inoculation enokitake substrates and spent substrates were obtained from Nagano Vegetable and Ornamental Crops Experiment Station (Nagano, Japan). The strain of mushroom and substrate used were *F. velutipes* (Garyu No.5) and YK2 medium, respectively. The YK2 medium was composed of 35% corncob meal, 33%

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* Department of Applied Microbiology, Forestry and Forest Products Research Institute (FFPRI), Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan; e-mail: tshimo@ffpri.affrc.go.jp

1) Department of Applied Microbiology, Forestry and Forest Products Research Institute (FFPRI)

2) Japan Atomic Energy Agency

rice bran, 10% beet, 5% cotton seed husk, 5% wheat bran, 5% grain sorghum, 4% fossil shell, and 3% okara (soy bean residues obtained after the extraction of soy milk during tofu preparation). The moisture content of the medium was adjusted to 65% and then 575 g of the moisture-adjusted substrate was packed into culture bottles before sterilization. Fruiting bodies were picked from the culture bottles after 52 days of cultivation. The spent substrates were crushed to pass through a 4-mesh (4.74 mm) screen and then dried at 60˚C until the moisture content of the sample became less than 10%. Xylan from birch wood was obtained from Fluka (Switzerland), and soluble xylan was prepared as described by Dupont et al. (1998). For irradiation, the dried samples were packed in polyethylene bags and irradiated with ^{60}Co gamma rays at a dose of 10 kGy/h giving a total dose of 500 kGy. The cellulase used was a commercial enzyme, Meicelase (Lot No. CEPB-5081, Meiji Seika, Japan) from *T. viride*. The xylanases used were also commercial enzymes, Cellulosin HC 100 (HBI, Japan) from *A. niger* and Cellulosin TP 25 (HBI) from *T. viride*. These commercial enzyme preparations were provided by Meiji Seika Co., Ltd. and HBI Co., Ltd., respectively

Pulverization: The spent substrates were pulverized with a blender (Wonder blender WB-1, Osaka Chemical, Japan) at 25,000 rpm before and after irradiation. The fragility of the samples was evaluated by measuring the powder fraction able to pass through a 100-mesh (150 μm) screen with sieving after grinding. The powder yield (%) indicates the ratio of the powder weight to the sample weight (Kumakura et al., 1986; a 100-mesh screen was employed in this study).

Analytical methods: The dried spent substrates (10 g) were grinded with the blender for 30 sec to give a powder yield of about 50%. Klason lignin was determined according to JIS 8008-1961 after extraction with ethanol-benzene. The acid-soluble lignin content was estimated spectrophotometrically from the absorbance of the hydrolyzate at 205 nm (Swan 1965) and the amount of total sugars in the hydrolyzates was measured using the phenol-sulfuric method (Dubois et al. 1956) with glucose and xylose as standards. To determine the relative neutral sugar composition, samples were hydrolyzed according to Effland (1977). Free neutral monosaccharides were analyzed by high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD; Dionex, CA) equipped with a CarboPac PA1 column (Dionex). The monosaccharides were eluted with 1 mM NaOH containing 0.3 mM acetic acid at 35˚C at a flow rate of 1 ml/min.

Isolation and characterization of the water extract: Spent substrate (0.5 g) with a powder yield of 50% was processed by extraction with 50 ml of hot water (40°C) for 3 h with stirring. The water extracts were then collected by filtration with GP 100 fritted glass crucibles. The total amount of sugar in the concentrated water extracts was measured using the phenol-sulfuric acid method with soluble xylan and glucose as standards. A small portion of the water extract was lyophilized and hydrolyzed with 2 M trifluoroacetic acid to form free glycoses. The sugar compositions of the hydrolyzates were analyzed by HPAEC-PAD. The molecular size distribution of the water extracts was determined by size exclusion chromatography (SEC) using a TSK gel G3000 PWXL column (300 × 7.8 mm i.d., Tosoh, Japan) equilibrated with deionized water at 60°C at a flow rate of 0.5 ml/min. The elution patterns were monitored using a differential refractometer.

Preparation and GPC analysis of holocellulose: The spent substrates were ground to pass through a 42-mesh (355 μm) screen. Chlorite holocellulose was prepared according to the procedure of Browning (1967). The volumes and weights required for in this procedure were scaled in proportion to the mass of the sample used in each preparation. Molecular size distributions of the holocellulose samples were analyzed by SEC of tricarbanilate derivatives (Wood et al., 1986) using two TSK gel GMH_6 columns (300 × 7.5 mm i.d., Tosoh, Japan) connected in series and equilibrated with tetrahydrofuran at 50˚C at a flow rate of 0.5 ml/min. Polystyrene F-128 (Mw: molecular weight 1.26×10^6), F-10 (1.07×10^5), A-2500 (2.8×10^3), and α-cellulose (Sigma, MO) were used as standards.

Enzymatic hydrolysis: The spent substrates were hydrolyzed with a commercial enzyme preparation, Meicelase, as described previously with a slight modification (Sudo et al., 1976). The reaction mixture consisted of 100 mg of sample and 25 mg of enzyme in 5 ml of 0.1 M sodium acetete buffer, pH 4.8. The mixture was incubated at 40˚C for 48 h in a Monod's shaking culture apparatus. The reaction was stopped by heating a portion withdrawn from the reaction mixture at 100˚C for 5 min. The amount of reducing sugars liberated into the reaction mixture was measured according to the method of Somogyi-Nelson (1952) with glucose and xylose as standards. Saccharification rate was calculated based on the weight of polysaccharides in the spent substrate. When Cellulosin HC 100 was dissolved at the same concentration, 25 mg of enzyme in 5 ml of buffer, the xylanase activity was 288 units. Here, one unit of enzyme activity was defined as the amount of enzyme required to liberate the equivalent of 1 μmol of xylose or glucose per min.

Enzyme assays: Exo-β-D-glucanase activity and endo-β-D-glucanase activity were determined by measuring the increase in reducing powers arising from the hydrolysis of filter paper No. 51A (Toyo Roshi, Japan) and sodium carboxymethyl cellulose (low viscosity, Sigma), respectively. Exo-β-D-glucanase reaction mixture consisting of 1 ml of enzyme solution (25 mg/ml) in 0.1 M sodium acetate buffer (pH 4.8), two 1-cm^2 pieces of filter paper (about 17 mg) and 4 ml of buffer was incubated in a L test tube at 40°C for 24 h. The amount of reducing sugars liberated

into the mixture was measured as described above. Endo-β-D-glucanase reaction mixture consisting of 50 µl of enzyme solution, 0.25 ml of 0.4% sodium carboxymethyl cellulose, and 0.2 ml of buffer was incubated at 40°C for 30 min; the reaction was stopped by heating at 100°C for 5 min. β-D-Glucosidase activity was assayed by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl-β-D-glucopyranoside. The assay mixture consisting of 50 µl of enzyme solution, 0.25 ml of 2 mM *p*-nitrophenyl-β-D-glucopyranoside and 0.2 ml of buffer was incubated at 40°C for 10 min; the reaction was stopped by adding 0.5 ml of 0.2 M sodium carbonate. Absorbance at 408 nm was measured. The Meicelase and Cellulosin TP 25 contained no appreciable amount of reducing sugars according to the method of Somogyi-Nelson. Since the Cellulosin HC 100 contained a detectable amount of sugars in the enzyme preparation, the sugars were filtered off with a biomax 5,000 ultrafiltration membrane (Millipore, MA) before use.

Enzymatic hydrolysis of enokitake mycelium: Enokitake mycelium was grown on potato dextrose agar medium (Difco, MI). The mycelium, along with the medium, was then collected and boiled in hot water three times for 5 min each to dissolve the agar and then collected by filtration with No. 2 filter paper (Toyo Roshi). The collected mycelium was suspended in water and disrupted by a blender, then lyophilized. The lyophilized sample was hydrolyzed with Meicellase, Cellulosein HC 100 and Cellulosein TP 25 as described above except that the amount of sample contained in the reaction mixture was 50 mg. The sugar composition of the hydrolyzates was analyzed by HPAEC-PAD.

Results and Discussion

Changes in the composition of the enokitake substrate

When the fruiting bodies of the enokitake mushroom were harvested after 52 days of cultivation, 34% of the original substrate was consumed. As shown in Fig. 1, almost all the lignin component in the substrate remained in the bottle during cultivation; therefore, the Klason lignin content of the spent substrate increased compared to that in the original substrate. Accumulation of lignin in the spent enokitake substrate has also been observed with sawdust as the main substrate ingredient (Masuno et al. 2000). The amounts of ethanol-benzene extract and carbohydrates, on the other hand, decreased remarkably in the spent substrate.

Effects of pulverization and irradiation on enzymatic hydrolysis

Pulverization pretreatment increased the enzymatic susceptibility of the spent enokitake substrate. The saccharification rate after 24 of incubation increased from 5 to 24% with 50 sec of grinding (data not shown). Thus the extent of enzymatic hydrolysis is markedly affected by the particle size of the substrate. The average saccharification rate of the irradiated samples was nearly 50% after 50 sec of grinding, which is about 2.0 times higher than that of the untreated samples (Fig. 2). In contrast to the untreated samples, irradiated samples showed much higher susceptibility against the cellulase preparation. Substrate particle sizes were evaluated as powder yields and the relationship between powder yield and operation time is shown in Fig. 3. The spent enokitake substrate before grinding showed a powder yield of 4%. The powder yields of both irradiated and untreated samples increased with grinding. After 28.1 sec of grinding, nearly half the untreated sample passed through the 100-mesh screen, indicating a powder yield of approximately 50%. The irradiated sample reached a 50% powder yield after 17.8 sec of grinding, indicating that the untreated sample required a 1.6 -times longer operation time to reach the same powder yield as the irradiated sample. These facts show that the irradiated spent substrates were ground

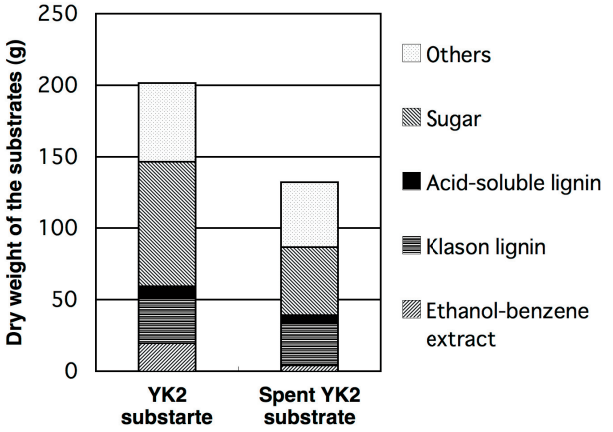

Fig. 1. Changes in the composition of the enokitake substrate during cultivation.

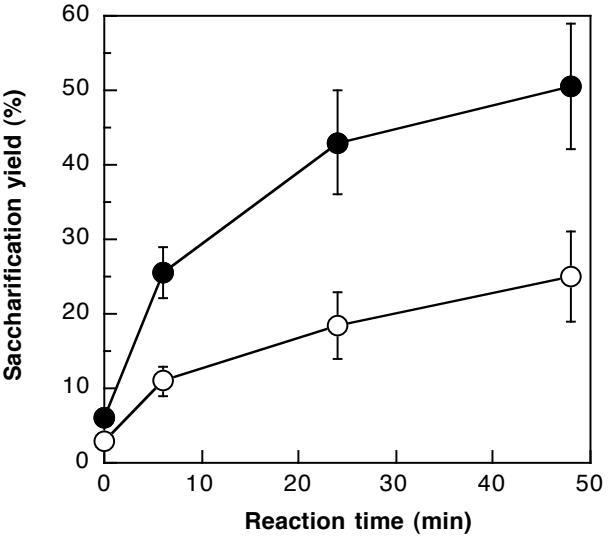

Fig. 2. The saccharification rates of the spent enokitake substrate before (○) and after (●) irradiation. The samples were ground for 50 sec before enzymatic hydrolysis. Mean values ± SE (n = 4) are given.

more easily than the untreated samples, as previously reported with sawdust, chaff and paper (Kumakura and Kaetsu 1982; Kumakura et al., 1986), and consequently the irradiated samples formed more fine particles than untreated samples after the same period of pulverization. Next, the irradiated and untreated samples with almost the same powder yields (50%) were comparatively hydrolyzed with the cellulase preparation. The saccharification rate of the irradiated sample was 45% and that of the untreated sample 22%. The irradiated sample was much more susceptible to the enzyme reaction than the untreated one beyond the effect of particle size.

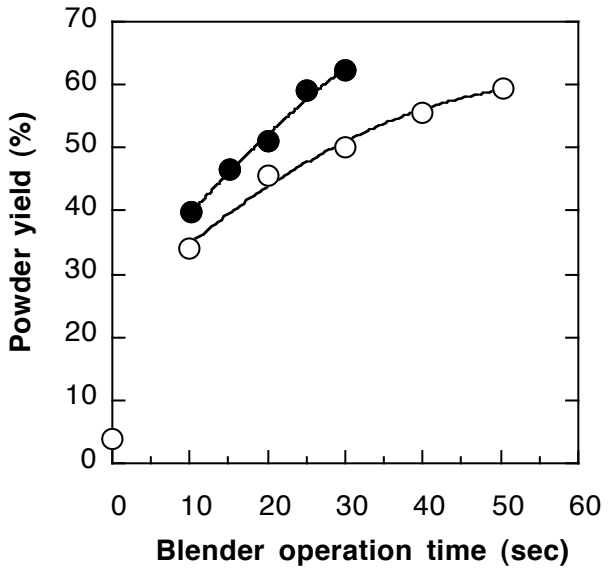

Fig. 3. Effects of grinding time on the powder yield of the spent enokitake substrate before (○) and after (●) irradiation. Results are expressed as the means of two independent experiments.

Effect of irradiation on the spent enokitake substrate

Changes in molecular size distributions of chlorite holocelluloses prepared from original, spent, and irradiated spent substrates were analyzed by SEC (Fig. 4). Although there was no significant effect of cultivation on holocellulose depolymerization, the peak of holocellulose from the irradiated spent substrate was shifted to the side of lower molecular size. The depolymerization of holocellulose in the spent substrate on irradiation might have caused deterioration in the strength of the sample, since cellulose plays an important role in maintaining the strength of wood and plant bodies.

The depolymerization of holocellulose is also considered to affect the solubility against water. In fact, amount of the water extracts of the irradiated sample was higher than that of the untreated sample. The water-soluble portion of the untreated and irradiated samples was 17.2 and 22.3%, respectively, and the sugar content in these water extracts was 24.8 and 37.2%, respectively. The water-soluble portion and sugar content of the water extract therefore increased with irradiation. The percentage of water-dissolved component in bagasse has reported to be increased with irradiation dose; however, this

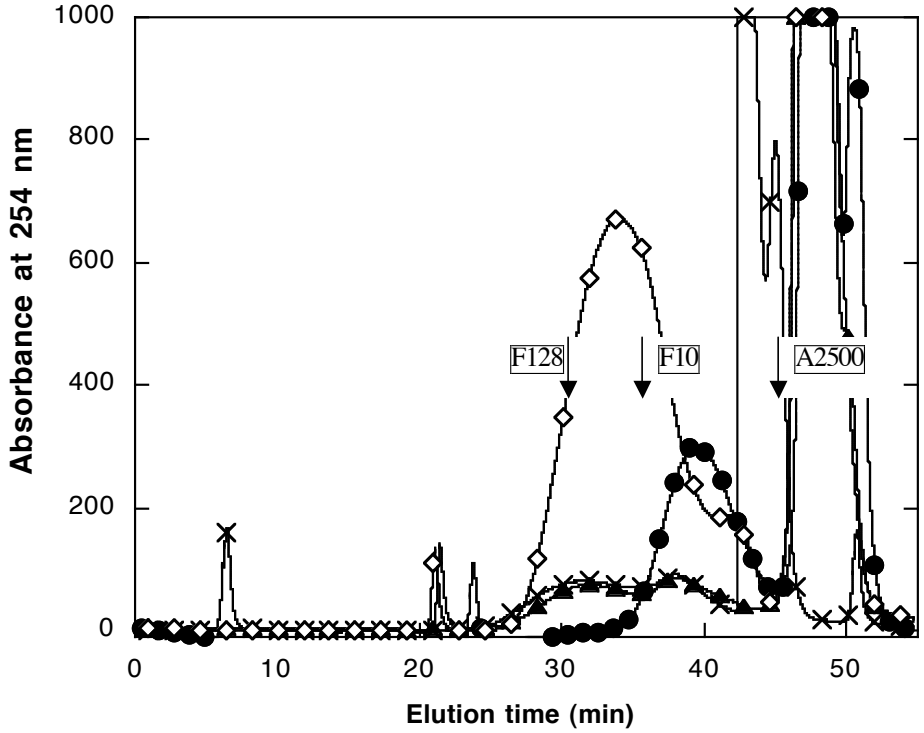

Fig. 4. Changes in the molecular size distributions of tricarbanilated holocelluloses prepared from original YK2 substrate (×), spent enokitake substrate (▲), irradiated spent enokitake substrate (●), and α-cellulose as standard (◇). Allows indicate the elution times of polystyrene standards.

Table 1. Relative sugar compositions of water extracts from untreated and irradiated samples

Extract	Glycosyl residue (mol%)				
	L-arabinose	D-xylose	D-glucose	D-galactose	D-mannose
Untreated	16.5	23.0	36.4	14.3	9.8
Irradiated	11.4	54.7	19.7	7.2	7.0

Table 2. Enzyme activities in the reaction mixtures. Enzyme prepareations used in this study were Meicelase (M), Cellulosein HC 100 (H), and Cellulosin TP 25 (T). MH and MT represent a combined enzyme preparations.

	Activity (unit/5 ml)				
	M	MH	H	MT	T
exo-β-D-glucanase	$4.93x10^{-2}$	$2.49x10^{-2}$	–	$3.77x10^{-2}$	$2.62x10^{-2}$
exo-β-D-glucanase	46.5	23.3	–	29.8	13.0
β-D-glucosidase	21.8	11.6	1.5	14.3	6.9
endo-β-D-xylanase	27	158	288	158	288
β-D-xylosidase	–	–	–	$1.11x10^{-1}$	$2.21x10^{-1}$

–: not detected

water-soluble component was not fully characterized (Kumakura and Kaetsu 1983).

The molecular size distribution of the water extracts were analyzed by SEC. Generation of oligosaccharides such as cellobiose and cellotriose by irradiation was limited (data not shown). The relative sugar compositions of the water extracts are shown in Table 1. Glucose was the major sugar component of the water extract from the untreated sample, while xylose was the main sugar in that from the irradiated sample. Xylose was not found to be present as a free monosaccharide in the water extract of the irradiated sample. These results suggest that irradiation treatment increases the water solubility of xylan from depolymerization of holocellulose. As described above, the saccharification rates of the irradiated and untreated samples were different in the same powder yield; this difference could be explained by the increased amount of water-soluble polysaccharide, especially xylan, during gamma-ray irradiation.

Effects of xylanase and xylosidase activities on saccharification rate

In addition to cellulase, the cellulase preparation Meicelase used in this study contained various glycosyl hydrolases such as xylanase (Sudo et al., 1976). It exhibited an endo-β-D-xylanase activity of 1.1 μmol xylose/mg min but no β-D-xylosidase activity when the enzyme assays were performed with soluble xylan and *p*-nitrophenyl-β-D-xylopyranoside as substrates, respectively. The effects of xylan-degrading activity on the saccharification rate of the spent enokitake substrate were examined using two xylanase preparations, Cellulosin HC 100 and Cellulosin TP 25, respectively. Since both xylanase preparations show not only xylanase activity but also cellulase activities, the enzyme activities related to cellulose and xylan hydrolysis in the two xylanase preparations as well as in the cellulase preparation and in mixtures of each were assayed; the results are shown in Table 2. Both of xylanase preparations were prepared to show the same endo-β-D-xylanase activities. Cellulosin HC 100 showed a β-D-glucosidase activity as well as an endo-β-D-xylanase activity but no exo- or endo-β-D-glucanase activity. Cellulosin TP 25 showed a specifically remarkable β-D-xylosidase activity. The highest saccharification rate was obtained when the irradiated sample was hydrolyzed with Meicellase and Cellulosin TP 25 (Fig. 5), which increased the saccharification rate up to 86%. Cellulosin HC 100, which was free of exo-β-D-glucanase and endo-β-D-glucanase activities could not hydrolyze the spent substrate more than

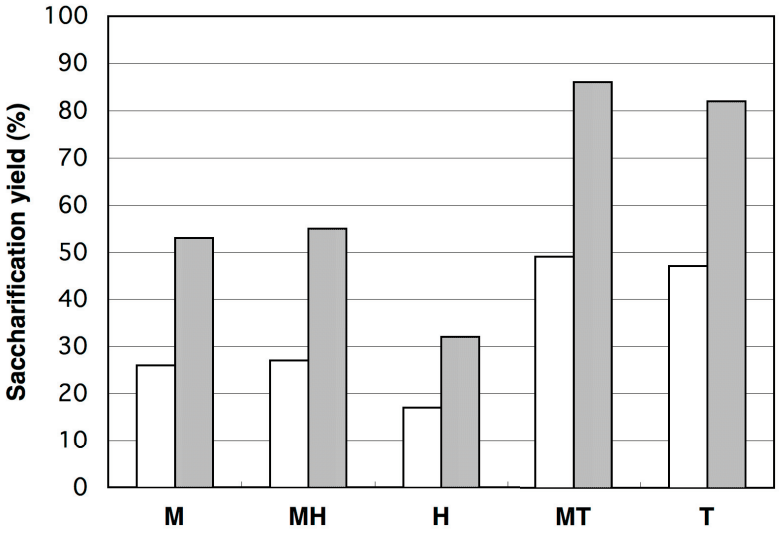

Fig. 5. The saccharification rates of the spent enokitake substrates before (white bar) and after (black bar) irradiation. Samples were hydrolyzed with Meicelase (M), Meicelase and Cellulosin HC 100 (MH), Cellulosin HC 100 (H), Meicelase and Cellulosin TP 25 (MT), and Cellulosin TP 25 (T). Results are expressed as the means of two independent experiments.

Table 3. The relative sugar composition of the spent enokitake substrate and monosaccharide composition of the enzymatic hydrolyzates from irradiated samples. Samples were hydlolyzed with Meicelase (M), Cellulosin HC 100 (H) , and Cellulosein TP 25 (T).

	Glycosyl residue (mol%)				
	L-arabinose	D-xylose	D-glucose	D-galactose	D-mannose
Spent substrate	12.72	37.43	39.81	6.40	3.64
M	6.92	31.25	59.65	1.11	1.07
H	7.75	35.14	53.88	1.86	1.37
T	2.84	49.55	45.21	0.73	1.66

32% even though the sample was irradiated. When the enzyme preparation Cellulosin HC 100 was combined with Meicellase, the saccharification rate of the irradiated substrate was 55%, which was slightly higher than that with Meicellace alone.

The spent substrate contained fungal mycelium to some extent as in the original substrate. The mycelium was enzymatically digested with Meicellase, Cellulosin HC 100 and Cellulosin TP 25. Though the rates of degradation depended on the enzyme preparations, glucose was detected in the hydrolyzates (data not shown), and therefore, at least part of the mycelium in the spent substrates were thought to have been saccharified by the enzyme preparations during the reaction periods.

The relative sugar composition of the spent enokitake substrate and monosaccharide composition of the enzymatic hydrolyzates from the irradiated spent substrates were analyzed by HPLC-PAD (Table 3). In the acid hydrolyzate of the spent substrate, glucose and xylose were detected as the major ingredients at a ratio of 1:1. Among the analyzed samples, the enzymatic hydrolyzate with Cellulosin TP 25 was composed of the highest content of xylose, and the value was close to the content of glucose, reflecting the sugar composition of the spent enokitake substrate (Table 3). Glucose was the main component in the enzymatic hydrolyzates of both Meicellase and Cellulosin HC 100, which were not efficiently convert xylan to xylose. A third of the YK2 medium consisted of corncob meal. Not only a large amount of xylan present in corncobs (Whistler and Tu 1952) but other components in the YK2 medium also contain a considerable amount of xylan; therefore, half of the constituted neutral monosaccharide in the spent substrate was xylose. β-D-Xylosidase activity seems to contribute to increased saccharification rate. Its role in xylan saccharification is postulated to be analogous to the role of β-D-glucosidase in saccharification of cellulose by cellulases (Dekker 1983).

Recent progress in technology has made it possible to coferment glucose and xylose (Ho et al., 1998; Walfridsson et al., 1997; Ingram and Doran 1995) to produce bioethanol. In the enzymatic saccharification process required to supply fermentable sugars, pretreatment of the lignocellulosic materials and selection of enzymes are important factors. The findings of this study broaden the understandings of using gamma-ray irradiation as a pretreatment and support the importance of xylan degrading enzymes in the enzymatic hydrolysis of lignocellulosic material containing xylan.

Acknowledgments

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エノキタケ廃菌床の酵素分解におけるガンマ線照射の影響

下川 知子[1)*]、中村 雅哉[1)]、長澤 尚胤[2)]、玉田 正男[2)]、石原 光朗[1)]

要 旨

コーンコブ、米ぬかを主原料とした菌床栽培により生じるエノキタケ廃菌床の、酵素分解処理におけるガンマ線照射の影響について検討を行った。菌床は培養過程で 34% 減少していたが、リグニンは残存していた。廃菌床の主な糖組成はグルコースとキシロースであった。500 キログレイのガンマ線照射処理により廃菌床の酵素糖化率はほぼ倍になり、一定時間のブレンダー処理によって生じる細粒子の量も増加した。照射処理によって廃菌床中のホロセルロースは明らかに低分子化しており、試料がもろくなった原因と考えられた。また、照射によって、キシラン由来の糖成分の水溶解性が高まった。セルラーゼの他に、キシラナーゼ、キシロシダーゼが含まれているトリコデルマ由来の酵素製剤セルロシン TP25 を用いることで、酵素糖化率は 80%にまで上昇した。

キーワード：ガンマ線照射、廃菌床、エノキタケ、酵素分解、キシラナーゼ、セルラーゼ

＊森林総合研究所きのこ・微生物研究領域 〒 305-8687 つくば市松の里 1　e-mail:tshimo@ffpri.affrc.go.jp
1) 森林総合研究所きのこ・微生物研究領域
2) 日本原子力研究開発機構