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Isolation and Characteristics of *Trichoderma harzianum* FJ1 Producing Cellulases and Xylanase

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Abstract Strain FJ1, a filamentous fungus isolated from rotten wood, showed high ability to hydrolyze cellulosic materials. To identify the strain FJ1, ITS sequencing analysis and morphological observation were performed. The strain FJ1 was identified as *Trichoderma harzianum*. The strain produced a large amount of CMCase, xylanase, β-glucosidase, and avicelase. Optimal culture conditions for the production of the enzymes, such as pH, temperature, and inoculation concentration, were initial pH 6.0–7.0, 25–30°C, and 10^4 ea-spores/ml in Mandel's medium, respectively. *T. harzianum* FJ1 utilized various cellulosic materials and organic nitrogen sources to produce cellulases and xylanase, and also considerably a crystalline and/or insoluble material like Avicel and rice straw. The highest levels of CMCase and xylanase were 41.2 and 65.6 U/ml in 7 days of cultivation using 2.5% of carbon source (Avicel+CMC) and 0.5% of nitrogen source (peptone), respectively.

Key words: *Trichoderma harzianum*, cellulase, CMCase, xylanase

Enormous amounts of agricultural, industrial, and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes. This has become a significant problem in the ecology and environment. Therefore, it has become of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon sources [2, 3, 46]. Cellulosic materials consisting of cellulose and hemicellulose are the most abundant renewable carbon sources which are converted to glucose and xylose by enzymatic hydrolysis [2, 19, 23, 32]. As a hydrolysis method, bioconversion technologies of cellulosic materials were largely studied by the use of filamentous fungi, such as *Trichoderma* sp. [1, 21, 38, 50], *Aspergillus* sp. [9, 20, 49], and *Penicillium* sp. [8, 51] with the effective cellulolytic enzyme system. The complete enzymatic hydrolysis of cellulosic materials needs three different types of cellulases: endoglucanase (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4) [48], exocellobiohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74) [5, 48], and β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) [7, 48]. The endoglucanase randomly hydrolyzes the β-1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by β-glucosidase [3].

Enzymatic hydrolysis processing of cellulosic materials could be accomplished through a complex reaction of these various enzymes. The significant points of these enzyme-based bioconversion technologies are the reaction condition and the production cost of the related enzyme system. Therefore, there has been much research aimed at obtaining new microorganisms producing cellulolytic enzymes with higher specific activities and greater efficiency [17, 25, 33, 41, 43]. The objectives of this study were to isolate a vigorous microorganism with high specific activities and to determine the optimal conditions for producing the enzymes.

MATERIALS AND METHODS

Isolation of Microorganism

To isolate a microorganism producing cellulases and xylanase, a piece of rotten wood was used as a screening source. The rotten wood was crushed and suspended with sterilized water, and then the suspension was cultivated in Mandel's medium [27] with sawdust (1.0%) instead of Avicel and CMC at 30°C for 5 days. The Mandel's medium contained Avicel 5.0 g, CMC (Carboxymethylcellulose sodium salt) 5.0 g, Urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $FeSO_4{\cdot}7H_2O$ 0.3 g, $CaCl_2$ 0.3 g, $FeSO_4{\cdot}7H_2O$ 5.0 mg, $MnSO_4{\cdot}7H_2O$ 1.5 mg, $ZnSO_4{\cdot}7H_2O$ 1.4 mg, and $CoCl_2$ 2.0 mg per 1.0 liter of distilled water. All fungi isolated from the

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culture broth were subcultured 3 times. Finally, isolates that efficiently degraded the sawdust were isolated on yeast malt extract agar (YMEA) medium, and grown in Mandel's medium to measure the enzyme activities. A filamentous microorganism, strain FJ1, which showed better growth and higher degradation ability in cellulosic material, was selected and used for further experiments. The strain was cultivated on YMEA medium at 30°C for 3 days, and then subcultured once a month and stored at 4°C. The YMEA medium contained yeast extract 4.0 g, malt extract 10.0 g, glucose 4.0 g, and agar 20.0 g, per 1.0 liter of distilled water.

Identification of Strain FJ1

To identify the strain FJ1, ITS (Internal transcribed spacer) sequencing analysis and morphological observation were performed. Morphological observations were made from cultures grown on YMEA medium at 30°C. The morphologic characteristics by SEM (scanning electron micrograph) were observed for the more complex conidiophores developing from the characteristic tufted or postulated areas of conidiation, usually 3 days after inoculation.

ITS sequencing analysis was carried out according to the methods of Hermosa *et al.* [14]. The determined sequences were aligned, and the similarity (%) was computed through comparison with other strain. The evolutionary distance of these were calculated by the Jukes and Cantor model [18], and a phylogenetic tree was drawn by Neighbor-Joining method [36].

Biomass Determination

Fungal biomass was indirectly estimated by mycelial protein content, a modified method of Marcel *et al.* [28]. The pellets of the culture broth were washed with distilled water and collected by centrifugation (10,000 ×g, 10 min), and then the pellets were resuspended in distilled water and sonicated (Sonics and Material Inc., Model VC 70, U.S.A.) at 70 watts for 30 min. The protein content in the supernatant of the sonicated solution was measured by the method of Lowry *et al.* [26]. The dry cell weight was determined by the correlation with the absorbance of the protein concentration (Y [g/l]=3.91·X [Abs.]+0.06, R^2: 0.98).

Enzyme Assay

The supernatant was separated from the culture broth by centrifugation (10,000 ×g, 10 min) and filtered through a 0.45 µm cellulose acetate membrane filter (Advantec Inc., MFS, Japan). The filtrate was used to measure enzyme activity. CMCase, β-glucosidase, and Avicelase activity were assayed according to Thomas and Bhat [47] and xylanase activity was assayed according to a modified method of Gawande and Kamat [13]. One unit of CMCase and xylanase activity was defined as the amount of enzyme required to release 1 µmol of glucose and xylose per min, respectively. One unit of β-glucosidase and Avicelase was defined as µmol of *p*-nitrophenol and cellobiose released per ml·min, respectively [47]. Reducing sugar was measured according to the DNS method [31] and the Somogyi-Nelson method [40].

Cultivations of Strain FJ1

Strain FJ1 cells grown on YMEA medium at 30°C for 3 days were collected, suspended in sterilized water, and then filtered through gauze, folded 5 times, to remove the mycelial debris. The filtrate was prepared as a spore suspension that was used for the inoculum of the liquid culture. The liquid cultivations were performed in 500-ml flasks containing 50 ml of Mandel's medium. The production of enzymes in various culture conditions such as pH, temperature, and inoculation concentration were examined. The initial pH in the culture medium ranged from 3.0 to 10.0, cultivation temperatures were from 25 to 50°C, and inoculation concentration of the spore suspension varied from 10^3 to 10^7 ea-spores/ml.

Effects of Carbon Sources on Enzyme Production

The enzyme activities were investigated in liquid culture using various commercial cellulosic and lignocellulosic materials as carbon sources in Mandel's medium. The commercial cellulosic materials employed were Avicel, CMC, xylan, cellobiose, and α-cellulose at a final concentration of 1% (w/v) instead of Avicel and CMC. For the use of lignocellulosic materials as carbon sources, rice straw, sawdust, pulp, and mixtures of these were employed under the same conditions. To enhance the production of enzymes, the effects of concentrations of the carbon source, using Avicel and CMC, were investigated in the Mandel's medium. The concentrations of carbon source ranged from 1.0 to 5.0%. The composition ratios of Avicel and CMC with each concentration of 2.5% were examined under the conditions of 5:1, 2.5:1, 1:1, 1:2.5, and 1:5.

Effects of Nitrogen Sources on Enzyme Production

The enzyme activities were investigated in the culture using various organic nitrogens. The nitrogen sources, such as bactopeptone (Difco, U.S.A.), beef extract (Difco, U.S.A.), malt extract (Merck, Germany), peptone from casein (Merck, Germany), peptone G (Acumedia, U.S.A.), peptone from meat (Merck, Germany), soybean flour (Sigma, U.S.A.), and yeast extract (Merck, Germany), were added to the Mandel's medium at final concentration of 0.1% (w/v). In addition, an optimal nitrogen concentration was examined in the range from 0.0 to 1.0% (w/v).

RESULTS AND DISCUSSIONS

Isolation and Identification of Strain FJ1

Six strains isolated from rotten wood showed some enzyme activities. Among the 6 strains, a strain FJ1 with the

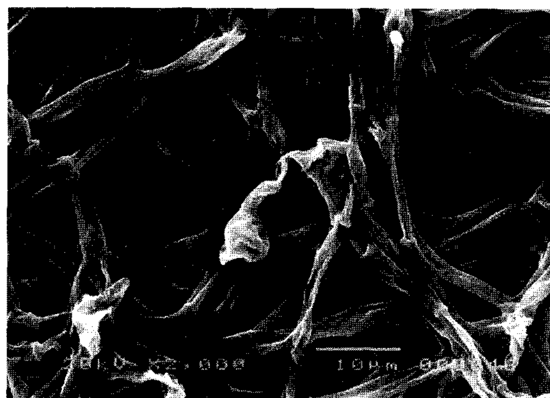

Fig. 1. Morphological appearance of strain FJ1 by SEM observation.

highest activity was selected as a candidate. To identify the strain FJ1, ITS sequencing analysis and morphological observation were performed. Morphologic construction of the strain FJ1 by SEM is shown in Fig. 1. The strain grew rapidly, attaining a diameter of 9 cm in 3 days of growth on YMEA medium. The colonial observation showed that conidiation with flat pustule entirely effused near the margin of the plate, and the bright white mycelium spread rapidly to dull green conidia, and reverse of the plate was colorless.

Results from ITS sequencing analysis indicated that the size of sequence was 597 bp, and similarities (%) to *T. inhamatum* and *T. harzianum* were 97.9% and 97.4%, respectively. The phylogenetic tree of strain FJ1 inferred by ITS sequencing analysis is shown in Fig. 2. The strain FJ1 was morphologically very similar to the *T. harzianum*, and its ITS sequences revealed that it was close in proximity to *T. inhamatum*. Finally, we named it as *T. harzianum* FJ1, and the genetic data were registered in GenBank (AF509962). *T. harzianum* species have been investigated as biological control agents (BCAs) [14] and melanin synthesis inhibitor [24], but the production of cellulolytic enzymes has never been studied.

The enzyme activities of *T. harzianum* FJ1 cultivated in the Mandel's medium were measured using the supernatant of the culture broth. The results are shown in Table 1. The enzyme activities were compared with those of *Trichoderma reesei* KCTC 6952, well known as a cellulase producer. The cellulase activities of FJ1 were similar to those of *T. reesei* KCTC 6952 under the same culture condition using the Mandel's medium. Changes of growth and enzyme activities of *T. harzianum* FJ1 in the shaken liquid culture are shown in Fig. 3. Strain FJ1 was exponentially grown in 2 days and in the secondary metabolism phase from the third day, when the cellulolytic enzymes appeared considerably, suggesting that the relation between the growth phase and the enzyme production phase is similar to a type III bioprocess [44]. The maximum activities of cellulolytic enzymes, CMCase, β-glucosidase, and Avicelase, were found after 5 days of incubation, and xylanase production peaked after 4 days, whereas *T. reesei* KCTC 6952 reached maximum activity after 7 days of incubation. This indicates that *T. harzianum* FJ1 has advantages over *T. reesei* KCTC 6952 in growth rate and xylanase production (Table 1 and Fig. 3).

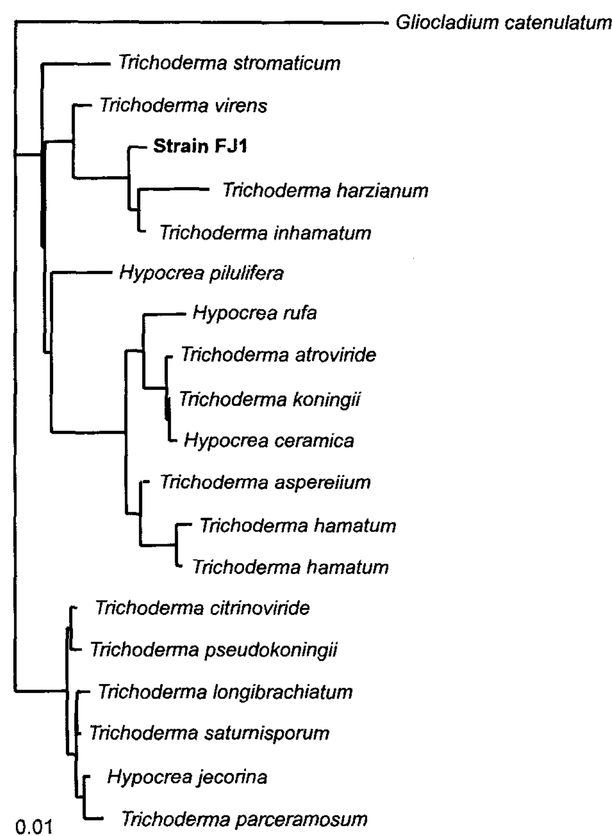

Fig. 2. Phylogenetic tree of strain FJ1 from analysis of ITS sequences.

Table 1. Comparison of the cellulases and xylanase activity of strain FJ1 and *Trichoderma reesei*.

	Cultivation time (in days)	Enzyme activities (U/ml)			
		CMCase	Xylanase	Avicelase	β-Glucosidase
FJ1[a]	5	9.07	12.56	0.21	0.54
T. reesei[ab]	7	8.60	9.95	0.43	0.48

[a]The cultivation of the strains were performed on Mandel's medium under the same condition s.

[b]*T. reesei* (KCTC 6952) was derived from the Korean Collection for Type Culture.

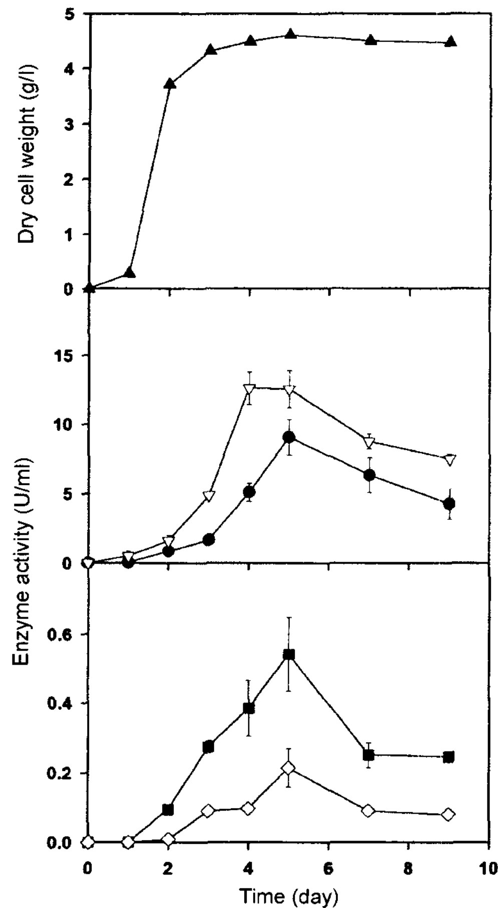

Fig. 3. Changes of dry cell weight and cellulolytic enzyme activities in the liquid culture of *T. harzianum* FJ1.
The bars show standard deviations. Symbols: dry cell weight (▲), CMCase (●), Xylanase (▽), β-glucosidase (■), Avicelase (◇).

Effect of Cultivation Factors

Effects of initial pH, temperature, and inoculation concentration of spore suspension on growth and enzyme activities in liquid culture were examined. Figure 4 shows the effect of inoculation concentrations on the production of enzymes. The highest enzyme activities appeared with inoculation of 10^4 ea-spores per liter in which the enzyme activities of CMCase, xylanase, β-glucosidase, and Avicelase were 9.41, 14.40, 0.71, and 0.24 U/ml, respectively. Jeenes *et al.* [15] showed that the inhibitory materials contained in the spore suspension repressed growth and enzyme production when high concentration of inoculums was added to liquid culture. Kang *et al.* [20] also reported the suitable concentration of inoculums size for the enzyme production.

The effect of the initial pH of the culture medium was examined. The relative enzyme activities after 5 days of incubation at 30°C are shown in Fig. 5. The optimum pH for the production of enzymes was found to be pH 6.0–7.0, and no enzyme activity appeared at above pH 8. CMCase and xylanase activities of the strain FJ1 were 9.3

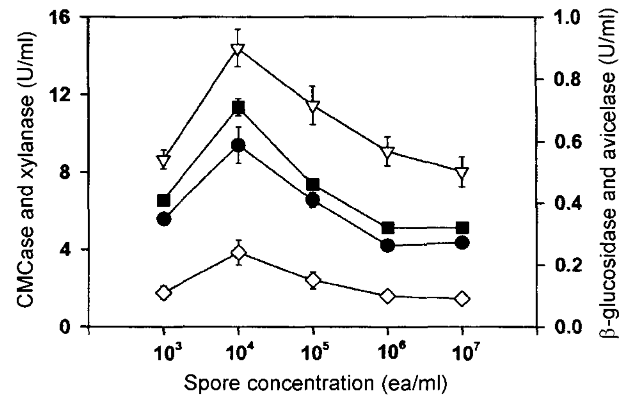

Fig. 4. Comparisons of enzyme activities in various inoculation concentrations of spore suspension.
The bars showed standard deviations. Symbols: CMCase (●), Xylanase (▽), β-glucosidase (■), Avicelase (◇).

and 13.3 U/ml at pH 6–7, respectively. As shown in Fig. 6, the optimum incubation temperature for the production of enzymes by *T. harzianum* FJ1 was observed at 25–30°C, but above 40°C, no growth or enzyme activity appeared.

Effect of Carbon Sources

The effects of carbon sources on production of extracellular enzymes were investigated using modified Mandel's medium. The medium was supplemented with 10.0 g/l of the commercial cellulosic or lignocellulosic materials as the carbon sources. A control medium contained no carbon source. The enzyme activities measured in the culture are shown in Figs. 7 and 8. CMC and cellobiose as soluble cellulosic materials and xylan (oat spelt, birchwood), α-cellulose, and Avicel as insoluble cellulosic materials were tested as inducers of the enzyme system in the strain FJ1 culture, and also lignocellulosic materials, such as rice straw, sawdust, and pulp with below 0.84 mm (20 mesh) particle size were used.

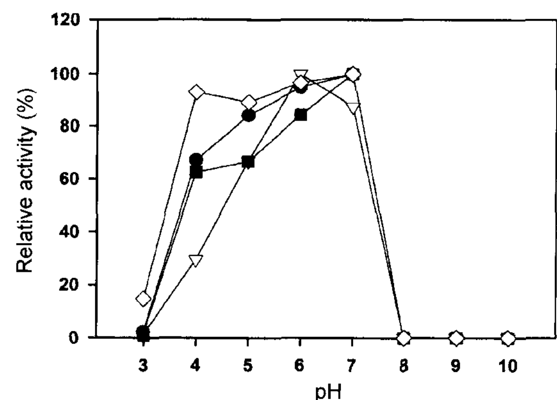

Fig. 5. Comparisons of relative enzyme activities in various initial pHs of culture medium.
Symbols: CMCase (●), Xylanase (▽), β-glucosidase (■), Avicelase (◇).

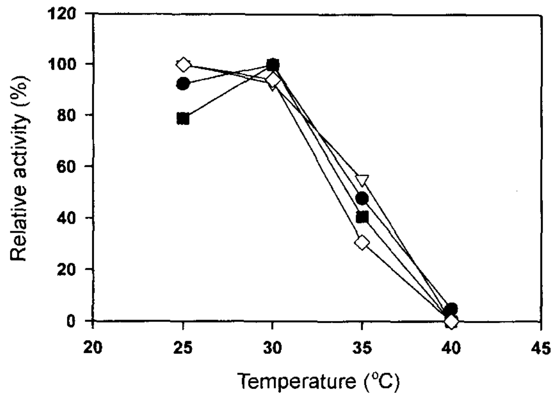

Fig. 6. Comparisons of relative enzyme activity in various cultivation temperatures (Cultivation conditon; static state culture, 7 days).
Symbols: CMCase (●), Xylanase (▽), β-glucosidase (■), Avicelase (◇).

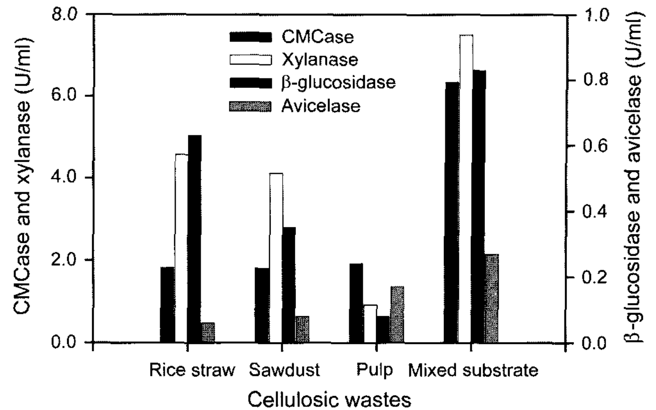

Fig. 8. Comparisons of enzyme activities in the cultures using various lignocellulosic materials (1.0%) in Mandel's medium instead of Avicel and CMC.

Growth was observed with all the commercial carbon substrates, although the enzyme activity appeared to be different in each substrate, as shown in Fig. 7. Cellobiose and CMC showed lower levels of enzyme activities than those of insoluble materials, such as α-cellulose and Avicel. The high enzyme activities were obtained when the strain was grown in the presence of crystalline cellulose, such as α-cellulose and Avicel, rather than hemicellulose, such as xylan of birchwood and oat spelt. That is, the production of enzymes was considerably dependent on the carbon source used and the highest activity was obtained in Avicel composed of cellulose of insoluble and crystalline form. No cellulase and xylanase were observed in the control culture which did not contain a carbon source, but a little growth caused by a carbon source contained in bacto peptone was observed (data not shown). Also, the cellobiose substrates induced less production of CMCase, Avicelase, and xylanase than that of the cellulosic substrates, but they produced a relatively large amount of β-glucosidase.

Figure 8 shows the cellulase and xylanase activities when the lignocellulosic substrates such as rice straw, sawdust, and pulp were used in the culture. In comparison with Figs. 7 and 8, the higher production of enzymes was observed in the lignocellulosic complex substrates than commercial cellulosic materials composed of pure material. In conclusion, the best production of enzymes were obtained in the presence of the following carbon sources: insoluble>soluble, cellulose >hemicellulose, raw lignocellulosic materials>pure commercial cellulosic materials.

To investigate the effect of concentration of carbon source, the concentrations of the mixture of Avicel and CMC were changed from 1.0 to 2.5%. As shown in Fig. 9, the highest level of enzymes was detected when a mixture of 2.5% was used, and inhibition was found in the high concentration

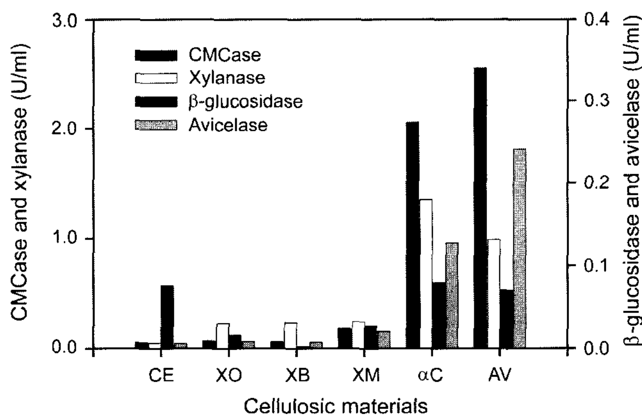

Fig. 7. Comparisons of enzyme activities in the cultures using various commercial cellulosic materials (1.0%) in Mandel's medium instead of Avicel and CMC.
Legends: cellobiose (CE), xylan of oat spelt (XO), xylan of birchwood (XB), CMC (CM), α-Cellulose (C), Avicel (AV).

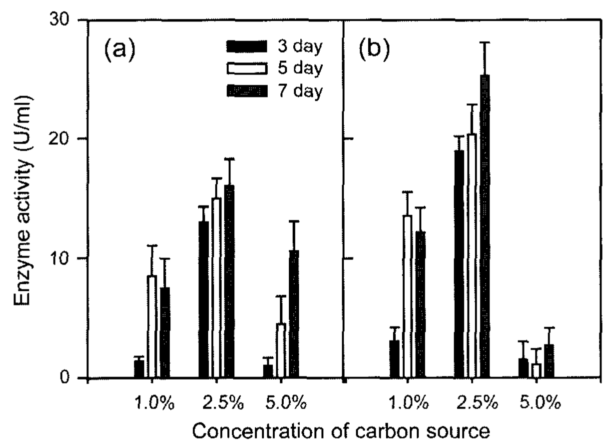

Fig. 9. Comparisons of enzyme activities in the culture using a mixture of Avicel and CMC.
The concentrations were varied from 1.0 to 5.0% in Mandel's medium. The bars show standard deviations. Legends: CMCase (a), xylanase (b).

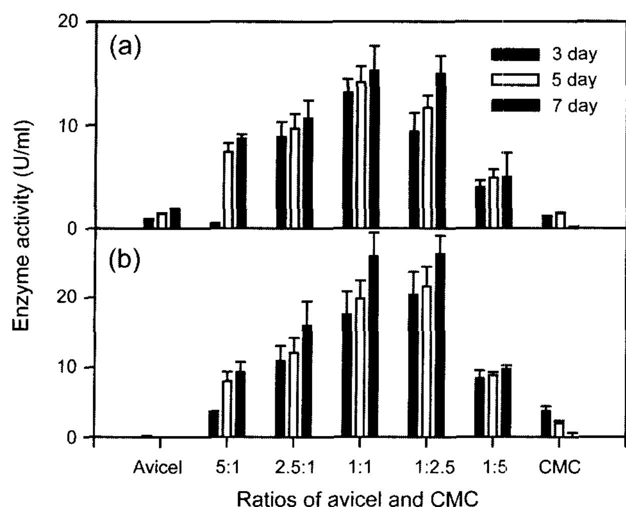

Fig. 10. Comparisons of enzyme activities in various composition ratios of Avicel and CMC with total concentration of 2.5% in Mandel's medium.

The bars showed standard deviations. Legends: CMCase (a), xylanase (b).

above 2.5%. The maximum CMCase and xylanase activities were 16.2 and 25.3 U/ml, respectively. Also, as the substrate concentration increased, a concentration of reducing sugar over 1.0 mg/ml in the culture broth after incubation was found, which probably inhibited the induction of the enzymes (data not shown). It has been known that the presence of decomposition products of low molecular weight like oligosaccharides in the substrates contributes to the repression of enzymes production, particularly when high substrate concentrations are used [12, 30, 37]. It was also possible that the production of enzymes of strain FJ1 were repressed in a similar pattern with the accumulator of hydrolysis products.

The results of enzymes production in various ratios of Avicel and CMC are shown in Fig. 10. The highest level of enzymes was detected in the 1:1 ratio of Avicel and CMC, which could serve as inducers. The maximum CMCase and xylanase activities were 15.2 and 25.9 U/ml, respectively. Although Avicel has been widely used in enzymes production [45], the enzyme induction in strain FJ1 cultures containing Avicel only did not occur, indicating that Avicel probably did not serve as a carbon source for strain FJ1 but played an important role in induction of enzymes (Fig. 10).

Binder and Ghose [4] suggested that the complete induction of a cellulase system required a physical contact between the mycelium and the cellulosic materials with different physical characteristics. Several reports suggested that the nature of carbon sources used played an important role in the production of enzymes [6, 22, 42]. In a similar case, *T. harzianum* FJ1 showed that the nature of carbon sources used for the induction of enzymes had a large influence on the hydrolytic activity. In addition, strain FJ1 was a good producer of β-glucosidase and Avicelase at cultivation, using Avicel and CMC as carbon sources.

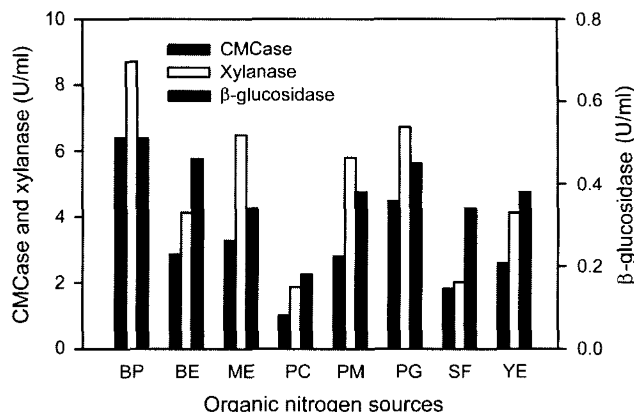

Fig. 11. Comparisons of enzyme activities in the cultures using various organic nitrogens in Mandel's medium.

Legends: bacto peptone (BP), beef extract (BE), malt extract (ME), peptone from casein (PC), peptone G (PG), peptone from meat (PM), soybean flour (SF), and yeast extract (YE).

Effect of Nitrogen Source

The effect of various organic nitrogen sources on the production of cellulolytic enzymes by *T. harzianum* FJ1 was investigated. The enzyme activities in the culture broth were examined after 5 days of incubation and the results are shown in Fig. 11. Although the enzyme activities were found in the cultures with all the organic nitrogen sources used, each nitrogen source had a considerable influence on the production characteristic of the enzymes. The differences were probably caused by a difference of the compositions, such as amino acids, peptides, vitamins, trace elements, and/or mineral salts contained in complex nitrogen sources as described by Phuong *et al.* [34]. Bacto peptone was a critical

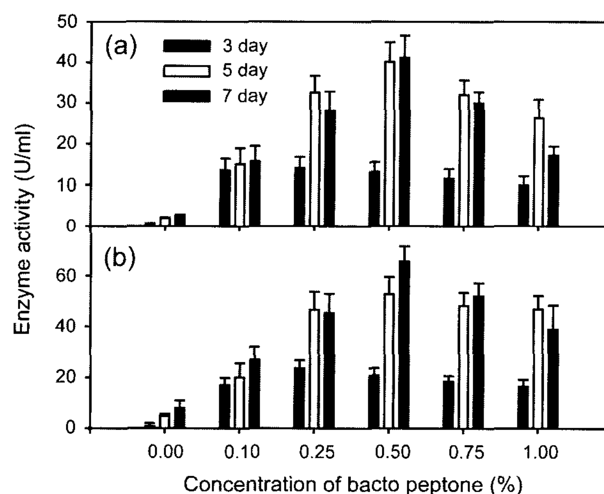

Fig. 12. Comparisons of enzyme activities in the culture according to the bacto peptone concentration.

The concentrations were varied from 0.0 to 0.1% and carbon source of 2.5% was used in Mandel's medium. The bars show standard deviations. Legends: CMCase (a), xylanase (b).

component for the growth and enzyme production of strain FJ1, as coincides with those in the previous reports [34, 35].

Therefore, bacto peptone was selected as a nitrogen source for further studies. To investigate an optimum concentration of the bacto peptone, the concentration in the growth medium was varied from 0.0 to 1.0% (w/v). Figure 12 shows the effect of concentration of the bacto peptone on the production of enzymes. Bacto peptone at 0.5% concentration gave the best production of enzymes, and the maximum activities of CMCase and xylanase were 41.2 and 65.6 U/ml in 7 days, respectively.

Consequently, the enhanced production of cellulases and xylanase of *T. harzianum* FJ1 in the optimized medium of carbon and nitrogen sources was compared with several well-known cellulases producers, such as *T. reesei* and *Aspergillus niger*. Yu *et al.* [50] reported the production of cellulase by *T. reesei* Rut C30 in a batch fermentor. When 5% of Solka Floc and 1% of wheat bran were included in the medium, CMCase and FPase activities were 232.4 U/ml and 21.25 U/ml, respectively. The production of cellulase and xylanase by *Aspergillus niger* KKS was investigated in an optimized medium by Kang *et al.* [20]; the activities of endoglucanase, β-glucosidase, β-xylosidase, and xylanase were 3.8, 4.2, 4.0, and 80.0 U/ml, respectively. In our study, the maximum activities of CMCase and xylanase of *T. harzianum* FJ1 were 41.2 and 65.6 U/ml in 7 days cultivation, respectively. In addition, strain FJ1 simultaneously produced not only cellulase and xylanase, but also amylase and pectinase with relatively high activities (data not shown). *T. harzianum* was widely used as biological control agents degrading cell wall by enzymatic reactions, such as chitinase [11], β-1,3-glucanase [39], and β-1,6-glucanase [16]. In the previous reports of *T. harzianum*, production of amylase [10] and pectinase [29] was dependent on the carbon source available, but study about its cellulases production was hardly known in our investigation.

For efficient saccharification of cellulosic wastes, the related enzymes, such as cellulases, xylanase, amylase, and pectinase, have to react reciprocally to the complex structure of cellulosic wastes. Therefore, strain FJ1 with its complex enzyme production system has a great advantage in the production and composition of the enzymes, compared with the fungi above. We concluded from these results that *T. harzianum* FJ1, capable of hydrolyzing cellulose and hemicellulose to glucose and xylose efficiently, could be applied to the industrial saccharification of cellulosic wastes such as food wastes and paper wastes.

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