博 士 学 位 論 文

Utilization of Corn Fiber for Sawdust-based Cultivation of Mushrooms

近畿大学大学院

農学研究科　応用生命化学専攻

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Doctoral Dissertation

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有用きのこ類の菌床栽培へのコーンファイバーの利用

近畿大学大学院　農学研究科
応用生命化学専攻
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ABBREVIATIONS

Avicel	crystalline cellulose
CMC	carboxymethyl cellulose
CNF	corn fiber
CNF-HWSF	hot-water soluble fraction from corn fiber
DMSO	dimethylsulfoxide
GPYL	glucose-peptone-yeast extract liquid
HPLC	high performance liquid chromatography
IFO	Institute for Fermentation, Osaka
MW	molecular weight
PDA	potato-dextrose agar
PDL	potato-dextrose liquid
PMML	partly modified matsutake liquid
TCA	trichloroacetic acid
TLC	thin-layer chromatography

CONTENTS

INTRODUCTION

Mushrooms are an important crop in Japan. Also, the mushroom industry is a global expanding industry with world production greater than 2 million tones annually (Kües and Liu 2000), and the mushroom production and consumption are increasing every year. The chief mushroom varieties produced in Japan are champignon (*Agaricus bisporus*), shiitake (*Lentinula edodes*) and the oyster mushroom (*Pleurotus ostreatus*).

Techniques using sawdust mixed with various ingredients as a substrate and growing under controlled environmental conditions, for the cultivation in sawdust-based culture gradually have been developed (Yamanaka 1995). Many kinds of mushrooms, except for *L. edodes*, are cultivated with sawdust-based cultivating methods. Indoor cultivation utilizing sawdust has the advantage of producing mushrooms throughout the year, and the potential for greatly increased yields through manipulation of substrates and environment. Although *L. edodes* traditionally has been cultivated on hard wood logs outdoors in a natural environment, alternatively, more intensive sawdust cultivation techniques recently have been developed because the shape produced by sawdust-based cultivation is more appealing than that produced by log cultivation.

However, the serious shortage of hardwood sawdust has happened as a result of the rapid expansion of mushroom cultivation and the rapid decrease in the amount of wood cuts. Hence, a few research groups have started to cultivate mushrooms with agricultural and industrial wastes or the other materials, but which had been hardly used on practical cultivation of edible mushrooms.

Sawdust-based culture generally is contains more nutrious than hardwood log-culture because nutrious supplements such as rice bran, wheat bran and the other carbon or nitrogen source are added to the sawdust-based culture. Therefore it is important for stable mushroom cultivation to understand the nutritional requirement of mushrooms in the sawdust-based culture. Also, there are a variety of extracellular enzymes among several fungi, and many enzymes are appeared in the mycelial growth and development processes of mushrooms. The changes in the amount or property of

various enzymes in the cultures of some basidiomycetes and fungi have been correlated with fungal growth and fruit-body formation. Ohga (1992) described that the cellulase and xylanase activities of the well-fruiting strains increased rapidly in the early exponential growth phases in the cultures of *L. edodes*, whereas these enzyme activities of non-fruiting strains maintained very low revel throughout the culture period. It was well known that the fructification-formation of *L. edodes* was enhanced with the treatment of lower temperature and osmotic pressure. Then it has been indicated that the treatment for fructification inducement changed the expression of laccase and cellulase in cultures of *L edodes* (Ohga and Royse 2001). Terashita et al. (1978) reported that the fruit-body yield of some mushrooms was enhanced by the addition of an acid-protease inhibitor (*Streptomyces*-Pepsin Inhibitor) to the culture medium. Thus, mycelial growth and fruit-body formation of mushrooms were regulates by some enzymes produced by mushrooms. Therefore, it is very valuable to increase the yield of fruit-body of mushrooms and to improve the quality of the fruit-body that the enzymes involved in the formation and quality of the fruit-body are clarified.

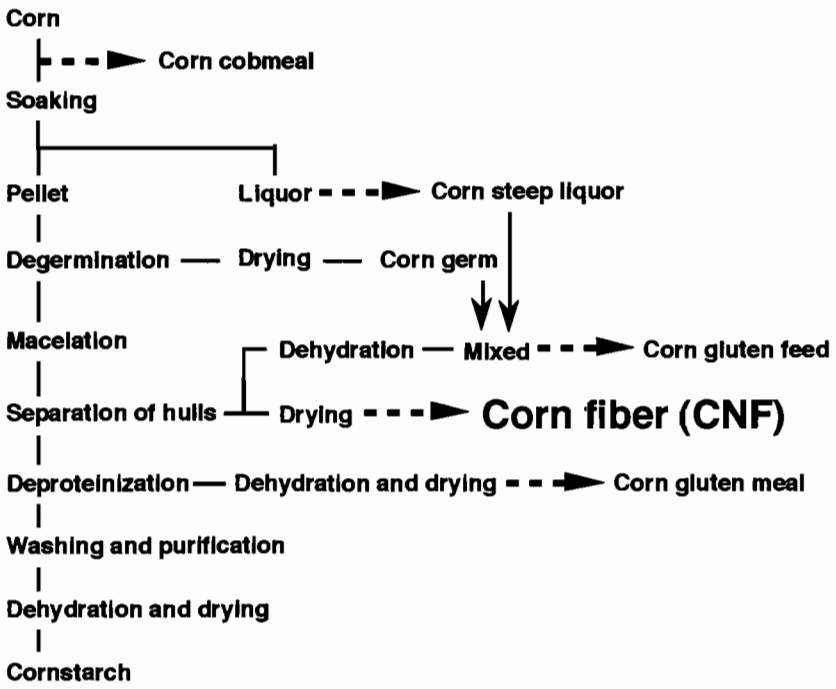


Fig. 1. Production of cornstarch by wet milling process

Corn fiber (CNF) is a by-product of the wet corn milling process used to produce

cornstarch in factories, as shown in Fig. 1. The amount of CNF as a by-product is about 200,000 tons per year in Japan, and 4 million tons in the world (Doner and Hicks 1997). However, at present CNF is not used efficiently.

Table 1. The main components of CNF

Ingredient	Content (%)
Hemicellulose	47
Cellulose	19
Starch	20
Crude protein	12
Crude fat and ash	2

The main components of CNF are shown in Table 1. CNF includes 50% hemicellulose, 20% each of cellulose and starch and 10% protein (a research by Oji cornstarch Co. Ltd.; unpublished data). Thus CNF has rich nutrients for culture substrate of mushrooms. Therefore, it seems that the CNF is a promising mushroom cultivation substrate.

The author has been attracted to the utilization of organic wastes and to develop a new culture substrate and an efficient cultivation method for mushroom production, and to research on utilization of CNF and clarify the components in CNF involved in mushroom cultivation. Moreover, observation on the mechanisms of the function of CNF to mushrooms was added.

Chapter I covers CNF for use as a substrate in the sawdust-based cultivation of three edible mushrooms including *L. edodes*, which is the most important and popular edible mushroom in Japan.

Chapter II deals with the effects of CNF hot-water soluble fraction (HWSF) on vegetative mycelial growth of edible mushrooms including mycchorizal fungi such as *Tricholoma matsutake* and *Lyophyllum shimeji*. The effect of CNF-HWSF on the rhizomorph formation of *Armillaria mellea* was also indicated.

In chapter III, the effects of CNF-HWSF on the fruit-body formation of mushrooms are described. This chapter includes the application studies on utilization of CNF-HWSF for sawdust-based cultivation of *P. ostreatus*.

Chapter IV describes the promoting mechanisms in the growth of mushrooms by CNF. Section 1 indicates the results in the chemical analysis of CNF-HWSF and the effect of each fractionated components from CNF-HWSF on the mycelial growth of mushrooms. Section 2 describes the stimulation of CNF-HWSF in the production of extracellular enzymes in cultures of edible mushrooms.

CHAPTER I

Waste by-product, Corn fiber (CNF) for Use as a Substrate in the Sawdust-based Cultivation of Edible Mushrooms

Section 1. Utilization of CNF for the cultivation of *Pleurotus* spp.

The indoor cultivation of many kinds of mushrooms using sawdust beds has an advantage in that mushrooms can be produced steadily throughout the year. In addition to the decrease in the amount of wood cut, the rapid expansion of mushroom production resulted in the shortage of wood sawdust, which is now a serious problem in mushroom cultivation. Hence, the usage of agricultural and industrial wastes as cultivation substrates of mushrooms has been increasing in recent years. For example, the cultivation of *Lentinula edodes* uses the residue of bamboo grass leaves (Katou et al. 1999); *Armillaria ostoyae* can be cultivated with carrot juice residue (Togashi et al. 1999); and utilized sake lees (Okumura et al. 1996), olive oil mill wastes (Zervakis et al. 1996), and coffee pulp are used for the culturing *Pleurotus ostreatus* (Gonzales et al. 1993).

Corn fiber (CNF) is a by-product of the wet corn milling process used to produce cornstarch in factories. By-products such as corn cobmeal (Corn cob.), corn steep liquor (CSL) and corn gluten meal are produced during this process. Recently, the utilization of these by-products has been examined. Corn cobmeal has been put to good use in the cultivation of mushrooms on a commercial scale. The amount of CNF as a by-product is about 200,000 tons per year in Japan, and four million tons worldwide (Doner and Hicks 1997). Most is used to make livestock feed after combining corn germ with corn steep liquor except for that utilized as functional food material, such as dietary fiber (Takeuchi 1997, Egashira 1999). The composition of CNF is about 50% hemicellulose, about 20% each of cellulose and starch and about 10% protein. Xylan is the major component of hemicellulose. Mushrooms have many strains that have high xylanase activity during the growth of fruit-bodies (Kawai 1973). Therefore, CNF is a possible alternative mushroom cultivation substrate.

The present section deals with the effect of CNF on the fruit-body formation of *Pleurotus* spp. in a sawdust-based cultivation.

Materials and Methods

Strains

The strains used in this study were *Pleurotus ostreatus* (Pc 89-1) and *P. cornucopiae* (Pc 98-3) stock culture of Hokkaido Forest Products Research Institute.

Culture media

Corn fiber was obtained from Oji cornstarch Co.. For mushroom production substrate, ingredients (sawdust of *Abies sachalinensis*, corn fiber and wheat bran) were mixed in the ratio as shown in Table I-1 and water was added to raise final moisture content to 65%. The mixture 460 g was packed into a 850 ml capacity polypropylene bottle. A hole of diameter 2.0 cm and depth 13 cm was made at the center of the upper surface of substrate. The bags were sterilized at 121°C for 30 min.

Table I-1. Compositions of sawdust based media in *Pleurotus ostreatus* and *P. cornucopiae*

Experimental plot	Control	a	b	c	d	e	f	g	h	i	j
CNF (%)	0	87.5	75.0	62.5	50.0	37.5	25.0	12.5	37.5	25.0	12.5
Sawdust (%)	50.0	0	0	0	0	12.5	25.0	37.5	50.0	50.0	50.0
Wheat bran (%)	50.0	12.5	25.0	37.5	50.0	50.0	50.0	50.0	12.5	25.0	37.5

Culture materials were curculated by dry weight bases.

Culture condition

In *P. ostreatus*, five grams of the spawn were inoculated into the hole of substrate in the bottle and incubated at 22°C, relative humidity 70% in the dark. When the mycelia were grown throughout in the bottle, the surface of mycelia was

removed (*Kinkaki*). Then the colonized substrate in the bottle was poured into water (2 h). Then fruit-body formation was performed at 12°C, relative humidity 85% and 350 lux illumination was provided for 12 h daily.

In *P. cornucopiae*, five grams of the spawn were inoculated into the hole of substrate in the bottle and incubated at 22°C, relative humidity 70% in the dark until formed of fruit-bodies primordia. Then fruit-body formation were performed at 16°C, relative humidity 85% and 350 lux illumination was provided for 12 h daily.

Evaluation of fruit-bodies

Fruit-body yields were measured as fresh weight grams, each average and standard division (SD) were calculated.

Results

Effect of CNF on sawdust-based cultivation of Pleurotus ostreatus

To examine the utilizable possibility of CNF for fruit-body production of mushroom, *Pleurotus ostreatus* was cultivated on sawdust-based medium with CNF. Fig. I-1 shows the picture of one example as to the fruit-body development of *P. ostreatus*. The fruit-bodies obtained from the medium with CNF were by no means inferior to control without CNF, moreover, the number of fruit-body development has increased by the addition of CNF.

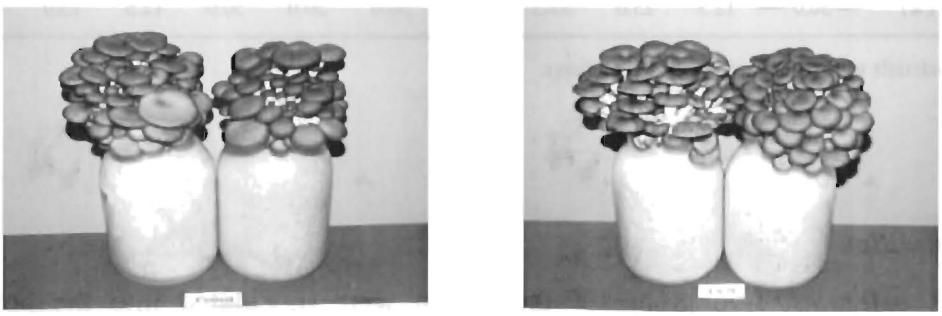


Fig. I-1. The effect of CNF on fruit-body formation of *Pleurotus ostreatus*
Left shows control. Right shows experimental plot c: 25% and 100% of CNF instead of wheat bran and sawdust, respectively.

The fruit-body yield and the cultivation period of *P. ostreatus* were shown in Fig. 1-2. When this mushroom was cultivated on medium of only CNF and bran without sawdust, the fruit-body yield was increased. Especially, on the medium of which the ratio of CNF to bran was 5 to 3 or 1 to1 as shown in experimental plot c and d, fruit-body yield increased about 1.6 times that of the control. Also, the terms up to germination were shortened about 3-9 days and the days required for harvest were reduced about 4-11 days by supplementation with CNF. On the medium in which 25-75% of bran was replaced by CNF (experimental plot h-j), however, the decrease of fruit-body yield and a delay of cultivation period were appeared.

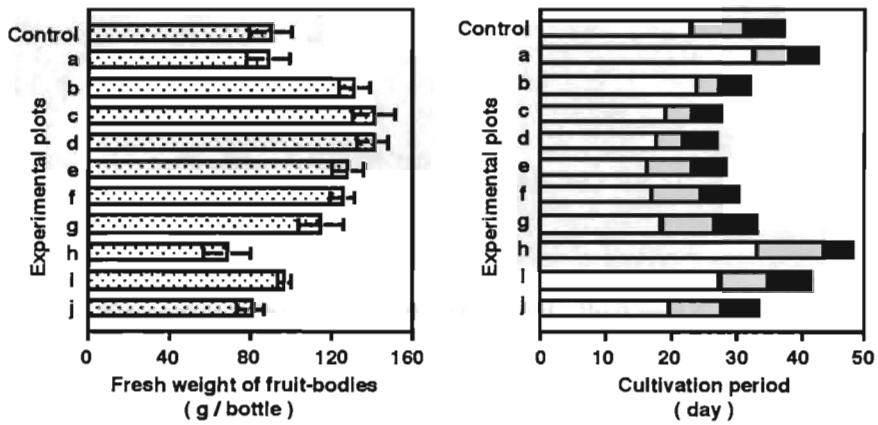


Fig. I-2. The effect of CNF on fruit-body yield and cultivation period of *Pleurotus ostreatus*

Left: fruit-body yields are expressed as means plus standard errors (n=16). Right: ☐ shows for spawn running days, ☐ shows the days from soaking to germination days, ■ shows the period of fruit-body growing. For symbols refer to Table I-1.

Effect of CNF on sawdust-based cultivation of Pleurotus cornucopiae

The effect of CNF on the production of *Pleurotus cornucopiae*, which is being cultivated mainly in Hokkaido and is on the market in Japan, was examined. Fig. I-3 shows the picture of one example as to the fruit-body development of *P. cornucopiae*. The fruit-bodies obtained from the medium with CNF were by no means inferior to

those obtained from the control medium without CNF. The fruit-body yield and the cultivation period of *P. cornucopiae*, are shown in Fig. I-4. The fruit-bodies yields on the medium in which 25-100% of sawdust was replaced by CNF (experimental plots c-g) increased to 88.2-107.7 g / bottle from 75.4 g / bottle of the control medium. Also, the terms required for germination were shortened about 1-3 days and the periods required for harvest were reduced about 2-4 days by supplementation with CNF. However, on the medium in which 25-75% of bran was replaced by CNF (experimental plot I and j), the fruit-body yields decreased the cultivation periods were delayed. While, no fruit-body formation was recognized on the medium 37.5% of bran was replaced by CNF (h).

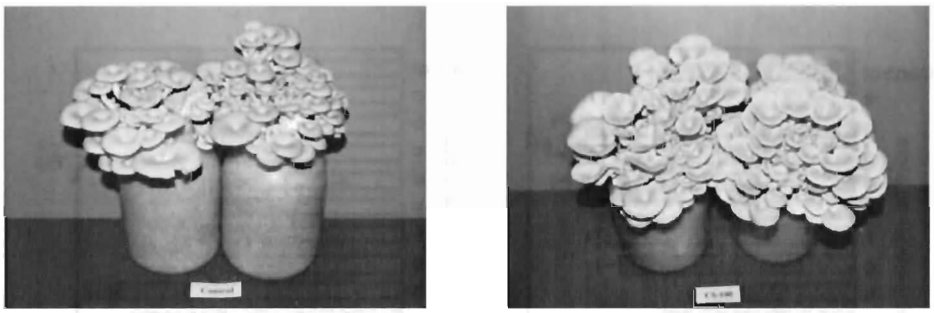


Fig. I-3. Effect of CNF on fruit - body formation of *Pleurotus cornucopiae*
(Left shows control, Right shows experimental plot f: 50% of CNF instead of sawdust).

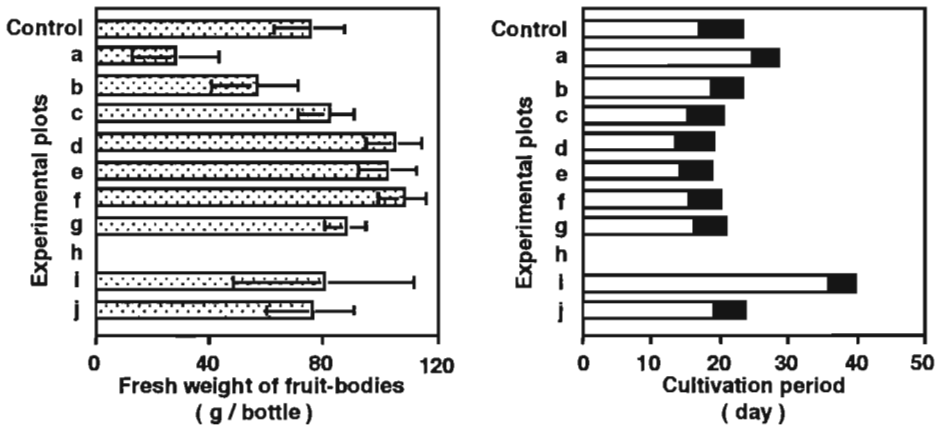


Fig. I-4. Effect of CNF on fruit-body yields and cultivation period of *Pleurotus cornucopiae*
Left: fruit-body yields are expressed as means plus standard errors (n=16). Right: □ shows for germination days, ■ shows the period of fruit-body growing. For symbols refer to Table I-1.

Discussion

Altering various factors, e.g., genotype, culture substrate, nutrient supplement and environmental condition have an influence on the fruit-body yield of mushrooms. Especially, carbon-nitrogen (C/N) ratio and concentration of nitrogen source in sawdust culture influence significantly fruit-body production (Suzuki 1979, Boyle 1998). In *P. ostreatus* and *P. cornucopiae*, about 30-40 of initial C/N ratio were most suitable for fruit-body production and their fungi were required much nitrogen source at fruiting developments (Harada et al. 1999). When 50-100% of CNF instead of sawdust, the fruit-body yield of these fungi increased markedly. And also in *P. ostreatus*, on the medium the ratio of CNF to bran was 3 to1 or 5 to 3 without sawdust, the fruit-body yield increased and shortened the cultivation period. Because CNF included much more protein than sawdust, it seemed that the increase of fruiting yields was owing to high concentration of nitrogen source in the medium.

In this section, the author described CNF is a useful culture material for sawdust-based cultivation of *P. ostreatus* and *P. corunicopiae*. Also, possibility that CNF has a component to promote the growth of fungi was indicated from reduction in cultivation period.

Summary

With *Pleurotus ostreatus* and *P. cornucopiae* the production of fruit-body on CNF based substrate were examined. The fruit-body yield increased at 1.2-1.6 times by using CNF instead of sawdust or wheat bran. In addition, the cultivation period was shortened 2-11 days compared to that of the control without CNF. CNF is useful in producing the fruit-body, which increases yield and shortens the cultivation period.

Section 2. Effect of CNF on the formation and yield of fruit-body in the sawdust-based cultivation of *Lentinula edodes*

Lentinula edodes, shiitake mushroom, is one of the traditionally important edible mushrooms cultivated in the world. In 1997, the worldwide production of *L. edodes* reached 1,564,400 ton, which was about 25.4% of the total mushroom supply in the world. Altering various factors, e.g., genotype, culture substrate (natural logs and sawdust-based substrate), nutrient supplement and environmental condition have improved cultivation efficiency of *L. edodes*. These improvements have contributed to a consistent market supply of high quality mushrooms. In particular, sawdust-based cultivation has contributed to expanding production and consumption of *L. edodes*. Sawdust-based substrates usually consist of hardwood sawdust, rice bran and wheat bran. The decrease in the amount of wood sawdust produced in the world and a rapid expansion of the mushroom production resulted in a shortage of hardwood sawdust. The shortage has being become a serious problem in mushroom cultivation.

In section 1, CNF was proved to be useful in the formation and yield of the fruit-body of *Pleurotus ostreatus* and *P. cornucopiae* in sawdust-based cultivation.

The present section deals with the effect of CNF on the fruit-body formation of *L. edodes* in sawdust-based cultivation.

Materials and Methods

Strain

A commercial strain of *Lentinula edodes* Hokken No. 600 (Hokken Co.) was used in this study.

Culture media

CNF was obtained from Oji cornstarch Co. (about 10% of moisture content). For mushroom production substrate, ingredients (sawdust of *Betula ermanii*, CNF and wheat bran) were mixed in the ratio as shown in Table I-2 and water was added to raise

final moisture content to 65%. The mixture 2.5 kg was packed into a polypropylene bag, forming the final size 12×12×15 cm. Two holes of diameter 2.5 cm were made at the center of the upper surface of the substrate. The bags were sterilized at 121°C for 30 min.

Table I-2. Compositions of sawdust based media in *Lentinula edodes*

Experimental plot	Control	A	B	C	D	E	F	G	H
CNF (%)	0	5.750	11.00	17.25	23.00	19.25	38.50	25.00	50.00
Sawdust (%)	77.00	77.00	77.00	77.00	77.00	57.75	38.50	75.00	50.00
Wheat bran (%)	23.00	17.25	11.50	5.75	0	23.00	23.00	0	0

Culture materials were curculated by dry weight bases.

Culture condition

Ten grams of the spawn were inoculated onto the substrate in the bag and incubated at 22°C and relative humidity 70% in the dark for 30 days, then 350 lux illumination was provided for 12 h daily for 60 days. At the end of the incubation period, the bags were removed, and placed at 16°C, relative humidity 85% and with alternate 12 h illumination (350 lux) and 12 h darkness treatment to induce fruit-body formation. After the 1-4 th flushes, the substrates were soaked in water and cultures were carry out until 5 th flush.

Evaluation of fruit-bodies

The size of fruiting was classified into five groups by the diameter of the pileus as follows: LS (less than 3 cm and malformation), S (3-4.5 cm), M (4.5-6 cm), L (6-8 cm) and LL (more than 8 cm). Fruit-body yields were measured as fresh weight grams, these averages and SD (Standard division) were calculated.

Results

Utilization of CNF for the cultivation of Lentinula edodes

The effects of CNF on the production of *L. edodes*, which is the most important and popular edible mushroom in Japan, were examined. Fig. I-5 shows the picture of one example as to the first flush of fruit-body development of *L. edodes*. The sizes of fruit-bodies developed from the medium supplemented with CNF (Right) were larger those from the control medium (Left).

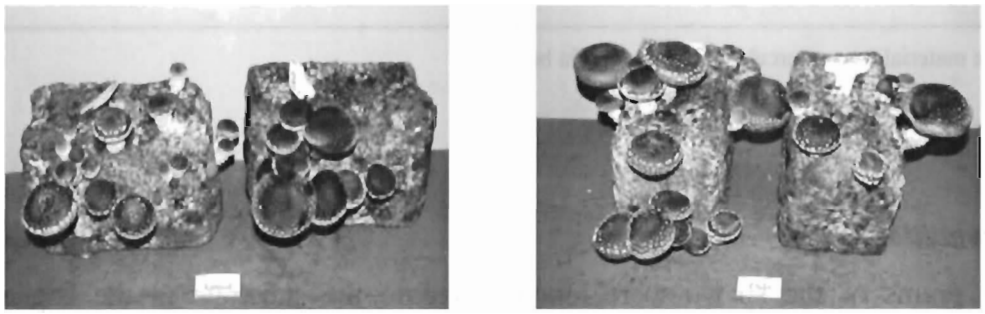


Fig. I-5. Effect of CNF on fruit - body formation of *Lentinula edodes*
(Left: control, Right shows experimental plot E: 25% of CNF instead of sawdust).

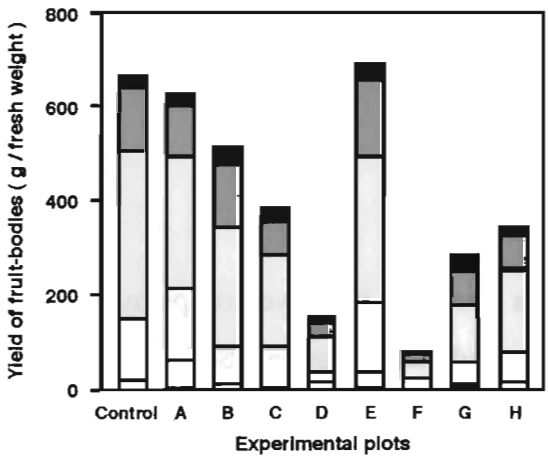


Fig. I-6. Effect of CNF on the fruit-body yield of *Lentinula edodes*
Size of pileus diameter □: LS (3 cm or less and monstrosity), □: S (3-4.5 cm), □: M (4.5-6 cm), □: L (6-8 cm), ■: LL (8 cm or more).
For symbols refer to Table I-2.

The effects of CNF on the total fruit-body yield and size of pileus diameter were shown in Fig. I-6. The total fruit-body weight up to fifth flush produced on the substrate with 25% CNF instead of sawdust (683.8 g) or wheat bran (628.6 g) was almost same as that on the control substrate (662.8 g). Moreover, many large size fruit-bodies such as LL and L were obtained from these substrates. However, when the concentration of CNF was over 50%, fruit-body yields were declined.

Discussion

The properties of CNF seem to be intermediate between those of sawdust and wheat bran in the sawdust-based cultivation because the protein content of CNF is mostly about 10%, this quantity is much larger than sawdust, less than that of wheat bran, and CNF is similar to a chip of wood in form, and has the ability to maintain water inside.

It was reported that the high concentration of nitrogen source was inhibited the growth of *L. edodes* (Katou et al. 1999). In cultivation of *L. edodes*, the fruit-body yield decreased little when 50% of CNF was used instead of wheat bran, while, that markedly declined when 50% of CNF was used instead of sawdust. The result may be caused by a rise of nitrogen source with CNF.

In this section, the utilization of CNF for sawdust-based cultivation of *L. edodes* was described. As the results, CNF was effective in improving the quality of fruit-body. The more remarkable effect of CNF on the reduction of the cultivation period required for the fruit-body development of *L. edodes* could be expected by the treatment to induce the fruit-body formation in an early stage because the cultivation period required for the fruit-body development of *P. ostreatus* or *P. cornicopiae* was shortened by adding of CNF to the culture substrates.

Summary

The effect of corn fiber (CNF), which is a by-product of the wet corn milling process of the production of cornstarch in factories on the fruit-body formation of *Lentinula edodes* in a sawdust-based cultivation, was examined. The increase in fruit-body yield was not observed, but the pileus diameter of fruit-body tended to enlarge with added CNF. CNF is effective in improving the quality of fruit-body of *L. edodes*.

CHAPTER II

Promoting Effect of the Hot Water Soluble Fraction (HWSF) from CNF on Vegetative Mycelial Growth in Edible Mushrooms

Section 1. Effect of the CNF-HWSF on vegetative mycelial growth in edible mushrooms including mycorrhizal fungi

In chapter I, the author showed that the CNF useful culture material for sawdust-based cultivation of *Pleurotus ostreatus*, *P. cornucopiae* and *Lentinula edodes*. Therefore, it was suggested that the growth promoting component for fungal in CNF material was contained from the reduction in cultivation period and the increase of fruit-bodies yields. Terashita et al (1997) have reported that the yield of fruit-bodies on *Pholiota nameko* and *Hypsizygus marmoreus* increased with CNF as a growth substrate for the sawdust based cultivation, and they suggested that the existence of mycelial growth promoting components in the hot water soluble extracts from CNF.

This section describes the promoting effects of CNF-HWSF upon vegetative mycelial growth containing mycorrhizal fungi.

Material and Methods

Strains

Lentinula edodes (Mori No. 465), *Hypsizygus marmoreus* (Takara No. 1), *Pleurotus ostreatus* (Kitamura; obtained from Kin-ki Nyugyo Co.), *P. eryngii* (axenic isolate from a commercial mushroom), *Flammulina velutipes* (IFO 7777), *Grifola frondosa* (Mogami), and *Pholiota nameko* (Meiji) were used in these experiments. In addition, two species fungi, *Lyophyllum shimeji* (MH 01721) and *Tricholoma matsutake* (IFO 30605), mycorrhizal mushrooms, were also used. These fungi were stored on potato dextrose agar medium (Nissui Co.) in a test tube.

Culture media

Potato-dextrose liquid (PDL) medium consists of potato extract (200 g boiled in 500 ml distilled water), 15 g glucose and 1mg thiamine hydrochloride per liter of distilled water. Partly modified matsutake liquid (PMML) medium was prepared according to the method of Terashita et al. (2000). This medium consists of 22.7 g glucose, 5 g dried beer yeast (Wako Pure Chem.), and 5 g Sunpeal CP (commercially available) per liter of distilled water. The mixture was heated for 30 minutes, and then 1.0 ml thiamine hydrochloride solution (0.1 g thiamine hydrochloride per 10 ml distilled water) was added after the residue was removed. The initial pH of this medium was adjusted to 5.1 with 1 N HCl.

Preparation of CNF- HWSF

Corn fiber (100 g) was mixed with 1L distilled water and extracted for 3 h at 80°C. After the residue was removed by centrifugation at 10,000 g (0°C, 10 min) the supernatant solution was concentrated at 40°C to 100ml by a rotary evaporator.

Inoculations and culture conditions

The PDL medium was supplemented with 5, 10, 20 and 30% (v/v) CNF-HWSF and dispensed in 16-ml aliquots in 100-ml Erlenmeyer flasks before autoclaving at 121°C for 10 min. As inoculum, a mycelial block (diameter 5 mm) was cut from a plate culture that had grown on a PDA medium for 14 days at 24°C in a Petri dish (diameter 90 mm). The incubation was carried out at 24°C for 15 days. For the mycorrhizal mushrooms that grew on the PDL and PMML media, the incubation was carried out at 24°C for 30 days (*L. shimeji*) or 60 days (*T. matsutake*).

Measurement of vegetative mycelial growth

The vegetative mycelia after incubation were separated from the medium by filtration and washed thoroughly with distilled water, after which they were dried at 80°C for 24 h. Their dry weight was measured after cooling in a desiccator.

Results

Effect of CNF-HWSF on the mycelial growth of edible mushrooms

Fig. II-1 illustrates the picture of growth obtained with CNF-HWSF tested. The vegetative mycelial growth of *L. edodes* on the PDL medium with added CNF-HWSF is shown in Fig. II-2. Mycelial growth was enhanced by adding 5-20% CNF-HWSF to the medium; but at 30% CNF-HWSF the results were almost identical to the control (without CNF-HWSF). In particular, addition of 20% CNF-HWSF to the medium increased the mycelial dry weight to 98.6 mg/flask. This weight was about 9.5 times more than that of the control (10.4 mg/flask).

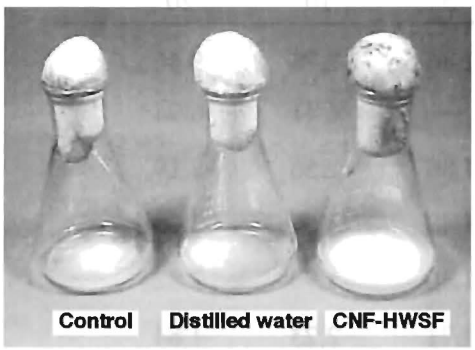


Fig. II-1. Mycelial growth of *Lentinula edodes* on a PDL medium with CNF-HWSF
The vegetative mycelia were cultured for 15 days at 24°C in a PDL medium with or without 20% of CNF-HWSF or disttiled

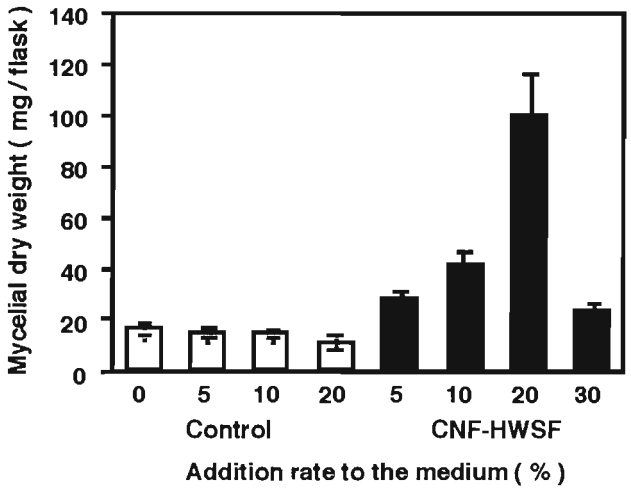


Fig. II-2. The Promotive effect of CNF-HWSF on the vegetative mycelial growth of *Lentinula edodes*
The vegetative mycelia were cultured for 15 days at 24°C. Values are means ± S. D. (n=8). *Open bars*, potato-dextrose liquid (PDL) medium with added distilled water (control); *filled bars*, PDL medium with added CNF-HWSF.

The promotive effect of CNF-HWSF on vegetative mycelial growth of several species of mushrooms is shown in Table II-1. When these fungi were cultured on PDL medium with CNF-HWSF added, the mycelial dry weight was greater than that of the control (without CNF-HWSF). The optimal experimentally derived concentrations for mycelial growth were 10% for *H. marmoreus* (2.56 times), *P. eryngii* (2.23 times) and 20% for *L. edodes* (9.46 times), *P. nameko* (6.94 times), *G. frondosa* (3.68 times), *P. ostreatus* (2.82 times) and *F. velutipes* (2.23 times).

Table II-1. Promotive effect of CNF-HWSF on the vegetative mycelial growth of several edible mushrooms

Mushroom	Concentration of supplement (%)		
	5	10	20
Lentinula edodes	1.86	2.78	9.46
Pholiota nameko	—	6.41	6.94
Hypsizygus marmoreus	1.86	2.56	2.19
Grifola frondosa	1.48	3.66	3.68
Pleurotus ostreatus	1.40	1.90	2.82
Pleurotus eryngii	1.71	2.23	1.84
Flammulina velutipes	1.48	1.70	2.23

Growth ratio obtained for control (distilled water) was set at 1.00.
Vegetative mycelia were cultured for 15 days at 24°C in potato-dextrose liquid (PDL) medium.
Data represent averages of triplicate experiments, with six Erlenmeyer flasks per experiment.

Effect of CNF-HWSF on mycelial growth of mycorrhizal mushrooms

Table II-2 shows the effect of mycelial growth on *L. shimeji* forming mycorrhiza. On the PDL medium for 30 days at 24°C, the dry weight of mycelia increased by 3.72 times when 5% CNF-HWSF was added to the medium. However, the promotive effect of CNF-HWSF in PMML medium was only 1.37-fold (5% supplemented) or 1.65-fold (10% supplemented).

Table II-2. Promotive effect of CNF-HWSF on the vegetative mycelial growth of *Lyophyllum shimeji*

Addition to the medium (%)	Dry weight of mycelium (mg / flask)	
	PDL medium	PMML medium
Distilled water (Control)		
5	22.42 ± 3.15	120.68 ± 14.81
10	23.20 ± 7.00	124.20 ± 20.76
20	29.35 ± 7.19	—
CNF-HWSF		
5	83.35 ± 33.86	165.93 ± 15.06
10	45.70 ± 17.06	205.54 ± 30.91
20	0	—

Vegetative mycelia were cultured for 30 days at 24°C in PDL and partly modified matsutake liquid (PMML) medium.
Data represent the average of duplicates with six Erlenmeyer flasks (means ± S. D).

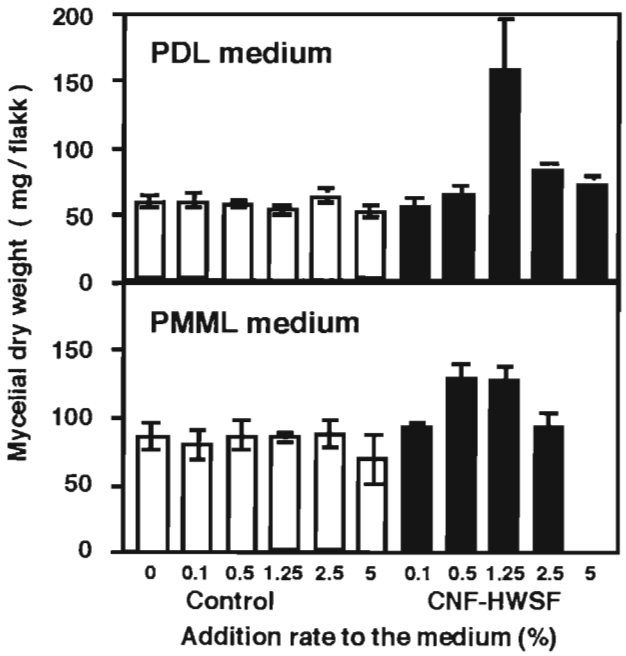


Fig. II-3. Promotive effect of CNF-HWSF on the vegetative mycelial growth of *Tricholoma matsutake*
The vegetative mycelia were cultured for 60 days at 24°C. Values means ± S. D. (n=6). *PMML*, partly modified matsutake liquid medium. For symbols refer to Fig. II-1.

The effects of CNF-HWSF on mycelial growth of *T. matsutake* are shown in Fig. II-3. The preliminary experiments examined the same concentrations (10% and 20%) of additives for this mushroom, but the hyphae did not grow with these CNF-HWSF concentrations. The effect of mycelial growth of *T. matsutake* was then tested again

at lower CNF-HWSF concentrations. As a result, the best promotive effect of CNF-HWSF was shown to be at a concentration of 1.25% (3.33 times that of the control) in PDL medium and at concentration of 0.5% (1.53 times) and 1.25% (1.51 times) in PMML medium.

Discussion

The effects of CNF-HWSF on mycelial growth of seven edible mushroom species were examined. The results showed that the mushrooms tested grew well when CNF-HWSF was added. The promotive effect of CNF-HWSF (10-20%) was more efficient on the slow-growing mushrooms such as *L. edodes* and *P. nameko* than in the rapid-growth species such as *F. velutipes* and *P. ostreatus*. However, mycelial growth was inhibited in the 30% of the CNF-HWSF sample of *L. edodes*. This effect was confirmed using mycorrhizal mushrooms (*L. shimeji* and *T. matsutake*). CNF-HWSF showed a stronger promotive effect on mycelial growth of these fungi in PDL medium than in PMML medium. It was thought that PMML medium is richer in nutrition for mycorrhizal species than PDL medium. Hence, these mushrooms did not necessarily utilize the CNF-HWSF (especially the macromolecular substances as nutritional substrate) in PMML medium. The increase in the mycelial dry weight of *T. matsutake* when 1.25% CNF-HWSF was added to the PDL medium can be explained by the promotive factor in the CNF-HWSF supplied.

In previous reports of growth-enhancing agents for mushrooms, lignin sulfonate of MW 1,000-2,000 daltons, which is fractionationed from sulfite pulp waste (Inaba et al. 1981, 1983), and lignin from commercial suppliers (Azuma and Kitamoto 1994) have demonstrated positive effects. Moreover, it has been recognized that lignin sulfonate is involved in the enhanced mycelial growth on *T. matsutake* (Inaba et al. 1993).

Generally, mushrooms use glucose as their primary carbon source; but when the glucose and nitrogen source concentrations are high in the medium, mycelial growth is inhibited (Azuma and Kitamoto 1994, Boyle 1998). The mycelial growth of *L. edodes* is also impeded by the presence of acetic acid, which is generated from beech

sawdust sterilized by autoclaving (Meguro et al. 1991). However, acetic acid enhances the hyphal growth on *G. frondosa*, *Wolfiporia cocos* and *Dendropolyporus umbellatus* (Mizutani 2000).

The effect of CNF-HWSF, as mentioned above, was greater on the slow-growing species. This result is similar to the reports from Terashita et al. (1997), who noted that the increased yield of fruit-bodies due to CNF supplement in sawdast-based cultivation was more remarkable on mushrooms that take a long time to cultivate. Kitamoto et al. (1971) reported that rapidly growing species rely on mycelium and medium nutrients, whereas slow-growing species depend almost entirely on nutrients in mycelia for fruit-body development. These differences of nutritional physiology were suggested to reflect mycelial growth.

Many studies have explored the relation between the mycelial growth and the carbon source of the medium. Azuma and Kitamoto (1994) reported that glucose, maltose and starch were the most suitable carbon sources for *L. edodes*, and fructose and sucrose regulated mycelial growth. They stated that the optimal glucose concentration was 2-3%. *Coriolus pubescens* and *L. tigrinus* are also reported to grow well on xylan and celluloses (avicel and CMC), and growth is almost the same as when glucose is added to the medium (Elisashvili et al. 1999). Moreover, in the case of *Volvariella volvacea*, when 13 carbon sources (e.g., glucose, starch and xylan) were added to the medium, only arabinose and sorbose could not be utilized (Cai et al. 1999).

CNF-HWSF is thought to contain numerous components which growth substrates for mushrooms because CNF includes protein, starch, arabinoxylan, cellulose and the other ingredients. Though it is also considered that the positive effect is shown for the growth of mushrooms by these components doing with the growth substrates, it is difficult to be considered that the so remarkable promoting effect only in this nutrient composition is obtained. It is thought that promoting component expect for the nutrition substrates may exist for CNF-HWSF.

Based on the results obtained here, it is thought that CNF-HWSF is sufficient for promoting vegetative mycelial growth of edible mushrooms. Further research is necessary concerning the isolation and characterization of promotive substances to reveal the mechanisms involved.

Summary

The effects of adding a hot water-soluble fraction (HWSF) from CNF to a medium on the vegetative mycelial growth of nine edible mushrooms such as *Lentinula edodes* and *Pholiota nameko* were investigated. The results showed that the mycelial growth of these fungi was markedly increased (1.4-9.5 times that of the control) by adding 5%-20% CNF-HWSF to the medium. These promotive effects were also showed on mycorrhizal mushrooms, such as *Tricholoma matsutake* (3.3-fold) and *Lyophyllium shimeji* (3.7-fold). The promotive actions were more effective on slow-growing mushrooms (*L. edodes* and *P. nameko*) than on rapidly growing mushrooms (*Pleurotus ostreatus* and *Flammulina velutipes*). The results obtained in this experiment suggest that CNF-HWSF can be used as a promotive substance for cultivating edible mushrooms.

Section 2. Rhizomorphs production on *Armillaria* genus by hot water soluble fraction from CNF

The vegetative mycelia of mushrooms were classified roughly into two groups by the difference of their organization and function. One is aerial hyphae which produced energy and the other is a submerged hyphae which has the abilities of decomposed and assimilated nutriment. It was difficult to distinguish the difference with visually in many mushrooms, but the mycelia of *Armillaria* genus on a synthetic medium such as potato dextrose agar medium is able to recognize between aerial hyphae and submerged hyphae that was called rhizomorphs. The term "rhizomorph" is means "having the form of a root".

In this section described the effect of CNF-HWSF on rhizomorph production of endomycorrhiza, *Armillaria mellea* and *A. tabescens*.

Material and Methods

Spawn strains

Armillaria mellea (axenic isolate from a commercial mushroom), *A. tabescens* (FPF-A. t. 9705 B and FPF-A. t. 0007 C; obtained from Fukuoka Pref. Forest Res.) were used in this study. These fungi were stored on potato dextrose agar (PDA) medium (Nissui Co.) in a test tube.

Preparation of CNF- HWSF

Corn fiber (100 g) was mixed with 1 L distilled water and extracted for 3 h at 80°C. After the residue was removed by centrifugation at 10,000 g (0°C, 10 min) the supernatant solution was concentrated at 40°C to 100 ml by a rotary evaporator.

Inoculations and culture conditions

The PDA medium was supplemented with 5%, 10%, 20% and 30% (v/v) CNF-HWSF, and dispensed in 13-ml aliquots in 20-ml test tube, before autoclaving at 121°C for 15min. As inoculum, a mycelial block of 5 mm square was cut from a

slant culture that had grown on a PDA medium for 30 days at 24°C in a test tube. The incubation was carried out at 24°C for 20 days.

Measurement of vegetative mycelial growth

After incubation, the agar in the medium was melted in boiling water and washed thoroughly with distilled water. Then aerial hypae and rhizomorphs were separated and their dried at 80°C for 24 h and dry weight measured after cooling in a desiccator.

Results

Effect of CNF-HWSF on the rhizomorphs productions of Armillaria mellea

Fig. II-4 illustrates the growth of *A. mellea* obtained with or without CNF-HWSF tested. The rhizomorphs of *A. mellea* were remarkably extended with CNF-HWSF. The aerial mycelial growth on the PDA medium was almost identical to the control at added CNF-HWSF. While, as shown in Fig. II-5, *A. mellea* produced abundant rhizomorphs when cultured on a PDA medium that is supplemented with 5%-20% of CNF-HWSF, and addition of 20% CNF-HWSF to the medium increased the rhizomorph dry weight to 133.52 mg/ test tube (control; 12.96 mg/test tube).

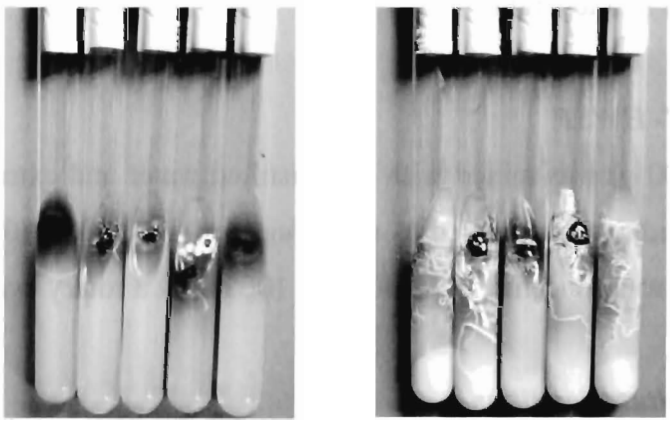


Fig. II-4. Effects of CNF-HWSF on mycelial growth of *Armillaria mellea*
The vegetative mycelia were cultured in PDA medium for 16 days at 24°C
(Left: control, right; 10% of CNF-HWSF was added).

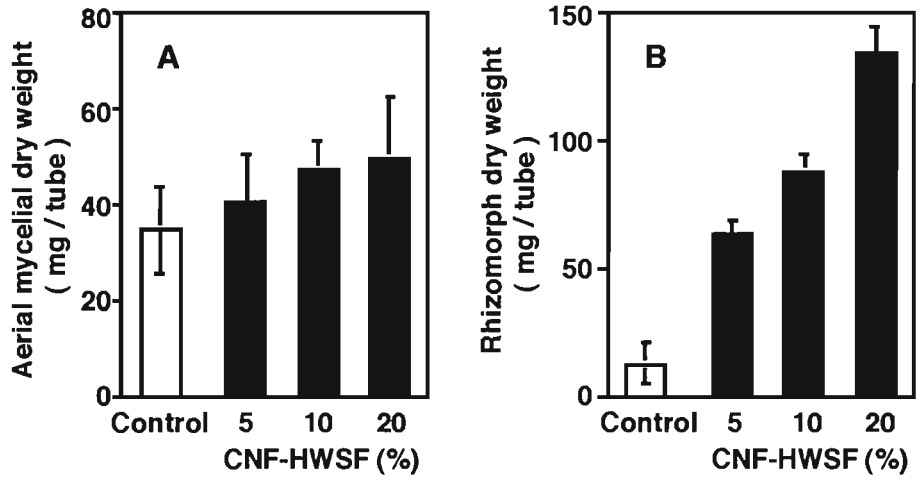


Fig. II-5. Effects of CNF-HWSF on aerial mycelial growth (A) and rhizomorph productions (B) of *Armillaria mellea*

The vegetative mycelia were cultured for 16 days at 24°C. Values are means ± S. D. (*n*=5). *Open bars*, potato dextrose agar (PDA) medium (control); *filled bars*, PDL medium added CNF-HWSF.

Effect of CNF-HWSF on the rhizomorphs productions of Armillaria tabescens

Table II-3. Effect of CNF-HWSF on aerial mycelial growth and rhizomorph production of *Armillaria tabescens*

Strain	Addition to the medium (%)	Dry weight of mycelium (mg / tube)	
		Aerial hypha	Rhizomorph
A. t. 9705 B	0	44.78 ± 11.98	24.05 ± 12.98
	5	47.90 ± 5.33	36.98 ± 5.50
	10	38.82 ± 9.41	74.85 ± 16.36
	20	36.66 ± 6.26	60.68 ± 15.62
	30	47.85 ± 6.17	96.50 ± 15.54
A. t. 0007 C	0	69.46 ± 14.84	9.10 ± 0.52
	5	69.92 ± 7.54	37.25 ± 15.41
	10	66.22 ± 14.33	41.84 ± 11.28
	20	61.82 ± 14.49	106.03 ± 22.14
	30	68.94 ± 12.96	70.40 ± 7.06

The vegetative mycelia were cultured for 21 days at 24°C in PDA medium.
Data represent averages of diplicate with 5 test tubes. means ± S. D. (n=5)

The promotive effect of CNF-HWSF on rhizomorphs production of *A. tabescens*, A. T. 9705-B and A. T. 0007-C is shown in Table II-3. When these fungi were cultured on PDA medium with CNF-HWSF added, the dry weight of rhizomorph was greater than that of the control, but no influence on aerial hypha was detected in two strains.

Discussion

A. mellea can be grown in culture on several media. The production of rihizomorphs in culture, however, has depended upon the presence of a complex substrate such as extract of malt, yeast, potato or wood of several tree species. This has prevented an investigation of the nutritional requirements for rhizomorph production. The determination of factors affecting their production is of particular interest since the rihizomorphs function in the penetration of host roots.

Previous reports has shown that *A. mellea* will grow vigorously and produce abundant rihizomorphs when cultured on a synthetic medium that is supplemented with relatively low concentrations of ethanol, propanol or butanol (Weinhold 1963), and the effects of these alcohols were appeared only at the existence of amino acids in the medium (Weinhold 1966). The growth-promoting effect of alcohol reported on *Fusarium solani* (Cohrane et al. 1963) and for certain aliphatic aldehydes and alcohols with several Hymenomycetes (Fries 1961).

The effectiveness of natural material in promoting growth and rhizomorph production by *A. mellea* is well known. Also, a substance produced by *Aureobasidium pullulans* has been shown to be very action in stimulating rhizomorph formation (Pentland 1965).

It is a common observation that many fungi supplied with a natural substrate respond by increased activities such as growth, sporulation, fruiting development and rhizomorph production. However, often the identity of the active ingredients and the mechanism of action are not knowm. As *A. mellea* and *A. tabescens* was pathogeneic fungi, an understanding of factors that affect the production and development of

rhizomorphs is also important.

Summary

The effect of CNF-HWSF on rhizomorph production of *Armillaria mellea* and *A. tabescens* in agar medium were examined. Although no growth-promoting affects of CNF-HWSF were recognized for aerial hyphae, when CNF-HWSF was present in PDA medium, the rhizomorphs were extended too much. From these results, CNF-HWSF was thought affecting the supply of the growth substrates of mushrooms.

CHAPTER III

Utilization of CNF-HWSF for Mushroom Cultivation

Section 1. Effect of CNF-HWSF on fruit-body development of *Lentinula edodes* and *Flammulina velutipes* in liquid medium

In chapter II, the author described that the hot water soluble fraction (HWSF) from CNF has a promoting effect on mycelial growth of various edible mushrooms (1.4-9.5 times that of the control) including mycorrhizal fungi such as *Tricholoma matsutake* (3.3-fold) and *Lyophyllium shimeji* (3.7-fold) (Arai et al 2003). In this section, effect of CNF-HWSF on the fruit-body formation of *Lentinula edodes* and *Flammulina velutipes* in a liquids medium were examined in laboratory scale with the object of utilizing of CNF and CNF-HWSF in practical culture application.

Material and Methods

Strains

Lentinula edodes (Mori No. 465) and *Flammulina velutipes* (IFO 7777) were used in this study. These fungal strains were transferred annually on PDA medium.

Culture media

The glucose-peptone-yeast extract liquid (GPYL) medium was used as the basic medium. It was composed of 50 g glucose, 2.5 g poly peptone, 2.5 g yeast extract, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.5 g $CaCl_2 \cdot 2H_2O$ in 1 liter distilled water and 20 ml of mineral solution (0.25 g $FeCl_2 \cdot 6H_2O$, 0.36 g $MnCl_2 \cdot 4H_2O$, 0.2 g $ZnCl_2$ and 0.05 g $CuSO_4 \cdot 5H_2O$ / L) was added.

Preparation of CNF- HWSF

Corn fiber (100 g) was mixed with 1L distilled water and extracted for 3 h at

80°C. After the residue was removed by centrifugation at 10,000 g (0°C, 10 min) the supernatant solution was concentrated at 40°C to 100 ml by a rotary evaporator.

Inoculations and culture conditions

The GPYL medium was supplemented with 10% (*F. velutipes*), 20 % (*L.edodes*) CNF-HWSF, and dispensed in 50-ml aliquots in a tall Petri dish (diameter 11 cm× 7cm), before autoclaving at 121°C for 5 min. For the mycelial inoculum, a mycelial block (diameter 10 mm) was cut from a plate culture which had been grown on a PDA medium at 24°C in a Petri dish (diameter 90 mm) for 14 days. The mycelial cultures were incubated statically for 23 days (*L. edodes*) or 16 days (*F. velutipes*) at 24°C, and they were then placed at 15°C to induce fruit-body formation. In another set of experiments in *L. edodes*, cultures were subjected to fructification at 16 days because mycelial growth was enhanced by the CNF-HWSF.

Results and Discussion

Effect of CNF-HWSF on fruit-body formation of Lentinula edodes

Fig. III-1 shows the picture of *L. edodes* at 16 days and 56 days after inoculation. The days required for reproduction growth period of *L. edodes* on with or without CNF-HWSF were 16 or 23 days, respectively. The cultivation process from low temperature treatment to fruit-body formation on the medium with CNF-HWSF were shortened about 7 days than that of the control, the total days to crop harvest were 56 days on the addition of CNF-HWSF and 70 days on the control. While, the cultivation period for fruit-body formation of the other medium with CNF-HWSF that temperature shift down were occurred at the same times as the control was in need of 70 days. This result suggested that the CNF-HWSF has positive effect not only vegetative mycelial growth stage but also on the fruit-body development process.

Table III-1 shows the effects of CNF-HWSF on fruit-body development of *L. edodes*. Fresh fruit-body weights on liquid medium with added CNF-HWSF of *L. edodes* increased to about 2-3 times than that of the control, and 80% of the culture

dish supplemented with CNF-HWSF were fruit-body formation as against 40% in the control. It was indicated that fruit-body formation induced by adding CNF-HWSF.

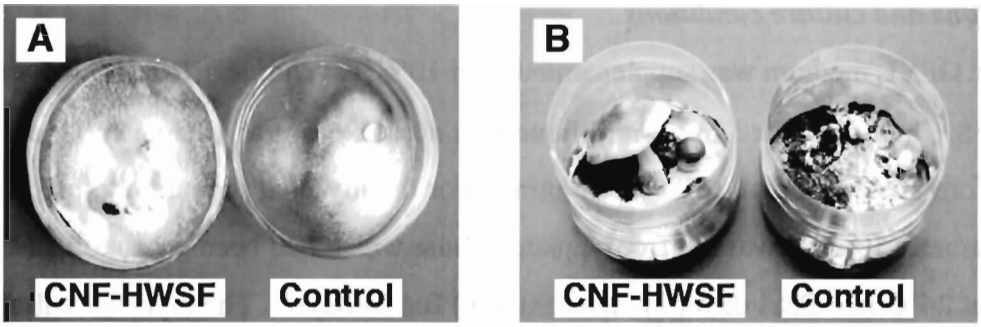


Fig. III-1. Mycelial growth (A) and fruit-body development (B) of *Lentinula edodes* on a liquid medium with or without CNF-HWSF
The days of the pictures were indicated at 16 days (A) and 56 days (B) after inoculation, respectively. Fructification was induced at 16 days (CNF-HWSF) or 23 days (control).

Table III-1. Effect of CNF-HWSF on the fruit-body development in liquid medium on *Lentinula edodes*

	Number of culture dish	Number of fruiting dish	Total yield of fruit-bodies (g)	Yield (g / dish)
Control	10	4	39.42	3.94 (1.0)
CNF-HWSF				
16 days	10	8	103.20	10.32 (2.6)
23 days	10	9	79.22	7.92 (2.0)

The vegetative mycelial growth were cultured for 23 days at 24°C.
Fructification was induced at 16 and 23 days after inoculation.
The yield of fruit-body represented fresh weight.

Also, to research for what stage appeared the effect of CNF-HWSH, we examined to the addition of CNF-HWSF to the medium at various stages. The resulting was shown in Table III-2. The crop of fruit-bodies and rate of fruit-body development increased only when CNF-HWSF was added at the stage of primordia

except for addition at before inoculation. No influence on the cultivation period for fruit-body formation were observed when the supplemented with CNF-HWSF at the stage of mycelial growth, temperature shift down treatment and primordia. Mushrooms are in need of many nutritional components at fruit-body development. It was reported to the fruit-body was formed after almost the carbon sources in the medium were consumed in *F. velutipes* (Kitamoto et al. 1976) and *Coprinus atramentarius* (Robert 1977), suggesting that these fungi might make use of the components in mycelium for fruit-body development. Although, Yoshida et al. (1986) were reported to the both constituent of mycelium and medium were utilized for fruit-body formation because the fruit-body weight were higher than decrease of mycelium weight and the quantity of glucose and nitrogen source in the medium decreased during developmental period on *P. ostreatus*. In this experiment, the increase of fruit-body weights was confirmed at not only before inoculation but also CNF-HWSF was added to the primordial formation stages. This result was suggested that CNF-HWSF components also acted as nutritional substrates for the fruit-body formation.

Table III-2. The influence of CNF-HWSF on fruit-body formation at different addition phases on *Lentinula edodes*

	Number of culture dish	Number of fruiting dish	Total yield of fruit-bodies (g)	Yield (g / dish)
Control	10	5	33.30	3.33 (1.00)
CNF-HWSF				
A	10	9	120.83	12.83 (3.85)
B	10	5	31.84	3.18 (0.96)
C	10	4	40.16	4.16 (1.25)
D	10	8	139.33	13.93 (4.18)

The vegetative mycelia were cultured for 23 days at 24°C. Symbols were indicated phase of supplemented CNF-HWSF: A; before inoculation (0 days), B; mycelial growth (13 days), C; inducement of fryiting (23 days), D; primordia formation (35 dayd). Fructification of symbol A was induced at 16 days after inoculation. The yield of fruit-body represented fresh weight.

Effect of CNF-HWSF on fruit-body formation of Flammulina velutipes

The same experiment as *L. edodes* was carried out using *F. velutipes*. As shown in Table III-3, the fruit-body yield with CNF-HWSF were increased about 1.5 times as against the control, but the cultivation process for fruit-body formation of the medium with CNF-HWSF was late than that of the control. In *F. velutipes*, the inducement of fruit-body formation as to all dishes was performed at the same time, because both mycelial growths with or without CNF-HWSF were almost the same on visually. However, the mycelial dry weight of with CNF-HWSF and control at 16 days after inoculation on *F. velutipes* were about 870.55 mg and 442.25 mg, respectively. Judging from the influence on difference of low temperature treatments was observed in *L. edodes*, it seems that the medium with CNF-HWSF on *F. velutipes* were also in need of treatment for induce of fruit-body at early times.

Table III-3. Effect of CNF-HWSF on the fruit-body formation in liquid medium on *Flammulina velutipes*

	Number of culture dish	Number of fruiting dish	Total yield of fruit-bodies (g)	Yield (g / dish)	Days required for fruiting
Control	10	9	11.32	1.13 (1.0)	30
CNF-HWSF	10	8	14.58	1.46 (1.3)	33

The vegetative mycelia were cultured for 23 days at 24°C.
Fructification was induced at 23 days after inoculation.
The yield of fruit-body represented fresh weight.

Summary

The effects of CNF-HWSF on fruit-body developments in liquid medium were tested using *L. edodes* and *F. velutipes*, which has been able to be made fruit-body

development in liquid medium easy. Growth-promoting effect of CNF-HWSF indicated not only the period in mycelial growth but also fruit-body formation phases. A bioactive substance for fruiting was existed in CNF-HWSF because of fruit-body developments of *L. edodes* were obviously increased on the medium supplemented CNF-HWSF than that of the control.

Section 2 Utilization of CNF-HWSF for sawdust based cultivation of *Pleurotus ostreatus*

Commercial production of most mushrooms is on synthetic substrate contained in bottles. The main material used for mushroom production is sawdust from mill yard. Rice bran, wheat bran and corncob meal are widely used as nutrient supplements. The rapid increase of mushroom production in Japan has focused the need to develop more efficient substrate formulas to improve yield and to shorten the crop cycle.

In section 1, the author described that the CNF-HWSF was proved to be useful in the formation and yield of the fruit-body of *Lentinula edodes* and *Flamulina veltipes* in glucose peptone yeast-extract liquid medium.

The present section deals with the effect of CNF-HWSF as nutrient supplements on the fruit-body formation of *Pleurotus ostreatus* in sawdust-based cultivation.

Materials and Methods

Strains

Pleurotus ostreatus (Kitamura; obtained from Kin-ki Nyugyo Co.) were used in this experiment. This fungus was stored on potato dextrose agar (PDA) medium (Nissui Co.) in a test tube.

Preparation of CNF- HWSF

Corn fiber (100 g) was mixed with 1 L distilled water and extracted for 3 h at 80°C. After the residue was removed by centrifugation at 10,000 g (0°C, 10 min) the supernatant solution was concentrated at 40°C to 100 ml by a rotary evaporator.

Culture media

For mushroom production substrate, ingredients (soft wood and rice bran) were

mixed in the ratio of 4:1 (g/g) and water was added to raise final moisture content to 70%. The mixed substrate was supplemented with 0.5 g, 1.0 g, 2.0 g and 3.0 g freeze-dried CNF-HWSF per a bottle, and the mixtures 130 g were packed into a 200 ml capacity glass bottle. A hole of diameter 1.5 cm and depth 7 cm was made at the center of the upper surface of substrate. The bottles were sterilized at 121°C for 30 min.

Culture condition

As inoculum, a test tube of mycelia for a bottle were cut from a slant culture that had grown on a PDA medium for 14 days at 24°C in a test tube. When the mycelia were grown throughout in the bottle, the surface of mycelia was removed (*Kinkaki*). Then the colonized substrate in the bottle was poured into water (2 h). Then fruit-body formation was performed at 16°C, relative humidity 70% and 350 lux illumination.

Evaluation of fruit-bodies

Fruit-body yields were measured as fresh weight grams, each average and standard division (SD) were calculated.

Results and Discussion

Effect of CNF-HWSF on sawdust-based cultivation of Pleurotus ostreatus

Table III-4 shows the effect of CNF-HWSF on fruit-body formation of *P. ostreatus* in sawdust-based medium. Fresh fruit-body weights on the medium with added 1.0 g and 2.0 g CNF-HWSF of *P. ostreatus* increased to about 1.3 times than that of the control, and 90-100% of the culture bottle supplemented with 0.5-2.0 g CNF-HWSF were fruit-body formation as against 70% in the control. But at 3.0 g of supplement the results were almost identical to the control.

Table III-4. Effect of CNF-HWSF on the fruit-body development in sawdust-based medium of *Pleurotus ostreatus*.

Addition to the medium (g)	Number of culture bottle	Number of fruiting bottle	Total yield of fruit-bodies (g)	Yield (g / bottle)
Control	10	7	79.39	7.94 (1.0)
0.5	10	9	82.81	8.28 (1.0)
1.0	10	9	103.43	10.34 (1.3)
2.0	10	10	100.7	10.07 (1.3)
3.0	10	7	78.94	7.89 (0.9)

The vegetative mycelia were cultured for 23 days at 24°C.
The yield of fruit-body represented fresh weight.

Summary

Pleurotus ostreatus was examined fruit-body production on sawdust-based substrate supplemented with CNF-HWSF. The yield of fruit-bodies and fruiting ratio increased by supplemented with CNF-HWSF. CNF-HWSF is useful as supplement for sawdust-based cultivation of *P. ostreatus* in producing the fruit-body which increases yield.

CHAPTER IV
Growth-Promoting Mechanisms on Edible Mushrooms by CNF-HWSF

Section 1. Chemical analysis of CNF-HWSF and the effect of each fraction from CNF-HWSF on the vegetative mycelial growth of mushrooms

In chapter II and III, the author described that mycelial growth and fruit-body development of edible mushrooms promoted with CNF-HWSF. Mycelial growth and fruit-body development of mushrooms were affects by several nutriments (carbon and nitrogen source and the others) in the medium. Many studies have explored the relation between the mycelial growth and the carbon source of the medium. Azuma and Kitamoto (1994) reported that glucose, maltose and starch were the most suitable carbon sources for *L. edodes*, and fructose and sucrose regulated mycelial growth. *Coriolus pubescens* and *L. tigrinus* are also reported to grow well on xylan, avicel and CMC (Elisashvili et al. 1999). Moreover, Cai et al. (1999) described that the only arabinose and sorbose could not be utilized in *Volvariella volvacea* when 13 carbon sources (e.g., glucose, starch and xylan) were added to the medium. Also, a few study about the effect of nitrogen source for growth of fungi were reported, that had complex nitrogen source was suitable for growth on mushrooms than simple nitrogen source (Boyle 1998). CNF-HWSF is also thought to contain nutritious components for growth of mushrooms because of the CNF is natural material.

In the present section, the author investigated its fractionation by organic solvent and molecular weight and by a chemical analysis that revealed the promotive substances of CNF-HWSF. Also, the growth-promoting activity of CNF-HWSF component(s) on the mycelium of *L. edodes* under the condition in the absence of carbon or nitrogen source was examined using the synthetic medium.

Materials and Methods

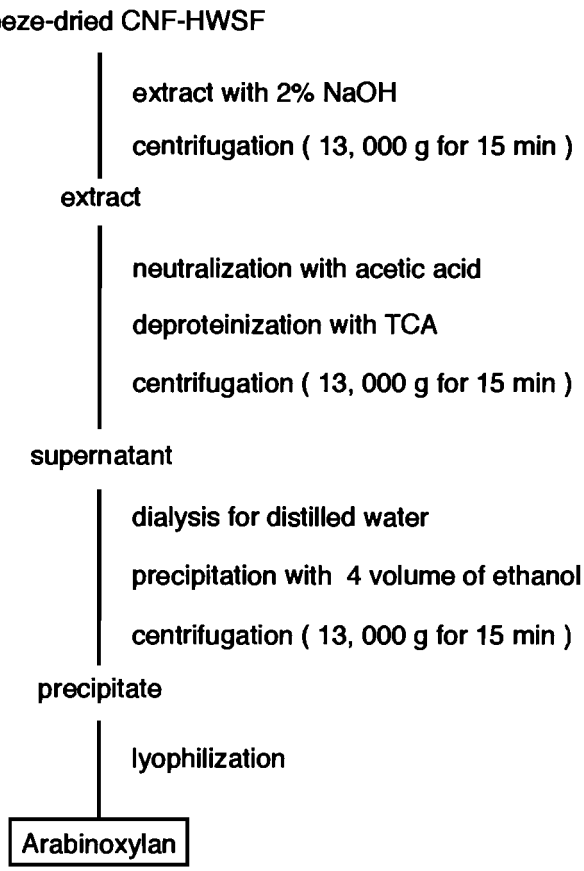
Strains

Lentinula edodes (Mori No. 465), *Hypsizygus marmoreus* (Takara No. 1), *Pleurotus ostreatus* (Kitamura; obtained Kin-ki Nyugyou Co.), *Flammulina velutipes* (IFO 7777), were used in these experiments. These fungi were stored on potato dextrose agar medium (Nissui Co.) in a test tube.

Preparation of CNF- HWSF

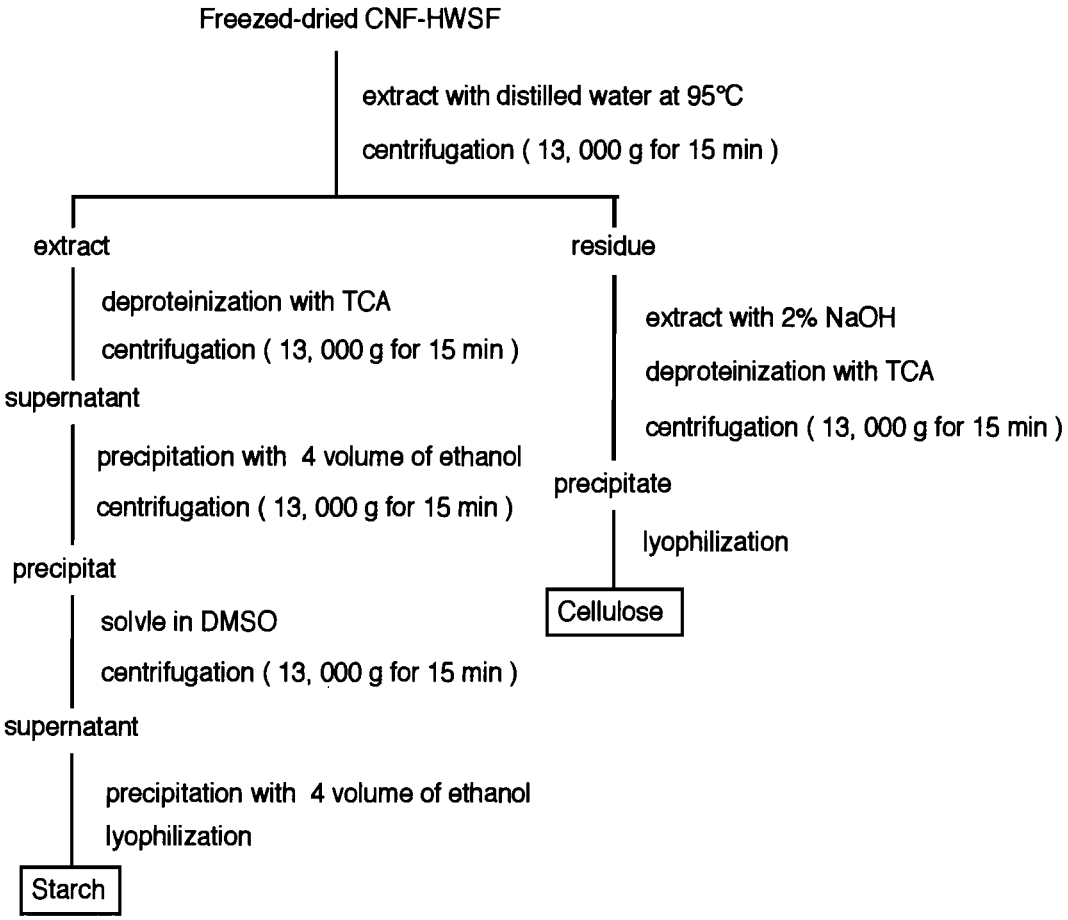
Corn fiber (100 g) was mixed with 1 L distilled water and extracted for 3 h at 80°C. After the residue was removed by centrifugation at 10,000 g (0°C, 10 min) the supernatant solution was concentrated at 40°C to 100 ml by a rotary evaporator.

Fractionation of polysaccharides from CNF-HWSF



Scheme IV-1. Fractionation of arabinoxylan from CNF-HWSF

As shown in Scheme IV-1, arabinoxylan from the CNF-HWSF was isolated by the methods of Takeuchi (1997). To 3.5 g freeze-died CNF-HWSF (this weight is equivalent to 100 ml of CNF-HWSF) was added 50 ml of 2% NaOH. The extraction was performed at room temperature for 18 h. After removing the residues by centrifugation at 13,000 g, 5°C for 15 min, the supernatant solution was neutralized with 1N acetic acid, and trichloroacetic acid (TCA) was added to make the final concentration 7% for deproteinization. The solution was dialyzed for 3 days at 4°C and added to 4 volumes of 99.5% ethanol. The precipitate was lyophilized.



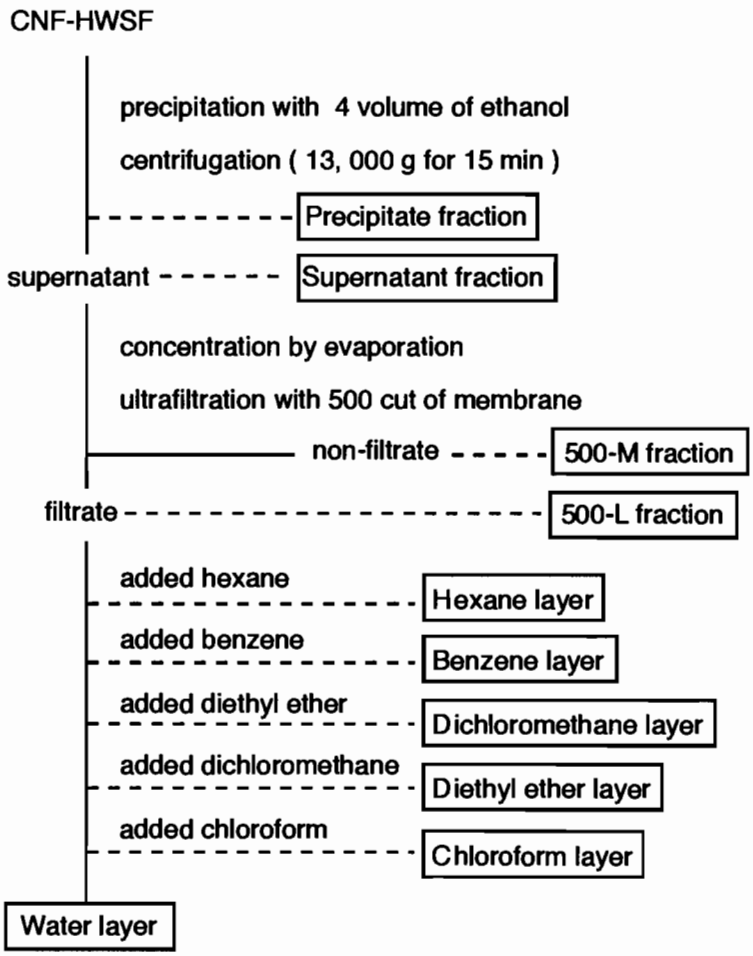
Scheme IV-2. Fractionation of starch and cellulose from CNF-HWSF

The preparation of cellulose was performed as follows: Dried CNF-HWSF 3.5 g was washed with 50ml of distilled water at 90°-95°C for 3 h and with 50 ml of 2% NaOH at room temperature for 18 h. This manipulation was repeated three times, and

the collected precipitate was then lyophilized.

Starch was obtained in a manner similar to the preparation of cellulose: 3.5 g of CNF-HWSF was extracted with 50ml of distilled water at 90°-95°C for 3 h. The extract solution obtained by filtration was deproteinized with TCA solution and then added to 4 volumes of methanol. The precipitate was dissolved in 20 ml of dimethylsulfoxide (DMSO) and the insoluble component was removed by centrifugation (13,000 g, 25°C, 15 min). The starch was then reprecipitated in 4 volumes of ethanol (Scheme IV-2).

Fractionation of promoting component in CNF-HWSF



Scheme IV-3. Fractionation of CNF-HWSF

The preparations of crude promoting components from CNF-HWSF were shown in Scheme IV-3. Four hundred ml of ethanol was added to 100 ml of CNF-HWSF,

and left overnight. The sample was separated by centrifugation (0°C, 15 min at 13,000 g) into the supernatant and the precipitate. The ethanol was removed by distillation from water, the mixture solution was further separated into MW 500 (molecular weight) or less and more fractions by molecular sieving using ultrafiltration membranes (YC 05, Amicon Co. Ltd.). Then the low-molecular-weight (MW 500 daltons or less) fraction was extracted successively two times with hexane, benzene, diethyl ether, dichloromethane and chloroform of twice the volume of 500-L fraction solution, and respective organic layers and water layer were obtained.

Gel filtrate chromatography

One ml of water layer, which concentrated the quantity by 20 times, was applied to a Sephadex G-10 (Amersham Pharmacia) column (1.5×100 cm) equilibrated with distilled water. The column was eluted by distilled water at flow rate of 1 ml/min and fractions of 3 ml were collected. The obtained solution was made a qualitative and quantitative analysis about sugars and amino acids by Somogyi-Nelson method, HPLC and ninhydrin reaction, and was separated five fractions for assay as follows: 1-20, containing oligosaccharides (A); 21-24, containing oligosaccharides (B); 25-34, containing monosaccharides and amino acids (C); 35-60, no-containing monosaccharides and amino acids (D); 60-80, non-containing monosaccharides and amino acids (E).

Culture media

Potato-dextrose liquid (PDL) medium consists of potato extract (200 g boiled in 500 ml distilled water), 15 g glucose and 1 mg thiamine hydrochloride per liter of distilled water.

The basic medium was composed of 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.5 g $CaCl_2 \cdot 2H_2O$, in 1 liter distilled water and 20 ml of mineral solution (0.25 g $FeCl_2 \cdot 6H_2O$, 0.36 g $MnCl_2 \cdot 4H_2O$, 0.2 g $ZnCl_2$ and 0.05 g $CuSO_4 \cdot 5H_2O$ / L) was added. Glucose and ammonium tartrate were added to give 50 g / liter and 5.0 g / liter, respectively.

Inoculations and culture conditions

The PDL medium was supplemented with 10% or 20% (v/v) CNF-HWSF or separated each fractions from it, soluble starch (Kanto Chem.), corn starch (Wako Pure Chem.), CMC (Wako Pure Chem.), avicel (Asahikasei Co. Ltd.), and xylan (Nakalai tesq.) solution of the same concentration as that of CNF-HWSF.

Mycelial growth of *L. edodes* by basic medium supplemented with fraction D (this fraction obtained from gel filtrate chromatography, and that has no sugars and amino acids) was tested with and without glucose as a carbon source or ammonium tartrate as a nitrogen source.

These medium were dispensed in 16 ml aliquots in 100 ml Erlenmeyer flasks, before autoclaving at 121°C for 10 min. As inoculum, a mycelial block (diameter 5 mm) was cut from a plate culture that had grown on a PDA medium for 14 days at 24°C in a Petri dish (diameter 90 mm). The incubation was carried out at 24°C for 15 days.

Measurement of vegetative mycelial growth

The vegetative mycelia after incubation were separated from the medium by filtration and washed thoroughly with distilled water, after which they were dried at 80°C for 24 h. Their dry weight was measured after cooling in a desiccator.

Chemical analysis of CNF-HWSF

The concentration of reducing sugars in the CNF-HWSF was analyzed by the Somogyi-Nelson method (Somogyi 1952) using a calibration curve obtained with glucose as a standard. Free amino acid was estimated using the ninhydrin reaction with leucine as standard. The crude protein was determined in freeze-dried CNF-HWSF by the micro- Kjeldahl method (Kamiya M 1992).

The reducing sugars were analyzed and characterized using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The TLC analysis was carried out with a Silicagel G chromatography plate (Analtech) at room temperature. Chloroform and methanol (6:4) were the developing solvents. The plate after development was sprayed with a dyeing reagent of 20% sulfuric acid in

the methanol solution, and kept at 110°C for 10-15 min. HPLC was performed using a Shimadzu type LC-10AS and a RID-10 differential refractive index detector. The separation by HPLC was performed in an Ultron PS-80P column (Shinwa Chemical Industries, 300×8 mm) using Milli-Q water as a development eluent at a flow rate of 1.0 ml/min and 80°C. Arabinose, xylose, galactose and glucose were used as internal standards. The qualitative analysis of amino acids was performed with an amino acid automatic analyzer (Hitachi 8500 Type).

Results

Chemical analysis of CNF-HWSF

Table IV-1 shows the results of the chemical analysis of the components from CNF-HWSF. The protein, free amino acid, and reducing sugar contents were 0.6%, 0.2% and 0.4%, respectively.

Table IV-1. Chemical components of CNF-HWSF

parameter	Content (mg / 100 ml) [a]		
	Protein	Free amino acid	Reducing sugar
CNF - HWSF	559.8	225.9	425.3
Supernatant obtained from ethanol treatment	—	219.7	330.0
Ultrafiltration [b]			
500-L	—	198.0	326.3
500-M	—	10.2	14.1

Protein, free amino acid, and reducing sugar contents were analyzed by the micro-kjeldahl method, ninhydrin method, and Somogyi-Nelson method, respectively.
500-L, MW ≦500 daltons fraction; 500-L MW >500 fraction; MW, molecular weight.
[a]The amount was converted to 100 ml (dry weight 3.5 g) CNF-HWSF.
[b]Ultrafiltration was done using membrane YC 05, (Amicon Co.) for the supernatant after the ethanol treatment.

Fig. IV-1 shows the resulting of analysis of reducing sugars by high performance liquid chromatography (HPLC), and amino acids analysis in CNF-HWSF with amino acid automatic analyzer is shown in Fig. IV-2. Also, the characterization of polysaccharides, amino acids and reducing sugars is shown in Table IV-2. CNF-HWSF contained 0.82 g starch, 0.70 g arabinoxylan and 0.07 g cellulose. The main components of the reducing sugars were glucose (126.78 mg), arabinose (123.74 mg) and xylose (75.48 mg). Among the amino acids, the fraction was rich in leucine.

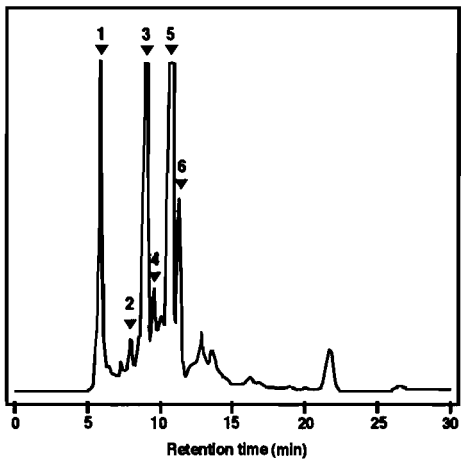


Fig. IV-1. HPLC analysis of CNF-HWSF
Ten microliters of sample was subjected to HPLC analysis as described in Materials and Methods. The elution tomes of the standards oligosaccharides (1), disaccharides (2), glucose (3), Xylose (4), arabinose (5), and fructose (6) are indicated.

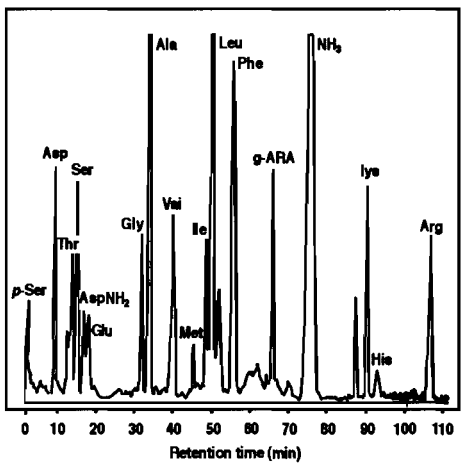


Fig. IV-2. Free amino acids analysis of CNF-HWSF
Five microliters of sample was subjected to analysis as described in Materials and Methods.

Table IV-2 Main components of polysaccharides, free amino acids, and reducing sugars in CNF-HWSF.

Component	Amount [a]
Polysaccharides (g) [b]	
Starch	0.82
Arabinoxylan	0.70
Cellulose	0.07
Reducing sugars (mg) [c]	
Glucose	126.78
Arabinose	123.74
Xylose	75.48
Free amino acids (mg) [d]	
p-serine	3.9
Asparagine	4.0
Threonine	3.2
Serine	3.2
Glutamic acid	3.3
Alanine	8.3
Valine	5.7
Isoleucine	3.6
Leucine	15.2
Tyrosine	5.8
Phenylalanine	1.4
Arginine	6.8

[a] Amount converted to 100ml (dry weight 3.5g) CNF-HWSF.
[b] Polysaccharides are reported as dry weight of component fractionated from CNF-HWSF.
[c] Reducing sugars were measured with thin-layer chromatography and high-performance liquid chromatography.
[d] Free amino acids were determined by amino acid automatic analyzer.

Effect of each fractions from CNF-HWSF on the vegetative mycelial growth of Lentinula edodes

After CNF-HWSF was separated into components by ethanol treatment and ultrafiltration, the promotive effect of the fractions on mycelial growth were examined. The results are shown in Fig. IV-3. The promotive effect was indicated in the supernatant solution after ethanol treatment and in low-molecular-weight components after ultrafiltration (500-dalton membrane). There were no effects when starch, cellulose, arabinoxylan fraction or chemical reagents were added.

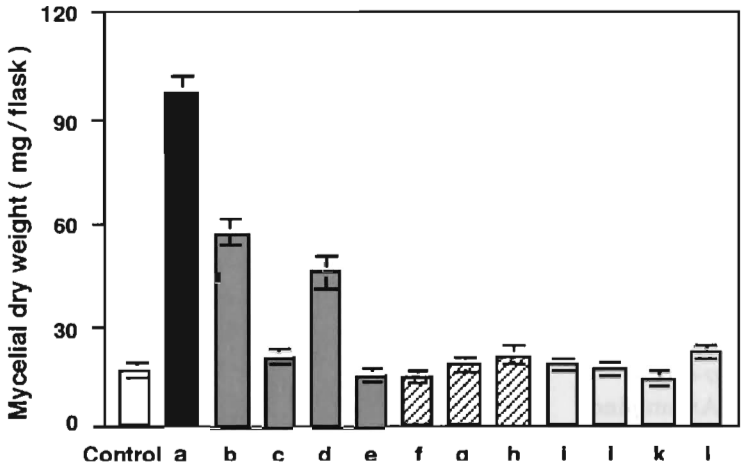


Fig. IV-3. Effect of CNF-HWSF components on the vegetative myceliak growth of *Lentinula edodes*

The vegetative mycelia were cultured for 15 days at 24°C in PDL medium with added CNF-HWSF (20%). Values are means ± S. D.. a, CNF-HWSF; b, supernatant of ethanol treatment; c, precipitate of ethanol treatment; d, 500-L by ultrafiltration; e, 500-M by ultrafiltration; f, g and h, starch, cellulose and xylan isolated polysaccharide from CNF-HWSF, respectively. I, j, k and l, commercial sample, soluble starch, corn starch, avicell and xylan, respectively. MW, molecular weight; 500-L, MW ≦ 500 fraction; 500-M > 500 fraction.

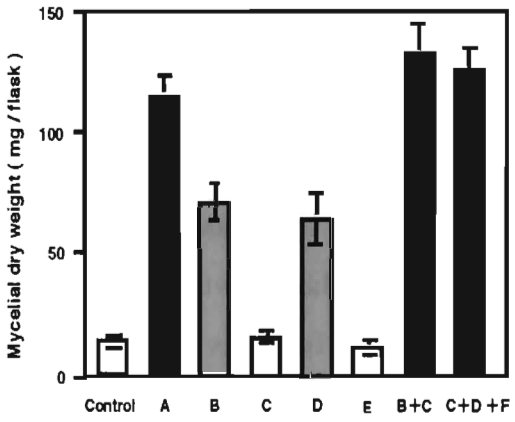


Fig. IV-4. Influence of CNF-HWSF components on the vegetative mycelial growth of *Lentinula edodes*

The vegetative mycelia were cultured for 15 days at 24°C in PDL medium with added CNF-HWSF (20%). Values are means ± S. D.. A, CNF-HWSF; B, supernatant after ethanol treatment; C, precipitate after ethanol treatment; D, 500-L by ultrafiltration; E, 500-M by ultrafiltration. MW, molecular weight; 500-L, MW ≦ 500 fraction; 500-M > 500 fraction.

To examine the promotive components of CNF-HWSF, mixture of two fractions (c and d) or three fractions (d, e and f) from CNF-HWSF were added again to the PDL medium; and *L. edodes* mycelia were then cultured. The results indicated that the

promotive effect on the vegetative mycelial growth recovered to the most effective level, corresponding to that of the CNF-HWSF (Fig. IV-4). These results suggested that the enhanced ingredients contained in CNF-HWSF were dispersed by fractionation.

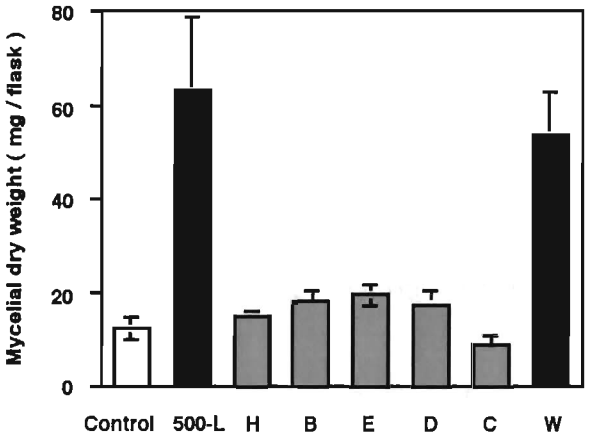


Fig. IV-5. Effect of each fraction from CNF-HWSF with organic solvent extracts on the mycelial growth of *Lentinula edodes*
The vegetative mycelia were cultured for 15 days at 24°C in PDL medium with added CNF-HWSF (20%). Values are means±S. D. (n=5). H, hexane; B, benzene; E, diethyl ether; D, dichloromethane; C, chloroform; W, water layer.

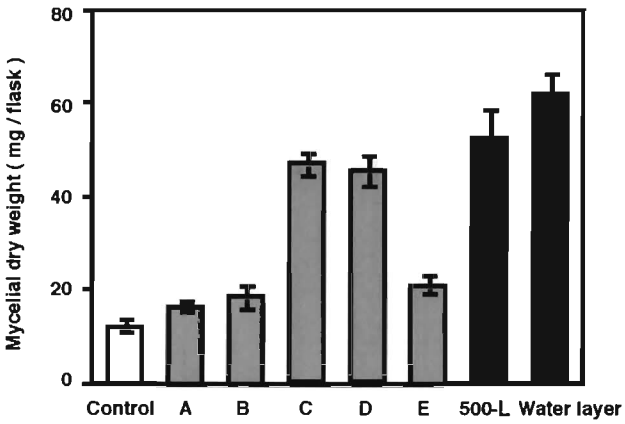


Fig. IV-6. Influence of the gel filtration fraction from CNF-HWSF components on the vegetative mycelial growth of *Lentinula edodes*
The vegetative mycelia were cultured for 15 days at 24℃ in a PDL medium added to each fraction (20%; 500-L and water layer, 40%; gel filtration samples). Values are means ± S. D. (n=5). Symbols indicate fraction No. by gel filtration using Sephadex G-10 (A: 1-20, B: 21-24, C: 25-34, D: 35-60 and E: 60-80). Experimental conditions of chromatography are described in Materials and Methods.

Also, to search for the promoting components of a 500-L fraction, the crude product was extracted with organic solvents. As the result, only the water layer removing an organic solvents soluble components from 500-L fraction, was included the promoting component (Fig. IV-5). Then the water layer was fractionated by gel filtration on Sephadex G-10 column, the effect of these fractions on the mycelial growth of *L. edodes* assay. The results are shown in Fig. IV-6. The fraction C and D obtained gel filtrate fractionation were increased the mycelial growth of *L. edodes*. The fraction C included reducing sugars and amino acids, while these were not detected in the fraction D.

Effect of D fraction from CNF-HWSF on mycelial growth of Lentinula edodes in synthetic medium

The author indicated that the D fraction by gel filtration has an enhanced component(s) except for sugars and amino acids. To investigate the growth-promoting effect of D fraction on *L. edodes*, the synthetic medium with and without glucose as a carbon source and ammonium tartrate as nitrogen source was used. The result was shown in Fig. IV-7. When the medium was supplemented with D fraction, glucose supported excellent mycelial growth of *L edodes*. However, when only D fraction or nitrogen + D fraction were present in the medium, mycelial growth was no enhanced.

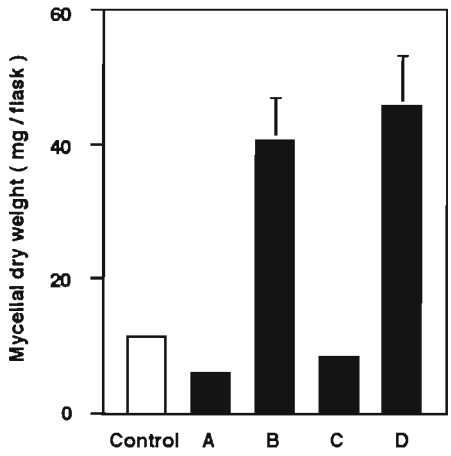


Fig. IV-7. Effect of D fraction on mycelial growth of Lentinula edodes. The vegetative mycelia were cultured for 15 days at 24°C in synthetic medium with added D fraction (40%). Values are means ± S. D. (n=5). Control, basic medium with glucose and ammonium tartrate; A, with D fraction; B, with D fraction and gluzose; C with D fraction and ammonium tartrate; D, with D fraction, glucose and ammonium tartrate

Discussion

Hemicellulose is a major component of CNF (about 50%); and as an arabinoxylan, it is a typical component of graminaceous monocot plants (Wolf et al. 1953, Hromádková et al. 1987). Arabinoxylan is a complex polymer consisting of β -1,4-linked xylosyl residues, which can be acetylated or have covalently linked arabinosyl and glucuronic acid side groups attached (Ebringerová et al. 1995). The sugar composition of the hemicellulose in CNF has been determined by several groups (Whistler and Bemiller 1956, Sugawara et al. 1994, Saulnier et al. 1995). The constitution reported for each sugar was as follows: D-xylose (48-54%), L-arabinose (33-35%), galactose (5-11%) and D-glucronic acid (3-6%), although here is considerable variation between research groups. Hemicellulose has a water-insoluble fraction (hemicellulose A) and a water-soluble fraction (hemicellulose B); fraction A has few side chains, whereas fraction B has many (Egashira 1999).

It is reported that physiological activities of soluble polysaccharide is higher than that of the insoluble fraction (Ebringerová et al. 1995). The hemicellulose of CNF has been linked to intestinal disorders and the regulation of cholesterol; and these effects are stronger in the soluble component than in the insoluble component (Takeuchi 1997, Egashira 1999). Ebringerová et al. (1995) reported that soluble arabinoxylan has a role in immunological control. They noted that the principal factor is a disaccharide (2-*O*- β -D-Xylp- α -L-Alaf) found in arabinoxylan alone.

The natural material CNF is thought to contain numerous components. The major constituents of CNF-HWSF extracted with hot water were protein, starch and arabinoxylan, which are sufficient growth substrates for mushrooms. It was shown that the promotive effects on mycelial growth of edible fungi were shown in low-molecular-weight fractions, which contained reducing sugars and free amino acids (MW 500 daltons or less) prepared from CNF-HWSF. On the other hand, the polysaccharide fractions fractionated from CNF-HWSF and the commercially available reagents used in our studies have not been linked to increased mycelial growth. A marked promotive effect of CNF-HWSF probably arises from components

other than the carbon sources because the low-molecular-weight carbohydrates accounted for only 0.4% of the fraction. The author expected that this promotive effect on the mycelial growth of mushrooms were due to the complex effects caused by the CNF-HWSF ingredients. Based on this experiment were approximately equal to that of the CNF-HWSF fraction when all the fractions from CNF-HWSF were pooled, added to PDL medium, and cultured. The promotive effect of the polysaccharide fraction is shown only when this fraction coexists with the low-molecular-weight fraction. The CNF-HWSF in this experiments seem to influence significantly the metabolic pathway concerning substrate utilization for vegetative growth in mushrooms.

Summary

To search for the promoting component in CNF-HWSF, chemical analysis of sample was performed. CNF-HWSF contained 0.6% protein, 0.2% free amino acid and 0.4% reducing sugar, and starch, arabinoxylan and cellulose contents were 0.8 g, 0.7 g and 0.07 g, respectively. There were no effects for mycelial growth when starch, cellulose, arabinoxylan fraction or their degradation components and their chemical reagents were added. The promoting effects on mycelial growth were shown in the low-molecular-weight fraction prepared from CNF-HWSF except for reducing sugars and amino acids.

Section 2. The stimulation of extracellular carbohydrases of edible mushrooms by hot-water extract from corn fiber

In chapter I, the author have shown that CNF is effective in producing the fruit-body of edible mushrooms that increases yield, shortens the cultivation period and increases the quality of mushrooms. Also, the hot water soluble fraction (HWSF) from CNF has a promoting effect on the mycelial growth of various edible mushrooms (1.4-9.5 times that of the control) by adding 5%-20% CNF-HWSF to the medium were described (chapter II). Their promoting effects were also apparent on mycorrhizal mushrooms, such as *Tricholoma matsutake* (3.3-fold) and *Lyophyllium shimeji* (3.7-fold), and remarkable rhizomorph productions were observed with *Armillaria mellea.*

The promoting component in CNF-HWSF was existed the low molecular weight fraction (MW < 500 daltons) prepared from CNF-HWSF, and active ingredient(s) indicated character soluble in water.

In this section, to reveal the promotive mechanisms on mushroom growth, we examined the effect of CNF-HWSF components on the stimulation of extracellular enzymes such as amylase, CMCase and xylanase.

Material and Methods

Strains

Lentinula edodes (Mori No. 465), *Hypsizygus marmoreus* (Takara No. 1), *Pleurotus ostreatus* (Kitamura; obtained from Kin-ki Nyugyou Co.), and *Flammulina velutipes* (IFO 7777) were used in this study. As inocula, a mycelial block was cut from a plate culture that had grown on a potato-dextrose agar (PDA) medium (Nissui Co.) for 14 days at 24°C in a petri dish (diam. 90 mm).

Preparation of CNF- HWSF

CNF-HWSF was prepared as the same manner described in section 1 in this chapter.

Isolation of polysaccharides from CNF-HWSF

Arabinoxylan from the CNF-HWSF was obteined by the methods of Takeuchi (1997), or cellulose and starch in CNF-HWSF were isolated according to the method described in section 1.

Fractionation of promoting component

CNF-HWSF was fractionated with ultrafiltration using the molecular sieving (500-dalton) membrane and several organic solvents as described in section 1.

Gel filtrate chromatography

A column chromatography with Sephadex G-10 (Amersham Pharmacia) was carried out as the same manner described in section 1. Then, fractions were separated into five groups (A-E).

Medium compositions, inoculations and culture conditions

The potato dextrose liquid (PDL) medium consists of potato extract (200 g was boiled in 500 ml distilled water), 15 g glucose and 1mg thiamine hydrochloride per liter of distilled water. The PDL medium was supplemented with CNF-HWSF and fractions from CNF-HWSF solution of the same concentration as that of CNF-HWSF and dispensed in 16-ml aliquots in 100-ml Erlenmeyer flasks, before autoclaving at 121°C for 10 min. As inoculum, a mycelial block (diam. 5 mm) was cut from a plate culture. The incubation was carried out at 24°C for 15 days.

Enzyme assays

After separation of mycelium by filtration, the culture filtrate was assayed for enzyme activities. The culture filtrate was dialyzed with 25 mM McIlvaine buffer at each optimum pH (*L. edodes*, pH 4.0; *H. marmoreus*, pH 6.0; *P. ostreatus* and *F. velutipes*, pH 5.0) and used as the crude enzyme. Amylase, cellulase and xylanase activities were determined by measuring the amount of glucose or xylose released from soluble starch, carboxymethy cellulose (CMC) and xylan by the Somogyi-Nelson

method (Somogyi 1952) with glucose or xylose as the standard. Reaction mixtures contained 90 μl of 100 mM McIlvaine buffer at each optimum pH, 90 μl of 0.5% (wt/vol) soluble starch, CMC and xylan solution, and 20 μl of crude enzyme solution. After incubation at 37°C for 60 min, the reaction was terminated by adding 200 μl of Somogyi reagent. The mixture was vortexed, placed in a boiling-water bath for 10 min, and cooled on ice, and then 200 μl of Nelson reagent was added. After being vortexed, the mixture was allowed to stand at room temperature for 20 min and centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 655 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of each reducing sugar per minute.

Results

Effect of CNF-HWSF on extracellular enzymes production of mushrooms

To search for the promoting mechanism on the growth of mushroom fungi by the addition of a CNF component in the culture medium, we examined the effect of extracellular enzyme productions from *L. edodes*. These results were shown in Table IV-3. Three kinds of carbohydrase activities derived from *L. edodes* were increased by the addition of CNF-HWSF, the production of enzymes rose to a maximum at the concentration of 20% CNF-HWSF. The author described that the mycelial growth of *L. edodes* was activated with CNF-HWSF and peaked at addition of 20% sample (chapter II), the extracellular enzyme productions by CNF-HWSF seem to correlated with growth promotion of mycelium.

Also, the stimulation of extracellular enzyme productions by CNF-HWSF was found with other edible mushrooms such as *H. marmoreuse*, *P. ostreatus* and *F. velutipes* as shown in Table IV-4. Three kinds of carbohydrase activities were obviously increased by the addition of CNF-HWSF than that of the control. From these results, it was suggested that enzymatic stimulation with the CNF-HWSF is a common phenomenon in mushrooms, and increased of enzymes production from slow-growing mushrooms such as *L. edosed* and *H. marmoreus* by the supplement markedly

appeared than rapid-growing fungi (*P. ostreatus* and *F. velutipes*).

Table IV-3. Effect of CNF-HWSF on extracellular enzyme production from *L. edodes*

Supplement concentration (%)	Enzyme activities (mU / ml)		
	Amylase	CMCase	Xylanase
0	8.51	1.14	0.38
10	55.34	14.77	6.79
20	90.10	34.42	17.47
30	44.90	11.90	3.65

Cultures were grown in 100-ml Erlenmeyer flasks containing 16 ml of PDL medium at 24°C for 15 days.
Carbohydrase activities were measured by Somogyi-Nelson method in 0.1M McIlvaine buffer pH 4.0 at 37 °C.

Table IV-4 Effect of CNF-HWSF on extracellular enzyme production from edible mushroom

	CNF-HWSF (%)	Enzyme activities (mU / ml)		
		Amylase	CMCase	Xylanase
H. marmoreus	0	1.08	n. d.	1.60
	10	4.08	2.33	4.72
P. ostreatus	0	34.31	1.10	1.68
	20	55.44	2.94	5.29
F. velutipes	0	18.62	2.15	2.27
	20	45.66	10.74	7.58

Cultures were grown in 100-ml Erlenmeyer flasks containing 16-ml of PDL medium at 24°C for 15 days.
Carbohydrase activities were measured by Somogyi-Nelson method in 0.1M McIlvaine buffer (*H. marmoreus*: pH 6.0, *P. ostreatus* and *F. velutipes*: pH 5.0) at 37 °C. n. d.: not detected.

Effect of fractions from CNF-HWSF on the extracellular enzyme productions of L. edodes

The fractions derived from CNF-HWSF were added in the PDL medium, and *L.* edodes was incubated. Then the extracellular carbohydrase (amylase, CMCase and xylanase) productions were measured at 15 days after inoculation. These results were shown in Fig. IV-8. Obviously high values resulted when the enzyme production of the medium, which fungi had been cultivated on a PDL medium, included low molecular weight fractions such as supernatant of ethanol treatment and less than an MW 500 fraction in CNF-HWSF. These results of *H. marmoreus*, *P. ostreatus* and *F. velutipes* also indicated tendency similar to that of *L. edodes* (data is not shown). There were no effect either mycelial growth or exo-carbohydrases production when chemical reagents of glucose, xylose, arabinose and substrates polysaccharides which including CNF-HWSF were added. Also, the fraction C and D obtained gel filtrate fractionation were increased the mycelial growth of *L. edodes* (see Fig. IV-6 in section 1), were stimulated xylanase activity in the medium (Fig, IV-9). The fraction C included reducing sugars and amino acids, while these were not detected in fraction D.

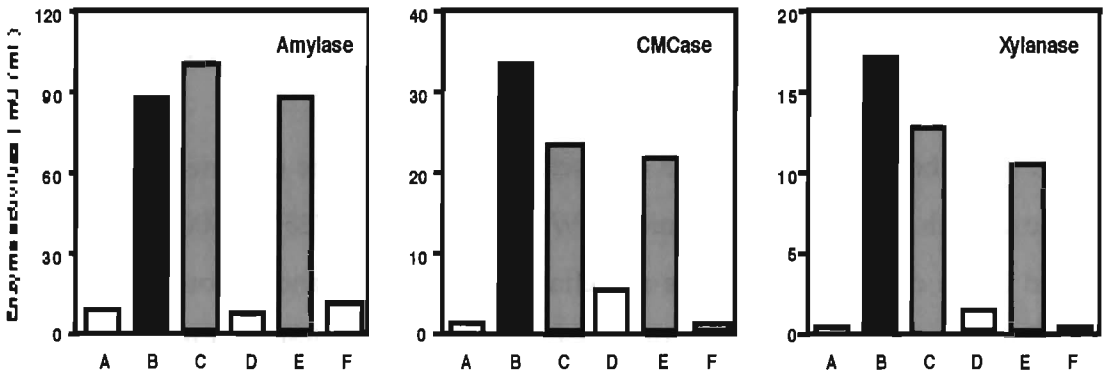


Fig. IV-8. The influence of CNF-HWSF components on the extracellular enzymes production of *Lentinula edodes*

The vegetative mycelia were cultured for 15 days at 24℃ in PDL medium added CNF-HWSF (20%). A: control, B: CNF-HWSF, C: supernatant of ethanol treatment, D: precipitate of ethanol treatment, E: 500-L by ultrafiltration, F: 500-M by ultrafiltration.

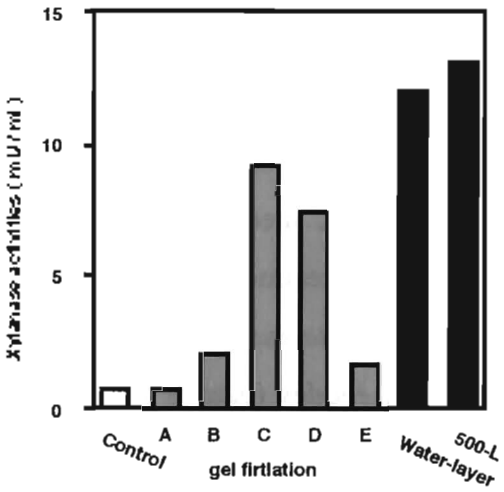


Fig. IV-9. The influence of the gel filtration fraction from CNF-HWSF components on the extracellular enzyme of *Lentinula edodes*

The vegetative mycelia were cultured for 15 days at 24℃ in a PDL medium added to each fraction (20%; 500-L and water layer, 40%; gel filtration samples). Xylanase activities were measured as described in the Materials and Methods. Enzyme activities were measured using a culture filtrate after 15 days of incubation. Symbols indicate fraction No. by gel filtration using Sephadex G-10 (A: 1-20, B: 21-24, C: 25-34, D: 35-60 and E: 60-80).

Changes in vegetative mycelial growth and the extracellular enzyme productions of Lentinula edodes

In general, mushroom fungi have many strains that have high xylanase activity during the growth. The author investigated the relationship between the mycelial growth and the xylanase production in the culture filtration of *L. edodes*, which is the most important and popular edible mushroom in Japan.

Fig. IV-9 shows the time course of mycelial growth and the enzyme production during the growth of *L. edodes* colonies. When the CNF-HWSF or 500-L fraction was added in the culture medium, the mycelial growth was enhanced from the initial stage of cultivation period. In particular, there was a great difference in mycelial dry weight with the addition of CNF-HWSF components during 6-15 days after inoculation. Furthermore, only the mycelial growth with CNF-HWSF observed a remarkable increase at the end of the incubation period (12-15 days).

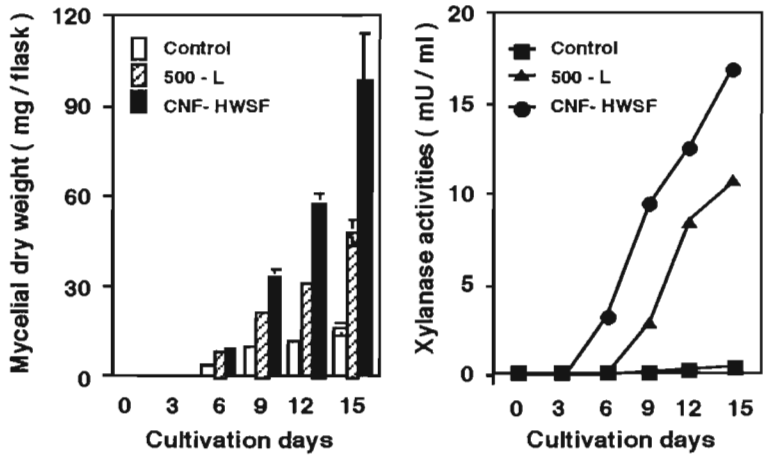


Fig. IV-10. Time course of vegetative mycelial growth and xylanase production in the PDL medium in *Lentinula edodes*
The vegetative mycelia were cultured at 24°C in a PDL medium added CNF-HWSF (20%). Error bars indicate the standard deviation (n=6).

The xylanase activities were detected at 3-6 days after inoculation on the CNF-HWSF or 500-L fraction supplemented. These activities increased rapidly during 6-15 days after inoculation. However, it was hardly detected in the control medium through the cultivation. The production of the other carbohydrases such as amylase and CMCase in this mushroom was also stimulated when cultured with CNF-HWSF components (data is not shown). Furthermore, these effects by CNF-HWSF shows on other mushroom fungi (*H. marmoreus*, *P. ostreatus* and *F. velutipes*).

Discussion

Generally, though it is possible that the mushroom utilizes various materials for their growth, the utilization ability is different by the species or strain of the mushrooms (Ohga 1992, Azuma and Kitamoto 1994). Moreover, mushrooms have fruit-body forming or non-forming strains in the same species (Ohga 1992, Terashita et al. 2000). The different abilities of an individual mushroom species to grow and fruit

are determined by both fungus- and substrate-associated factors (Cai et al. 1999). These include the ability of the mushroom to produce the decomposition enzymes necessary to degrade individual components of the growth substrate. The elucidation of cultivation and growth physiology mechanism of mushrooms seems to be possible by examining property and behavior of these degradation enzymes.

The author examined the effects of the CNF-HWSF ingredient on extracellular enzymes in *L. edodes*. As a result, CNF-HWSF was stimulated to produce degradation enzymes in the fungus and the effects shown on other three fungal species. In section 1, the promotive effects upon the mycelial growth were shown from the low molecular weight fractions (fraction C and D by gel filtrate chromatography in less than M.W. 500) prepared from CNF-HWSF was indicated, and the increase of carbohydrase activities on a medium was also shown in the low molecular weight fraction from CNF-HWSF. These results suggest that a C and D fraction involving the main promoted component(s), and the enzyme activation by CNF-HWSF, concerns the promotive effect on mycelial growth with CNF-HWSF.

The secretion of xylanase and cellulases from fungi were induced with degradation substrate of these enzymes and the production of substrates, were regulated by the presence of glucose (Eriksson and Hamp 1978, Canevascini et al. 1979. Chow et al. 1994, Yagüe et al. 1997, Ozcan et al. 1997). However, the production of enzymes from fungi were not stimulated with the polysaccharide substrates and their degradation components, has no catabolite repression with glucose (data is not shown). De Groot et al. (1998) reported that the expression of the xylanase and cellulase gene is regulated by components of compost rather than by the substrates they act upon on *Agaricus bisporus*.

The mycelial weights in *L. edodes* were higher with CNF-HWSF and with a 500 or lesser fraction from the initial stage, but the biomass value on CNF-HWSF of the 15th day after inoculation remarkably rose beyond that on the addition of the 500-L fraction. The mycelial dry weights of fungi were not increased when only the ethanol treatment precipitate (polymer) fraction from CNF-HWSF was added on a culture medium. However, the mixture of the high and low molecular fraction enhanced the mycelial growth. Because CNF-HWSF contained rich nutrients (monomer and

polymer) and the 500-L fraction included no or few polymer, growth enhancements also had relation to polymer components of CNF-HWSF as the nutritive components.

From the results presented above, the promoting mechanism on mycelial growth was discussed as follows. The mycelial growth was enhanced by free amino acids (alanine, leucine, arginine and others) and monosaccharides (glucose, arabinose and xylose) in CNF-HWSF, reported previously from fast development and second promotion caused from increase of supply with utilizable growth substrate by activation and stimulation of growth-substrate degradation enzymes by some ingredient(s) of CNF-HWSF.

Summary

In section 1 on chapter II, the hot water soluble fraction (HWSF) from CNF has a promoting effect on the mycelial growth of various edible mushrooms including mycorrhizal fungi described. To reveal the promoting mechanisms, the effect of CNF-HWSF on the stimulation of extracellular enzymes was examined. As a result, the production of extracellular carbohydrases such as amylase, CMCase and xylanase were markedly enhanced by the addition of low molecular weight fractions (less than M.W. 500) prepared from CNF-HWSF. The enzymatic stimulations and enhancement of mycelial growth appeared during the 3-15 days after inoculation. From these results, the promoting effect by CNF-HWSF seems to be a 2 step reactions. The first step could be achieved by rich nutrients such as free amino acids and monosaccharides from CNF-HWSF. The second step (during 3-15 days) considered that the marked promoting effect was caused by the stimulation of extracellular enzymes.

SYNOPSIS

Mushrooms have been long attracting a great deal of interest in many areas of foods and biopharmaceuticals, and the demand of which are increasing every year. Under these circumstance, study on mushrooms as foods that physiological or biochemical investigation in order to efficiency and rational cultivation methods for fruit-body production, breeding with genetic techniques and, screening of new strains, are advanced. While, to study regarding global utilization of mushrooms as non-foods for medical materials and bio-remediation, had also developed.

Commercial production of most edible mushrooms is on synthetic substrate contained in polypropylene bags and bottles. A common substrate used for commercial production of mushrooms is supplemented sawdust, and bran, derived from cereal grains, such as rice, wheat and oats, are widely used as nutrient supplements. However, the sawdust suitable for the mushroom cultivation is insufficient. Also, because the greatest purpose on mushroom cultivation was to improve yield and quality and to shorten the crop cycle, improvement of the cultivation technique for producing high-quality fruit-body and the development of the new culture medium for mushroom cultivation is desired.

The author investigated in order to utilize corn fiber, which is a by-product of the wet corn milling process, for the mushroom cultivation. The main object of this research was to make sawdust-based cultivation of edible mushrooms use of corn fiber. In addition, the author investigated its fractionation by organic solvent and molecular mass and by a chemical analysis that revealed the promotive substances of corn fiber components. The promotive mechanisms of vegetative mycelial growth of edible mushroom cultivations are also discussed.

The results obtained in the research are collected in the present thesis, which is made up of four chapters. The findings obtained in each chapter are summarized as follows.

In chapter I, study on the fruit-body production of mushrooms by CNF used for the substrates in the sawdust-based cultivation are described. In this study, it indicated that CNF is a useful culture material for sawdust-based cultivation, and

which is effective in producing the fruit-body to increases yield, shortens the cultivation period and increases the quality of mushrooms. From these obtained results, it was suggested that the growth promoting component for fungal in CNF material was contained.

In chapter II, the author prepared the extract that is hot-water soluble fraction from CNF (CNF-HWSF), and examined the effects of the CNF-HWSF on vegetative mycelial growth of edible mushrooms. Growth-promoting effects of CNF-HWSF on mycelium of mushrooms appeared for all fungi used in this study, the effect was more efficient in the slow-growing mushrooms. Mycelial growth of mycorrhizal fungi also were activated with added CNF-HWSF, the affects were remarkably indicated for submerged hyphae.

Chapter III is concern with fruit-body production of edible mushrooms using CNF-HWSF. At first, fruit-body formation of *L. edodes* and *F. velutipes* in liquid medium supplemented with CNF-HWSF were examined. In both fungi, yield of fruit-body were obviously increased by added with CNF-HWSF. Moreover, development rate of fruit-body in *L. edosdes* rose, and cultivation period were reduced by CNF-HWSF. Also, the author examined to effect of CNF-HWSF on fruit-body development of *Pleurotus ostreatus* in a sawdust-based cultivation, and indicated to increases of fruit-body yield by supplemented CNF-HWSF. These results suggested that CNF-HWSF has positive possibility as supplement for mushroom production.

In chapter IV, the author described the growth-promoting mechanism on mycelium of mushrooms by CNF-HWSF. As growth of mushrooms (mycelial growth and fruit-body development) has affected by some nutriments, the author analyzed components of CNF-HWSF. Although CNF-HWSF has a rich nutriments for mushrooms, growth-promotion of mycelium was not appeared only the nutritional components in CNF-HWSF and the positive effect shows in low molecular weight fraction which except for sugars and amino acids. However, to removing their nutriment substrates from CNF-HWSF resulted in a decline of growth-enhancement, it suggested that polymer substrates were also concerned in growth-promotion of mushrooms.

In order to reveal the promoting mechanisms, the effect of CNF-HWSF on the

stimulation of extracellular enzymes was examined. As a results, to the production of extracellular carbohydrases were markedly enhanced by the addition of CNF-HWSF, and mycelial growth was correlated closely with the increases of enzyme productions. From these obtained results, the author surmised as fellow; the first step could be achieved by rich nutrients such as free amino acids and monosaccharides from CNF-HWSF. The second step considered that the marked promoting effect was caused by the stimulation of extracellular enzymes. However, further research is necessary concerning the isolation and characterization of promotive substances to reveal the mechanisms involved.

The results of collect in the present thesis are not only to develop the way for the utilization of corn fiber with the meager application, and it was also able to contribute to the elucidation of the fruit-body formation mechanisms of the edible mushrooms.

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