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https://doi.org/10.5109/20317

出版情報：九州大学大学院農学研究院紀要. 56 (2), pp.249-254, 2011-09. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **56** (2), 249–254 (2011)

Increase in Yield of the Straw Mushroom (*Vovariella volvacea*) by Supplement with *Paenibacillus* and *Bacillus* to the Compost

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(*Received April 22, 2011 and accepted May 9, 2011*)

Two bacterial isolates N10 and B2, isolated from the dry paper mulberry bark and from the air of indoor–farmed straw mushroom, respectively, were identified as *Paenibacillus polymyxa* and *Bacillus subtilis* by the conventional and Biolog method. The antagonism of these bacteria against plant pathogens and various kinds of edible mushrooms were investigated *in vitro* to determine its potential for compost application. *P. polymyxa* N10 inhibited plant pathogenic fungi, i.e., *Fusarium oxysporum, Aspergillus niger, Alternaria* sp., *Sclerotium rolfsii* and some edible mushrooms, i.e., *Volvariella volvacea, Pleurotus ostreatus, P. abalonus, Lentinus squarrosulus* and *Auricularia auricular*. *P. polymyxa* N10 was also inhibitive to commonly contaminated fungi found in compost, such as *Trichoderma* sp. and *Monilia* sp.. *B. subtilis* B2, on the other hand, did not inhibit the growth of *V. volvacea* but was antagonistic to almost all of tested fungi, except *Trichoderma harzianum* and *Monilia* sp.. Our preliminary test showed that yield of straw mushroom was increased by supplementation of cultured broth in compost in outdoor cultivation. They were grown in shaken nutrient broth at room temperature for 24 hrs (ca.1.5×10^7 and 1.0×10^7 cfu/ml for *P. polymyxa* N10 and *B. subtilis* B2) and sprayed to mushroom compost at the rate of one liter per 4 m^2 area of 45 kg dry wt., then inoculated with straw mushroom spawn. Seven crops were done per year and each crop was accomplished by two indoor farms consisting of three replications each. Results of experiments of the first two years clearly indicated that mushroom yields from the bacterial supplemented compost were significantly higher than those of the control and nutrient broth treated one. On the third year, soybean milk or cow milk were used as growing medium for bacteria instead of nutrient broth to simplify the bacterial preparation technique and *B. subtilis* B2 was chosen as the only bacterial strain used for experiment based on experimental results of the previous two years. Overall results of the 3 year experiments strongly indicated that supplementation of *P. polymyxa* N10 and *B. subtilis* B2 to straw mushroom compost increased the mushroom yield over the non supplemented one.

Keywords: *Bacillus subtilis* B2, *Paenibacillus polymyxa* N10, straw mushroom

INTRODUCTION

Straw mushroom has been the most popular mushroom in Thailand for the past 60 years. The demand is always high and the production has been dramatically increasing year by year. The biggest producer in the world is China (about 150,000 tons /year). Thailand is the second according to the consideration as one of the most suitable countries for straw mushroom cultivation. Yield of straw mushroom production may fluctuate due to certain factors such as nutritional property of raw material, pest and disease outbreak in the case of indoor farming, geographic region and cultivating season. To increase the yield of straw mushroom under indoor–farmed conditions many experiments have been carried out with varying success. Thermophilic fungi are used to improve the pre–fermentation of compost production before steaming at 45–50 ˚C. Fermor *et al.* (1985) reported that steaming at 50–55 ˚C could stimulate growth of thermophilic fungi or actinomycetes that were useful microorganisms in the decomposition process. Payapanon and Pitukpriwan (2003) reported that straw mushroom yield increased by 1–4% over the control by supplementation of *Scytalidium thermoplilum* to the compost. Our preliminary test on straw mushroom production by supplementing cultured broth with *Bacillus* sp. B2 and another unidentified bacterial strain N10 resulted in increasing yields over the non supplemented compost.

The purpose of the study was to carry out the taxonomical identification of the two bacteria as well as antagonistic activity against various kinds of fungi, subsequently detailed investigation on effects of supplementation to the compost on yield increase of straw mushroom by the indoor cultivation method.

MATERIALS AND METHODS

Bacterial strains used

Two *Bacillus* species, a pectinase–producing strain N10, originally isolated from the dry paper mulberry

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bark (Sittidilokratna *et al*., 2007), and strain B2 which was isolated from the indoor–farmed straw mushroom by air trapping on agar plate, were used. Both strains were endospore forming rod–shaped bacteria, and were morphologically and physiologically studied by following the methods for identification of *Bacillus* sp. (Claus and Berkeley, 1986; Gordon, 1989), and they were also confirmed by the Biolog data based on 95 carbon source utilization patterns.

Antagonistic effects of bacteria against fungi

Both strains were tested against various kinds of plant pathogenic fungi, compost contaminated fungi and other popular edible mushroom in Thailand. Antagonism between bacteria and fungi was observed on potato dextrose agar (PDA) plate. Each fungal mycelia plug was cut by cork borer and placed on the center of PDA plate. Each bacterium was grown on nutrient agar slant for 24 hrs before inoculated by streaking two horizontal lines on PDA at the position of 20 mm from the rim of Petri dish upper and lower of inoculated fungi. Inhibition activity was measured in mm at the distance from the edge of bacterial colony to the nearest fungal mycelia within 1–15 days depending on each fungal growth rate. The results were determined on the day of control fungi occupied all plate surface.

Straw mushroom spawns preparation

The straw mushroom spawn was prepared from two groups of spawn materials, the pre–composted horse manure and lotus seed coat (2:3 by wt.) and the pre–composted mungbean pod, sawdust and cotton waste (1:5:5 by wt.), equal part of the two groups were mixed and further added with 3% rice bran and 60–65% water (by wt.). The prepared compost–spawn was filled into polypropylene bags about 500 g per bag and then being pasteurized for 45 minutes at 11 psi. The bags were then inoculated with mycelial plugs, followed by incubated at room temperature. Mycelia completely grew throughout the compost within two weeks.

Bacterial cell preparation

Two bacterial strains N10 and *Bacillus* sp. B2 were grown separately in nutrient broth with shaking at room temperature (30–35 °C) for 24 hrs. Nutrient broth (NB) was used as growing media for both bacterial strains for the first two years of this experiment. However, on the third year some modification was made, only *Bacillus* sp. B2 was selected and grown either on UHT cow milk or soybean milk instead of NB.

Preparation of compost and cultivation method

The raw materials used for producing compost were rice straw, cotton waste, gypsum, $CaCO_3$, CaO, urea, glutinous rice flour and rice bran. They were mixed at the ratio of 300: 250:2:2:1:1.5:2:2.5 (dry wt.). After composting process was completed, the compost was spread on the soaked rice straw on shelf and steam was blown into the house and maintained temperature at 60–65 °C for three hrs. When the temperature of steamed compost decreased to 35 °C one liter of each bacterial suspension (ca. 1.5×10^7 cfu/ml for *P. polymyxa* N10 and 1.0×10^7 cfu/ml for *Bacillus* sp. B2) was sprayed onto surface layer of the compost (size area 4 m^2 per 45 kg dry wt.), subsequently the spawn of straw mushroom was laid down on top of the compost. Three replications per farm and two farms per crop were performed. NB was sprayed as a control and compared with above treatments and normal compost. Straw mushroom from each treatment was harvested 6–10 days after inoculation according to the season. The production yield was determined by weight and the averages of biological efficiency yield of seven crops per year were compared.

The only simplified technique for the farmers is to use UHT cow milk or soybean milk to prepare *Bacillus* sp. B2 suspension instead of using NB.

RESULTS AND DISCUSSION

Identification of selected bacteria

As shown in Table 1 the strain N10 was clearly identical to *Paenibacillus polymyxa*. Colony appearance on nutrient agar was thin and often with amoeboid spreading as same as described by Priest (2009). In addition *P. polymyxa* can hydrolyze pectin, pullalan, starch and xylan but activity on cellulose is weak (Priest, 2009). These properties of strain N10 had been confirmed by Shompoosang (2007).

Metabolic pattern in Biolog test showed that strain N10 could utilize β–cyclodextrin, dextrin, glycogen, L–arabinose, arbutin, D–cellobiose, D–fructose, D–galactose, α–D–glucose, α–D–lactose, lactulose, maltose, maltotriose, D–mannitol, D–mannose, D–melibiose, β–methyl–glucoside, palatinose, raffinose, D–ribose, salicin, starchyose, sucrose, D–trehalose, turanose, D–xylose, glycerol and thymidine. This was strongly confirmed that strain N10 to be identified as *P. polymyxa* by Biolog database. Further, a partial 16S rRNA gene sequence (1263 bp) of N10 was analyzed. The homology in the database was 98.9%, 98.8% and 98.8% with *P. polymyxa* strains EBL2071 (EF545556), L1–9 (FJ178378), and G–14 (EU434620), respectively (data not shown).

On the other hand, morphological and physiological studies of *Bacillus* sp. B2 indicated that it was similar to *B. subtilis* (Table 1). By the Biolog method, this bacterium was also identified as *B. subtilis* because of its positive growth on utilization of dextrin, N–acetyl–D–glucosamine, L–arabinose, arbutin, D–cellobiose, D–fructose, gentibiose, D–gluconic acid, α–D–glucose, maltose, maltotriose, D–mannose, 3–methyl glucose, palatinose, D–psicose, D–ribose, salicin, sucrose, D–trehalose, turanose, D–xylose, L–malic acid, pyruvic acid, L–asparagine, L–glutamic acid, L–serine, glycerol, inosine, uridine, and D–L–α–glycerol phosphate. However, current classification of *B. subtilis* is so complex because a few new species and subspecies had been splitted from *B. subtilis* by differences in DNA relatedness and some chemotaxonomic characteristics. No distinguishable traits among *B. mojavensis*, *B. subtilis* subsp. *spizizenii*

Table 1. Phenotypic characteristics of selected bacterial strains N10 and B2

Character	N10	B2	*P. polymyxa*[a)]	*B. subtilis*[a)]
Cell width, μm	0.8–1	0.7	0.6–0.8	0.7–0.8
Cell length, μm	2–3	2	2–5	2–3
Spores				
ellipsoidal	+	+	+	+
central or para central	–	+	V	+
subterminal or terminal	+	–	V	–
swelling the sporangium	+	–	+	–
Motility	+	+	+	+
Catalase	+	+	+	+
Anaerobic growth	+	–	+	–
V–P reaction	+	+	+	+
pH in V–P broth	5.5	5.4	4.5–6.8	5.4–8.0
Growth in				
media at pH 5.7	+	+	+	+
5% NaCl	–	+	–	+
7% NaCl	–	+	ND	+
10% NaCl	–	+	–	d
Acid from				
D–glucose	+	+	+	+
L–arabinose	+	+	+	+
D–xylose	+	+	+	+
D–mannitol	+	+	+	+
Hydrolysis of				
starch	+	+	+	+
casein	+	+	+	+
gelatin	+	+	+	+
oat spelt xylan	+	ND	ND	ND
colloidal chitin	–	ND	ND	ND
carboxymethyl cellulose	+	ND	ND	ND
lecithin in eggyolk	–	–	–	–
Reduction of nitrate to nitrite	+	+	+	+
Utilization of				
citrate	–	+	–	+
propionate	–	–	–	–

[a)] Cited from Gordon (1989) and Ash *et al.* (1993)

and *B. vallismortis* by conventional phenotypic tests had reported by Logan and De Vos (2009). Although only above phenotypic characteristics are not enough to indicate the critical taxonomic position of *Bacillus* sp. B2, we designate tentatively strain B2 as *Bacillus subtilis* afterward.

Antagonistic effect of bacteria against fungi

P. polymyxa N10 had potential to inhibit all kinds of plant pathogenic fungi tested *in vitro* as shown in Fig. 1 and all kinds of tested edible mushroom in Fig. 2. In the case of commonly contaminated compost fungi, N10 strain inhibited well *Trichoderma* sp. but rarely did against *Monilia* sp. On the other hand, *B. subtilis* B2 showed the similar response (Fig. 3 and Fig. 4), however, when it was tested with straw mushroom *in vitro*, antagonistic activity was not shown but many small pin heads were formed (Fig. 5a). Appearance of pin heads formation was also evident from the co–cultured plate of *P. polymyxa* N10 and straw mushroom (Fig. 5c) but not found from cultivation of straw mushroom alone (Fig. 5b). The evidence about pin heads formation on compost which had been usually evaluated as the parameter to determine the quality of various substrates as reported by Chang and Quimio (1982) and Hu *et al.* (1976). However, pin heads formation on PDA

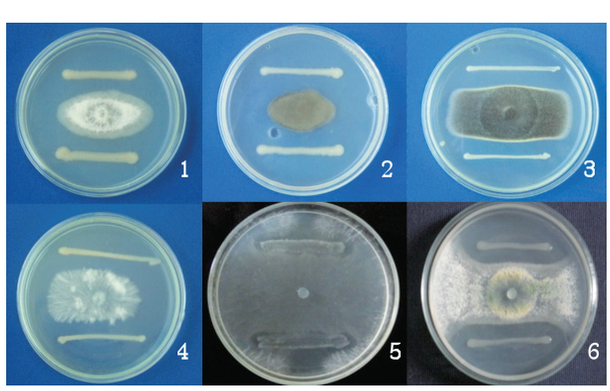

Fig. 1. Inhibition activity of *Paenibacillus polymyx*a N10 to plant pathogenic fungi (1–4) and commonly compost contaminated fungi (5 and 6). 1 *Fusarium oxysporum*, 2 *Alternaria* sp., 3 *Aspergillus niger*, 4 *Sclerotium rolfsii*, 5 *Monilia* sp., 6 *Trichoderma harzianum*.

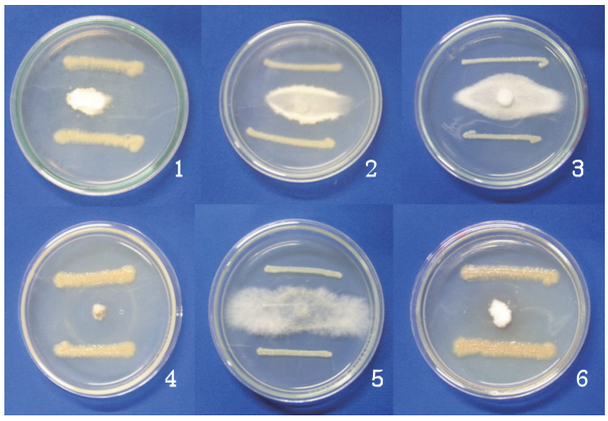

Fig. 2. Inhibition activity of *Paenibacillus polymyxa* N10 to various kinds of edible mushroom mycelia. 1 *Pleurotus ostreatus* (cream), 2 *Pleurotus ostreatus* (black), 3 *Lentinus squarrosulus* Mont. 4 *Auricularia auricular* (Hook.) Underw 5 *Volvariella volvacea* (Bull. Ex Fr.) Sing., 6 *Pleurotus ablonus* Han.

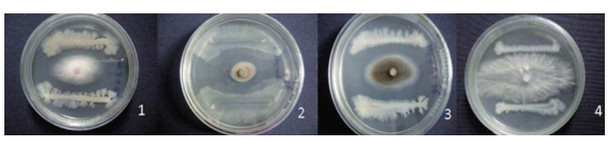

Fig. 3. Antagonistic activity of *Bacillus subtilis* B2 to plant pathogen fungi. 1 *Fusarium oxysporum*, 2 *Alternaria brassicola*, 3 *Aspergillus niger,* 4 *Sclerotium rolfsii*.

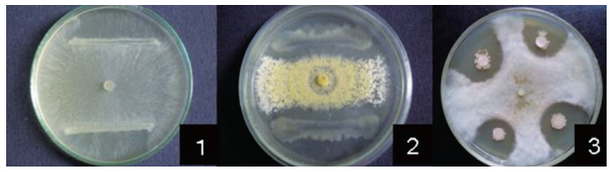

Fig. 4. Antagonistic activity of *Bacillus subtilis* B2 to commonly compost contaminated fungi. 1 *Monilia* sp., 2 *Trichoderma harzianum*, 3 *Trichoderma* sp.

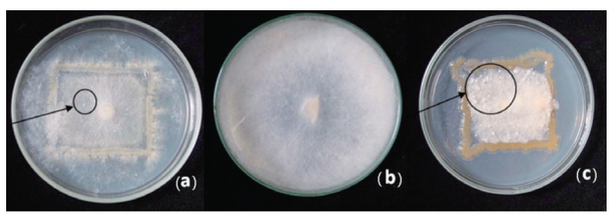

Fig. 5. Effect of *Bacillus subtilis* B2 and *Paenibacillus polymyxa* N10 on growth of *Volvariella volvacea*. B2 was not antagonistic (a), whereas N10 inhibited growth of *V. volvacea* (c) compared with normal growth of *V. volvacea* (b). Many small pin heads were formed as shown (arrow) in both cases (a) and (c).

plate was not mentioned before. To select the suitable bacteria for gaining the yield of straw mushroom, *P. polymyxa* N10 seem to have higher potential than *B. subtilis* B2 as *P. polymyxa* N10 could inhibit almost of our tested fungi including all kinds of edible mushroom. Our endeavor will further investigate on how and why these bacteria could enhance the yield of straw mushroom observed from our preliminary test.

Application of bacteria to compost for straw mushroom production

Yields of straw mushroom from the composts supplemented by either *P. polymyxa* N10 or *B. subtilis* B2 were higher and significantly different from those supplemented by nutrient broth and the control in both 2007 and 2008 experiments (Tables 3 and 4). Interestingly, *P. polymyxa* N10 and *B. subtilis* B2 gave the same result. Moreover, *B. subtilis* B2 suspension prepared from cow milk or soybean milk gave higher yield than

Table 2. Antagonistic activities of *Paenibacillus polymyxa* N10 and *Bacillus subtilis* B2

Fungi	Inhibition distance (mm)		Days of growth
	P. polymyxa N10	*B. subtilis* B2	
Plant pathogenic fungi			
Fusarium oxysporum	12	4	6
Alternaria brassicola	ND	7	4
Alternaria sp.	12	ND	2
Aspergillus niger	9	5	6
Sclerotium rolfsii	8	2	5
Commonly compost contaminated fungi			
Trichoderma sp.	ND	5	5
Trichoderma harzianum	6	0	4
Monilia sp.	0	0	1
Edible mushroom			
Pleurotus ostreatus (cream)	13.1	ND	9
Pleurotus ostreatus (black)	11.5	ND	5
Pleurotus abalonus Han	11.5	ND	15
Lentinus squarrosulus Mont.	9.3	ND	4
Auricularia auricular (Hook.)	*	ND	13
Volvariella volvacea (Bull. Ex Fr.) Sin	10.1	0	3

ND : Not determined.
* : Completely inhibited.

Table 3. Biological efficiency on yield of straw mushroom production from the compost supplemented with bacteria during Oct. 2007–Sep. 2008

Treatments	Yield of fresh mushroom (kg) per weight of dry substrates 100 kg[a]							
	Winter		Summer			Rainy		
	Dec. 2007	Jan.–Feb. 2008	Mar. 2008	Apr. –May 2008	May – Jun. 2008	Jul. 2008	Aug. –Sep. 2008	Year 2008
Paenibacillus polymyxa	35.43 a	46.65 ab	38.58 a	31.33 a	48.87 a	42.32 a	21.12 a	37.45 a
Bacillus subtilis B2	37.78 a	47.92 a	40.36 a	31.24 a	42.32 b	37.70 ab	21.03 a	36.45 a
Nutrient broth (NB)	35.54 a	30.88 c	30.02 b	23.65 b	30.80 c	29.67 c	18.76 a	30.01 b
Control	34.73 a	40.84 bc	27.53 b	25.31 b	32.11 c	30.10 bc	19.28 a	30.54 b
C. V. (%)[b]	6.1	8.5	9.7	7.4	5.7	10.6	13.7	7.1

[a] Mean followed by a common letter are not significantly different at 5% level by LSD.
[b] Coefficient of variation

Table 4. Biological efficiency on yield of straw mushroom production from the compost supplemented with bacteria during Oct. 2008–Sep. 2009

Treatments	Yield of fresh mushroom (kg) per weight of dry substrates 100 kg[a]							
	Winter		Summer			Rainy		
	Dec. 2008	Jan.–Feb. 2009	Mar. 2009	Apr. –May 2009	May – Jun. 2009	Jul. 2009	Aug. –Sep. 2009	Year 2009
Paenibacillus polymyxa	25.74 c	33.07 a	44.72 a	38.28 a	31.23 a	27.37 a	33.31 a	32.64 a
Bacillus subtilis B2	42.24 a	34.55 a	43.78 a	34.61 ab	29.20 a	27.85 a	32.32 a	34.82 a
Nutrient broth (NB)	32.07 b	26.35 b	39.37 b	27.96 bc	25.34 b	23.12 a	27.71 b	28.28 b
Control	33.60 b	28.62 ab	38.14 b	25.96 c	27.64 a	23.78 a	26.98 b	29.19 b
C. V. (%)[b]	4.5	9.5	5.5	13.3	6.1	10.2	4.1	5.5

[a] Mean followed by a common letter are not significantly different at 5% level by LSD.
[b] Coefficient of variation

Table 5. Biological efficiency on yield of straw mushroom from the compost supplemented with *Bacillus subtilis* B2 grown in soybean milk during Dec. 2009–May 2010

Treatments	Yield of fresh mushroom (kg) per weight of dry substrates 100 kg[a]				
	Winter		Summer		
	Dec. 2009– Jan. 2010	Jan.–Feb. 2010	Mar. –Apr. 2010	Apr. –May 2010	Year 2010
Soybean milk+ *B. subtilis* B2	33.12 a	41.01 a	42.32 a	25.14 a	35.40 a
Soybean milk	33.35 b	35.24 b	35.18 b	22.19 a	31.49 b
Control	26.90 c	36.56 b	38.23 ab	21.32 a	30.75 b
C. V. (%)[b]	4.9	4.4	7.9	7.0	6.1

[a] Mean followed by a common letter are not significantly different at 5% level by LSD.
[b] Coefficient of variation

Table 6. Biological efficiency on yield of straw mushroom from the compost supplemented with *Bacillus subtilis* B2 grown in cow milk during Dec. 2009–May 2010

Treatments	Yield of fresh mushroom (kg) per weight of dry substrates 100 kg[a]				
	Winter		Summer		
	Dec. 2009– Jan. 2010	Jan.–Feb. 2010	Mar. –Apr. 2010	Apr. –May 2010	Year 2010
Cow milk+ *Bacillus subtilis* B2	33.77 a	39.76 a	36.29 a	22.74 a	33.14 a
Cow milk	32.92 a	32.94 b	34.14 b	21.70 a	30.43 b
Control	27.43 b	33.98 b	29.07 c	17.94 a	27.11 b
C. V. (%)[b]	3.7	8.2	8.4	9.4	7.4

[a] Mean followed by a common letter are not significantly different at 5% level by LSD.
[b] Coefficient of variation

that of cow milk, soybean milk and control (Tables 5 and 6).

The mushroom compost which was sprayed with either *P. polymyxa* N10 or *B. subtilis* B2 tended to have less incidence of fungal contamination of commonly found in compost such as *Trichoderma* sp., *Monilia* sp, *Aspergillus* sp., *Penicillum* sp., and so on. The results showed that *P. polymyxa* N10 could inhibit the growth of almost all of tested fungi. Moreover, it also affected to many species of plant pathogenic bacteria, especially in *Xanthomonas* spp. (Shompoosang, 2007). Mechanisms of fungal inhibition might be related to various hydrolytic enzyme productions by bacteria that had been reviewed by some researchers. Cellulase, mannanase, xylanase and caseinase producing strains of *P. polymyxa* and *B. pumilus* could inhibit *Rhizoctonia solani, Aphanomyces cochlioides* and *Pythium ultimum* on PDA plate were reported (Nielsen and Sorensen, 1997). Budi *et al*. (2002) also found that cellulose, protease, chitinase and pectinase producing *Paenibacillus* sp. B2 could inhibit *Phytophthora parasitica* and *F. oxysporum*. Our strain of *P. polymyxa* N10 produces pectinase, cellulase, protease, amylase and xylanase (Shompoosang, 2007; Sittidilokratna *et al*., 2007). On the other hand, Nalisha *et al*. (2006) explained that *B. subtilis* produced antifungal compound which had inhibitory effects on wide range of fungi, including *S. rolfsii*. These results indicated that both *P. polymyxa* N10 and *B. subtilis* B2 had potential to increase yield of straw mushroom. Investigations on the actual mechanisms of the two bacterial strains enhancing the mushroom yield are going to be performed.

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