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Evaluation of Disinfectants on Growth of Bacteria Associated with Pinning Stage of Paddy Straw Mushroom Cultivation

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Abstract: Pinning stage is extremely crucial for mushroom cultivation, many bacteria infect mushroom in this stage due to its proteinaceous nature. In the present study, eight bacteria were isolated from the pinning stage of paddy straw mushroom *Volvariella volvaceae*. The bacterial isolates were tentatively identified by morphological, physiological and standard biochemical tests. Out of the eight bacteria were isolated, *Staphylococcus saprophyticus*, *Bacillus pumilis*, *Bacillus sp*. and *Kingella* sp. were inhibiting growth of *Volvariella volvaceae.* These contaminating bacterial isolates showed resistance to most of the antibiotics used. Then, the bacterial isolates were subjected to various disinfectants such as calcium hypochlorite, hydrogen peroxide and calcium hydroxide added at different concentration. It was observed that, 300 ppm of calcium hydroxide solution is economic and reduces growth of contaminating bacterial isolates. Thus, concluded field study and nutritional quality analysis mushroom is essential to use calcium hydroxide as a cost effective control measures to increase paddy straw mushroom production.

Key words: Pinning, Biochemical, Contaminating, Nutritional, Disinfectant.

Introduction

India has large number of agro-climatic regions that offer congenial climatic conditions for tropical mushroom cultivation**.** Mushrooms are the fleshy, fruiting bodies of various macro-fungi growing on organic material, compost, fallen leaves, damp wood and dead plant or animal matter. Edible mushrooms contains 20-35 % protein, rich in vitamin such as riboflavin, niacin, pantothenic acid and essential minerals like selenium, copper, potassium respectively. These are now getting significant importance due to their nutritional, medicinal value and today their cultivation is being done in about 100 countries. Though 20 mushroom varieties are domesticated but oyster, paddy straw, button, shiiake and wood ear mushrooms contribute 99 % of the total world production [1]. The total mushroom production in India reached 0.04million tons/year by 2009 [2, 3]. Moreover, total tropical mushroom production in Odisha is 12, 120.3 tones/year, of which paddy straw mushroom (*Volvariella volvaceae*) only contribute 8007.2 tones [1]. Paddy straw mushroom is dominating whole of Odisha, available in every nook and corner of the state. It is the 6th mushroom of the world in production [4] due to its fast growing nature, better consumer demand and relatively high profit to the growers [5]. Moreover, efficient re-integration of agricultural residues is the remarkable ecological advantage of paddy straw mushroom cultivation has been documented.

Like all other crops, mushrooms are also affected adversely by various biotic and abiotic factors or agents. Among the biotic agents, fungi, bacteria, viruses, nematodes, insects and mites cause damage to mushrooms directly or indirectly. Several pathogenic bacteria induced varieties of symptoms like blotch, soft rot, yellowing and

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immature browning in paddy straw, oyster and button mushroom cultivation due to lack of suitable control measures as well as bactericides [6]. The most common bacterial problem encountered by growers are species of *Pseudomonas*, which reduceself-life period as well as yield of mushroom. Bacterial brown blotch diseases of button and oyster mushroom, caused by *Pseudomonas tolaasii* and *Pseudomonas reactans*, are endemic and reduce significantly the quality and quantity of mushroom. Paddy straw mushroom cultivation is mainly affected by bacterial rot disease caused by species of *Pseudomonas* in India and Indonesia [7]. The causative agent produces toxin that disrupt the cell membrane via the formation of membrane pores [8, 9]. The expression of disease symptoms in mushroom depends upon the stage of development of the fruit body; however bacterial infection at the pinning stage is most crucial for mushroom cultivators. On account of that, the present study elucidate the complex bacterial activity associated with pinning stage of the tropical edible mushroom *Volvariella volvaceae* and their cost effective control measures to increase mushroom production.

Materials and methods

Collection of Samples

Paddy straw mushroom samples were collected aseptically in a sealed, sterile plastic bag during the pinning stage of cultivation, from Central Tropical Mushroom Research and Cultivation Centre (CTMRCC), Orissa University of Agriculture and Technology, Bhubaneswar. Samples were then transported to the laboratory by using a thermo cool box and processed immediately in the laboratory for bacteriological analysis. Fungal culture and chemical & culture media used in the study were procured from CTMRCC and Hi-media laboratories Pvt. Ltd., Mumbai respectively.

Aerobic heterotrophic bacteriological analysis

Aerobic, heterotrophic and mesophilic bacteria were isolated using serial dilution followed by spread plate method on Nutrient Agar and incubated at 37°C for 24 hours. Triplicates were made for samples and colonies were compared before processing for pure cultures. Enumeration of bacterial load was done by plate count method and pure culture of the isolates was made on Nutrient agar media slant. Identification of bacterial isolates was carried out on the basis of their colony characteristics, Gram's reaction, standard biochemical tests, sugar utilization tests and enzymatic activities as required by Bergy's manual of determinative bacteriology[10]and PIBWin software [11].

Antagonistic activity of bacterial isolates

This study was carried out for screening of bacterial isolates inhibiting growth of mushroom *Volvariella volvaceae*. Antagonistic activity of bacterial isolate were done by standard well diffusion method. Lawn culture of *Volvariella volvaceae* was prepared using Sabouraud's Dextrose Agar plates. Then 0.1 ml young active culture of bacteria was added in the well made with the help of gel puncher, the plate was incubated at 28-30^{o}C for 48-72 hrs and zone of inhibition was observed.

Antibiotic sensitivity assay

The selected bacterial isolates were screened for the antibiotic resistance at 37°C for 24 hours in Muller Hinton Agar by following disc diffusion method [12]. The isolate were subjected to different antibiotics like Amoxicillin, Amoxyclav, Bacitracin, Rifampicin, Ofloxacin, Cefotaxime, Vancomycin, Amikacin, Gatifloxacin, Fluconazole, Fosfomycin and Methicillin to observe the effect of these antibiotics on the physiology of the bacterial isolates. After incubation, the zone of inhibition was measured.

Estimation of disinfectant tolerance

In this test various disinfectants such as calcium hypochlorite (bleaching powder), hydrogen peroxide and calcium hydroxide (lime) were taken to evaluate the best disinfectant against contaminating bacterial isolates. Estimation of chlorine tolerance level of contaminating bacterial isolates were conducted using Nutrient Broth medium supplemented with filter (0.22 µm)

sterilized chlorine in form of calcium hypochlorite solution ranging from 100-1000 ppm with an increase in 100 ppm. Then, 100 µl of fresh culture was added to the respective concentration and incubated at 37°C/24 hours. Finally, the isolates were streaked onto the Nutrient Agar plates, incubated at 37°C/24 hours and bacterial growth was observed. Then, tolerance level of hydrogen peroxide and calcium hydroxide were observed by the method described earlier. Similarly the disinfectants tolerance levels of fungal isolate *Volvariella volvaceae* was also studied using Sabouraud's Dextrose Broth with an incubation temperature of 30°C for 24-48 hours.

Result and discussion

Aerobic heterotrophic bacteriological analysis

A total of eight aerobic heterotrophic bacteria were obtained from the pinning stage of mushroom *Volvariella volvaceae*. Apart from all, two were Gram negative and six were Gram positive bacterial isolates. The probabilistic identified bacterial isolates were *CDC Group IIj, Kingella sp., Bacillus sp., Bacillus cereus, Bacillus pumilis, Taxon*41, *Micrococcus luteus* and S*taphylococcus saprophyticus*. These isolates were able to utilize various sugars like glucose, mannitol, maltose, galactose and trehalose as source of energy. Moreover, majority of bacterial isolates showed Amylase, Cellulase, Caesinase and DNase enzyme activity. Association of various bacteria like *Micrococcus roseus, Bacillus pumilis, Bacillus licheniformis, Pseudomonas aeruginosa, Bacillus cereus* and *Escherichia coli* was also observed [13,14] in edible mushrooms. It is noteworthy to mention that, dominance of the genus *Bacillus, Pseudomonas* [7] and *Micrococcus* due to supplementation of contaminated substrate, paddy straw and water used during cultivation of mushroom.

Antagonistic activityof bacterial isolates

Antagonistic activity result revealed that, *Staphylococcus saprophyticus, Bacillus* sp., *Kingella* sp. and *Bacillus pumilis* are inhibiting the growth of edible mushroom *Volvariella volvaceae*. Among the four bacterial isolates *Staphylococcus saprophyticus* and *Bacillus pumilis* showed higher zone of inhibition than *Bacillus* sp. and *Kingella* sp. against the edible mushroom. Several species of *Pseudomonas* has the capability to inhibit growth of *V. volvaceae* [7] during mushroom cultivation. Similarly species of *Bacillus* are also inhibiting growth of *Tricoderma* sp. as reported earlier [3]. This may be due to production of different secondary metabolites by bacterial isolates, that are inhibiting growth of mushroom *Volvariella volvaceae*.

Antibiotic sensitivity profile of bacterial isolates

The antibiotic sensitivity profile indicated that *Staphylococcus saprophyticus* was sensitive to Cefotaxime, Amoxyclav, Gatifloxacin and Ofloxacin and resistant to Methicillin. *Bacillus pumilis* was ensitive to Amikacin and Gatifloxacin and showed resistance to Bacitracin, Cefotaxime, Gatifloxacin, Fluconazole & Methicillin. Similarly, *Kingella* sp. showed sensitiveness to most of the antibiotics used in the study and *Bacillus* sp. was sensitive to Amoxyclav, Gatifloxacin and Amoxyclav respectively.

Disinfectant tolerance of bacterial and fungal isolates

Disinfectant tolerance study revealed that *Staphylococcus saprophyticus, Bacillus* sp., *Kingella* sp., and *Bacillus pumilis* grew luxuriantly up to 500 ppm and moderate growth was also observed up to 700 and 1000 ppm of chlorine respectively (Table 1). But fungal species *Volvariella volvaceae* grew luxuriantly up to 300 ppm and could tolerate up to 900 ppm of chlorine. Moreover, application of 300 ppm of chlorine was unable to reduced growth of these contaminating bacterial isolates. Thus, other disinfectants like hydrogen peroxide and calcium hydroxide were selected for further study.

Correspondingly *Staphylococcus saprophyticus* and *Bacillus* sp., grew luxuriantly up to 200 ppm and *Kingella* sp., and *Bacillus pumilis* grew up to 100 ppm and could tolerate up to 300 ppm and 100 ppm of hydrogen peroxide respectively (Table 2). However, fungal species *Volvariella volvaceae* grew luxuriantly up to 300 ppm and could tolerate up to 600 ppm of hydrogen peroxide

Table 1. Chlorine tolerance profile of microbial isolates

Chlorine (ppm)	Growth profile				
	Volvariella volvaceae	*Staphylococcus saprophyticus*	*Bacillus sp.*	*Kingella sp.*	*Bacillus pumilis*
Control	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++
200	+++	+++	+++	+++	+++
300	+++	+++	+++	+++	+++
400	++	+++	+++	+++	+++
500	+	+++	+++	++	+++
600	+	++	+++	++	+++
600	+	++	+++	++	+++
700	+	++	+++	++	+++
800	+	-	++	-	+++
900	+	-	++	-	++
1000	-	-	+	-	+

+++ Luxuriant growth, ++ Moderate growth, + Mild growth and - No growth

Table 2. Hydrogen peroxide tolerance profile of microbial isolates

H_2O_2 (ppm)	Growth profile				
	Volvariella volvaceae	*Staphylococcus saprophyticus*	*Bacillus sp.*	*Kingella sp.*	*Bacillus pumilis*
Control	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++
200	+++	+++	+++	+	+
300	+++	++	+++	-	-
400	++	-	-	-	-
500	+	-	-	-	-

+++ Luxuriant growth, ++ Moderate growth, + Mild growth and - No growth

in the medium. Thus, application of 300 ppm hydrogen peroxide solution reduces growth of these contaminating bacterial isolates. This result corroborated the results of [15], who observed the most common bacterial problem encountered by growers are *Bacillus* sp.and *Pseudomonas* sp. and treatment of paddy straw or water with 3 % hydrogen peroxide kills contaminants without encouraging new resistant strains. Similarly *Staphylococcus saprophyticus*, *Bacillus* sp., *Kingella* sp., and *Bacillus pumilis* grew luxuriantly up to 100 ppm and could tolerate up to 300 ppm and 200 ppm respectively (Table 3). However fungal species *Volvariella volvaceae* grew luxuriantly up to 300 ppm and could tolerate up to 500 ppm of lime water in form of calcium hydroxide. Thus, application of 300 ppm lime water solution is reducing growth of these contaminating bacterial isolates. Antimicrobial activity of calcium hydroxide is related to the release of hydroxyl ions in an aqueous environment. Hydroxyl ions are highly oxidant free radicals that show extreme reactivity, reacting with several biomolecules[16].

Conclusion

The spawn and other materials are used for

Table 3. Lime water tolerance profile of microbial isolates

Lime water (ppm)	Growth profile				
	Volvariella volvaceae	***Staphylococcus saprophyticus***	***Bacillus sp.***	***Kingella sp.***	***Bacillus pumilis***
Control	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++
200	+++	+++	+++	+++	+++
300	+++	++	++	++	++
400	++	+	-	-	+
500	+	-	-	-	-

+++ Luxuriant growth, ++ Moderate growth, + Mild growth and - No growth

cultivation of edible mushroom are nutritionally and biologically active to support growth of mushrooms as well as undesirable microbes. This is because mushroom growing processes are always been intruded and spoilt by bacteria leading to low yield and thereby causing economic losses for mushroom growers. Among all the disinfectant studied, lime water is economic and effectively reduces growth of these contaminating bacterial isolates. Thus the above experiment concluded that, field study and nutritional quality analysis of mushroom is essential before considering use of calcium hydroxide as a cost effective control measures to increase paddy straw mushroom production.

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