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Morphological and genetic characteristics of Oyster mushrooms and conditions effecting on its spawn growing

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Abstract: In Vietnam, oyster mushrooms are particularly interesting as one kind of popular foods. In this research, 18 isolated strains were obtained from 6 different samples of fresh oyster mushrooms. Of these pure isolates, three groups performing significantly different ability of starch degradation at the 95% confidence level could be distinguished. One representative strain of each group was selected for further study of morphological and genetic characteristics, including strains no. 1.2 (TCT), 2.3 (TBT) and 5.2 (NBT). The morphological and molecular identification methods gave the similar results, in which two strains of white oyster mushrooms were *Pleurotus floridanus* and one strain of Japanese oyster mushroom was *Pleurotus cystidiosus*. Because of its highest performance in starch degrading activity, *Pleurotus floridanus* (strain 1.2) was selected for the study of conditions affecting on its spawn growing. Based on the statistical analysis, the favorable conditions for spawn growing were found at 57% humidity and at 27^{o}C incubation temperature.

Keywords: Oyster mushroom, morphological characteristics, genetic characteristics, ITS

Introduction

Mushroom cultivation is a profitable agribusiness world-wide. There are more than 5000 mushroom varieties could be employed for foods and medicines. In the fungal classification system proposed by Ainsworth and followed by J. Webster (Sharma, 1989), almost edible mushrooms are members of the subdivision Basidiomycotina and Ascomycotina (Dung, 2007).

In Vietnam, especially the Mekong Delta of the south, mushroom production has been developing significantly due to various advantages, including appropriate climate, prosperous labors, and abundant raw materials (Dong *et al*., 1997). Among many kinds of edible mushrooms, oyster mushrooms have been commercialized and consumed remarkably. They have not only excellent taste but also high-content nutritious components, including proteins, carbohydrates, vitamins and minerals. In addition, many research projects have revealed that oyster mushrooms could prevent and reduce several serious diseases, including high blood pressure and cholesterols (Agrawal *et al*., 2010), and breast cancer, prostate cancer (Jedinak and Sliva, 2008).

Three primary factors affecting the yield of oyster mushrooms are temperature, compost component and humidity. The process of cultivating oyster mushrooms has 3 main steps: isolating mushroom from fruiting bodies, preparing primary and secondary spawn, and cultivating mushrooms from these spawns to harvest fruiting bodies (Dung, 2003).

Although oyster mushrooms are produced and consumed popularly, their different strains are still distinguished simply basing on the fruiting body color and taste, or the location producing them. Few strains of contemporary cultivated oyster mushroom are identified at genus and species level. Besides, it is necessary to establish a process producing mushroom spawn efficiently and stably to provide an ample source for mushroom production.

The objectives of this study are to isolate oyster mushrooms, identify their capacity of degrading starch, study morphological and genetic characteristics of pure isolates, and propose appropriate conditions about the humidity, temperature and compost component in oyster mushroom spawn production.

Materials and Methods

Collection of samples and isolation of oyster mushroom

Samples of different oyster mushroom varieties were purchased from small-scale factories and representative wholesale markets in four provinces and cities (Can Tho, Vinh Long, Phung Hiep, Ben Tre) in the Mekong Delta region. Collected samples should be fresh, intact and lack of injures. Under aseptic lab conditions, samples of mushroom fruiting bodies were washed and then wiped with alcohol 70^{o}. Small pieces of the core part of caps and stipes were separated, transferred into PGA medium, and incubated at 30^{o}C for fungal growth covered with mycelium. The culture transfer was conducted many times until obtaining the pure isolates that were store in PGA tubes at 4^{o}C.

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Screening test of starch degradation

Based on the starch degrading method described in a research of Dung *et al*. (2006), all pure isolates were tested for their ability to degrade soluble starch, in rice starch agar medium, containing 0.5% of soluble starch, 0.1% of peptone and 1.5% of agar. After 6 days of incubation at 30°C, visualization of starch degradation was done by flooding with a 0.25% iodine solution. Clearing of the typical blue coloration of the starch with iodine indicated starch degradation

Morphological and genetic characterization of selected isolates

The representatives of isolates were selected from the previous screening test of starch degradation for further morphological and genetic characterization. The morphological properties were examined and classified according to taxonomic keys and descriptions (Sharma, 1989; Dung, 2003). For genetic characterization, of all selected strains, genomic DNA was extracted following the procedure of Liu *et al*. (2000) with some modifications. PCR amplification was conducted with the Thermal Cycler C1000, CC5566, Bio – Rad, USA. Each PCR tube contained 2.5 µl DNA template, 5 µl buffer IV 10X, 4 µl $MgCl_2$ 50 mM, 2 µl dNTPs 200 µM, 0.25 µl of primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990), and 0.5 µl Taq polymerase 5U, totally 50 µl for one tube. PCR products were tested by gel electrophoresis. The amplified DNAs were then sequenced and compared with database on the website *http://blast.ncbi.nlm.nih.gov/Blast.cgi* to identify the species of strains.

Conditions affecting mushroom spawn growing

A factorial design (3 factors at 3 levels) was used: compost humidity (55%, 60% and 65%), incubation temperature (20°C, 25°C and ambient temperature: 28-33°C); and raw materials of compost component (formula 1: 93% paddy, 2% $CaCO_3$, 5% sawdust, formula 2: 93% paddy, 3% $CaCO_3$, 4% sugar, formula 3: 98% paddy, 2% gypsum dust). Each treatment had triplicates. During the incubation period, the growth and development of the fungal mycelia in all treatments were daily observed and evaluated. The period for the complete spawn growing was recorded. The appearance of any undesirable contamination was also monitored and detected.

Statistical analysis

Experimental data were collected and statistically analysed using StatGraphics Plus Version 5, Manugistics, Inc., Rockville, USA.

Results and Discussion

Mushroom isolation and starch degrading capacity

Eighteen pure isolated strains were obtained from six different varieties of oyster mushroom. Isolates of white oyster mushrooms appeared in identical white mycelia, whereas isolates of Japanese oyster mushrooms got some black pots containing spores sticking on their hyphae system. The appearance and the growth of the mycelium system on the Petri dish were described in Figure 1.

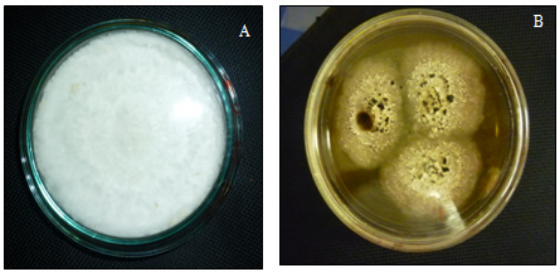

Figure 1. Mycelium appearance of white oyster mushroom TCT (A) and Japanese oyster mushroom NBT (B)

The results of the mould growth (indicated as mycelium appearance) and the diameters of the starch degradation zones as well as the places of collection and the names of mushrooms varieties were presented in Table 1. Most strains grew well on starch medium, but this does not automatically imply that they are good at starch degradation. However, it can be concluded that strains that actively degraded starch, always grew well. Statistical analysis revealed significant ($p < 0.05$) differences in degrading ability. Three groups of mould isolates performing significantly different degrading activity could be distinguished. One representative of each group was selected for studies of morphological and genetic characterization. Besides, the strain TCT (nr. 1.2) that had the highest degrading capacity was further employed in experiment of conditions affecting mushroom spawn growing.

Morphological characterization of selected mould isolates

All the three selected isolates had septa and clamp connections in their filaments. According to Sharma (1989), clamp connections were formed during cell division of secondary hyphae in most of the genera of Basidiomycetes. Their hyphae were white when observed with naked eye, but transparent and colorless under optical microscope. The two isolates TBT and TCT got homologous white mycelia, while NBT isolate had many sticks containing black liquid

Table 1. Performance of mould isolates in starch degradation activity

Names of mushrooms	Notation	Places collected	Isolate nr.	Mycelium appearance [1]	Diameter of clear zone in iodine test (cm)	
					Mean values [2]	SD [3]
White oyster mushroom Can Tho	TCT	Can Tho	1.1	+++	6,22a	0,83
			1.2	**+++**	**6,45a**	**0,65**
			1.3	+++	6,25a	0,69
White oyster mushroom Ben Tre	TBT	Ben Tre	2.1	+++	4,28b	0,64
			2.2	+++	4,30b	0,30
			2.3	**+++**	**4,35b**	**0,44**
Japanese oyster mushroom Phung Hiep	NPH	Phung Hiep	3.1	+	3,33c	0,10
			3.2	+	3,35c	0,05
			3.3	+	3,35c	0,22
Japanese oyster mushroom Vinh Long	NVL	Vinh Long	4.1	++	4,88b	0,04
			4.2	++	4,87b	0,07
			4.3	++	4,89b	0,12
Japanese oyster mushroom Ben Tre	NBT	Ben Tre	5.1	++	3,38c	0,17
			5.2	**++**	**3,43c**	**0,24**
			5.3	++	3,37c	0,24
Japanese oyster mushroom Can Tho	NCT	Can Tho	6.1	++	3,28c	0,06
			6.2	++	3,27c	0,06
			6.3	++	3,25c	0,08

[1] Levels of mycelium appearance ranging from + (little) to +++ (very much).
[2] Means of triplicates with different subscripts are statistically different at the 95% confidence level. Strains bold are those selected for further study.
[3] Standard deviation

drops with abundant spores inside. The appearance of mycelium and hyphae growing of three isolates was illustrated in Figure 1 and Figure 2.

Oyster mushrooms were saprophytic and edible. In life cycle, they formed a specific sexual structure called fruiting body, or sporocarp, by plaiting the hyphae tightly. Except some differences in color, the sporocarps of three isolates shared many similar characteristics: they were offset, fleshy, and unable to revive when remoistened as well as release milky liquid when injured. The upper stipites were smooth, but the lower were pilous. The caps were hairless, lack of outer membrane and laterally stuck to the stipe. At the lower surface of caps, there were many gills radiating from the end of the stipe to the outer side of the cap. On these gills but not their edges, there was a layer tissue producing cells which would develop into spores called hymenium. Sticking on this hymenium layer, there were a lot of basidia with short stem and 4 basidiospores. Each basidiospore would develop into a fungal spore and release into the air when mature. Oyster mushroom spores were uncolored, transparent and inswept like a bean-shape. When gathering, they formed a white spore-printing feature. According to the morphological classification system of fungi (Sharma, 1989; Dung, 2003), three isolates of TCT 1.2, TBT 2.3 and NBT 5.2 belonged to the Subdivision *Basidiomycota*, Class *Hymenomycetes*, Order *Agaricales*, Family *Pleurotaceae* and Genus *Pleurotus*.

Genetic characterization of selected mould isolates

The extracted DNAs of selected mould isolates were determined in the range of the typical ratios of absorbance at 260 nm and 280 nm (1.8 - 2.0), and the final DNA concentrations were about 600 - 900 ng/µl.

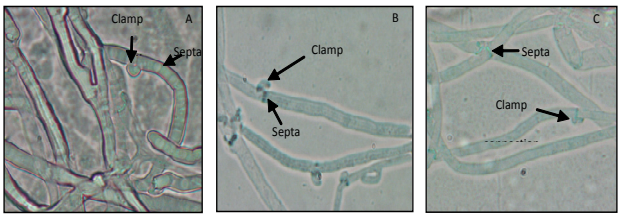

Figure 2. Hyphae appearance of three oyster mushroom isolates at x100 magnification. (A) white oyster mushroom TCT; (B) white oyster mushroom TBT; and (C) Japanese oyster mushroom NBT

These results indicated that the extracted DNAs obtained were qualified and available for further molecular determination. PCR products from the couple of primers ITS1 and ITS 4 provided a clear single band between 600 bp and 700 bp. Their sequences were recorded as below.

The DNA sequence of TCT 1.2 was 632 bp in length as following:

01 AGAATTACTA TGGAGTTGTT GCTGGCCTCT
AGGGGCATGT GCACGCTTCA 61 CTAGTCTTTC
AACCACCTGT GAACTTTTGA TAGATCTGTG
AAGTCGTCTC 111 TCAAGTCGTC
AGACTTGGTT GCTGGGATTT AAACGTCTCG
GTGTGACTAC 161 GCAGTCTATT
TACTTACACA CCCCAAATGT ATGTCTACGA
ATGTCATTTA 211 ATGGGCCTTG TGCCTTTAAA
CCATAATACA ACTTTCAACA ACGGATCTCT
261 TGGCTCTCGC ATCGATGAAG
AACGCAGCGA AATGCGATAA GTAATGTGAA
311 TTGCAGAATT CAGTGAATCA
TCGAATCTTT GAACGCACCT TGCGCCCCTT
361 GGTATTCCGA GGGGCATGCC
TGTTTGAGTG TCATTAAATT CTCAAACTCA
411 CTTTGGTTTC TTTCCAATTG TGATGTTTGG

ATTGTTGGGG 461 GCTGCTGGCC TTGACAGGTC GGCTCCTCTT AAATGCATTA GCAGGACTTC 511 TCATTGCCTC TGCGCATGAT GTGATAATTA TCACTCATCA ATAGCACGCA 561 TGAATAGAGT CCAGCTCTCT AATCGTCCGC AAGGACAATT TGACAATTTG 611 ACCTCAAATC AGTAGGACTA CCCGCTGAAC TTAAGCATAT GACCT

The DNA sequence of TBT 2.3 was 632 bp in length as following:

01 AGTCTTCCCAACCACCTGTG AACTTTTGAT AGACAGTGAA GTCGTCTCTC AAGTCGTCAG 61 ACTTGGTTGC TGGGATTTAA ACGTCTCGGT GTGACTACGC AGTCTATTTA 110 CTTACACACC CCAAATGTAT GTCTACGAAT GTCATTTAAT GGGCCTTGTG 161 CCTTTAAACC ATAATACAAC TTTCAACAAC GGATCTCTTG GCTCTCGCAT 210 CGATGAAGAA CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTCA 260 GTGAATCATC GAATCTTTGA ACGCACCTTG CGCCCCTTGG TATTCCGAGG 310 GGCATGCCTG TTTGAGTGTC ATTAAATTCT CAAACTCACT TTGGTTTCTT 360 TCCAATTGTG ATGTTTGGAT TGTTGGGGGC TGCTGGCCTT GACAGGTCGG 410 CTCCTCTTAA ATGCATTAGC AGGACTTCTC ATTGCCTCTG CGCATGATGT 460 GATAATTATC ACTCATCAAT AGCACGCATG AATAGAGTCC AGCTCTCTAA 510 TCGTCCGCAA GGACAATTTG ACAATTTGAC CTCAAATCAG GTAGGACTAC 560 CCGCTGAACT TAAGCATATC AATAGACGGA GGAAGGA

The DNA sequence of NBT 5.2 was 661 bp in length as following:

01 ATACATTCAA CCACTTGTGC ACTTTTGATA GATTCGCAGA GTTGCCCTCT CAGGTCAGTA 61 AATGACTTGG TTGGTCGGGA TTGTCACAGT CCTGGCTTTG ACTTTGTGGG 110 TCTATTATCT TATACACACT TGTATGTCCA TGAATGTTAT TTTCTTGGGC 161 CATGTGCCTA TAAAACCTAA TACAACTTTC AACAACGGAT CTCTTGGCTC 210 TCGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG 261 AATTCAGTGA ATCATCGAAT CTTTGAACGC ACCTTGCGCC CCTTGGTATT 310 CCGAGGGGCA TGCCTGTTTG AGTGTCATTA AATTCTCAAA TCTATAGAGC 360 TTTTTTGTGA TATAGATTTG GATTGTTGGG GGCTGCTGGC TTTTTACCAA 410 GTTGGCTCCT CTTAAATGCA TTAGCGGGAC TTTATTGCCT CTGCGCACAG 460 TGTGATAATT ATCTACGCTG GCCGACATGC AATGACTTTA CAAGTCCAGC 510 TTTCTAACTG TCTTTCAAGA CAATGACTTG ACAATTTGAC CTCAAATCAG 560 GTAGGACTAC CCGCTGAACT TAAGCATATC AATAAGCGGA GGAAAGATCA 610 TTAATGAATT ACTCATGAAG CTGATGCTGG TCTCTCGGGA CATGTGCACG 661 C

When compared with database in gene bank *(http://blast.ncbi/nlm.nih.gov/Blast.cgi)*, sequences of TCT and TBT samples were homologous with partial sequence of 18S rRNA; complete sequence of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2; and partial sequence of 28S ribosomal RNA gene of the species *Pleurotus floridanus*, accession number GU721058 (99%) and FJ810170.1 (98%), respectively. They were classified in the Eukaryota; Fungi; Dikarya; *Basidiomycota*; *Agaricomycotina*; *Agaricomycetes*; *Agaricomycetidae*; *Agaricales*; *Pleurotaceae*; *Pleurotus* and species *Pleurotus floridanus*.

Separately, NBT sequence was 100% homologous with partial sequence of 18S rRNA; complete sequence of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2; and partial sequence of large subunit ribosomal RNA gene of the species Pleurotus cystidiosus, accession number DQ978222.1. This mushroom isolate was classified in the Eukaryota; Fungi; Dikarya; *Basidiomycota*; *Agaricomycotina*; *Agaricomycetes*; *Agaricomycetidae*; *Agaricales*; *Pleurotaceae*; *Pleurotus* and *Pleurotus cystidiosus*.

In general, the morphological and molecular identification methods offered basically similar results. All three isolates belonged to the subdivision *Basidiomycota* and genus *Pleurotus*. However, the molecular identification system provided some more different taxonomic units (Subdivision *Agaricomycotina*, Class *Agaricomycetes*, and Subclass *Agaricomycetidae*). In addition, oyster mushrooms belonged to Family *Agaricaceae* according to morphological identification system, yet Family *Pleurotaceae* as in molecular identification method. These dissimilarities were due to different taxonomic systems between the two methods. Morphological taxonomic system was built based on classification of Ainsworth and followed by Webster (Sharma, 1989). Molecular taxonomic system was created by comparing gene sequences of enormous

fungi strains, especially the genes coding for rRNA. These comparisons were analyzed, and a new classification system was proposed (David *et al*., 2007). Overall, this classification system added and rearranged some taxonomic units above genus, but the genus and species units were remained.

Factors affecting mushroom spawn growing

Based on the experience of leading local producers and the few available literature references (Dong *et al*., 1997; Dung, 2007), three factors at three levels affecting mushroom spawn growing were selected for the experimental design. The aim was to find out the favourable conditions for the completion of mushroom spawn growing in the shortest incubation time. Figure 3 shows a surface plot based on the statistical analysis of the results in testing conditions affecting spawn growing during mushroom production.

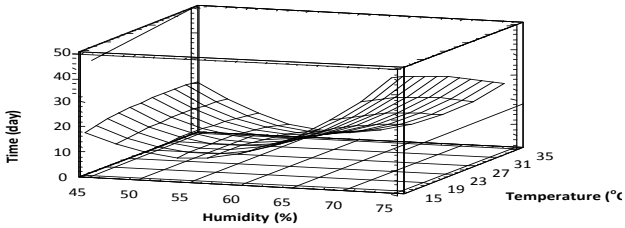

Figure 3. Analysis results of optimal conditions in producing oyster mushroom spawn

As an indicator harvesting the complete spawn growing, the total days of fungal mycelia filling up compost bags were recorded. No microbial contamination was found in all treatments. The incubation time to get completely full-fill spaw bag was different sighnificantly with 95% confidence interval among three different incubation temperatures 20°C, 25°C and ambient temperature (28-33°C); they were 12.7 days, 11.2 days and 8.8 days in average, respectively. Thus, the ambient temperature (28-33°C) was the favourable temperature to produce spaw bags with shortest incubation time. There was no sighnificantly different results of incubation time between two levels of moisture content at 55% and 60% (9.4 days for both); however, at the humidity level of 65% the incubation time was significantly different (13.8 days). Consequently, it could indicate that the levels of moitsture content at 55% and 60% were the most suitable to produce oyster mushroom in short time. It was also showed that three different fomulars of compost component offered no significant difference on incubation time. Overall, according to the analysis of interactions among the three testing factors (incubation temperature, compost humidity and compost component), the optimum conditions to produce oyster mushroom spawn were at 57% of compost humidity and at 27°C of incubation temperature for 9 days of incubation time. In the research of Zadrazil and Kurtzman (1982), it was also found that the hyphae growing of *P. Floridanus* could effectively develop at the incubation temperature from 25 – 30°C.

Conclusions

The representatives of selected target strains of oyster mushrooms were cultivated at laboratory-scale to determine the purification and the accuracy of the mushroom isolation as well as to identify the morphological characteristics. For genetic study, genomic DNA of selected strains was extracted. A couple of primer ITS1 and ITS4 was used to amplify the gene coding for ITS1, ITS2 and 5.8S rRNA. The similarities between these amplified genes and databases of DNA revealed that white oyster mushroom was the *Pleurotus floridanus*, and Japanese oyster mushroom belonged to the *Pleurotus cystidiosus*. For the favourable conditions affecting mushroom spawn growing it was indicated at 57% of compost humidity and at 27°C of incubation temperature for 9 days of incubation time.

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