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RESEARCH ARTICLE

Chemically and physically induced mutagenesis in basidiospores of oyster mushroom *Pleurotus ostreatus* var. *florida*

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Abstract

The oyster mushroom *Pleurotus ostreatus* is the third important edible mushroom in the world, following the button and shiitake mushrooms. There is an increasing interest to utilize mutation in order to obtain desirable characteristics in edible mushrooms, including *P. ostreatus*. However, there is a scientific shortage of information available on accurate experimental details of mutation induction in *P. ostreatus*, particularly while it comes to basidiospores. Therefore, the objective of this study was to obtain a reliable method to induce physical and chemical mutations in *P. ostreatus* var. *florida* basidiospores through ultraviolet (UV) irradiation and ethyl methane sulphonate (EMS). The findings revealed that the UV exposure at a distance of 45 cm for 120 seconds and the EMS treatment at 1.5% caused a maximum killing effect (over 95%) in the basidiospores, as compared to the untreated spores. Moreover, the 50% lethality dose (LD_{50}) was seen to be at 41.73±2.5 seconds of the U.V exposure and the concentration 0.51±0.05 % (v/v) of EMS. The findings of this study warrant further research to investigate morphological and developmental characteristics in fruiting bodies generated by the putative mutant spores. In addition, the experimental conditions established in this study may be useful for inducing mutation through UV radiation or EMS treatment in basidiospores of *P. ostreatus*.

Introduction

The global production of oyster mushrooms, *Pleurotus* spp, have been increased over recent decades (Chang, 1996) so that it occupied the third position of the global mushroom production in 1997 (Chang and Miles, 2004). The significance of these mushrooms is mainly due to having a high biological efficiency and significant levels of proteins (Hernández et al., 2003; kalmis et al., 2008). In addition to nutritional values, therapeutic and biological properties such as anti-bacterial, anti-virus, anti-tumor, etc, have been well demonstrated in oyster mushrooms (Cohen et al., 2002; Kues and Liu, 2000).

Mutation has long been exploited in crop breeding programs in order to improve both quality and productivity. Various methods of inducing mutation may be used, including chemical, biological, and physical agents such as ultraviolet (UV) and gamma irradiations (Fahad and Salim, 2009). Similar to crops, mutation has been recognized as an effective breeding tool for both parasitic fungi and edible mushrooms (Shivanna and Govindarajulu, 2009; Jaivel and Marimuthu, 2010; Lee et al, 2011; Liu et al, 2011). Accordingly, there are several

reports on mutagenesis induction through UV or chemical agents such as ethyl methane sulphonate (EMS) in various species of *Pleurotus*. Isolated protoplasts from mycelium of a *P. erynjii* strain were exposed to UV radiation, resulting in identification of a mutant dikaryon that was entirely sporeless (Obatake et al, 2003). In another study, protoplasts of a wild strain of *P. ostreatus* were subjected to UV irradiations for several periods and survival rates of protoplasts were determined. Abnormal vegetative mutants with unusual morphological characteristics were also isolated and fruiting bodies formation of normal mutants was evaluated (Joh et al, 2004).

In addition to protoplasts, limited studies have reported induction of mutation in basidiospores of *Pleurotus* spp. In this regard, basidiospores and mycelium of two strains of *P. florida* and *P.sajor-caju*, were exposed to UV radiation in order to develop low sporing strains (Ravishankar et al., 2006). Another study reported that UV-induced mutations in spores of *P. ostreatus* resulted in an appressed mycelial growth type, slower spawn run, or creamish white sporophore (Sharma and Sharma; 2014). Induction of mutation for improving characteristics such as high productively and adaptability to the wide range of temperature were also investigated in mycelium and basidiospores of five strains of oyster mushrooms, using gamma and UV irradiation (Beejan and Nowbuth, 2009). In addition, laccase production and growth of *P.florida* and *P.sajor-caju* treated with gamma radiation were examined (Abo-state et al, 2011). There are also very limited studies undertaken to investigate mutagenic effects of EMS on basidiospores of *P. ostreatus* (Sharma and Sharma; 2014).

As opposed with other edible mushrooms, the available studies with UV or EMS on induction of mutation in basidiospores of *P. ostreatus* lack information on important experimental details; such as number of basidiospores exposed to mutagenic agents and dose-dependent curves of spore killing percentage, sources and concentrations of original stocks of EMS, UV wavelengths, distances and times of UV exposure, authentication of tested mushrooms, and proper statistical analysis. Therefore, the present study intended to establish a reproducible method to induce mutations in basidiospores of *P. ostreatus* var. *florida* using EMS and UV radiation. The findings of this study may be applicable in further investigations on using UV or chemical mutagenesis for strain improvement in different varieties of *P. ostreatus.*

Material and Methods

Samples

A cultivated strain of ***Pleurotus ostreatus* var. *florida* stocked at Industrial Fungi Biotechnology Research Department of Iranian Academic Centre for Education**, Culture and Research (ACECR)-Mashhad Branch, was used. This strain has previously been **authenticated morphologically and confirmed through Internal Transcribed Spacer (ITS) sequence analysis by mycologists in our institute. T**he mushroom mycelia were purified in potato dextrose agar (PDA) medium, and subjected to spawn production and mushroom growing on wheat straw based on procedures adapted in the department. The resultant fresh fruiting bodies were then used for spore preparation. The sterile filter papers containing basidiospores were cut out aseptically and added to one ml of sterilized distilled water to produce spore suspension.

Chemicals

Chemicals used for this study were freshly prepared before use. PDA media and EMS (Cat no M0880) were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Germany), respectively.

Mutagenesis by EMS

The spores were treated by EMS using a modified method of Liu et al. (2011). In brief, a stock solution of EMS (1.167 g/mL) in water was made. Then, various EMS doses were taken and added into one mL of the spore suspension to reach the following dilutions as follow: 0.25%, 0.50%, 0.75%, 1.00%, and 1.5% (v/v). The EMS-treated spores were then incubated at 30°C for one hour with shaking. The EMS residue (as supernatant) was removed after centrifugation at 9000 × g for five minutes, and the spores (as pellet) was carefully washed twice with sterile distilled water. The spores were then counted using a hemocytometre so that 500 spores/200 µL were seeded onto an 8-cm plate containing PDA media. The same procedure was performed with untreated spores to serve as negative controls. The plates were placed at 25°C for 21 days, with daily observations. The spore viability percentages were measured based on the amount of living spores in treated spores relative to negative controls (defined as 100% viability), followed by conversion to spore killing percentages (through subtracting from 100). The 50% lethality dose (LD_{50}) was estimated by regression analysis of the dose-response curve.

Mutagenesis by UV radiation

The spores were prepared as described above for EMS treatment. The cultured spores were exposed to UV radiation at 254 nm (G30T8 Germicidal 3FT 30W T8 UVC , Philips, The Netherlands) under a laminar air flow hood for six different periods (30, 45,60,75, 90 and 120 seconds), at various distances of 10, 20, and 45 cm. This procedure was carried out in darkness to inhibit photoreactivation (Chan and Miles, 2004), as it has been demonstrated that photoreactivation may repair UV induced-DNA damage. After UV irradiation, the plates were incubated at 25°C for 21 days. Spore killing percentages were determined as described above for EMS treatment.

Statistical analysis

Each experiment was independently repeated at least three times. GraphPad Prism version 6 was utilized to conduct statistical analyses and one-way analysis of variance (ANOVA) tests. Means were compared using Tukey method with a significance level (*p* value) of 0.05. Graphs were drawn using Microsoft Office word 2007.

Results and Discussion

Impact of EMS on lethality of basidiospores

Overall, our findings revealed that EMS caused lethality in the basidiospores in a dose-dependent manner. The maximal and minimal EMS-caused lethality were found to be at the concentrations of 1.5%, v/v (over 99% spore lethality) and 0.25%, v/v (only 13.6% spore lethality), respectively (Figs. 1 and 2). Interpolation of the EMS lethality curve showed that the 50% lethality point is at the concentration 0.51±0.05 %, v/v. Besides, our findings revealed that high levels of EMS (2%-10%, v/v) completely prevented the spores from germinating (data not shown).

Statistical comparisons showed significant differences in spore lethality between the lowest concentration (0.25%, v/v) and the higher concentrations ($p<0.01$). Similarly, such meaningful differences in spore lethality were observed between the highest concentration (1.5%, v/v) and the rest. Also, the ability of EMS at concentration 0.5 % (v/v) to kill the spores differed significantly from that at concentration 0.75 % (v/v) ($p<0.05$). However, no meaningful difference was observed between 0.75 % and 1% (v/v) ($p>0.05$).

A similar study reported a much lower concentration of EMS (0.005%) to block mycelia growth of spores of *P. ostreatus* (Sharma and Sharma, 2014), as compared to our data. However, the concentration of original stock of EMS, number of spores treated and the dose-response curve of spore lethality have not been given. Thus, there is no adequate information to accurately compare our findings with similar reports on basidiospores of *P. ostreatus*. On the contrary, our findings generally agree with those from a study on *Volvariella volvacea* that applied the same methodology as ours; where it was found that EMS at 1% caused 72% lethality in basidiospores of *V. volvacea* (Liu et al., 2011). Thus, our findings may suggest that a proper methodology is required to use EMS or other mutagenic agents for induction of mutation in basidiospores of edible basidiomycetes, where the spore load, centrifugation and washing of spores, concentrations of chemical agent and dose-response curves should be taken into account.

Basidiospores lethality rate through UV radiation

Prior to UV irradiation, the spores were accurately counted to reach 500 spores/200 µL. Similar to the EMS findings, the lethality of basidiospores treated by UV radiation exhibited a time-dependent manner. As such, the spore killing percentage rose with increasing periods of UV exposure (Figs. 3 and 4). At a distance of 45 cm, the maximal effect of UV was seen at the time 120 seconds, while the LD_{50} was found to be at 41.73±2.5 seconds. Furthermore, UV exposure times less than 30 seconds could not kill spores effectively (data not shown), whilst no spore germinated when UV lasted for more than 120 seconds (Fig. 3). In addition to the optimized distance 45 cm, two distances 10 cm and 20 cm were also evaluated. The results showed that no spore was recovered from UV irradiation at the afore-mentioned distances up to 21 days (data not shown).

Statistical analysis revealed that there were significant differences ($p<0.05$) in spore killing percentage between the shortest time of UV exposure (30 seconds) and the longer ones. The longest time of UV radiation (120 seconds) did not significantly differed from that of 90 seconds; however, the both were seen to be significantly different from the rest of periods ($p<0.01$). In addition, a significant difference was observed between the UV exposure at 45 seconds and that of 60 seconds ($p<0.05$).

Ravishankar et al. (2006) utilized UV for a much longer time (75 minutes) to induce a lethality dose of 99% in basidiospores of *P. ostreatus* var *florida*, as compared to our findings. However, no further information was given regarding distance of UV exposure, the wavelength of UV, and number of spores treated with UV. In addition, another study reported results with UV radiation that were different from ours (Sharma and Sharma, 2014), where an UV radiation at distance of 10 cm for 15 minutes was reported to induce intended mutations in spores of *P. ostreatus*. Again, type and wavelength of UV and number of spores treated were not indicated. Therefore, we could not make an accurate comparison with the afore-mentioned findings. On the other hand, our findings are in line with survival ratios observed in protoplasts of *Pleurotus*. spp, where an UV exposure of 17-22 seconds at 10 cm distance and 50-70 seconds caused mutations in protoplasts from mycelium of a *P. erynjii* strain (Obatake et al., 2003) and *P. ostreatus* (Joh et al., 2004), respectively.

Figures

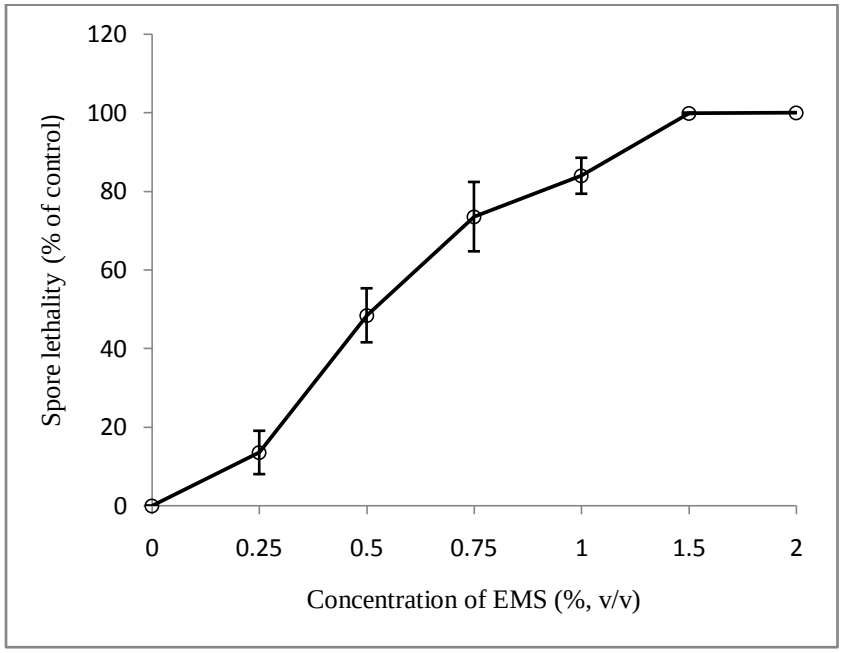

Figure 1. Mutagenic effects of various doses of EMS (v/v in water) on spore germination in *P. ostreatus* var. *florida*. Values represent means ± standard deviation. Each experiment was independently repeated as least three times from which standard deviations have been derived. Spore lethality was scored based on the amount of germinating spores treated with EMS relative to 500 untreated spores, followed by subtracting from 100.

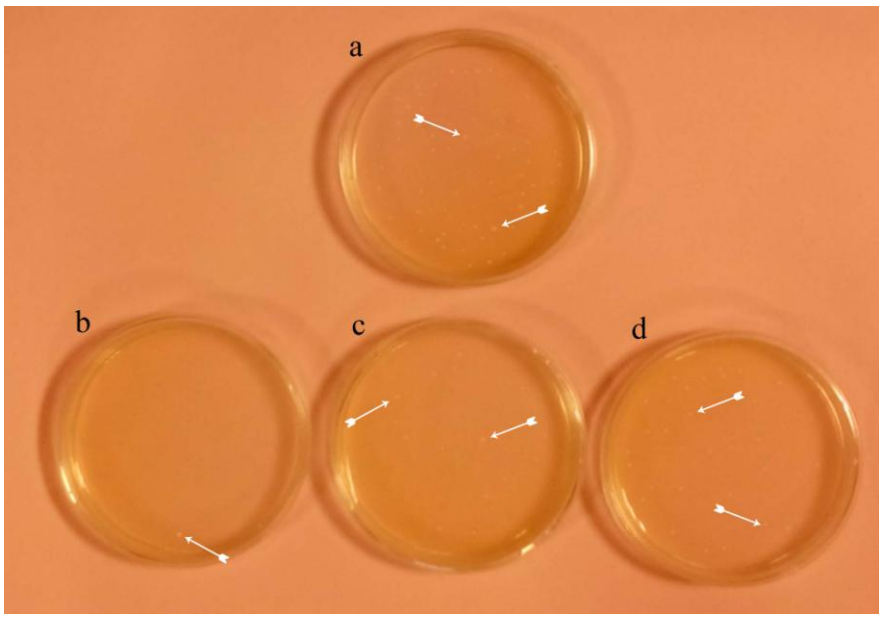

Figure 2. Illustration of the effect of EMS on germination of basidiospores of *P. ostreatus* var. *florida* seeded in petridishes. Each experiment was independently repeated as least three times. The photos were taken 21 days after treatment. a, untreated basidiospores; b, germinating spores treated with a high dose of EMS (1.5% v/v); c, germinating spores treated with EMS at 0.5% v/v; d, germinating spores treated with a low dose of EMS (0.25% v/v).

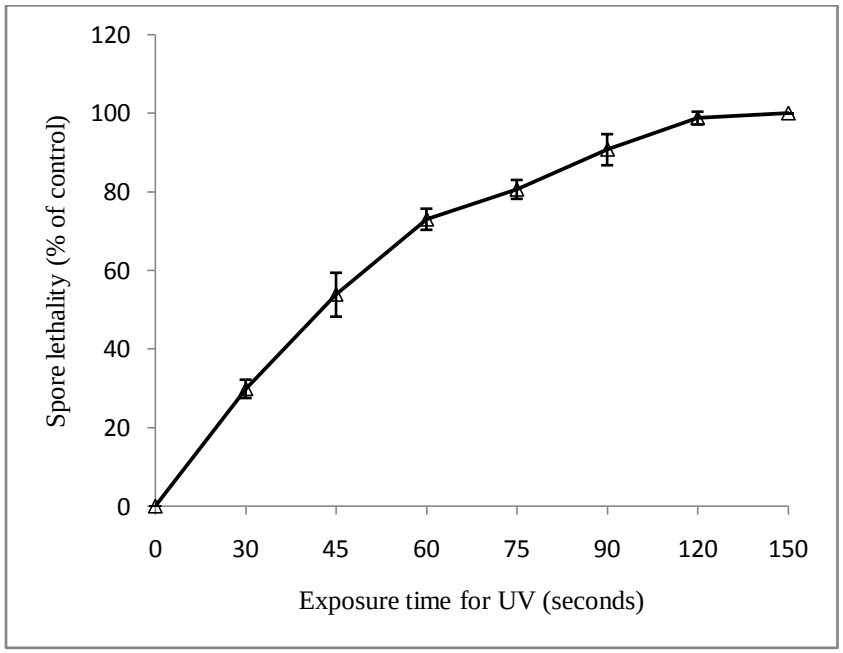

Figure 3. Effect of UV radiation at various times on the survival of *P. ostreatus* var. *florida* basidiospores. Values represent means ± standard deviation. Each experiment was independently repeated as least three times from which standard deviations have been derived. Spore lethality was scored based on the amount of germinating spores treated with UV light relative to 500 untreated spores followed by subtracting from 100.

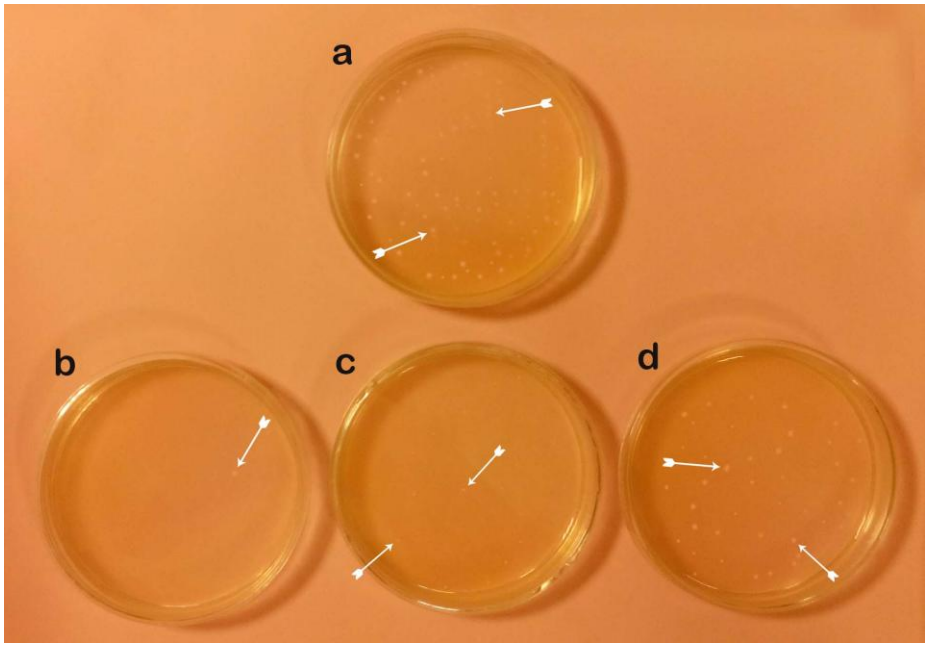

Figure 4. Illustration of the effect of UV radiation on germination of basidiospores of *P. ostreatus* var. *florida*. Each experiment was independently repeated as least three times. The photos were taken 21 days after treatment. a, untreated basidiospores; b, germinating spores exposed to UV for a long time (90 seconds); c, germinating spores exposed to UV for 45 seconds; d, germinating spores exposed to UV for a short time (30 seconds).

Conclusions

Reproducibility and accuracy of methods for using chemical or physical agents for induction of mutation in mushrooms are prerequisites for further investigations on strain improvement. However, such data is still inadequate in the literature for mutation induction in basidiospores of *P. ostreatus*, particularly while it comes to chemical mutagenic agents. Therefore, we attempted to establish an accurate and reproducible methodology to use UV irradiation and EMS for induction of putative mutations in *P. ostreatus* var. *florida* basidiospores. Experimental conditions evaluated in this study may be useful for inducing mutation through UV radiation or chemical treatment in spores of basidiomycete mushrooms. The findings of this study may also warrant further research to investigate morphological and developmental characteristics in fruiting bodies generated by the putative mutant spores.

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Declaration of interest

There is no conflict of interest.

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