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# CHEMICALLY INDUCED MUTAGENESIS IN THE KING OYSTER MUSHROOM *Pleurotus eryngii* TO GENERATE HIGH-TEMPERATURE TOLERANT STRAIN

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*Pleurotus eryngii*

Mutation

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Methyl methane sulfonate

Tolerance

**ABSTRACT**

In this study, a high-temperature-tolerant strain of the king oyster mushroom ***(****Pleurotus eryngii****)*** was generated by chemical mutagenesis**.** Cultivation of ***P. eryngii*** generally involves incubating the mycelia at 25°C and then moving the spawns for further incubation at 18°C for fruitification**.** However, in tropical countries, the temperature is a major concern in the production of oyster mushroom where the average temperature is 32°C**.** In the current study, the mycelia were treated with ethyl methane sulphonate ***(***EMS***)*** or methyl methane sulphonate ***(***MMS***)*** for chemical-induced mutation**.** Seven mutants ***(***EMS 1, 2, 6, 26, 35, 36, and 38***)*** from EMS mutagenesis exhibited higher growth rates than the wild-type strain at 32°C**.** However, mutant strains from MMS mutagenesis showed a low growth rate when compared with wild-type. On sawdust substrate, the spawn running conditions for these strains were performed at 32°C, and fruitification occurred at 18°C**.** The yield and biological efficiency of EMS 36 and 38 mutants were higher than those of the wild-type strain**.** The activities of cellulase and xylanase of EMS 36 and 38 mutants showed that both these mutants had higher activities than the wild-type strain which may influence mushroom production**.** Therefore, these EMS 36 and 38 mutants can be cultivated in tropical countries, which could provide a high yield and reduce the cost during spawn running step.

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## 1 Introduction

The king oyster mushroom (*P. eryngii*) belongs to the order Agaricales, the family Apiaceae (Zervakis et al., 2014). Recently, the industrial cultivation of *P. eryngii* has been increasing in Europe, Asia, and North America (Estrada & Royse, 2007). For mushroom cultivation, the mycelial growth and primordia formation steps are performed at temperature ranges of 25°C-28°C and 10°C-17°C, respectively (Ruhl & Kues, 2007). Hence, the cultivation of *P. eryngii* in tropical countries, where the average temperature (about 32°C) is higher than the temperature needed for cultivation. In these countries, optimum production of *P. eryngii* requires the use of a temperature controller, which resulted in higher costs or cost-inefficiency. Therefore, this study was conducted to generate a high-temperature-tolerant strain of *P. eryngii* to overcome this problem.

Recently, several studies have been conducted on mutagenesis in fungi to develop new strains with desired characteristics using various methods such as transformation and chemical and physical mutagenesis. To improve yield, mushrooms have been mutated by various methods. Bangyeekhun et al. (2020) exposed *Volvariella volvacea* by UV and reported that mutant strains showed a 30% higher yield than the parental strain. Romruen & Bangyeekhun (2017) mutated the *P. eryngii* by using *Agrobacterium* mediated transformation. These researchers obtained four mutants but among these four, only one mutant has significantly higher fresh weight and biological efficiency (BE) as compared to the wield strains.

Further, chemical mutagenesis is considered to be convenient and easy because it involves very few steps. Previous studies have been suggested a tremendous success of chemical mutagenesis in mushrooms cultivation. Ethylmethanesulphonate (EMS) and methylmethanesulphonate (MMS) are categorized as alkylating agents and these induced mutations via DNA damage (Griffiths et al., 2005; Lundin et al., 2005). EMS and MMS have been successfully used for mutagenesis in various fungi. EMS has been successfully employed to induce the mutagenesis in various mushroom verities such as *Volvariella volvacea*, *Flammulina velutipes,* and *P. ostreatus* (Liu et al., 2011; Lin-Zhi et al., 2013; Sharma & Sharma, 2014; Teimoori et al., 2014) while MMS has been successfully induced mutation in *Hypsizygus marmoreus* (Lee et al., 2011).

The current study was carried out to evaluate the mutagenesis properties of two chemical mutagens (EMS and MMS) in *P. eryngii*. Further, the yield, biological efficiency (BE), enzymatic activities (cellulase and xylanase), and the day to harvest were investigated in the mutant and wield types.

## 2 Materials and Methods

### 2.1 Chemical mutagenesis

*P. eryngii* cultured on potato dextrose agar (PDA) at 25°C for 10 days. Then, the agar was cut for 120 pieces with a diameter of 0.2 cm and treated with 50 mL of EMS and MMS as mutagens under appropriate conditions (based on the results of preliminary experiments of the same author). For EMS mutagenesis, the mycelia were treated with 0.25% (v/v) EMS and incubated at 32°C and 180 rpm for 1 h. For MMS mutagenesis, the mycelia of *P. eryngii* were treated with 0.2% (v/v) MMS at 32°C and 180 rpm for 45 min. After mutagenesis, the mycelial plugs were filtered and washed with sterile distilled water three times.

### 2.2 Selection and growth rate of mutants

The mutants that survived were transferred to PDA and incubated at 32°C for 5 days to determine the relative growth rate by measuring the colony diameter. The relative growth rate was computed as follows: growth rate of mutant strain/growth rate of the wild-type strain. Mutants whose relative growth was higher than that of the wild-type strain were chosen for further study.

### 2.3 Yield and biological efficiency (BE)

The wild and mutant strains were inoculated into sawdust substrate, which was prepared by mixing 10 kg of para rubber wood sawdust, 0.5 kg of rice bran, 0.1 kg of lime (calcium oxide), 0.25 kg of calcium sulphate, 20 g of Epsom salt (magnesium sulphate), and 65-70 L of water. The substrate was packed into heat-resistant bags, containing 900 g per bag. These bags were sealed and autoclaved at 121°C for 20 min.

The mycelial plug was inoculated and incubated at 32°C until grains became fully colonized. Then, the bags were inoculated with the spawn (10 g/bag) and divided into four conditions as described in Table 2. The bags were incubated until the mycelium covered the substrate bag completely and were kept open for the formation of fruit bodies (80-90% relative humidity). The weight of freshly harvested mushroom was measured (wet weight), and the BE was calculated using the total mushroom yield from each flush as follows:

BE (%) = (fresh fruiting body yield/dry weight of substrate) × 100

### 2.4 Cellulase and Xylanase activities

The mutants were grown in a liquid medium (Lee et al., 2001) containing 2% sawdust and incubated at 32°C and 180 rpm for 18 days. Cultures were harvested by filtering through cheesecloth. Cellulase and xylanase activities were measured by the 3,5-dinitrosalicylic acid (DNSA) method (Miller, 1972). For this, 0.5 mL

of the culture filtrate was mixed with 0.5 mL of 1% carboxymethyl cellulose (CMC) or 1% xylan in 0.05 M sodium acetate buffer (pH 5.0). For blank, 1% CMC or 1% xylan was added after incubation. The reaction mixtures were incubated at 45°C for 60 min, and 1 mL of DNSA reagent was added to each tube. The reaction mixtures were incubated in a vigorously boiling water bath for 10 min and then transferred to a cold-water bath for 3 minutes. The developed color was measured using a spectrophotometer at 540 nm. The absorbance of the sample tubes was translated into glucose production during the reaction using a glucose standard curve. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar per minute under the conditions assayed.

Table 1 The relative growth of mutants by using EMS and MMS as mutagens

| EMS | | MMS | |
|---|---|---|---|
| Strains | Relative growth | Strains | Relative growth |
| Wild type (PE) | 1.00 | Wild type (PE) | 1.00 |
| EMS 1 | 1.17 | MMS 1 | 0.85 |
| EMS 2 | 1.42 | MMS 2 | 0.85 |
| EMS 6 | 1.75 | MMS 3 | 0.65 |
| EMS 26 | 1.23 | MMS 4 | 0.46 |
| EMS 35 | 1.35 | MMS 5 | 0.46 |
| EMS 36 | 1.35 | MMS 6 | 0.39 |
| EMS 38 | 1.16 | MMS 7 | 0.39 |

### 2.5 Statistical analysis

The data from three independent experiments were analyzed using one-way ANOVA and Tukey's test using the program SPSS 11.0.

## 3 Results

### 3.1 Selection of mutants

EMS and MMS are alkylating agents which can cause DNA damage by the ethylation and methylation of DNA, respectively. These chemicals have been used as a mutagen in various studies (Shah et al., 2016; Volkova et al., 2020). After the induction of chemical mutagenesis, the relative growth rates of the mutants were determined by incubating these wild and mutant strains at

Table 2 Fresh weights, biological efficiency (BE) and day to harvest of wild type (PE) and mutant strains (EMS 1, 2, 6, 26, 35, 36 and 38) when were cultivated in selected condition

| Conditions | Temp. of spawn run (°C) | Temp. of fruiting (°C) | Strains | Fresh weight (g) | BE (%) | Day to harvest (d) |
|---|---|---|---|---|---|---|
| 1 | 25 | 18 | PE | 82.67±0.58$^{abc}$ | 19.00±0.13$^{abc}$ | 66.33±2.31$^{def}$ |
| | | | EMS 1 | 80.00±8.66$^{bcd}$ | 18.39±1.99$^{bcd}$ | 68.33±3.06$^{cde}$ |
| | | | EMS 2 | 31.67±2.89$^{f}$ | 7.28±0.66$^{f}$ | 73.33±2.89$^{abc}$ |
| | | | EMS 6 | 31.67±2.89$^{f}$ | 7.28±0.66$^{f}$ | 63.67±0.58$^{ef}$ |
| | | | EMS 26 | 70.00±8.66$^{cde}$ | 16.09±1.99$^{cde}$ | 62.00±0.00$^{f}$ |
| | | | EMS 35 | 96.67±5.77$^{a}$ | 22.22±1.33$^{a}$ | 68.67±2.52$^{cde}$ |
| | | | EMS 36 | 63.33±2.89$^{e}$ | 14.56±0.66$^{e}$ | 62.67±1.15$^{ef}$ |
| | | | EMS 38 | 65.00±5.00$^{de}$ | 14.94±1.15$^{de}$ | 66.33±2.31$^{def}$ |
| 2 | 32 | 18 | PE | 65.00±3.00$^{de}$ | 14.94±0.69$^{de}$ | 72.00±1.00$^{bcd}$ |
| | | | EMS 1 | 26.67±2.89$^{f}$ | 6.13±0.66$^{f}$ | 73.00±1.73$^{abc}$ |
| | | | EMS 2 | 21.67±1.89$^{f}$ | 4.98±0.66$^{f}$ | 74.00±3.46$^{abc}$ |
| | | | EMS 6 | 71.67±7.64$^{bcde}$ | 16.48±1.76$^{bcde}$ | 74.33±1.15$^{abc}$ |
| | | | EMS 26 | 71.67±7.64$^{bcde}$ | 16.48±1.76$^{bcde}$ | 79.00±2.00$^{a}$ |
| | | | EMS 35 | 26.67±2.89$^{f}$ | 6.13±0.66$^{f}$ | 75.00±2.00$^{ab}$ |
| | | | EMS 36 | 96.67±7.64$^{a}$ | 22.22±1.76$^{a}$ | 71.00±1.73$^{bcd}$ |
| | | | EMS 38 | 86.67±2.89$^{ab}$ | 19.92±0.66$^{ab}$ | 72.33±0.58$^{bcd}$ |
| 3 | 32 | 25 | All strains | - | - | - |
| 4 | 32 | 32 | All strains | - | - | - |

*The values are the means of triplicate experiments, error bars were SD and the letter indicates the statistically significant difference ($p < 0.05$).*

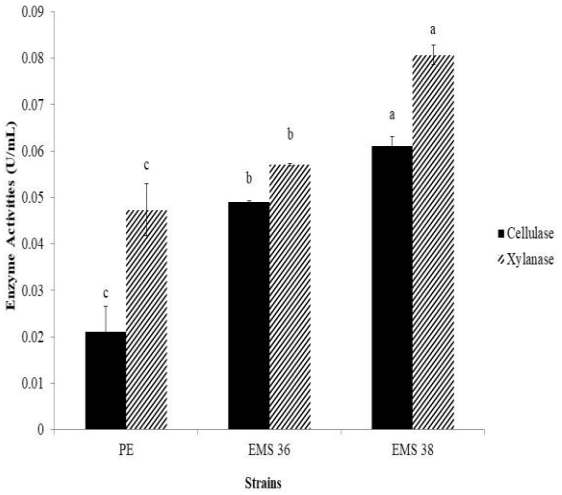


Figure 1 The cellulase and xylanase activities of wild type (PE) and are the means of triplicate experiments, error bars were SD the letter indicates the statistically significant difference ($p < 0.05$).

32°C for 5 days. From the selected 120 pieces of mycelial plugs, 48 and 53 pieces were survived after treatment with MMS and EMS, respectively. Furthermore, in MMS-induced mutagenesis, all mutants showed a relatively lower growth rate as compared to the wild strains (Table 1). However, the relative growth rates of seven EMS-induced mutant strains (EMS 1, 2, 6, 26, 35, 36, and 38) were higher than that of the wild-type strain (Table 1). Thus, these seven strains from EMS mutagenesis were selected for further characterization.

### 3.2 Yield, BE and day to harvest

The seven strains (EMS 1, 2, 6, 26, 35, 36, and 38) were cultured on sawdust substrate using the bag culture method. The inoculated bags were divided into four conditions as described in Table 2. The yield (fresh weight), BE, and the day to harvest were compared with those of the wild-type strain. The results showed that the spawn running step performed better at 25 and 32°C, while the induction of fruiting bodies occur only at 18°C (conditions 1 and 2; Table 2). In other conditions, the mycelia could be grown, but there was no fruiting body production (conditions 3 and 4; Table 2). However, the morphology of mutant and wild-type strains was not different.

In general, *P. eryngii* can be cultivated at 25°C for spawn running and 18°C for fruiting body production. In condition 2, the fresh weight and BE of two mutants (EMS 36 and 38) were significantly higher than those of the wild-type strain. Regarding the day to harvest, EMS 36 and 38 mutants were similar to the wild-type strain (condition 2; Table 2).

### 3.3 Cellulase and xylanase activities

EMS-induced mutagenesis not only influenced the growth rates and mushroom production but might also affect the enzymatic activities of the treated mushroom. To confirm this hypothesis, the activities of cellulase and xylanase were investigated in this study. Mycelia were grown in the liquid medium containing 2% sawdust as substrate. The cultures were incubated at 32°C for 18 days. Then, the enzyme activities were measured from the culture filtrate. The results demonstrated that the activities of both these enzymes from EMS 36 and 38 mutants were enhanced when the mycelia were grown at 32°C (Figure 1). Regarding cellulase activity, EMS 38 produced the highest activity (0.061 U/mL), followed by EMS 36 (0.049 U/mL) and wild-type (0.020 U/mL) strains. Similarly, in the case of xylanase, EMS 38 showed the highest at 0.081 U/mL, whereas those of EMS 36 and wild-type strain were 0.057 and 0.047 U/mL, respectively.

## 4 Discussion

Chemical mutagenesis has been successfully used for induced mutation in mushroom to obtain the desired characteristics. Lee et al. (2011) treated the basidiospores of *H. marmoreus* with MMS and obtained two mutant strains that exhibited increased fruiting body production. Further, *V. volvacea* and *P. ostreatus* have been mutated using EMS to obtain a cold-tolerant and a low-sporing strain, respectively (Liu et al., 2011; Sharma & Sharma, 2014; Teimoori et al., 2014). Interestingly, the high-temperature-tolerant strain of *F. velutipes* was successfully generated using EMS, and the mutant strains could be grown with fruiting body formation at high temperatures. Moreover, the BE of the mutants was increased under this condition (Lin-Zhi et al., 2013). Since *P. eryngii* has been generally cultivated at 18-25°C, these conditions increased the cost of cultivation in tropical countries due to high energy consumption. Therefore, this study was carried out to reduce the cost, for this, the mutation was performed at 32°C which is the average temperature of the tropical countries.

In this study, the mycelia of seven mutant strains from EMS mutagenesis could be grown at 25°C and 32°C. However, the fresh weight and BE of EMS 36 and 38 mutants were higher than those of the wild-type strain when cultivated at 32°C and fruitification at 18°C. However, the day to harvest was longer for EMS 36 and 38 mutants under this condition than that for wild-type under condition 1, but the spawn was incubated at 32°C for spawn running, the ambient temperature in tropical countries. The use of a temperature controller was eliminated in this step, thus reducing the cost.

Induction of mutagenesis in *P. eryngii* using the *Agrobacterium*-mediated transformation and UV irradiation method has been previously reported (Obatake et al., 2003; Kim et al., 2010;

Romruen & Bangyeekhun, 2017). In the present study, the yields of EMS 36 and 38 mutants were successfully enhanced. However, the yields of EMS 1, 2, and 35 were lower than wild-type strain in condition 2.

Subsequently, the activities of cellulase and xylanase of both these mutants were investigated. For this, *P. eryngii* was cultivated on a lignocellulosic substrate that contains cellulose (50%), hemicellulose (30%), and lignin (20%), it is required to produce lignocellulosic enzymes such as cellulase and xylanase (Sajith et al., 2016). It has been reported that the production of mushroom such as *Pleurotus* spp. and *V. volvacea* is related to the activities of these enzymes, and high enzyme activity was found in high-yield strains (Kurt & Buyukalaca, 2010; Zhao et al., 2010). In this study, the activities of cellulase and xylanase of EMS 36 and 38 mutants were higher than those of the wild-type strain, which may be the reason for the high yield of these mutant strains.

Lui et al. (2020) generated new mushroom strains by crossbreeding between monokaryon of *P. tuoliensis* or *P. eryngii*. Two new strains were obtained and show higher biological efficiency than parents. However, the dikaryotic strain of *P. eryngii* was used in this study. The current study resulted in the production of high-temperature tolerant strain. Therefore, both monokaryotic and dikaryotic strains could be used to create new strains.

**Conclusions**

High-temperature tolerant strain of *P. eryngii* was obtained by treatment with EMS but not from MMS. The fresh weight and BE of two mutant strains (EMS 36 and 38) were higher than wild type when spawn running at ambient temperature (32°C) and fruiting at 18°C. In addition, the enzyme activity of *P. eryngii* was successfully enhanced by chemical mutagenesis for the first time. This study showed cellulase and xylanase activities of 2 strains were improved. From the results of the present study, the energy consumption and cost in the spawn running step was reduced. There was an advantage for *P. eryngii* industrial cultivation in a tropical country.

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**Conflict of Interest:** Nil

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