RESEARCH ARTICLE

Improvement of small oyster mushroom and golden mushroom based on protoplast fusion breeding method

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Traditional mushroom breeding faces many limitations in improving strains such as insufficient genetic diversity and long breeding cycles. To solve these problems, this study utilized protoplast fusion technology to combine the excellent shapes of different parents to generate strains with new genetic characteristics. The study first optimized the protoplast isolation and fusion conditions of small oyster mushroom and golden mushroom, and then screened and analyzed strain fusions to identify strains with excellent agronomic shapes. The results showed that the protoplast yields of small oyster mushroom X01 and golden mushroom J01 were excellent. These two samples then served as the matrix for strain fusion experiments, and the obtained equations had high reliability. The regeneration rates of small oyster mushroom under single-layer plate culture and liquid-solid phase combined culture were 0.44% and 0.41%, respectively. The regeneration rates of golden mushroom under these two culture conditions were 0.41% and 0.44%, respectively. Based on the results and the convenience of experiment, the single-layer plate culture method was selected as the culture method. In addition, the best inactivation effect of protoplasts was obtained by bathing in water at 50°C for 25 minutes. The fusion was significantly better than the parent strain in terms of growth rate and yield. The results indicated that protoplast fusion technology was an effective tool for improving edible fungi, which could effectively improve the breeding of small oyster mushroom and golden mushroom.

Keywords: protoplast fusion; improved breeding; small oyster mushroom; golden mushroom; preparation; inactivation; fusion; identification.

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Introduction

In the field of modern agricultural biotechnology, strain improvement is a keyway to improve the yield and quality of edible mushrooms. As popular edible mushroom varieties, oyster mushrooms and golden mushrooms are of great significance in increasing production and improving quality to meet market demand. However, traditional breeding techniques are often limited by issues such as lack of genetic diversity, long breeding cycles, and low breeding efficiency [1, 2]. Therefore, developing efficient and rapid new breeding technologies is crucial for the edible mushroom industry. With the development of molecular biological technology, protoplast fusion technology, as a new type of cell engineering method, provides new strategies for genetic improvement of edible fungi [3, 4]. Protoplast fusion technology is an emerging cell engineering technique that can recombine genes at the cellular by fusing protoplasts from different parents, creating strains with new

genetic characteristics. Compared with traditional hybrid breeding, protoplast fusion breeding can overcome inter-specific hybridization barriers and greatly achieve genetic improvement. In addition, it can also shorten the breeding cycle and improve breeding efficiency [5]. Therefore, protoplast fusion is gradually becoming a powerful tool for genetic improvement of edible fungi, especially suitable for strains that are difficult to hybridize through conventional methods [6]. Although protoplast fusion technology has great potential in theory, it still faces many challenges in practical applications. The current technology has complexity in operation, low fusion efficiency, and insufficient stability and predictive ability for genetic characteristics after fusion. In addition, it is necessary to further optimize the screening and identification methods of fusion genes to ensure effective production of the required traits in the strains.

To solve the slow growth rate, long cultivation cycle, and difficulty in balancing the market supply of golden mushroom, this research was proposed to improve the small oyster mushroom and golden mushroom based on protoplast fusion. The study conducted in-depth research on strain fusion technology and attempted to optimize the screening process of fusions to improve strain breeding. Through in-depth investigation, it was expected to provide an innovative methodology for the genetic improvement of edible fungi and contribute to the sustainable development of the edible fungi industry.

Materials and methods

Protoplast preparation

The *Pleurotus ostreatus* strains were obtained from Weiyuan strain farm of Sichuan Neijiang Edible Fungi Research Institute (Neijiang, Sichuan, China) and named as X01, X02, and X03, respectively. The strains of golden mushrooms were obtained from the School of Food Science, Sichuan Agricultural University (Chengdu, Sichuan, China) and were named as J01, J02, and J03, respectively. Three basic media including potato dextrose agar medium (PDA), PDA optimized regeneration medium, and liquid potato medium were prepared for the experiments of this study, which were commonly used in microbiology experiments with PDA being a common solid culture medium and liquid potato medium being used for liquid culture. PDA optimized regeneration medium was used in experiments requiring more specific nutrients such as plant tissue culture. The strains with high protoplast yield and fast growth rate were chosen as the experimental parents. The yield of protoplasts and growth rate of mycelium from the parent were taken into account [10, 11].

(1) Liquid culture

The liquid cultures were prepared by activating and inoculating the strains of small oyster mushroom and golden mushroom onto PDA agar plates for 4 days. Afterwards, 2-3 fresh colonies of approximately 0.5 cm^2 were taken and inoculated into liquid potato glucose medium and were cultured at 24℃ and 30℃ for 7 to 10 days before an appropriate amount of mycelium was selected in the culture medium and transferred to a 250 mL conical flask containing 120 mL of the same medium [12]. The culture was then continued for at least 4 days under suitable temperature conditions until flocculent mycelium was obtained.

(2) Enzyme preparation

The cellulase, lysozyme, and snail enzyme were accurately weighed and hydrolyzed in 0.6 M sterile mannitol solution according to the enzyme preparation formula followed by centrifugation to remove impurities and filtration through a 0.22 μm organic phase micro-filtration membrane.

(3) The preparation of protoplasts for small oyster mushroom and golden mushroom

100 mg previously cultured flocculent mycelium was placed in a 2 mL Eppendorf tube. 1 mL of 0.6 M mannitol was added as an osmotic pressure

Table 1. Experimental design plan for optimization of protoplast preparation conditions.

Plan	X1 (%)	X2 (%)	X3 (%)	X4 (minutes)	X5 (days)	X6 (℃)
1	0	0.5	1.0	140	9	31
2	0.5	1.5	2.5	80	7	30
3	1.0	2.5	0.5	160	5	29
4	1.5	0	2.0	100	10	28
5	2.0	1.0	0	180	8	27
6	2.5	2.0	1.5	120	6	26
7	3.0	3.0	3.0	200	11	32

Notes: X1: cellulase. X2: wall lytic enzyme. X3: helicase. X4: enzymatic hydrolysis time. X5: bacterial age. X6: enzymatic hydrolysis temperature.

stabilizer. The sample was centrifuged at 5,439×g for 15 minutes. The precipitate was collected and washed 2 to 3 times. 1 mL of prepared enzyme solution was added to every 100 mg of mycelium. The mixture was placed in a constant temperature water bath for enzymatic hydrolysis treatment. The reaction tube was gently shaken every 30 minutes to promote the reaction. After enzymatic hydrolysis, sterile four layers wiping paper was used to filter the enzymatic hydrolysis mixture and remove undecomposed hyphae [13, 14]. The resulting filtrate was then centrifuged at 639×g for 3 minutes, and the precipitates were collected and cleaned by using 0.6 M and 0.5 M mannitol solutions for small oyster mushroom and golden mushroom, respectively. After centrifugation 3 times, the protoplasts were resuspended using an equal volume of mannitol as the enzymatic hydrolysate. The blood cell count board was then used to calculate the yield of protoplasts as below.

$$\text{Protoplast yield} = N \times V \times 400 \times 10^4 \quad (1)$$

where N was the average number of cells in each small cell of the blood cell counting plate. V was the dilution ratio of the sample. The growth rate of mycelium was determined as follows.

$$\text{Mycelial growth rate} = \frac{\text{Mycelial growth length (cm)}}{\text{growth days (d)}} \quad (2)$$

Optimization of protoplast preparation conditions for small oyster mushroom and golden mushroom

During the preparation of protoplasts, the yield of protoplasts was affected by enzyme type and concentration, bacterial age, enzymatic hydrolysis time, and enzymatic hydrolysis temperature [15]. The experiment of this study was based on the results of current available references. The protoplast preparation conditions of *Tricholoma gigas* and *Pleurotus pleurotus* were optimized through the uniform design method. The U_7 (7^6) uniform design table was used for the experiment. The main influencing factors were cellulase (X1, %), wall lytic enzyme (X2, %), helicase (X3, %), enzymatic hydrolysis time (X4, min), bacterial age (X5, d), and enzymatic hydrolysis temperature (X6, ℃). The experimental design plan for optimizing the protoplast preparation conditions of small oyster mushroom and golden mushroom was shown in Table 1. The protoplast yield under different conditions was measured. The regression analysis was conducted with the protoplast yield as the objective function to determine the protoplast preparation conditions for small oyster mushroom and golden mushroom.

Protoplast inactivation

1 mL each of the suspension from the protoplasts of golden mushroom and small oyster mushroom were transferred to 1.5 mL sterile Eppendorf tubes, respectively, and placed in a constant temperature water bath for heat treatment to achieve deactivation. The tubes were regularly shaken during the process to ensure uniform heating. The different treatment durations and temperature ranges were set as 5

to 65 minutes with an interval of 10 minutes and 45 to 55°C with a temperature interval of 5°C. The samples were then appropriately diluted to 10^5-10^6 cells/mL and were spread on 200 μL of regeneration medium agar plates and cultured in darkness at 25°C for 5 to 10 days. By observing the growth of protoplasts and calculating the lethal rate, the protoplast inactivation degree was determined as:

$$\text{Lethal rate (\%)} = \frac{A - B}{A} \times 100\% \quad (3)$$

where A was the number of regenerated colonies on the agar plate. B was the number of regenerated colonies after inactivation treatment.

Protoplast fusion

The protoplast concentrations of golden mushroom and small oyster mushroom were adjusted to 1×10^6 per milliliter and completely inactivated. 0.5 μL each of inactivated golden mushroom and small oyster mushroom protoplasts were placed in 2 mL sterile Eppendorf tubes for subsequent experiments [16, 17]. The initial conditions for the experiment were set to fuse at 30°C for 20 minutes. The results of the previous screening were used as the conditions for the subsequent experiment. The concentration of polyethylene glycol (PEG) gradually changed from 10% to 50% at intervals of 10%. The fusion time was 5 to 30 minutes with intervals of 5 minutes. The fusion temperature was set as 20-40°C with intervals of 5°C. The fusion rate was calculated using equation (4).

$$\text{Fusion rate (\%)} = \frac{C \times 2}{D} \times 100\% \quad (4)$$

where C was the number of regenerated colonies in hypertonic culture medium. D was the total number of parental protoplasts.

Protoplast regeneration

Two methods were used for protoplast regeneration. For single layer plate culture method, 100 μL each of protoplast samples from golden mushroom and small oyster mushroom were uniformly coated on a plate of PDA solid regeneration medium [18]. The plates were incubated under constant temperature conditions for 6-10 days. Subsequently, the number of colonies and regeneration rate were recorded and calculated. For liquid-solid culture method, 100 μL each of protoplast samples from golden mushroom and small oyster mushroom were transferred to liquid regeneration medium for 4 days of static cultivation. Afterwards, the cultured samples were coated onto solid regeneration medium and continued to be cultured for 4 days. The number of colonies and regeneration rate were recorded and calculated. The regeneration rate was determined using equation (5).

$$\text{Protoplast regeneration rate} = \frac{E - F}{G} \times 100\% \quad (5)$$

where E was the high osmotic regeneration plate colony count. F was the common regeneration plate colony count. G was the blood cell count plate protoplast count.

Identification of fusion strains

Both fused strains and their parent strains were cultured on PDA solid culture medium. The appearance of the produced colonies was observed and recorded [19]. The slide insertion method was used for cultivation. After the mycelium covered the entire slide, it was subjected to lactic acid carbolic acid staining treatment. The morphology of the mycelium was then examined and recorded using a 16 × 40-fold microscope. Preliminary identification was conducted based on the morphological characteristics of the fusion site, resistance test results, and mycelial growth rate [21]. After identification, strains with significant morphological differences from their parents, antagonistic effects, and mycelial chain connections were selected. Meanwhile, the mushroom emergence tests were conducted, and the mushroom emergence time and commercial characteristics were recorded and compared.

Table 2. Primers for ISSR-PCR amplification.

Primer name	Sequence (5' to 3')	Number of bases	Annealing temperature (℃)
IPL8	GAGAGAGAGAGAGAGACC	18	57.3
IPL11	AGCAGCAGCAGCAGCAGCG	19	64.0
IPL17	AGAGAGAGAGAGAGAGT	17	52.2
IPL29	TCTCTCTCTCTCTCTCC	17	54.6
IPL31	ACACACACACACACACC	17	54.6
IPL32	ACACACACACACACACG	17	54.6
IPL33	TGTGTGTGTGTGTGTGA	17	52.2
IPL34	TGTGTGTGTGTGTGTGC	17	54.6

Genomic DNA extraction

The fungi genomic DNAs were extracted by using Fungal DNA kit (Shanghai Haoran Biotechnology Co., Ltd., Shanghai, China) following manufacturer's instructions. All extracted genomic DNAs were checked by using 1.5% agarose gel electrophoresis.

Inter-simple sequence repeats (ISSR) - polymerase chain reaction (PCR)

The primers for ISSR-PCR are short, single-stranded DNA fragments that are composed of nucleotides (A, T, C, G) arranged in a specific sequence. In this study, a total of 8 primers were designed and synthesized for ISSR-PCR (Table 2). The ISSR-PCR reaction was composed of 1 µL of DNA template, 1.5 µL of 10 mM primer, 2.5 µL of 10× PCR buffer, 2 µL of 2.5 mM dNTPs, 0.2 µL Tag DNA polymerase, and dH_2O to 25 µL. The ISSR-PCR was conducted as pre-denaturation at 94°C for 2 mins followed by denaturation at 94°C for 30 s, annealing at 52°C to 58°C according to the annealing temperatures of different primers for 1 min, and final extending at 72°C for 10 min. The reaction was terminated at 4°C. The PCR products were checked by using 1.5% agarose gel.

Statistical analysis

All experimental data were processed using SPSS 26.0 (IBM, Armonk, NY, USA). Variance was analyzed by using ANOVA test. Quadratic polynomial stepwise regression analysis was used to process data with uniform design.

Results and discussion

Selection of parental strains

The yields of small oyster mushroom X01, X02, and X03 were 2.69×10^6, 1.88×10^6, and 2.45×10^6 pieces/mL, respectively. Based on the actual yield of protoplasts, X01 and X03 were selected as the parental strains. The yield of golden mushroom J01, J02, and J03 were 2.39×10^6, 2.31×10^6, and 2.03×10^6 pieces/mL, respectively. The yield difference between golden mushroom strains J01 and J02 was not significant, but both strains showed significant difference to J03. Therefore, J01 and J02 were selected as candidates for parental strains. The growth rate of mycelium can not only describe the quantity of mycelium, but also can reflect the health status of mycelium and the suitability of the growth environment. Therefore, correctly calculating the average growth rate of mycelium is of great significance for studying and mastering the growth characteristics of fungi. The mycelial growth rates of X01, X02, and X03 of small oyster mushroom were 0.48, 0.33, and 0.48 cm/d, respectively. According to mycelium growth rate, X01 and X03 were also the ideal parent strains. The mycelial growth rates of golden mushroom J01, J02, and J03 were 0.30, 0.18, and 0.27 cm/d, respectively. There was no significant difference in the growth rate between strains J03 and J01, but a significant difference was observed between both J01 and J03 strains and J02. Therefore, J03 and J01 were used as candidates for parent strains. After considering the protoplast yield and the mycelium growth rate of each strain, J01 was selected as the

Table 3. Optimization of protoplast preparation conditions for golden mushroom and small oyster mushroom.

Schemes	X1 (%)	X2 (%)	X3 (%)	X4 (minutes)	X5 (days)	X6 (°C)	Golden mushroom protoplast yield (10^6 pieces/mL)	Small oyster mushroom protoplasts yield (10^6 pieces/mL)
1	0.0	0.5	1.0	140	9	31	2.24 ± 0.12^d	2.26 ± 2.17^d
2	0.5	1.5	2.5	80	7	30	2.89 ± 0.08^b	2.67 ± 0.74^b
3	1.0	2.5	0.5	160	5	29	2.94 ± 0.09^a	3.10 ± 0.20^a
4	1.5	0.0	2.0	100	10	28	2.10 ± 0.20^e	1.91 ± 0.15^f
5	2.0	1.0	0.0	180	8	27	2.04 ± 0.23^e	2.02 ± 0.20^e
6	2.5	2.0	1.5	120	6	26	3.07 ± 0.19^a	2.45 ± 1.23^c
7	3.0	3.0	3.0	200	11	32	2.50 ± 0.06^c	2.17 ± 0.08^d

Notes: X1: cellulase. X2: wall lytic enzyme. X3: helicase. X4: enzymatic hydrolysis time. X5: bacterial age. X6: enzymatic hydrolysis temperature. The different superscripted letters in the same column indicated significant differences.

parent of golden mushroom, while X01 was selected as the parent of small oyster mushroom, which both were used for strain fusion experiments.

Optimization of protoplast preparation conditions for golden and small oyster mushrooms

The optimization results of protoplast preparation conditions for golden mushroom and small oyster mushroom were shown in Table 3. The yield of golden mushroom protoplasts was used as the objective function (Y). SPSS software was used to analyze the data. The quadratic polynomial stepwise regression analysis method was applied and resulted in the correlation coefficient test value R = 0.99994. Therefore, there was a close correlation between protoplast yield and various experimental factors in the regression equation. The significance test showed F = 40548.168 with a significance of $P < 0.01$, a residual standard deviation S = 0.0026, and a corrected correlation coefficient Ra = 0.99982. In addition, the *P* values of all factors in the equation were all less than 0.01, indicating that the effects of bacterial age, lysozyme concentration, enzymatic hydrolysis time, and snail enzyme concentration on the protoplast yield of golden mushroom had very significant effects. Taking the protoplast yield of small oyster mushroom as the objective function (Y), the correlation coefficient test value R was 0.9999, indicating a close correlation between the protoplast yield and various experimental factors. The significance test value F was 9999.85000 with a very significant difference ($P < 0.01$), a residual standard deviation S = 0.0007, and a corrected correlation coefficient Ra = 999999.

Protoplast regeneration rates of two parental strains under different cultivation methods

The regeneration rates of two parental strains under different cultivation methods showed that there was no significant difference between the two parental strains under different regeneration conditions. Specifically, the regeneration rates of small oyster mushroom in single-layer plate culture and liquid-solid phase combination culture were 0.44% and 0.41%, respectively, while the regeneration rates of golden mushroom under these two cultivation conditions were 0.41% and 0.44%, respectively. For the sake of simplicity, the single-layer flat plate cultivation method was selected as the more optimal cultivation method.

The inactivation of protoplasts

After heat treatment and inactivation, the protoplasts were inoculated onto PDA medium for cultivation. The results showed that, even after 60 minutes of inactivation at 45°C, a 100% complete inactivation effect couldn't be realized. However, raising the temperature to 50°C and performing a 25-minute heat treatment, or the same time length at 55°C, 100% inactivation effect could be achieved (Figure 1). The microscopic results showed that, after being

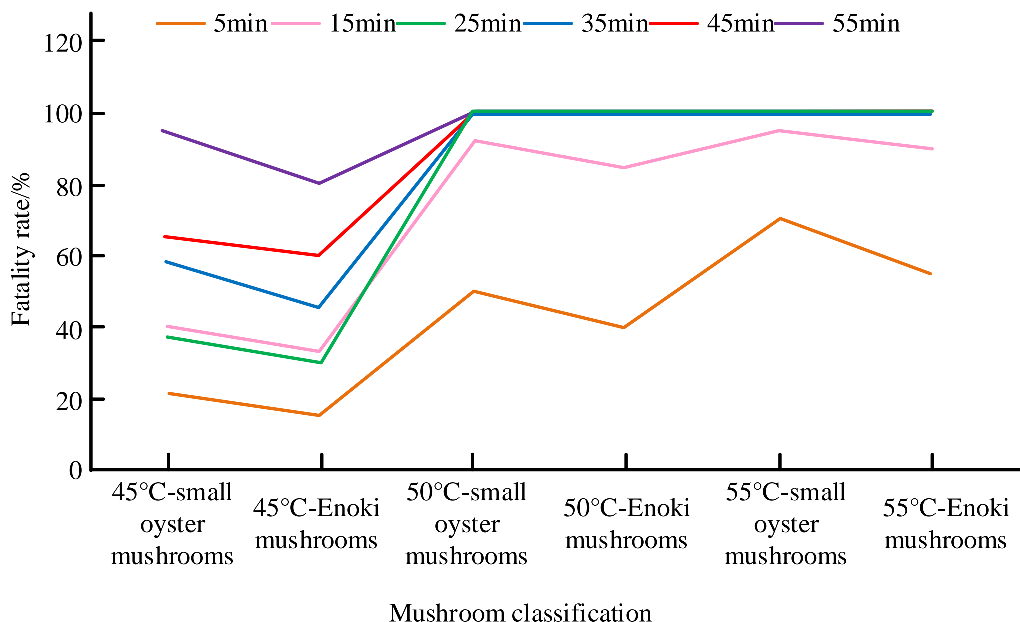


Figure 1. Thermal inactivation results of protoplasts.

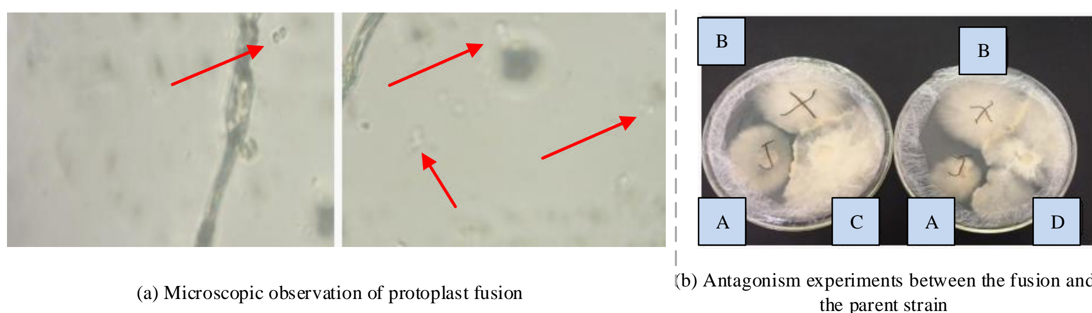


Figure 2. Microscopic examination and antagonistic experiment.

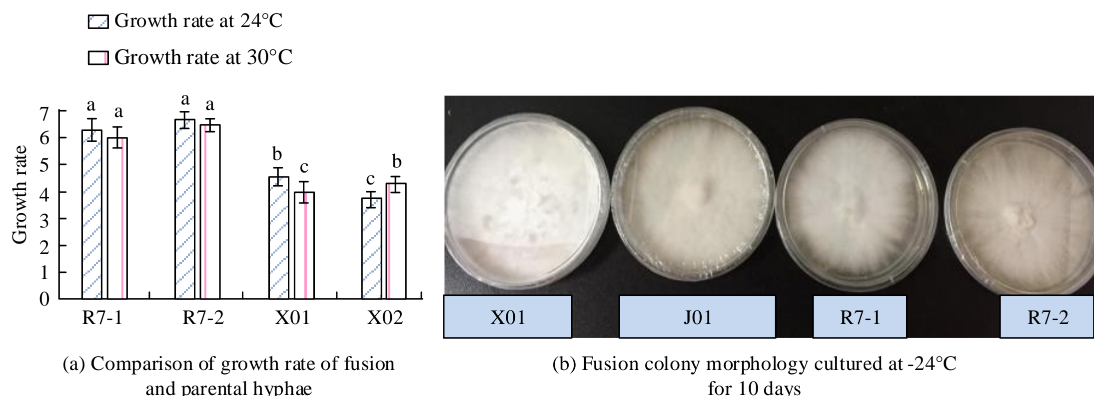
inactivated at 55°C for 25 minutes, the protoplasts were severely damaged, which might have adverse effects on subsequent fusion experiments. Therefore, the optimal inactivation treatment condition was ultimately chosen as 25 minutes at 50°C.

Fusion process optimization for protoplasts of golden and small oyster mushroom

According to the optimization results of protoplast fusion process, the fusion rate of protoplasts was used as the objective function (Y) to obtain the regression equation. The correlation coefficient R value was 0.99999, indicating a strong correlation between the fusion rate of protoplasts and the experimental factors included in the model. The significance was F = 5555.4722, and the P value was 0.0033, indicating that the model had statistical significance. The residual standard deviation was S = 0.0075, while the adjusted correlation coefficient was Ra = 0.99999.

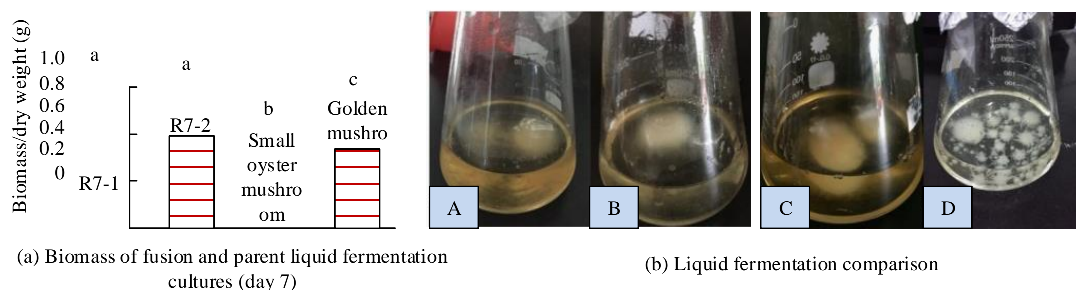
Screening of fusion elements

The fusion of protoplasts was observed under microscopy. In the early stage of protoplast fusion, the number of protoplasts fusing with each other was relatively small. But over time, under the action of polyethylene glycol (PEG) fusion inducer, adjacent two or more protoplasts



(a) Comparison of growth rate of fusion and parental hyphae (b) Fusion colony morphology cultured at -24°C for 10 days

Figure 3. Comparison of growth rate and colony morphology.



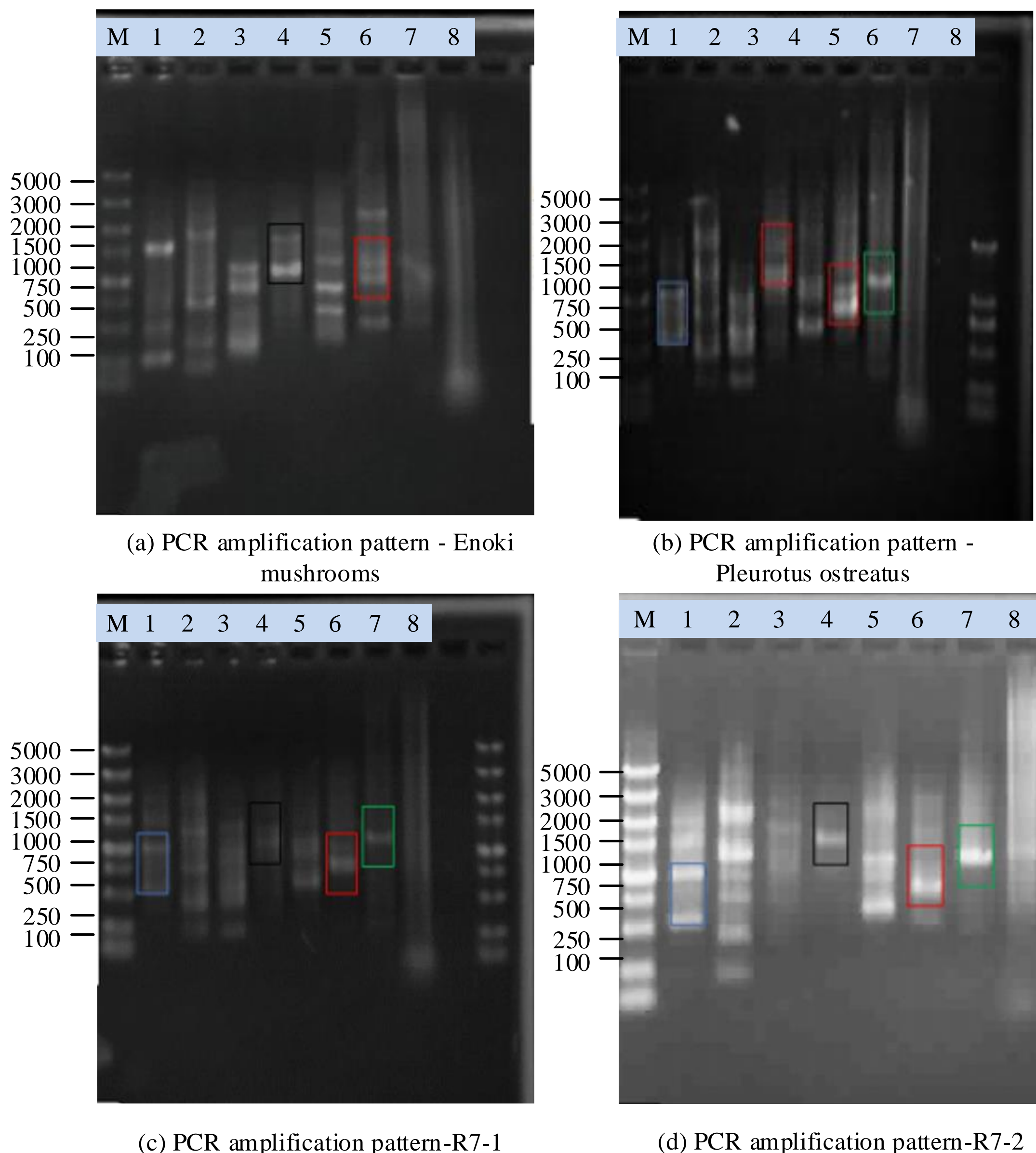
(a) Biomass of fusion and parent liquid fermentation cultures (day 7) (b) Liquid fermentation comparison

Figure 4. Liquid fermentation rate between fusants and parents. A. golden mushroom. B. small oyster mushroom. C. Fuson R7-1. D. Fuson R7-2.

gradually approached each other. The cell membranes of the strains began to come into contact and fuse with each other, forming a shape similar to an "8". As the fusion region of the strains expands, substances within the protoplast began to exchange, ultimately forming new individuals (Figure 2a). The results of the antagonistic experiment showed that there was a clear antagonistic line between the fusion offspring and the parents (Figure 2b). The growth rate of the bacterial strain was then measured, and the results showed that there was a significant difference in the growth rate between the fusion genes R7-1 and R7-2 and the parent strain (Figure 3a). The mycelium of the parent golden mushroom grew rapidly under a constant temperature of 30°C, while the mycelium of small oyster mushroom grew faster in a constant temperature of 24°C. There was no significant difference in the growth rate of fusion subunits R7-1 and R7-2 between 24°C and 30°C. The changes in colony morphology demonstrated that the colony edges of the fusion strains R7-1 and R7-2 were uneven, and the hyphae were relatively sparse. The specific colony morphology was observed between the small oyster mushroom and the golden mushroom (Figure 3b). The biomass of liquid fermentation culture between the fusion offspring and the parents was compared (Figure 4). The dry weight of fermentation biomass produced by fusion genes R7-1 and R7-2 during the liquid fermentation process on the 7th day significantly increased compared with the parents, which indicated that fusion genes had an advantage in biomass generation.

Identification of fusions

The ISSR-PCR results were shown in Figure 5. There were some common DNA characteristic bands between the fusion gene and its parents. There were new DNA bands that the parents did not possess also showing up. In addition, some DNA bands that were originally shared by the



(a) PCR amplification pattern - Enoki mushrooms

(b) PCR amplification pattern - Pleurotus ostreatus

(c) PCR amplification pattern-R7-1

(d) PCR amplification pattern-R7-2

Figure 5. ISSR-PCR amplification. Lanes 1-8: the PCR products using IPL8, IPL11, IPL17, IPL29, IPL31, IPL32, IPL33, and IPL34 primers, respectively. M: the molecular marker.

parents disappeared in the fusion site, indicating that the DNA structure underwent variation during the fusion process.

Conclusion

To improve the agronomic properties and commercial potential of edible fungi, the protoplast fusion breeding technology was used to improve the breeding of small oyster mushroom and golden mushroom. The study first optimized the isolation and fusion conditions of protoplasts followed by verifying the breeding value of the fusion through a strict screening process. Based on the experimental results, the strains of X01 (small oyster mushroom) and J01 (golden mushroom) with better performance were selected as the parent strains. According to the results of the

regeneration rates, the single-layer solid flat plate culture was selected for both small oyster mushroom and golden mushroom. In addition, the inactivation results showed that inactivation effect was the best at 50°C for 25 minutes. The study also found that some fusions showed better traits than that of the parent strain with the clear opposition between the two, which included the growth rate, biomass, and other aspects of the fusion that were significantly better than the parent strain. The study suggested that the protoplast fusion breeding could effectively improve the characteristics of small oyster mushroom and golden mushroom, and indirectly increase the commercial value of the mushrooms. However, the research did not cultivate the obtained fusion under actual production conditions. The scope of breeding needs to be further expanded in the future to obtain more adaptive breeding.

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