

An Enzymolysis-Assisted *Agrobacterium tumefaciens*-Mediated Transformation Method for the Yeast-Like Cells of *Tremella fuciformis*

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ABSTRACT

Agrobacterium tumefaciens-mediated transformation (ATMT), as a simple and versatile method, achieves successful transformation in the yeast-like cells (YLCs) of *Tremella fuciformis* with lower efficiency. Establishment of a more efficient transformation system of YLCs is important for functional genomics research and biotechnological application. In this study, an enzymolysis-assisted ATMT method was developed. The degradation degree of YLCs depends on the concentration and digestion time of Lywallzyme. Lower concentration ($\leq 0.1\%$) of Lywallzyme was capable of formation of limited wounds on the surface of YLCs and has less influence on their growth. In addition, there is no significant difference of YLCs growth among groups treated with 0.1% Lywallzyme for different time. The binary vector pGEH under the control of *T. fuciformis* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter was utilized to transform the enzymolytic wounded YLCs with different concentrations and digestion time. The results of PCR, Southern blot, quantitative real-time PCR (qRT-PCR) and fluorescence microscopy revealed that the T-DNA was integrated into the YLCs genome, suggesting an efficient enzymolysis-assisted ATMT method of YLCs was established. The highest transformation frequency reached 1200 transformants per 10^6 YLCs by 0.05% (w/v) Lywallzyme digestion for 15 min, and the transformants were genetically stable. Compared with the mechanical wounding methods, enzymolytic wounding is thought to be a tender, safer and more effective method.

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1. Introduction

Tremella fuciformis is a famous culinary-medicinal jelly mushroom cultivated in China and other Southeast Asia countries [1,2]. The yeast-like cells (YLCs) of *T. fuciformis* are asexually reproduced from basidiospores by budding. The YLCs are ideal material for genetic manipulation and gene functional research, they could also be used as a microbial cell factory system [3–7]. To this end, an efficient genetic transformation protocol for YLCs is increasingly needed.

In previous attempts, several transformation methods have been developed for the YLCs including electroporation [4,8,9], restriction enzyme mediated integration (REMI) [3], polyethylene glycol (PEG)-mediated protoplast transformation (PMT) [6,10] and *Agrobacterium tumefaciens*-mediated transformation (ATMT) [5,7,11–13]. Among these methods, ATMT is a relatively simple and effective genetic transformation system. However, the transformation efficiency is still lower. A possible reason is that the intact YLCs have a thick cell wall

consisting of layers of chitin, β -glucan, and glycoproteins [14], which increases the difficulty to absorb the T-DNA.

It was reported that the efficiency of ATMT was improved by wounding the receptor cells. Mechanical wounding of mycelia by vortexing with the existence of aluminium oxide particles obtained relatively higher transformation efficiency in several medicinal mushroom species, such as *Ganoderma lucidum*, *Lentinula edodes*, *Grifola frondosa*, *Schizophyllum commune*, and *Lyophyllum decastes* [15]. Transformation efficiency was also improved by wounding the YLCs of *T. fuciformis* via vortexing [12]. In addition, chemical wounding by NaOH treatment was able to generate cell surface wounds in *T. fuciformis*, which made them susceptible to ATMT [13].

Cell wall lyticases have been successfully applied to protoplast preparation in a variety of fungi [16]. The degradation degree of fungal cell wall depends mainly on the concentration and digestion time of the lyticase selected. Limited wounds of cell walls by

enzymolysis might have less influence on cells' growth but facilitate T-DNA transfer, which would improve the transformation efficiency. Here we investigated a suitable degradation condition for limited wounds to establish a relatively efficient enzymolysis-assisted ATMT method for the YLCs of *T. fuciformis*.

2. Materials and methods

2.1. Strains, plasmid, and growth conditions

T. fuciformis isolate Y32 (YLCs), preserved in the laboratory of Food Microbiology, Huazhong Agricultural University, was maintained on potato dextrose agar (PDA) and sub-cultured into the complete medium (CM) (20 g glucose, 1.32 g (NH₄)₂SO₄, 0.25 g MgSO₄·7H₂O, 0.5 g KH₂PO₄·3H₂O, 0.2 mg vitamin B1, 2 mg ZnSO₄·7H₂O, 0.5 g CaCl₂·2H₂O, 0.02 mg ammonium molybdate per liter) at 25 °C on an orbital shaker (150 rpm) (Max Q800; Thermo Fisher Scientific, Waltham, MA) in the dark. A binary plasmid pGEH, carrying the enhanced green fluorescent protein gene (*egfp*)-hygromycin B phosphotransferase gene (*hph*) fusion expression cassette under the drive of the native glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter, was constructed in our previous study [7]. *A. tumefaciens* strain EHA105 harboring pGEH, grown in YEB medium (5 g tryptone, 1 g yeast extract, 5 g nutrient broth, 5 g sucrose, 0.49 g MgSO₄·7H₂O per liter) containing kanamycin (50 µg/ml) and rifampicin (50 µg/ml), was used to transform the YLCs. The transformed colonies were maintained on the potato dextrose selected agar (PDSA) under selection pressure (50 µg/ml hygromycin B and 200 µg/ml cefotaxime).

2.2. Influence of lyticase to YLCs growth

Lywallzyme (Guangdong Institute of Microbiology, Guangdong, China), an effective complex cell wall lyticase used for edible fungal protoplast isolation [17,18], was resolved in double distilled water and sterilized by a 0.22-µm bacterial filter membrane. Three-day-old YLCs were diluted by double distilled water to 10⁶ cells/ml. Lywallzyme concentration and digestion time were chosen to evaluate the influence of Lywallzyme to YLCs growth by using single factor test. Equal volume of YLCs suspension and different concentrations of Lywallzyme solution (0, 0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1.6%) were mixed and then digested in a 32 °C water bath for 15 min. Subsequently, fixed the Lywallzyme concentration, YLCs were digested at 32 °C for different time (0, 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, 120 min). The survived YLCs was enumerated on PDA plates after 7 days' cultivation at 25 °C.

2.3. Observation of wounded YLCs by scanning electron microscope

According to the aforementioned influence result of lyticase to YLCs growth, three-day-old YLCs were treated with the maximal effective concentration of Lywallzyme and then collected by centrifugation at 8000 rpm for 5 min and washed with PBS three times. After treatment with gradient dehydration and critical point drying, the YLCs were observed under a field emission scanning electron microscope (SEM) (Model SU8010; Hitachi, Tokyo, Japan).

2.4. Enzymolysis-assisted *Agrobacterium tumefaciens*-mediated transformation

Lywallzyme concentration and digestion time were chosen to evaluate the influence of enzymolysis wounds to transformation efficiency using single factor test. The manipulation steps were the same as the methods described in 2.3. YLCs (3 days) were diluted into 10⁶ cells/ml by IM. Equal volume of YLCs suspension and different concentrations of Lywallzyme solution (0, 0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%) were mixed and then digested in a 32 °C water bath for 15 min. Subsequently, fixed the Lywallzyme concentration, YLCs were digested at 32 °C for different time (0, 15 min, 45 min, 90 min, 120 min).

A. tumefaciens strain EHA105 harboring pGEH was cultivated at 28 °C in YEB medium containing kanamycin (50 µg/ml) and rifampicin (50 µg/ml) to an OD₆₀₀ of 0.6–0.8. Then bacterial cells were resuspended in induced medium (IM) with 200 µM acetosyringone (AS) (Sigma-Aldrich, St. Louis, MO) to an OD₆₀₀ of 0.15–0.2 and incubated for another 6 h at 28 °C on an orbital shaker (180 rpm) for virulence pre-induction. 500 µl of wounded YLCs suspension were mixed with 500 µl of virulence pre-induced *A. tumefaciens* suspension and spread onto the mixed cellulose esters membrane (MCEM) on solid IM (with 200 µM AS) at 28 °C for 3 days. After incubation, the membranes with fungal and bacterial colonies were transferred onto PDSA, which was added with 200 µg/ml cefotaxime to kill the *A. tumefaciens* and 50 µg/ml hygromycin B to select the putative transformants. After 15 d, the colonies grew on the membrane were identified as putative transformants.

2.5. Verification of transformants

2.5.1. DNA isolation and PCR

Genomic DNA was extracted from five-round sub-cultured putative transformants by using the cetyltrimethyl ammonium bromide (CTAB) and a

Table 1. Primers for PCR amplification in this work.

Names	Sequences (5' to 3')	Descriptions
egfp-F	ATGGTGAGCAAGGGCGAG	Primers for <i>egfp</i>
egfp-R	CTTGTACAGCTCGTGGA	
hph-F	ACATGCATGCATATGAAAACTGAACTCAC	Primers for <i>hph</i>
hph-R	CCGCTCAGCGGCTATTCCTTTGCCCTCGGAC	
tubulin-F	GATGACCATTTCCTGCTTC	<i>tubulin</i> primers for qRT-PCR
tubulin-R	GTTCTGACATTTGCTACCG	

phenol-chloroform DNA extraction protocol omitting the β -mercaptoethanol [19]. To confirm the existence of *hph* gene and *egfp* gene in transformants, PCR analysis was performed by using the following primer pairs: hph-F and hph-R; egfp-F and egfp-R, respectively (Table 1). The amplification procedures were carried out as follows: an initial denaturation at 94 °C for 3 min; 34 cycles of 94 °C denaturation for 30 s, 55 °C annealing for 30 s, 72 °C elongation for 1 min; and a final extension at 72 °C for 10 min. PCR products were analyzed by 1.0% agarose gel electrophoresis.

2.5.2. Southern blotting

To analyze the number of T-DNA copies integrated into the YLCs genome, putative transformants were chosen randomly to perform the Southern blot analysis. Wild-type Y32 was used as the negative control and the pGEH extracted by using the E.Z.N.A.TM Plasmid mini Kit I (OMEGA Bio-tek, Norcross, GA) was used as the positive control. The *hph* fragment labeled with DIG was used as a specific probe for detecting. Total DNA (approximately 10 μ g per sample) was digested with *Xho*I (Takara, Shiga, Japan) for 12 h at 37 °C. Then the product was separated on a 1% agarose gel (Biowest, Barcelona, Spain) and transferred onto the nylon membranes. Hybridization and detection were conducted according to the instructions of the DIG-High Prime DNA Labeling and Detection Kit (Roche, Mannheim, Germany).

2.6. Expression of reporter gene in transformants

2.6.1. Quantitative real-time PCR analysis

To test the relatively expression ratio of the *egfp* gene, total RNA was extracted from T-DNA integrated transformants by means of RNaiso plus (Takara) according to the manufacturer's protocol. The first strand of cDNA was prepared with 5 μ g of total RNA using the M-MLV RTase cDNA synthesis kit (Takara). Quantitative real-time PCR (qRT-PCR) was performed to test the relatively expression ratio of the *egfp* gene with the ABI ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA). β -*tubulin* gene was as the endogenous control and the qRT-PCR conditions were 95 °C for 1 min,

followed by 40 cycles of 95 °C for 30 s, and 60 °C for 30 s. The primer sequences for *egfp* gene and β -*tubulin* gene are listed in Table 1. Each reaction was replicated three times to estimate error and the gene expression was normalized by the $2^{-\Delta\Delta C_t}$ analysis.

2.6.2. Fluorescence microscopy

For eGFP detection, different *egfp* expression level transformants were selected and mounted in double distilled water and then were analyzed by using a Nikon Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan) with a B-2A filter. The samples were excited at 460–500 nm wavelengths at automatic exposure time. Photomicrographs were captured under 40x objective for random transformants and processed with NIS-Elements BR 3.0 imaging software (Nikon). Wide-type Y32 was used as a negative control.

2.7. Detection of the stability of transformants

To determine whether the inserted T-DNA was integrated into the transformants genome stably, the transformants were cultured on PDA for five successive generations. Thereafter, the transformants were transferred to PDA containing 50 μ g/ml hygromycin B to observe whether the colonies survived under the antibiotic pressure again.

3. Results

3.1. Enzymolytic wounding conditions for YLCs

The cell growth was influenced by higher concentration Lywallzyme treatment seriously. The majority of YLCs cannot grow on the PDA with above 0.1% Lywallzyme treatment (Figure 1(a)). In addition, the cell survival rate did not show significant differences when the YLCs were treated with 0.1% Lywallzyme for different digest time (Figure 1(b)). The degradation degree of YLC wall depends on the concentration and digestion time of Lywallzyme. No wounds can be observed on the surface of YLCs without Lywallzyme treatment (Figure 2(a)). The YLCs were elliptic or fusiform observed under a SEM and the size of these cells were approximately 9 μ m in diameter (Figure 2(a)). However, lower concentration (\leq 0.1%) Lywallzyme treatment led to the formation

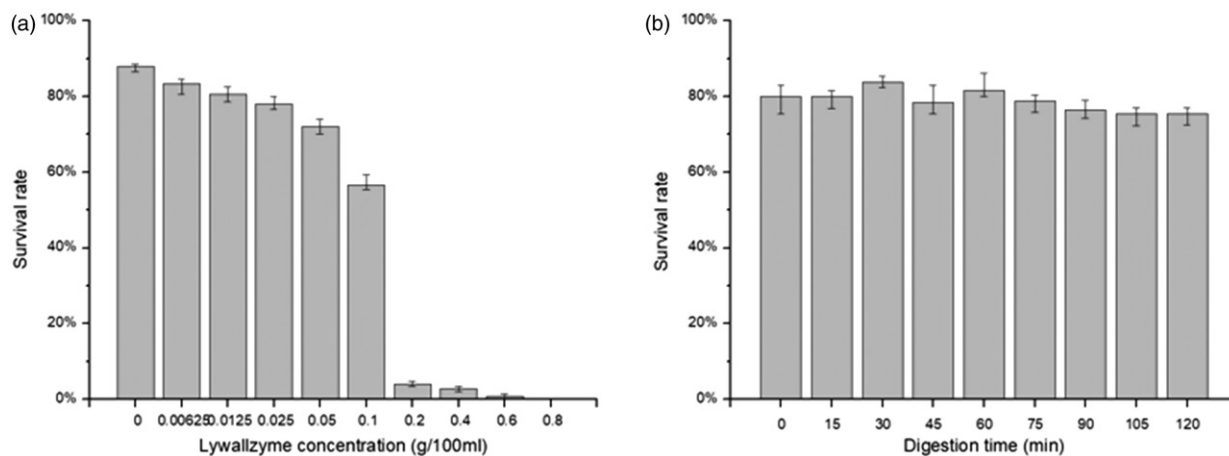


Figure 1. Survival rate of YLCs under different conditions. (a) Survival rate of YLCs occurred on the PDA medium after being treated by lywallzyme for 15 min with different concentration. (b) Survival rate of YLCs occurred on the PDA medium after being treated by 0.1% lywallzyme for different time.

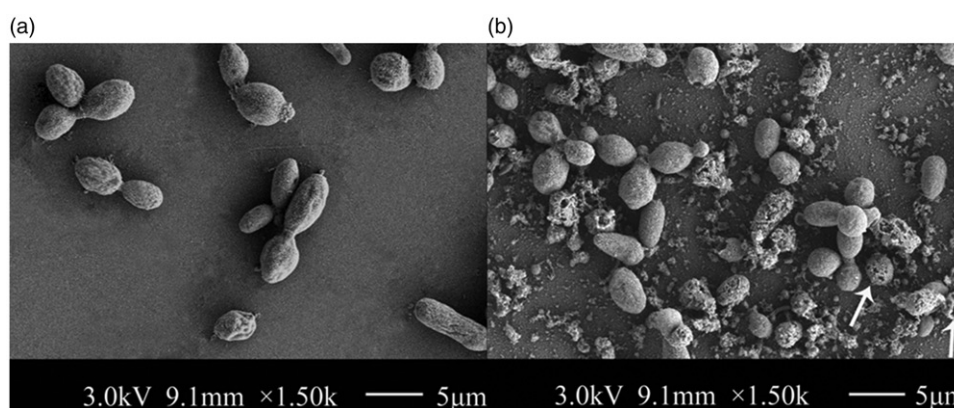


Figure 2. Observation of YLCs of *T. fuciformis* by Scanning electron microscope (SEM). (a) YLCs without Lywallzyme treatment. (b) YLCs with 0.1% Lywallzyme treatment. The wounds on the surface of YLCs were marked by the arrow.

of limited wounds on the surface of YLCs and had little influence on YLCs growth (Figure 2(b)).

3.2. Integration of T-DNA in transformants

PCR analysis showed that the 7 transformants yielded the expected 717 bp and 1026 bp target bands (Figure 3). From the results of Southern blot, we found that no hybridizing fragment was produced on the membrane in blank group, Y32 wild type. In 8 randomly selected transformants, most of transformants yielded single band of different sites, indicating random and single-copy insertion of T-DNA into the fungal genome. Meanwhile, only one double-copy transformant and one triple-copy transformant were obtained (Figure 4). The results of quantitative real-time PCR (qRT-PCR) indicated that *egfp* gene was expressed with different levels and the copy number is not proportional to the expression level (Figure 5).

3.3. Transformation efficiency of the enzymolysis-assisted ATMT

Transformation efficiency increased with the increase of the enzyme concentration ($\leq 0.05\%$). The

highest efficiency, 1200 transformants per 10^6 YLCs was obtained when the Lywallzyme concentration is up to 0.05%, which is 3-fold than that of the control group. However, the efficiency declined sharply when higher concentration of Lywallzyme ($\geq 0.1\%$) was used (Figure 6(a)), no significant differences were found for different treatment time (Figure 6(b)).

3.4. Expression of eGFP in transformants

Green fluorescence was not found in wild-type strain, but observed in individual transformed colonies under the dark-field microscope (Figure 7). Observing strong green fluorescence revealed that transformation was achieved successfully.

3.5. Stability of transformants

8 transformants selected randomly were transferred onto the non-selection PDA medium and then back onto the selection one, they were still able to grow and their colony morphologies were no discernible

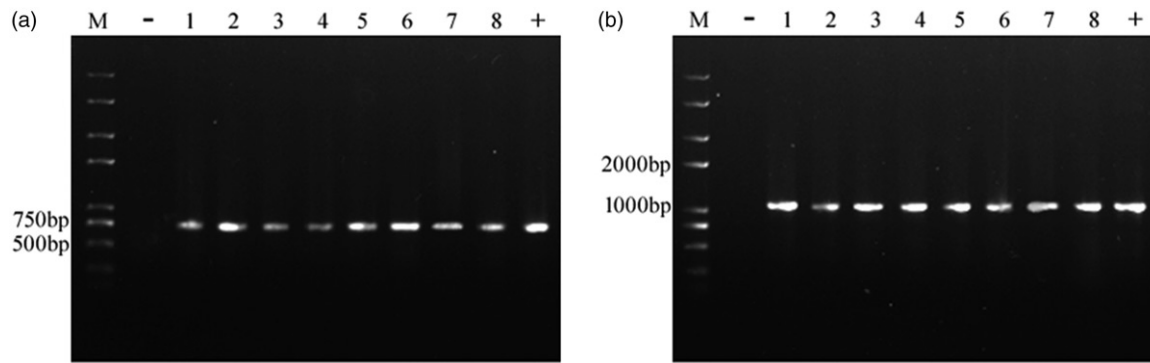


Figure 3. PCR electrophorogram of YLCs of *T. fuciformis* transformants. (a) *egfp* fragments amplified by PCR. (b) *hph* fragments amplified by PCR M: *Trans2K plus #* DNA marker (Transgen, Beijing, China); -: wild-type *T. fuciformis* strain Y32 (negative control), +: vector pGEH (positive control); 1–8: randomly selected transformants.

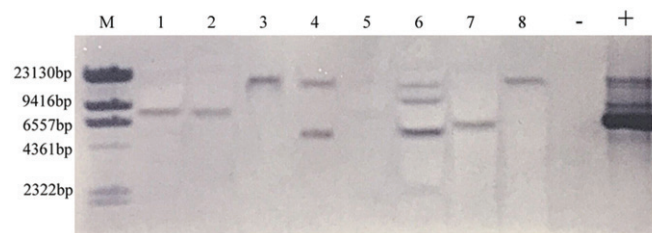


Figure 4. Southern blot analysis of transformants of *T. fuciformis* YLCs. Genomic DNA isolated from hygromycin-resistant transformants (10 µg) was digested with *XhoI* and probed with a fragment of the *hph* gene. M: λ -*Hind* III digest DNA marker (Takara, Japan); -: wild-type *T. fuciformis* strain Y32 (negative control); +: vector pGEH (positive control); 1–8: hygromycin-resistant transformants.

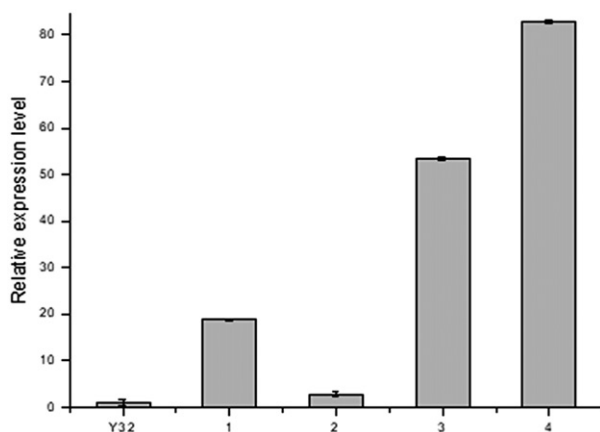


Figure 5. Relative expression ratio of the *egfp* in transformants of *T. fuciformis* YLCs and wild-type strain Y32. 1, 2: single-copy transformant; 3: double-copy transformant; 4: triple-copy transformant. Data were presented as mean values of three replicates with the corresponding standard deviations.

difference. It indicated that these transformants preserved the *hyh* gene and were mitotically stable.

4. Discussion

Numerous efforts have been made to increase the transformation efficiency of YLCs in *T. fuciformis*. Mechanical wounding-assisted ATMT method obtained near 350 transformants per 10^6 YLCs [12]. NaOH treatment ATMT method produced 40–50

transformants per 10^6 YLCs in average [13]. However, the transformation efficiency by enzymolytic wounding reached more than 1200 transformants per 10^6 YLCs in current research. It is approximately 3-fold higher than that of control group using intact YLCs. The results demonstrated that Lywallzyme treatment is effective to increase the transformation frequency. A reasonable interpretation is that the wounds digested by Lywallzyme increase the possibility of the T-DNA intake. Lywallzyme was usually used for protoplast preparation via dissolving the fungal cell walls. In this study, Lywallzyme was resolved in double distilled water to avoid the formation of protoplast survived in iso-osmotic solution. Lower concentration and shorter time treatment of Lywallzyme lead to limited wounds of YLCs, which was not enough to produce protoplasts and had less influence on the growth of YLCs. The limited wounds on the surface of YLCs make them susceptible to ATMT after Lywallzyme treatment.

With the rise of the Lywallzyme concentration, the survival rate of the YLCs decreased. It was attributed to the accumulation of wounds in YLCs. However, less than two-hour incubation of 0.1% Lywallzyme, the survival rate showed no significant decrease with the prolonged incubation.

Different promoters on the binary vectors also have impact on the transformation efficiency. CaMV35S promoter, widely used in plant

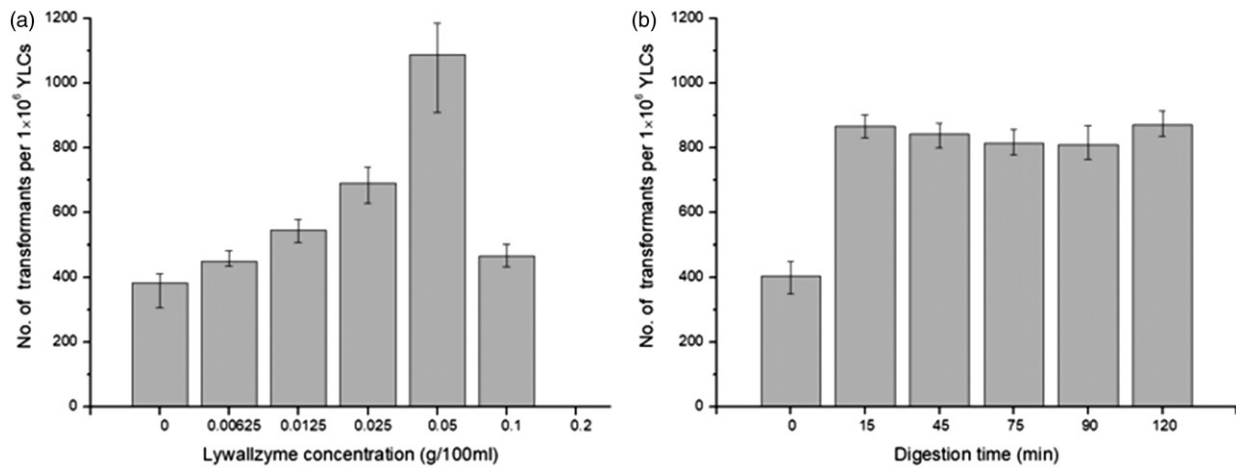


Figure 6. Transformation efficiency of YLCs under different conditions. (a) The transformation efficiency of YLCs after being treated by lywallzyme for 15 min with different concentrations. (b) The transformation efficiency of YLCs after being treated by 0.05% lywallzyme for different time.

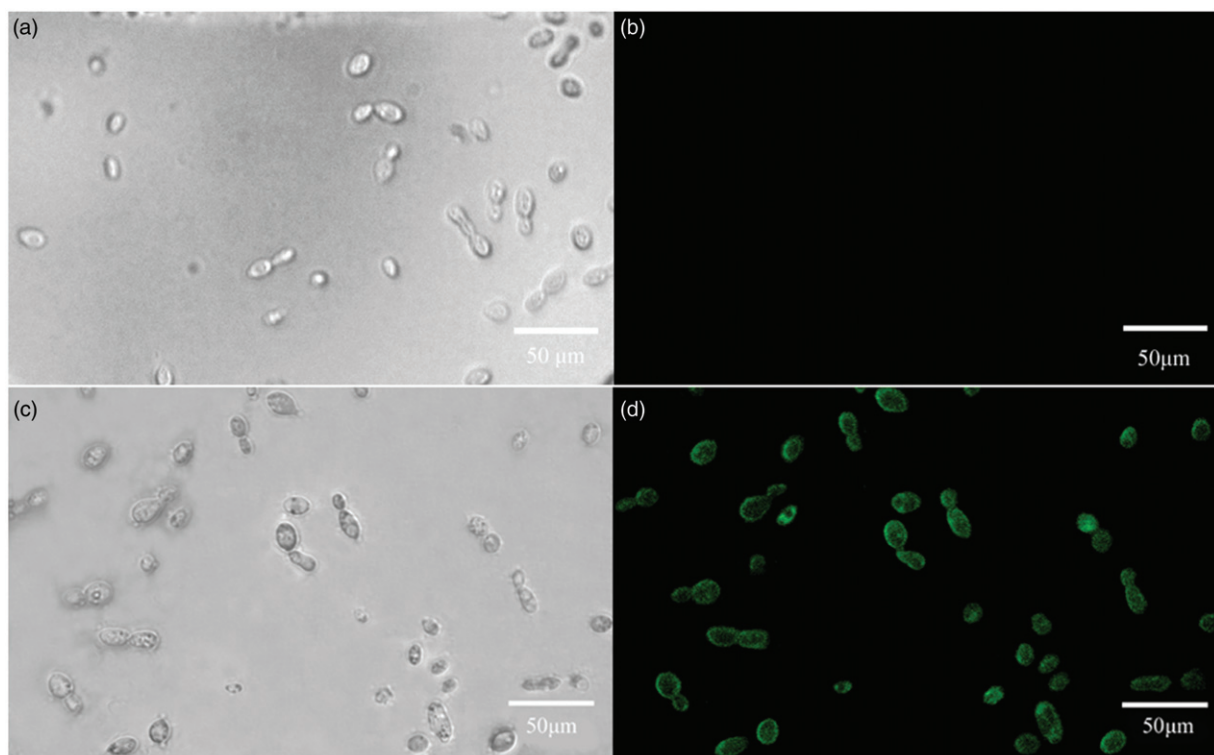


Figure 7. Expression of eGFP in individual transformants of *T. fuciformis* YLCs with hygromycin. (a) Wild type *T. fuciformis* strain Y32 (bright field). (b) Wild type *T. fuciformis* strain Y32 (corresponding fluorescence field). (c) Transformant (bright field). (d) Transformant (corresponding fluorescence field). Detection for bright field micrographs and corresponding fluorescence under UV light are shown with the same microscope equipment of excitation filters at 450–490 nm, a dichroic filter at 505 nm and an emission filter at 520 nm. Images were taken with 40x fields of view, bar = 50 μm.

transformation, is a heterogenous promoter for *T. fuciformis*. No transformants were obtained via pCAMBIA 1300 carrying *hph* gene under the drive of CaMV35S in YLCs transformation [12]. *gpd* promoter is an enhanced constitutive promoter [20]. In our study, a native *gpd* promoter from *T. fuciformis* was used to carry out successful transformation. It was reported that homologous *gpd* promoter region is effective in directing the heterologous genes expression among several mushrooms. The *Lentinus edodes gpd* promoter and *hph* gene

were fused together to allow a strong expression of a heterologous gene in *L. edodes* [21]. *gpd* promoter isolated from *Flammulina velutipes* was used to construct vector for high-level heterologous genes expression [22]. The *hph* gene under control of the promoter *gpd-Le* from *L. edodes* was more effectively integrated into the genome in *T. fuciformis* than that with the promoter *gpd-An* from *Aspergillus nidulans* [10]. It revealed that suitable match between promoters and host cells was beneficial for effective expression of foreign genes in host

cells. Homologous promoter or promoter from organisms close in genetic relationship is an efficient *cis*-element that can be easily recognized by regulatory factors of recipient hosts, resulting in methylation reduction, integration promotion and foreign gene expression.

5. Conclusion

In this study, an efficient enzymolysis-assisted *A. tumefaciens*-mediated transformation method for the YLCs of *T. fuciformis* was established. The *hph* and *egfp* gene were successfully introduced into the genomes of the YLCs. The transformation efficiency reached 1200 transformants per 10⁶ YLCs with 0.05% (w/v) Lywallzyme digestion for 15 min, and the transformants were genetically stable. Compared with the mechanical wounding methods, enzymolytic wounding is thought to be a tender, safer, and more effective method.

Disclosure statement

The authors declare no conflict of interest related to any part of the research.

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