An Economical and Sustainable Non-sterilization Cultivation Method of Oyster Mushroom (Pleurotus spp.) and its Key Influencing Factor

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Abstract

Natural processes inspire human innovation. For example, leafcutter ants inhabiting rainforests of South America cut plant debris, transport them to underground nests, dissociate them into small pieces, and use them to cultivate a specific fungus, which serve as the main food source for the whole colony. Inspired by this, Giant Juncao grass (Cenchrus fungigraminus, name used before: Pennisetum giganteum z.x.lin) was used as the raw material. It is a C4 tall grass with rapid growth, high yield, wide-spread adaptability, nitrogen fixation, low-quality soil requirements, biosafety, and can be cultivated on a large scale in many low value and idle lands such as saline and alkali soils, desert and barren mountains. On this basis, we screened out a non-sterilization-based cultivation method of oyster mushroom with fresh Giant Juncao grass. Using our novel method, the bottleneck technologies in the popularization and application of various oyster mushroom cultivation methods in underdeveloped areas were improved. Took Wei W.'s report as reference that the highest annual output of Giant Juncao grass was 450 tons per hectare in southern China. And according to the conversion rate of the oyster mushroom in this research which was 95% in average (Ratio of total weight of fresh mushroom to dry weight of culture material), and the water content which was 91.5%, about 119.7 tons of fresh oyster mushroom could be produced in 2-3 months. Took the nutrient contents of Pleurotus sapidus cultivated by sterilization cultivation method with fresh Giant Juncao grass reported by Zhixiang X. as reference, it could provide 2.37 tons of high-quality protein. In addition, this study found that, the key to the success of this method was the reduction of the soluble substances in culture materials, which significantly reduced the contamination by Mucor circinelloides and provided an enabling environment for the growth of oyster mushroom. These findings provided an index for quality control and a theoretical basis for further study. If this technology is applied appropriately, it will be able to greatly alleviate malnutrition and food shortages in an economical, organic, and sustainable manner in underdeveloped areas.

Introduction

Malnutrition and food shortages continue to be major socio-economic issues in underdeveloped areas of the earth. Therefore, there is a pressing need to develop sustainable and economically viable food resources that are enriched with nutrition and active compounds. Leafcutter ants from the tropical rain forests of South America dissociate plant debris to use as part of a culture system to mass cultivate a fungus that serve as their main food source [1, 2]. This natural paradigm can be mimicked for mass production of edible fungi for human populations. Accordingly, some methods already can cultivate edible fungi, such as oyster mushroom with some herbs and woody plants [3]. And the edible fungi can grow fast to high fecundity with many nutrients [4, 5] and several active compounds that can improve immunity [6, 7], anti-tumor [8] and confer anti-oxidative properties, etc [9, 10]. However, several factors hamper large-scale cultivation of edible fungi. Some of these limitations include insufficient supply of wild forage and wood, unsustainable large-scale deforestation [3], high cost of technology and equipment, high energy consumption, difficulty in drying fresh grass in humid environment, untimely drying, leading to fermentation deterioration and reduction in cultivation efficiency [11–13]. Prolonged

raw material treatment cycle and low utilization rate of traditional fermentation cultivation methods [14–16], as well as the need for a large number of toxic antibacterial agents for conventional non-sterilization cultivation approaches [17, 18]. Consequently, the conventional cultivation methods employed for oyster mushroom production have not been popularized or applied on a large scale in underdeveloped areas.

Therefore, in this study, we aimed to design and select a non-sterilization cultivation method of oyster mushroom with fresh Giant Juncao grass, in view of the shortcomings of various conventional existing methods. Firstly, Giant Juncao grass was used as the raw material. Giant Juncao grass (*Cenchrus fungigraminus*, name used before: *Pennisetum giganteum z.x.lin*), a C4 tall grass with rapid growth (It could grow to about 3m in three months) [19–21], high yield (The highest annual output of fresh grass in South China was 450 tons per hectare) [22], wide-spread adaptability (It can grow at an altitude of 200–1500 m, temperature of -2 ~ 45 ℃) [23–25], nitrogen fixation [26], low-quality soil requirements, and can be cultivated on a large scale in many low value and idle lands such as saline and alkali soils [27–29], desert and barren mountains [30–32]. Therefore, provided an economical, environmentally friendly, sustainable and stable supply of raw materials (It could be harvested continuously for 15–20 years in southern China) with biosafety (which is reproduced by stem, not seeds) and can absorb large amounts of carbon dioxide (In southern China, the annual absorption of carbon dioxide was up to 90 tons per hectare) [23, 33]. Therefore, it had been widely promoted in many parts of the world. [32, 34]. Further, compared with sterilization cultivation method [11, 35, 36], our present approach did not require high-temperature and high-pressure sterilization, thus, eliminating the need for high technical requirements, the use of large equipment and high energy consumption associated with sterilization. After directly preparing and loading, the samples can be inoculated directly in the open air. For our approach excluded the need for aseptic inoculation, which removed the need for high inoculation technical requirements and large investment in inoculation equipment. Compared with the fermentation cultivation method [14–16], the technique described herein solved the problems of long fermentation time, strict temperature and size requirements, large loss of weight in the fermentation process, and the uncertainty in determining whether the fermentation process was adequate/complete. Compared with the conventional non-sterilization cultivation method [17, 18], this approach did not require the use of multiple antibacterial agents with certain toxicity and had a wider tolerance to temperature range, which was the same as that of the sterilization cultivation method. In addition, this method involves crushing the fresh Giant Juncao grass and using it directly, which solves the problems associated with laborious drying procedures of raw materials in humid environment and the relatively slow drying phase, which was easy to cause fermentation deterioration and reduced cultivation efficiency [11, 12]. Therefore, we have obtained a short-cycle, recyclable, green, organic and environmentally friendly oyster mushroom cultivation method with high efficiency, fast speed, low technical threshold and low investment, which closely mimicked the leafcutter ants' cultivation of fungus using plant debris.

Additionally, we observed that this method was highly related to the volume of saturated lime water treatment and the infestation degree of a white mold. In the first three days, the mycelia of oyster mushroom in each group recovered normally and grew vigorously. The mycelia recovery and extent of growth in either the non-treatment or low saturated lime water treatment groups were slightly higher than

that of the high saturated lime water treatment groups. However, following the third day, the white mold grew rapidly near the oyster mushroom mycelia and some other areas of the mushroom bag. The oyster mushroom mycelia in the no saturated lime water treatment group were covered with this type of white mold in a few days, which led to its complete disappearance. Treatment with lime water was associated with sparser white mold levels in a dose dependent manner, and a concordant increase in oyster mushroom mycelia growth rate. In the equal weight saturated lime water treatment group, we observed small populations of oyster mushroom mycelia that did not disappear. In the four times weight saturated lime water treatment group, this white mold had been very sparsely distributed, which did not affect the growth of oyster mushroom, as the oyster mushroom mycelia could grow to full range/extent normally and produce mushrooms normally. In each treatment group, the addition of corn flour was associated with a slight increase in white mold preponderance and the oyster mushroom mycelia were slightly stronger, but it had no significant effect on the results.

Therefore, it was speculated that saturated lime water treatment would dissolve, dilute and take away some soluble substances in the fresh Giant Juncao grass powder that otherwise promoted the growth of this white mold. In further experiments, the white mold was isolated, purified, and identified through the methods of morphology and molecular biology. By measuring the concentration of soluble substances in each group of culture materials with a refractometer, it was found that the key to the success of this method was the reduction of the soluble substances in culture materials, which significantly reduced the contamination caused by *Mucor circinelloides* and provided an enabling environment for the growth of oyster mushroom. These findings provided an index for quality control and a theoretical basis for further study. Upon optimization and application, the technology described herein can be applied towards alleviating the burden of malnutrition and food shortages in underdeveloped areas.

Materials And Methods

Materials and instruments

Materials

The mature and fresh Giant Juncao grass with average height of 3-6 m and growth period of more than 6 months, corn flour, quicklime (calcium oxide) powder, deionized water, 12 cm×24 cm polypropylene mushroom cultivation bags, breathable covers and the spawn of *Pleurotus sapidus* P969 strain, fresh potatoes, centrifuge tubes were provided by China national engineering research center of Juncao technology; Disposable petri dish (90 mm×20 mm) were purchased from Beijing Labgic; HP Fungal DNA Kit D3195, Gel Extraction Kit D2500 was purchased from Omega Bio-Tek; DNA Marker were purchased from Tiangen Biotech; 2×EasyTaq PCR SuperMix (+dye), GelStain nucleic acid dye, Trans 2K plus DNA Marker, Trans 2K DNA Marker, pEASY-T5 Zero Cloning Kit, Trans1-T1 Phage Resistant Chemically Competent Cell were purchased from Transgen Biotech.

Instruments

The instruments used in this article are shown in the Table 1:

Table 1

Instruments

Methods

Screening out the non-sterilization cultivation method of oyster mushroom with fresh Giant Juncao grass

Depending on the applied mechanism and previous studies [3, 11, 13, 35-38], the following raw materials were used: Fresh Giant Juncao grass, corn flour and quicklime (CaO), which can also be easily obtained in many underdeveloped areas. This formula provided a highly alkaline environment that was well tolerated by oyster mushroom (*Pleurotus sapidus* P969 strain). Nonetheless, few pathogenic microbes can survive in CaO. Gradient experiments were conducted to compare the proportion of corn flour and the volume of saturated lime water used to treat fresh Giant Juncao grass as shown in Table 2, after which we selected the best performing groups. The steps were as follows:

1. Cut down the fresh Giant Juncao grass and shred it into filament fibers or small pieces within 2 cm with a pulverizer.
2. Added saturated lime water of different volumes in each lime water-treated group and soaked for 30 min.
3. Squeezed the excess water out of each lime water-treated group and adjusted the water content of each group to 65%.
4. Added every component to each group as shown in Table 1 and mixed thoroughly.
5. Put the substrate into mushroom cultivation bags, 250 g for each bag, and nine bags for each group.
6. Inoculated the spawn of the oyster mushroom on the surface of the substrate in the open air.
7. Screwed down the breathable covers that came with the mushroom cultivation bags and placing the bags at 25 ℃ for mycelia culture.
8. Selected the best-performing group, conducted mushroom cultivation experiment, and collected relevant data.

Table 2

Formula of each experimental group

Name	Model	Brand
Clean bench	SW-CJ-1F	Suzhou Antai
Vertical pressure steam sterilizer	YXQ-LS-100SII	Shanghai Boxun
Scientific research-grade positron fluorescence microscope and imaging system	Ni-U	Nikon Corporation
High-speed freezing centrifuge	5415R	Sigma Laborzentrifugen
PCR instrument	S1000	Bio-Rad
Gel imaging system	GBoxF3	Gene Company
Electrophoresis apparatus	PowerPacTM Basic	Bio-Rad
Biochemical incubator	LRH-250F	Shanghai Bluepard
Green gel cutter/Blue light transmission meter	OSE-470	Tiangen Biotech
Vortex mixer	MX-S	Scilogex
Microwave oven	MZC-2070M	Qingdao Haier
Pipettes	Research Plus	Eppendorf
Thermostatic water bath	HH·S21-6-S	Shanghai Xinmiao
Ice maker	IMS-20	Changshu Xueke
Ultra-pure water machine	WP-RO-30B	Sichuan Vortel
Analytical balance	BSA124A	Sartorius
Constant temperature culture oscillator	ZWY-2102	Shanghai Zhicheng
Fridge	BCD-216SDN	Haier Zhijia
Pulverizer	DFY-300	Wenling Linda
pH meter	PB-10	Sartorius
Ultra-low temperature freezer	Forma 900 series	Thermo Fisher Scientific
Refractometer	LH-Y12	Lohand Biological

Sample	Weight multiples of saturated lime water for raw material treatment (LW)	Fresh Giant Juncao grass powder after treatment %	Corn flour %	CaO %
LW×0	0	98	0	2
		97.75	0.25	
		97.5	0.5	
		97.25	0.75	
		97	1	
LW×1	1	98	0	2
		97.75	0.25	
		97.5	0.5	
		97.25	0.75	
		97	1	
LW×2	2	98	0	2
		97.75	0.25	
		97.5	0.5	
		97.25	0.75	
		97	1	
LW×4	4	98	0	2
		97.75	0.25	
		97.5	0.5	
		97.25	0.75	
		97	1	
LW×8	8	98	0	2
		97.75	0.25	
		97.5	0.5	
		97.25	0.75	
		97	1	

Isolation, purification and identification of white mold

We prepared PDA medium and picked out the culture material with mold from the position where the white mold grew in each treatment group. Next, placed them on PDA medium and made three repetitions for each group. After growing them at 25 ℃ for 5 days, we cut the end parts of the growing mycelia into small squares of 5 mm^2 and placed them on new PDA medium petri dishes to purify the mycelia and observed their growth. After growing to cover more than 2/3 of the petri dish, we observed the morphology of mycelia and sporangia through naked eye and microscopy.

Next, we extracted mycelial gDNA from the white mold by using HP Fungal DNA Kit D3195, used the gDNA, 2×EasyTaq PCR SuperMix (+dye) and ITS primers (ITS-5: 5'-GGAAGTAAAAGTCGTAACAAGG-3', ITS-4: 5'-TCCTCCGCTTATTGATATGC-3') to perform PCR amplification of ITS bar code sequence, ran the PCR products through agarose gel electrophoresis, used UV gel imaging, performed gel extraction of PCR products by using Gel Extraction Kit D2500, ligated the gel extraction products into the cloning vector by using pEASY-T5 Zero Cloning Kit, carried out transformation of *E. coli* competent cells with ligation products by using Trans1-T1 Phage Resistant Chemically Competent Cell and identification of positive clones by performing PCR amplification to complete the TA cloning. Next, we sent the positive clones to Fuzhou Sunya Biotechnology for ITS sequencing. The sequencing results were compared to the nucleotide data base in GenBank via NCBI BLAST. Detailed operation steps are shown in the Supporting Information of Supplementary Information.

Measuring the concentration of soluble substances in each group of culture materials with a refractometer

We squeezed out the liquid in each group of culture materials, and measured the concentration of soluble substances with a refractometer. After that, compared the result with the contamination caused by *M. circinelloides* and the growth of oyster mushroom mycelia in each group.

The performance of reducing the concentration of soluble substances in culture materials to 2%Brix with Giant Juncao grass in other growth periods

We crushed the tender green Giant Juncao grass growing for three months, the Giant Juncao grass growing for one year with high lignification degree, and the dead branches and leaves of Giant Juncao grass separately. Treated them with saturated lime water, used them as the raw materials for non-sterilization culture materials, mixed them with 2% quicklime powder and 0.5% corn flour, and controlled the concentration of soluble substances to 2%Brix measured by refractometer, made mushroom bags as experimental groups. Used the powder of Giant Juncao grass of these three growing periods without treatment as the raw materials for non-sterilization culture materials, mixed them with 2% quicklime powder and 0.5% corn flour, made mushroom bags as control groups, and measured the concentration of their soluble substances by refractometer. Other operations and the spawn were the same as in screening out the non-sterilization cultivation method of oyster mushroom with fresh Giant Juncao grass.

Results And Discussion

Screening out the non-sterilization cultivation method of oyster mushroom with fresh Giant Juncao grass

The Giant Juncao grass that we chose are shown in Figure 1, and the grass powder are shown in Figure 2. The results showed that the mycelia of oyster mushroom (*Pleurotus sapidus* P969 strain) in each group recovered normally in the first three days. The mycelial growth speed of the oyster mushroom in the no saturated lime water treatment group (LW×0) and the low-weight lime water treatment groups were even slightly faster than that in the high-weight lime water treatment groups. However, as shown in Figure 3, from the third day onwards, in the LW×0 group, the mycelia of a white mold began to spread near the oyster mushroom mycelia and across some places of the mushroom cultivation bags. At subsequent stages, the mycelia of this white mold became more preponderant, the growth of the oyster mushroom mycelia was hampered and the oyster mushroom mycelia were covered by the white mold mycelia. After 21 days, the oyster mushroom mycelia were almost entirely disappeared, as shown in Table 3. In the same-weight saturated lime water treatment group (LW×1), the mycelia growth of white mold was less than that of the LW×0 group, and the inhibition on the growth of the oyster mushroom mycelia was also alleviated. In the inoculated part, some oyster mushroom mycelia would not be covered by the white mold mycelia, and a small part of the oyster mushroom mycelia remained even after 21 days, as shown in Table 3. The mycelial growth of the white mold was further diminished in the treatment group subjected to twice the weight of lime water (LW×2), which was associated with enhanced growth of the mycelial growth of the oyster mushroom. After 21 days, about 1/3 of the oyster mushroom mycelia did not subside, as shown in Table 3. Starting with four times the weight of the saturated lime water treatment group (LW×4), the white mold only appeared sporadically in the form of small mycelial plaque or weak gauze, which did not affect the growth of the oyster mushroom mycelia. In this group, after approximately 21 days, the oyster mushroom mycelia could grow to full extent in the mushroom cultivation bags and did not subside, as shown in Table 3. There were no significant differences observed between the eight times the weight of saturated lime water treatment group (LW×8) and LW×4 group, and each repetition grew normally, with high stability, as shown in Table 3. Therefore, these groups can facilitate normal growth of the oyster mushroom mycelia.

As shown in Table 3, In each treatment group, addition of corn flour was associated with a slight increase in the white mold abundance and moderately stronger oyster mushroom mycelia, which was not significant. Based on these observations and previous studies, we deduced that addition of appropriate amount of corn flour is beneficial to improve the mycelial growth of oyster mushroom in this non-sterilization cultivation method. As such, the formula consisting of 0.5% corn flour in the middle of LW×4 group was selected for mushroom production experiments. As shown in Figure 4, the mushroom production experiment revealed that after the oyster mushroom mycelia grew to full extent by the average growth speed of 0.43 cm/d in bags, and was incubated for approximately 14 days, the mushroom bags would turn predominantly white and began to exhibit mushroom primordia. Mushrooms grew three or four times in total, the conversion rate was 95% in average (Ratio of total weight of fresh mushroom to dry weight of culture material), and the water content was 91.5%.

Isolation, purification and identification of white mold

Morphological identification of the white mold

Sequencing and comparison of ITS sequence of the white mold

As shown in Figure 5, when the white mold grew to approximately 2/3 of the plate on PDA medium, black sporangia became preponderant in most of the area, with the aerial mycelia exhibiting a curly morphology and the peripheral mycelia appearing white. Microscopy was used to discern the morphology of sporangium, spore and mycelia as well as the interval between mycelia, as shown in Figure 6. The genomic DNA was extracted from the white mold as shown in Figure 7, the ITS fragment was obtained through PCR as shown in Figure 8, and the positive clones of TA cloning were identified through PCR as shown in Figure 9. Compared the ITS sequence to the nucleotide data base in GenBank via NCBI BLAST, the comparison results corresponded to *Mucor circinelloides*. The ITS sequence is available in the Genbank, MN744376.1, and the length is 662 bp. This sequence-based data corroborated the results obtained via morphological identification. Full-length gels are presented in Supplementary Fig. 7, Fig. 8 and Fig. 9 of the Supplementary Information.

Measuring the concentration of soluble substances in each group of culture materials with a refractometer

Compared the concentration of soluble substances in each group of culture materials with a refractometer (The result is presented in Supplementary Chart 1 of the Supplementary Information) with the result of screening out the non-sterilization cultivation method of oyster mushroom with fresh Giant Juncao grass, we found that, with the increase of saturated lime water in treatments, the concentration of soluble substances and the contamination caused by *M. circinelloides* decreased, and the growth of oyster mushroom mycelia increased accordingly. For all groups with the concentration of soluble substances measured by refractometer equal to or less than 2%Brix, the *M. circinelloides* grew very week, which did not affect the growth of the oyster mushroom mycelia, and after approximately 21 days, the oyster mushroom mycelia could grow to full extent and did not subside.

The performance of reducing the concentration of soluble substances in culture materials to 2%Brix with Giant Juncao grass in other growth periods

According to the results as shown in Table 4, in the control groups, the group with Giant Juncao grass growing for 3 months and the group with Giant Juncao grass growing for 1 year still had high soluble substances, reached 11%Brix, the *M. circinelloides* grew vigorously, and the oyster mushroom mycelia could not grow effectively. The group with branches and leaves of dead Giant Juncao grass, had relatively low soluble substances, only 5%Brix, the *M. circinelloides* grew like gauze, the oyster mushroom mycelia could grow to full but week and had big black patches. In all the experimental groups with the treatment of saturated lime water, the *M. circinelloides* only appeared sporadically and the oyster mushroom mycelia could grow vigorously to full extent and did not subside in each group.

Conclusions

This study provided a non-sterilization cultivation method for oyster mushroom with fresh Giant Juncao grass. It saved time, labor, equipment and technical costs, and was green and organic. And the raw material Giant Juncao grass had the advantages of tall plant, high yield, wide adaptability, nitrogen fixation, bio-safety and strong sustainability.

Further study showed that the soluble substances of the formula promoted the growth of *M. circinelloides*, and this substantial growth resulted in the inhibition of oyster mushroom mycelial growth. These findings provided an index for quality control and a theoretical basis for further study. With proper optimization and application, this method can be an efficacious in alleviating the burden of malnutrition and food shortages.

Declarations

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Authors’ Contributions

Yulong Zhang wrote the main manuscript text, designed of the study and collection, analysis, and interpretation of data, and performed experiments, wrote original draft and edited the final manuscript. Zhijun Li conducted the experiments, and assisted with manuscript writing, and checked the final edition. Dongmei Lin contributed to the conception and design of the study and supervised the manuscript. Jingsi Chen performed the early experiments and collected some data. Linsong Shen, Fengmei Wen and Mingjun Xie prepared the figures and tables. Hui Lin, Xingsheng Lin and Hailing Luo contributed to funding acquisition and supervised the manuscript. Fan Yan edited the language. Eyalira Jacob Okal analyzed data and performed some experiments. Yingxing Lin did some experiments. Zhanxi Lin was the supervisor, who administrated this project, and contributed to the conception and design of this study, edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available upon request to corresponding author. The datasets generated and/or analyzed during the current study are available in the GenBank repository.

Gene data:

ITS sequence data: GenBank accession number MN744376.1.

Ethics approval and consent to participate

The authors declare all experimental research and field studies on plants (either cultivated or wild), including the cultivation and collection of plant material, comply with relevant institutional, national, and international guide-lines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 3 and 4 are available in the Supplementary Files section

Figures

Figure 1

Mature Giant Juncao grass

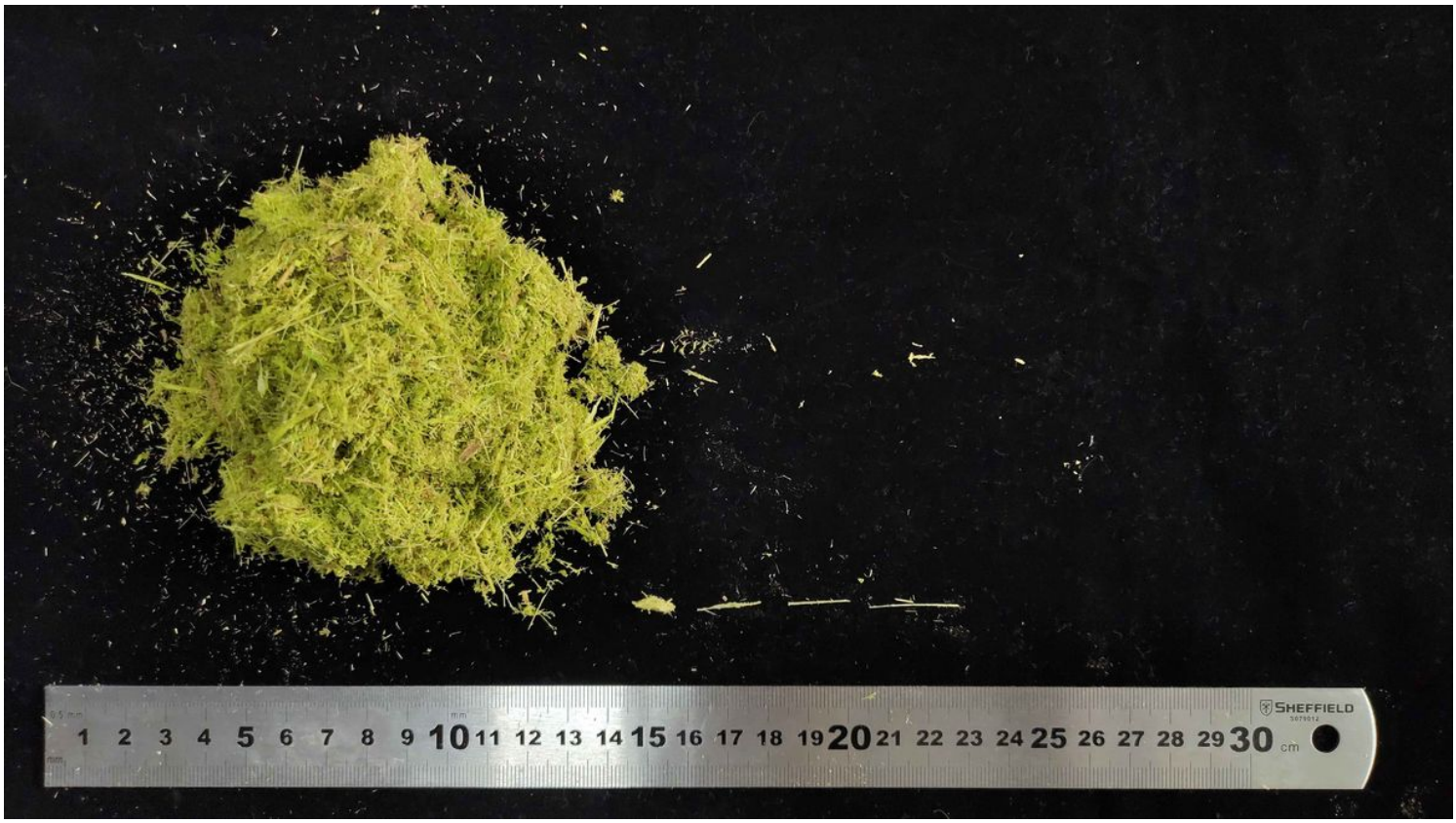

Figure 2

Fresh Giant Juncao grass powder

Figure 3

White mold pollution 6 days after inoculation, 0.5% corn flour, LW×0 group

Figure 4

Oyster mushroom growth in LW×4 group with 0.5% corn flour

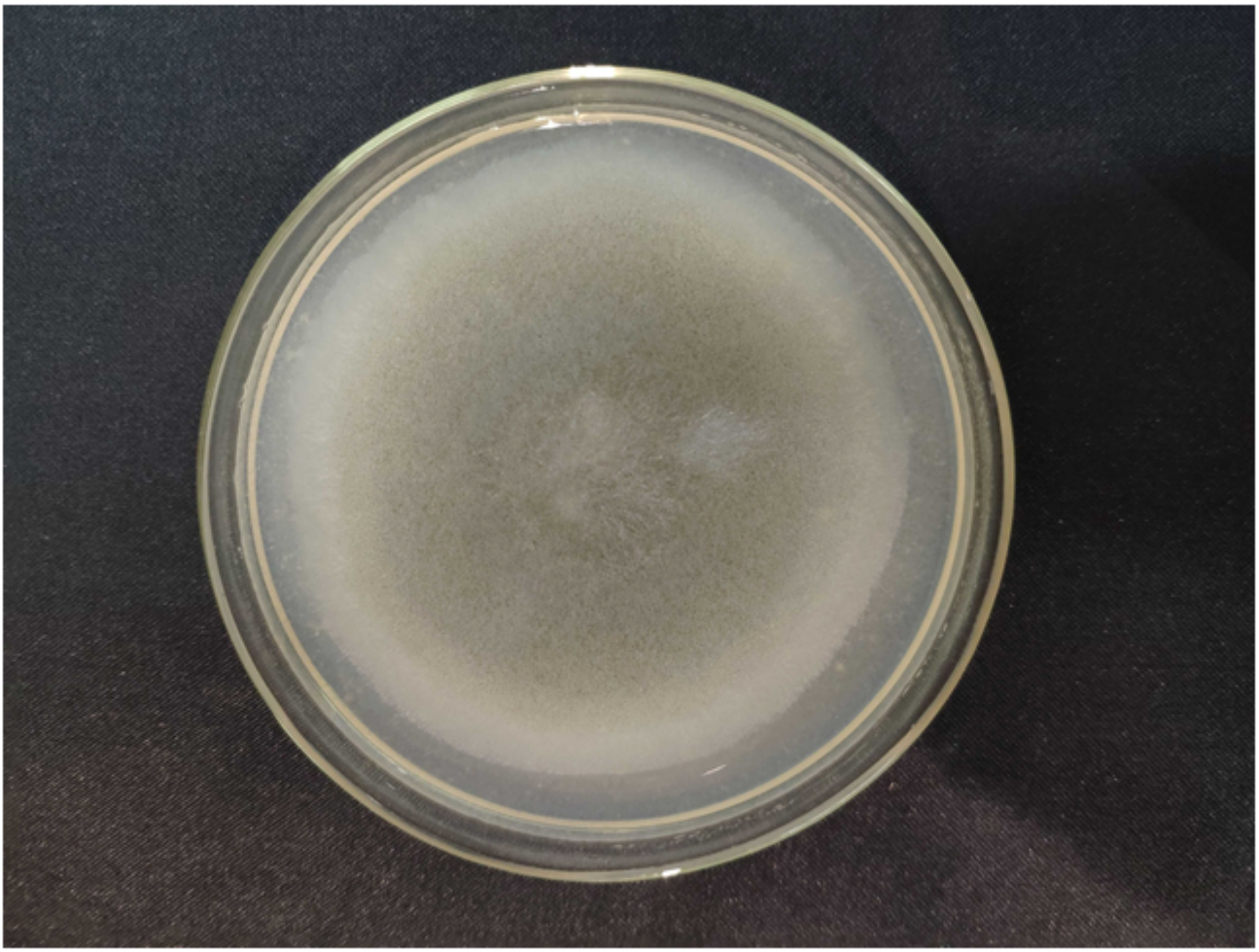

Figure 5

The white mold grown on PDA medium

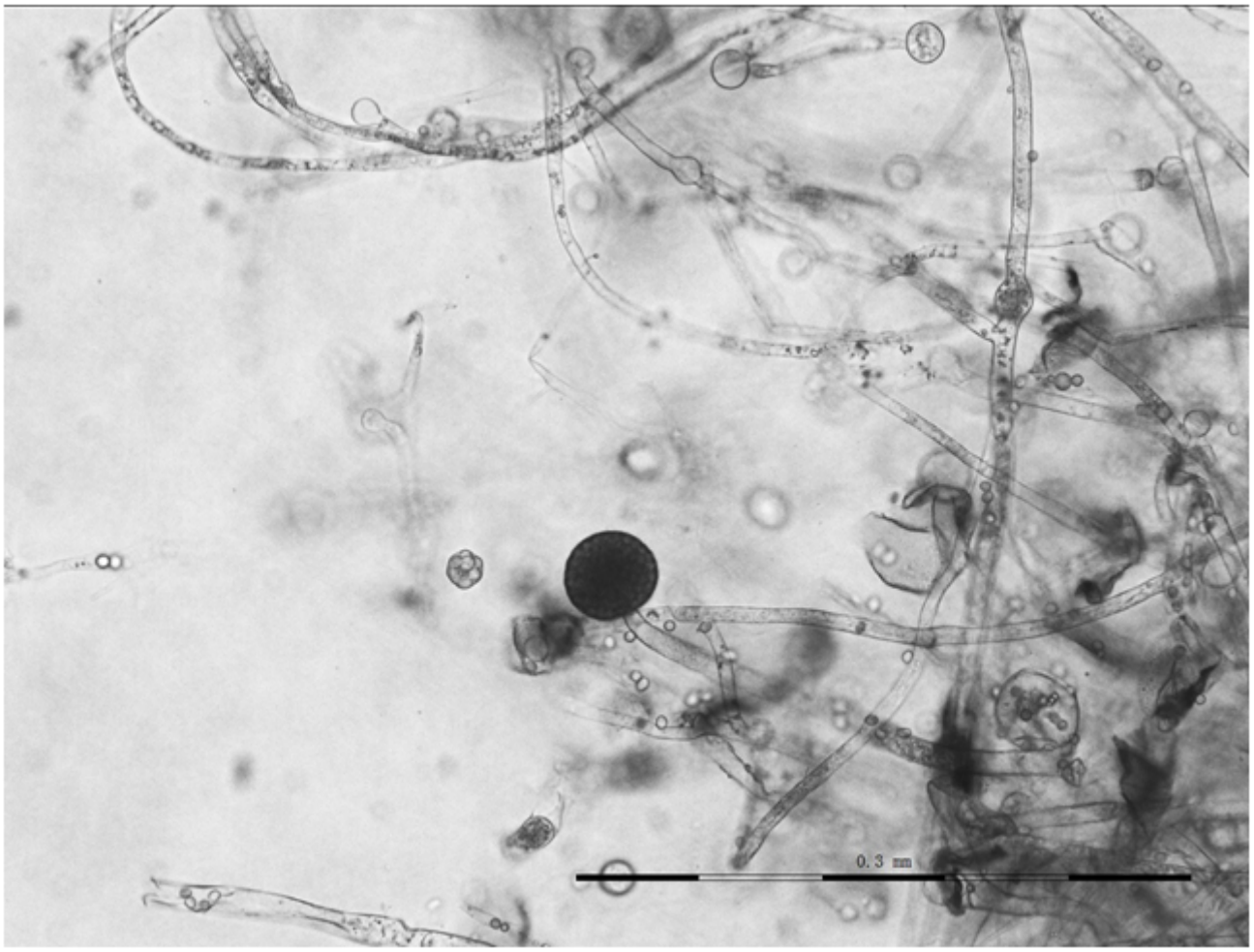

Figure 6

The white mold observed under a microscope

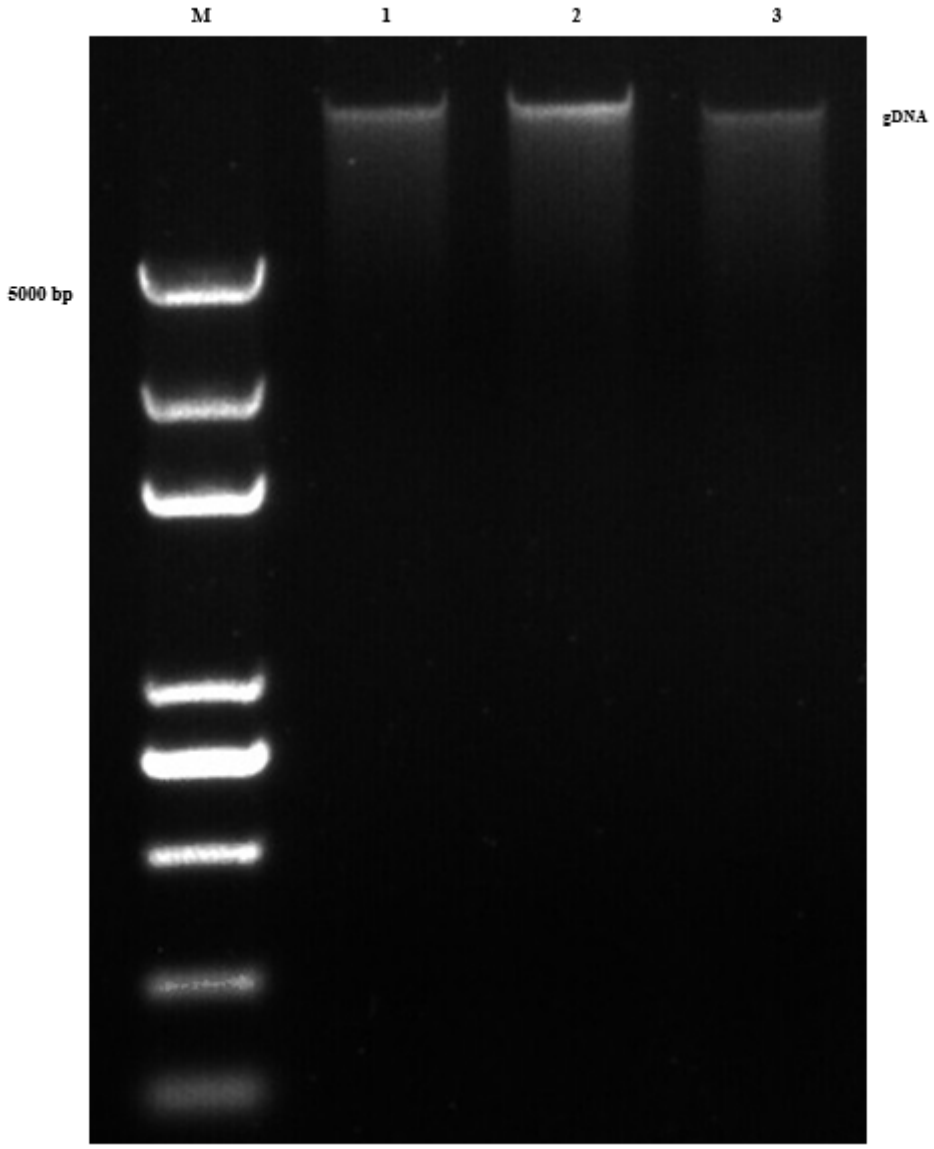

Figure 7

The genomic DNA extracted from the white mold

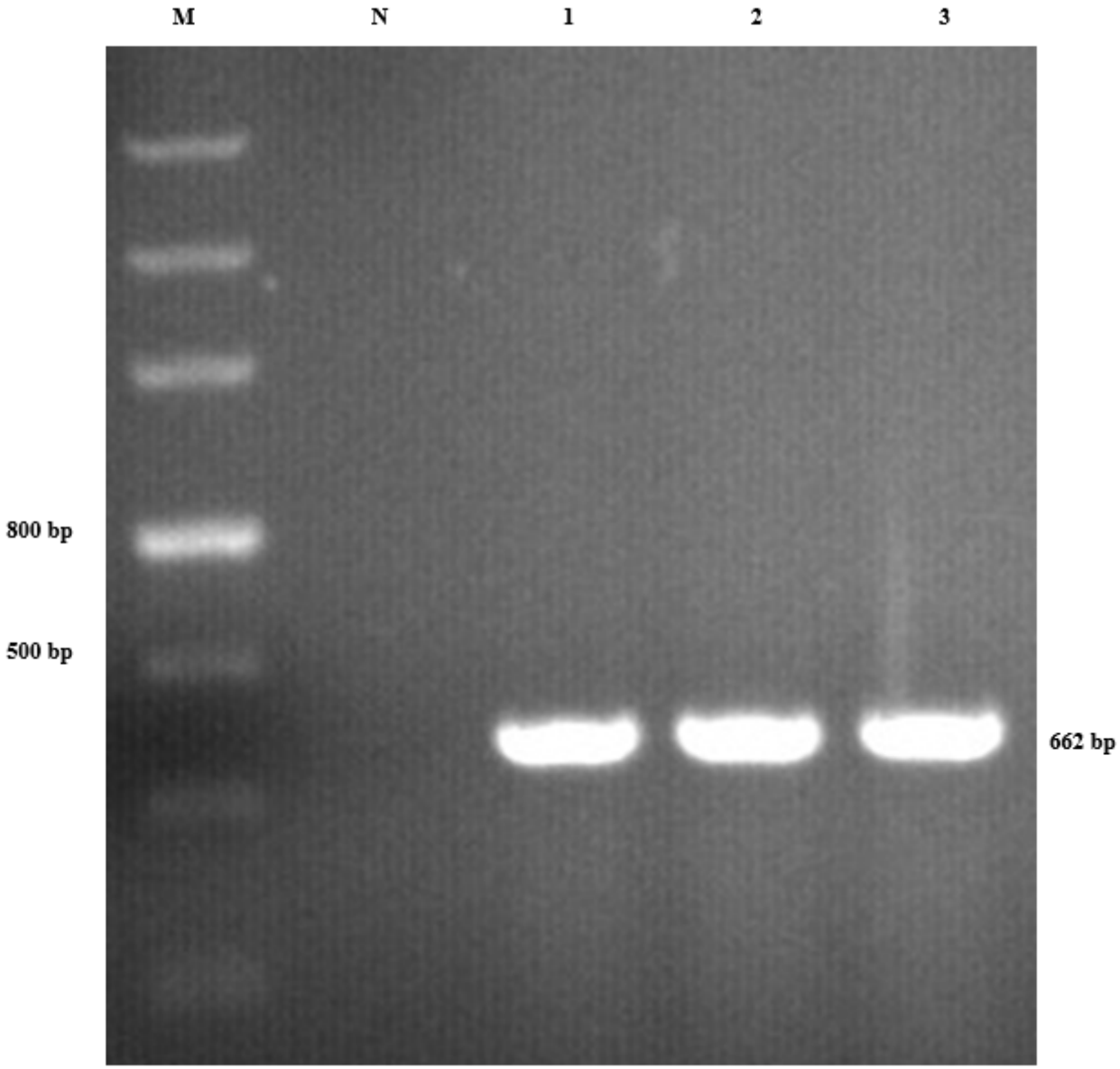

Figure 8

The ITS fragment of the white mold

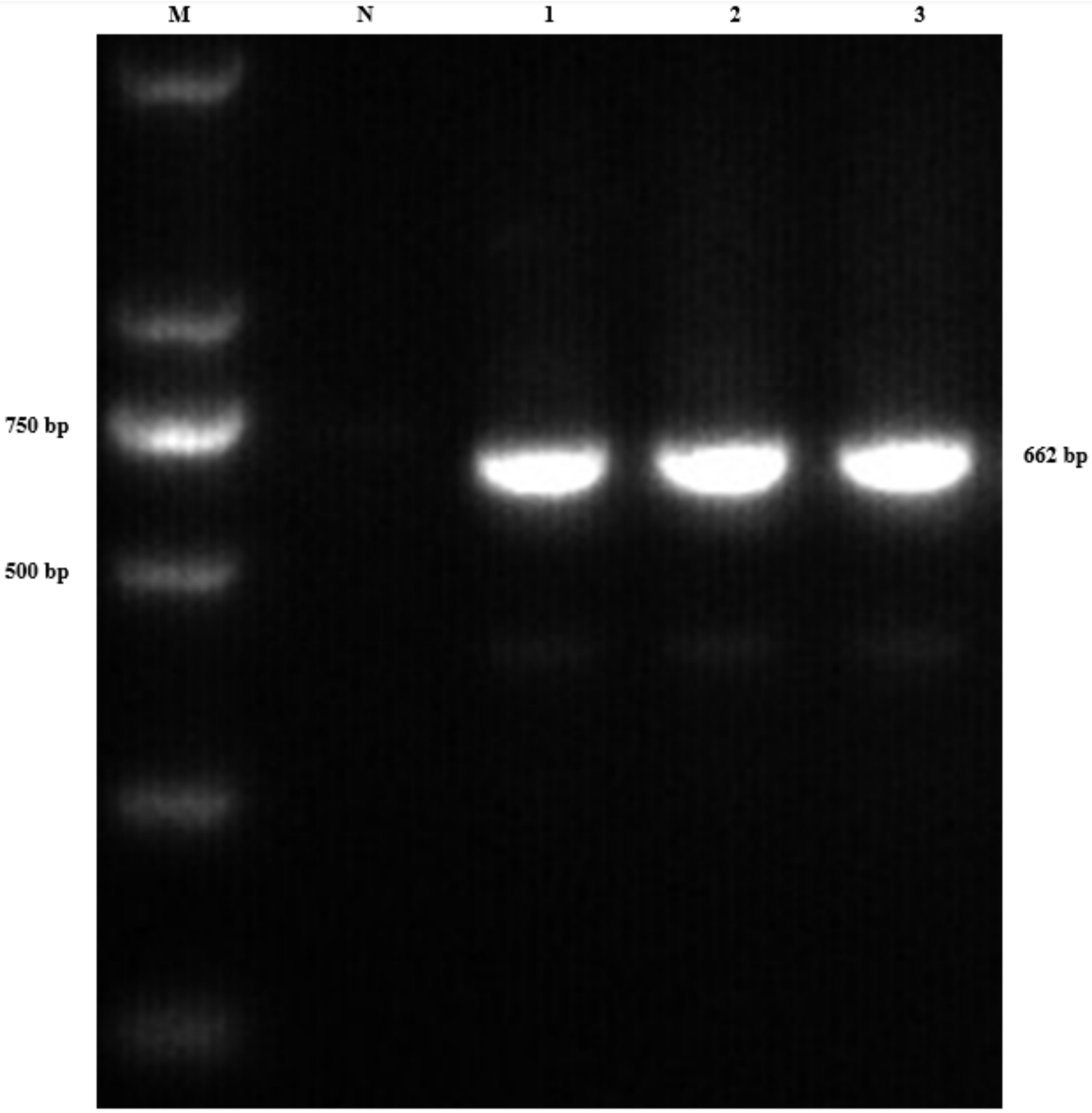

Figure 9

Identification of positive clones

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Concentrationofsolublesubstancesineachgroupofculturematerials.xls
- Table3and4.docx
- SupplementaryInformation.zip