Enhancement of pigment and proximate values of intergeneric hybrid of protoplast fusion of Monascus ruber and Pleurotus ostreatus

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Research Article

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Abstract

Protoplast fusion is a crucial technique for strain enhancement that brings genetic recombination to filamentous fungus and creates hybrid strains. In this present study, the intergenic fusion between *M. ruber* and *P. ostreatus* has been carried out to enhance pigments, secondary metabolites, and proximate values. Protoplasts from *M. ruber* and *P. ostreatus* were isolated and an equal amount of protoplast was taken and performed protoplast fusion. The regenerated colonies were plated in potato dextrose agar with selected biological markers (nystatin and fluconazole) for the confirmation of the hybrid. In a standardized condition, the protoplast yield of *M. ruber* and *P. ostreatus* was 2.31×10^7 cells/ml and 2.33×10^7 cells/ml, respectively. Acidic pH yielded high amounts of protoplasts than neutral and alkaline pH. KCl showed a high yield of protoplasts 2.36×10^6 cells /ml from *M. ruber* and 2.46×10^7cells/ml from *P. ostreatus*. The colony resistance to nystatin and fluconazole was confirmed that the obtained regenerated colonies were fusant. The fusant has no septation in its transverse walls with lipid droplets. Fusion of protoplast from *M. ruber* and *P. ostreatus* resulted in genetic inference at the metabolic levels, explaining the elevation of pigment in fusant colonies. The proximate composition of fusant strains was higher. In conclusion, we have developed a new strain with genetic variance capable of producing an increased level of pigment with nutritional value through protoplast fusion that allows the generation of fusant with the characteristics of parent strains and elucidates the effectiveness of fusant for commercial applications.

Introduction

Monascus fungi *(M. aurantiacus, M. pupureus, M. ruber)* are nonpathogenic filamentous ascomycetes that have been used in Asia for over 1000 years [44]. They are mainly used to produce food components, natural colors, and food supplements that have beneficial effects on human health [32]. *Monascus* fermented substrates have their colored appearance (red, orange, or yellow) due to their various oligoketide pigments produced by a combination of polyketide and fatty acid synthases. The principal pigments are yellow (ankaflavin and monascin), orange (rubropunctatin and monascorubrin), and red (rubropunctamine and monascorubramine) chemicals, although more than 20 colored compounds have recently been identified from fermented rice or culture medium [32]. *Monascus* can synthesize monacolin, mycotoxin, and citrinin, which have amino groups responsible for non-specific biological actions such as antimicrobial, anticancer, and immunomodulation. Monacolins are comprised of β-hydroxy acids that inhibit the key enzyme hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase in the cholesterol biosynthesis [32].

Pleurotus ostreatus mushrooms, commonly known as oyster mushrooms, are members of the *Apiaceae* family [11, 39]. They are very healthy foods, low in calories and fat, and rich in protein, chitin, vitamins, and minerals. *P. ostreatus* is a β-glucan-rich functional oyster mushroom that may help to regulate blood pressure, body weight, glucose and lipid metabolism, and appetite sensations [16]. *P. ostreatus* exhibits anticancer [24], immunomodulatory [28], antioxidant [3], anti-inflammatory [23], hypocholesterolemic [12], and antihypertensive [2]. Both *M. ruber* and *P. ostreatus* can produce the essential fungal metabolite

called lovastatin, a competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme in the biosynthesis of cholesterol [7, 20].

Protoplast fusion is a crucial technique for strain enhancement that brings genetic recombination to filamentous fungus and creates hybrid strains (interspecific or intergeneric) [19, 33]. A protoplast fusion is a powerful gene manipulation tool that removes the obstacles to genetic exchange imposed by traditional mating systems [19, 33]. The protoplast fusion approach offers much promise in genetic analysis and strain enhancement. It is essential for microorganisms with industrial applications [30]. The most commonly used method for preparing protoplast is the enzymatic digestion of cell walls [4]. Intergeneric protoplast fusion had been carried out between *Kluyveromyces and Saccharomyces cerevisiae* for increased sorbitol production [45]. Intraspecific protoplast fusion was conducted between recombinant strains of *A. niger* for glucoamylase production [5].

Food has long been used to alleviate hunger and meet nutritional requirements. Food is utilized to treat ailments and improve our quality of life in today's culture. In recent years, food consumers have been more concerned about how foods and supplements affect their health [13]. The functional food industry, which produces superfoods, nutraceuticals, and dietary supplements, has been booming recently [46]. As a result, the notion of food functioning governs the food markets of the major industrialized countries [27, 46]. Dietary supplements have been used to augment essential nutrients and bio-functionality, and functional foods are predicted to impact industrial demand worldwide due to their health-promoting qualities.

In this present study, the intergenic fusion between *M. ruber* and *P. ostreatus* has been carried out to enhance pigments and secondary metabolites for industrial purposes and to check the proximate values of fusant obtained from the fusion.

Materials And Methods

Microorganisms

Monascus ruber and *Pleurotus ostreatus* were purchased from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. All the strains were maintained in potato dextrose agar (PDA) for further analysis. The anti-hexamyco disc was used for the identification of biological markers.

Protoplast isolation

Protoplasts from *M. ruber* and *P. ostreatus* were isolated and treated with different osmotic stabilizers and enzymes then the mixture was examined under the light microscope for the release of protoplast.

Protoplast fusion and regeneration

According to Anne and Peberdy [6] method, an equal amount of protoplast was taken from *M. ruber* and *P. ostreatus* and washed with an osmotic stabilizer. One milliliter of protoplast was incubated with

polyethylene glycol (PEG) (4000–6000 molecular weight, 50% v/v) with 10 mM $CaCl_2$ and 50 mM glycine for 30 minutes. The mixture was serially diluted with an osmotic stabilizer (0.6 M KCl) and spread over to analyze regenerated colonies in a selective regeneration media. The regenerated colonies were plated in potato dextrose agar (PDA) with selected biological markers (nystatin and fluconazole) for the confirmation of the hybrid.

Extraction of pigment from Monascus ruber and fusant

Extracellular pigment estimation

A small amount of pigment was taken from the medium to estimate extracellular pigment using 90% ethanol. For this, 5 ml of solvent was taken per milliliter of fermented broth, and the samples were kept on a rotary shaker at 200 rpm for 1 hour and filtered through Whatman No.1 filter paper. For this, ethanol extract was considered a control medium for pigment analysis. The pigment analysis was carried out using the spectrophotometer (Shimadzu UV-1800) at a wavelength of 350–600 nm. Each supernatant was read at 400, 550, 520, and 540 nm. Pigment yield was expressed as optical density units per gram, and the absorbance was converted into pigment units using color values [17].

Intracellular pigment extraction

One gram of the fresh mycelial mat was taken from the seven-day-old culture and washed thoroughly with distilled water. To this, 5ml of 90% ethanol was added into a test tube. The mycelia mat was washed and heated in a boiling water bath for 30 minutes for the pigment release. Then it was kept on a rotary shaker at 200 rpm for 1 hour, allowing for 15 minutes, and filtered using Whatman No.1 filter paper. The pigment obtained from the filtrate was measured (optical density) using the same method used to measure extracellular pigment.

Estimation of biomass

The broth was filtered through Whatman No. 1 filter paper, and the mycelial mat was washed twice with distilled water. Then it was dried at 80°C in a hot air oven to constant weight and measured as biomass [43].

Proximate analysis of parental strain and fusant

Micronutrients

The moisture and ash content (method no. 942 -05), crude protein (method no 948 − 13), crude fat (method no 978 − 39), and crude fiber (method no 978 − 10) content in the mycelia sample were estimated according to the AACC procedure [1].

Minerals

Minerals such as sodium, potassium, calcium, phosphorus, zinc, iron, copper, manganese, and magnesium were determined using the dry ash method by AOAC procedure [8]. Ash was dissolved in an

acidic solution and was analyzed using a flame photometer and atomic absorption spectrophotometer.

Sugar and vitamins

Total sugar is determined by the volumetric method (the Lane- Eynon method) as mentioned in AOAC[8]. Vitamin content was estimated using 2, 6-dichlorophenol dye according to the prescribed method of AOAC [8].

Result

Protoplast isolation and *f*usion

Protoplast preparation was the most important step for fusion and regeneration. The mycelium age, enzyme mixture, and osmotic stabilizer were crucial to obtaining a protoplast. The mycelium age was an essential factor in the isolation of protoplasts. We ensured all the above conditions during the protoplasts isolation. In a standardized condition, the protoplast yield of *M. ruber* and *P. ostreatus* was 2.31×10^7 cells/ml and 2.33×10^7 cells/ml, respectively (Table 1).

Table 1
Effect of age of culture on the production of protoplasts

	Name	Age of Culture	Protoplast yield/ml
1	*Monascus ruber*	3 days	2.31×10^7 cells /ml
		4 days	1.91×10^7 cells /ml
		5 days	1.36×10^7 cells /ml
2	*Plerotus oystretus*	3 days	2.33×10^7cells /ml
		4 days	2.02×10^7cells /ml
		5 days	1.64×10^7cells /ml

The effect of pH on the release of *M. ruber* and *P. ostreatus* protoplasts was studied at a pH of 4 to 8. The result suggested that acidic pH yielded high amounts of protoplasts than neutral and alkaline pH. At pH 6, 2.21×10^6 cells/ml and 2.24×10^7 cells /ml of protoplast were released from *M. ruber* and *P. ostreatus*, respectively (Table 2).

Table 2
Effect of pH on the release of protoplasts

S.No	Name	pH	No. of Protoplast /ml
1	*Monascus ruber*	4.0	1.36×10^7 cells /ml
		6.0	2.21×10^6 cells /ml
		8.0	1.19×10^7 cells /ml
2	*Plerotus oystretus*	4.0	2.06×10^7 cells /ml
		6.0	2.24×10^7 cells /ml
		8.0	1.21×10^7 cells /ml

Lytic enzymes are also crucial factor in the protoplast isolation. In the present study, macerozyme (0.1 mg/ml), cellulase (10 mg/ml), pectinase (0.2 mg/ml), chitinase (0.2 mg/ml) and β-glucuronidase (0.1 mg/ml) were the lytic enzymes used for the protoplast isolation. *M. ruber* was treated with chitinase (0.2 mg/ml) and β-glucuronidase (0.1 mg/ml) that yielded 2.36×10^6 cells /ml of protoplasts. Similarly, *P. ostreatus* was treated with macerozyme (0.1 mg/ml), cellulase (10 mg/ml), pectinase (0.2 mg/ml) that yielded 2.01×10^7 cells/ml of protoplasts (Table 3).

Table 3
Effect of lytic enzymes on the production of protoplasts

S.No	Name	Enzyme	Concentration	No. of Protoplast /ml
1	*Monascus ruber*	Macerozyme	0.1 mg/ml	2.06×10^6 cells /ml
		Cellulase	10mg/ml	
		Pectinase	0.2mg/ml	
		Chitinase	0.2mg/ml	2.36×10^6 cells /ml
		β-glucuronidase	0.1mg/ml	
2	*Plerotus oystretus*	Macerozyme	0.1 mg/ml	2.01×10^7 cells /ml
		Cellulase	10mg/ml	
		Pectinase	0.2mg/ml	
		Chitinase	0.2mg/ml	1.41×10^6 cells /ml
		β-glucuronidase	0.1mg/ml	

Osmotic stabilizer plays a vital role in the isolation of protoplast. Different concentrations of the KCl, NaCl, and sucrose were used as osmotic stabilizers in protoplast isolation. Among these, using 0.6M, KCl

showed a high yield of protoplasts 2.36×10^6 cells /ml from *M. ruber* and 2.46×10^7cells /ml from *P. ostreatus* (Table 4).

Table 4
Effect of osmotic stabilizers on the production of protoplasts

S.No	Name	Osmotic Stabilizer	Concentration	No. of Protoplast /ml
1	*Monascus ruber*	KCl	0.6 M	2.36×10^6 cells /ml
			0.7 M	1.03×10^6 cells /ml
			0.8 M	1.01×10^6 cells /ml
		NaCl	0.6 M	1.11×10^6 cells /ml
			0.7 M	1.04×10^6 cells /ml
			0.8 M	1.03×10^6 cells /ml
		Sucrose	0.6 M	1.09×10^6 cells /ml
			0.7 M	1.14×10^6 cells /ml
			0.8 M	1.31×10^6 cells /ml
2	*Plerotus oystretus*	KCl	0.6 M	2.03×10^7 cells /ml
			0.7 M	1.04×10^7 cells /ml
			0.8 M	1.11×10^7 cells /ml
		NaCl	0.6 M	2.46×10^7cells /ml
			0.7 M	2.01×10^7 cells /ml
			0.8 M	1.51×10^7 cells /ml
		Sucrose	0.6 M	2.07×10^7 cells /ml
			0.7 M	1.11×10^6 cells /ml
			0.8 M	1.08×10^6 cells /ml

Regeneration and screening of fusant

The culture medium is a significant factor in the regeneration of fused protoplast. In the present study, PDA amended with 0.6 M KCl was used as the culture for protoplast regeneration and the regeneration frequency was 71%. The regenerated colonies were screened for a resistant character to confirm fusant

(Table 5). The colony resistance to nystatin and fluconazole was confirmed that the obtained regenerated colonies were fusant (Fig. 1). It was also confirmed by the mycelia patterns of both parental and fusant strains (Fig. 2).

Table 5
Selection of biomarker using antimycotic disc (HEXA Antimyco-01 HX104-1Pk)

S.No	Disc	Symbol	Concentration	Zone of Inhibition in Diameter	
				M.ruber	P.oyster
1	Amphotericin-B	AP	100 units	10 mM	R
2	Clotrimazole	CC	10 mcg	R	20 mM
3	Fluconazole	FLC	25 mcg	R	22 mM
4	Itraconazole	IT	10 mcg	R	22 mM
5	Ketaconazole	KT	10 mcg	R	R
6	Nystatin	NS	100 units	28 mM	R

The colony morphology has dramatically differed in the fusion products for the identification process. In *M. ruber*, hyphae are thread-like tubular filamentous structures septated with lipid droplets in their transverse walls. In contrast, the fusant has no septation in its transverse walls with lipid droplets (Fig. 3). In the present study, mycelial growth pattern and colony morphology were also used as markers for fusant identification.

Extracellular and intracellular pigment from Monascus ruber and fusant

The extracellular and intracellular pigment production of *M. ruber* and fusant were presented in Table 6. The pigment levels were increased in the fusant strain than in the parental strain. Fusion of protoplast from *M. ruber* and *P. ostreatus* resulted in genetic inference at the metabolic levels, explaining the elevation of pigment in fusant colonies.

Table 6
Extracellular and intracellular pigment yield of Monascus ruber **and fusant**

	Extracellular pigment yield U/g	Intracellular Pigment yield U/g
Monascus ruber	2.606	2.419
Fusant	2.946	2.642

Proximate analysis o *f* parental and *f*usant strain

The proximate composition of parental and fusant strains was analyzed, and the proximate analytes were listed in Table 7. The fusant strain was comprised of 7.28% of moisture, 4.87% of ash content, 11.01% of cured protein, 7.16% of crude fat, 51.01% of carbohydrates, and 8.43% of crude fiber.

Table 7
Micronutrients minerals, sugars, vitamins analysis of *Monascus ruber*, *Pleurotus ostreatus* and fusant

Micronutrients	*Monascus ruber*	*Pleurotus ostreatus*	Fusant
Moiture content	8.34%	16.42%	7.28%
Ash content	5.43%	6.91%	4.87%
Curde protein	10.07%	28.91%	11.01%
Crude fat	3.84%	12.06%	7.16%
Total carbohydrate	21.71%	57.06%	51.01%
Curde fibre	6.37%	12.21%	8.43%
Potassium	21.70 mg	141.23 mg	28.8 mg
Sodium	4.40 mg	22.75 mg	10.41 mg
Phosphorus	2.81 mg	18.72 mg	4.46 mg
Calcium	8.30 mg	10.58 mg	9.89 mg
Iron	14.71 mg	296.54 mg	21.11 mg
Magnesium	6.23 mg	15.241 mg	6.51 mg
Sugar	7.46 mg	196.68 mg	8.50 mg
Zinc	1.42 mg	9.292 mg	2.39 mg
Manganese	0.13 mg	1.172 mg	0.7 mg
Copper	0.34 mg	1.892 mg	0.6 mg
Vitamins B1	-	0.42 mg	0.13 mg
Vitamin B2	-	0.20 mg	0.05 mg

The freshness, flavor, and storage performance of edible fungus are usually affected by moisture content. A considerable difference was observed in the micronutrients and minerals present in the fusant than the parental strain, and it was confirmed that the hybrid has the characteristics of *M. ruber* and *P. ostreatus*.

Discussion

Our present study demonstrated that a new strain engineered with genetic variance was able to produce an increased proportion of pigments with nutritional value through protoplast fusion. Protoplast fusion allowed the generation of fusant with the properties of parental strains and illustrated the effectiveness of fusant for commercial applications.

The production of protoplasts was a critical step for fusion and regeneration. The age of the mycelium, the combination of enzymes, and the osmotic stabilizer are all essential elements to obtain protoplasts. In the present study, young mycelium was used to isolate protoplasts. The major reason is that the lytic action of digestive enzymes is more sensitive to young mycelium than to old mycelium [21]. Protoplast fusion, gene transfer, and metabolite productions are examples of physiological and genetic research that can benefit from fungal protoplasts. A recent study optimized several critical variables for the synthesis and regeneration of protoplasts from strain CH1. The highest yield of protoplasts was produced using an enzyme mixture consisting of cellulase, glusulase, and driselase in an osmotic stabilizer [37]. Similarly, another study indicated that in an osmotic stabilizer (0.6 M KCl), *Trichothecium roseum* protoplasts were extracted using the combining the lytic enzyme. A maximum number of protoplasts was achieved at pH 5.5. Protoplast regeneration and reversal attempts revealed a maximum regeneration (60.8 %) ina complete PDA media [9]. Consistent with the above findings, protoplast fusion and regeneration showed maximum regeneration. In the present study, the protoplast yield of *M. ruber* and *P. ostreatus* was 2.31×10^7 cells /ml and 2.33×10^7 cells/ml under standardized conditions. At pH 6, higher amounts of protoplasts were released from *M. ruber* (2.21×10^6 cells /ml) and *P. ostreatus* (2.24×10^7 cells /ml). *M. ruber* treated with chitinase (0.2 mg/ml) and β-glucuronidase (0.1 mg/ml) yielded 2.36×10^6 cells /ml of protoplasts and for *P. ostreatus* treated with macerozyme (0.1 mg/ml), cellulase (10 mg/ml), pectinase (0.2 mg/ml) yielded 2.01×10^7 cells/ml of protoplasts. 0.6M KCl showed a high yield of protoplasts 2.36×10^6 cells /ml from *M. ruber* and 2.46×10^7 cells /ml of protoplast from *P. ostreatus*.

Osmotic stabilizers, including inorganic salts, sugars, and sugar alcohols, were used to stabilize protoplasts isolated from mycelium. The concentration, molarity, and the type of stabilizer can impact the yield of the protoplast [26, 35]. Depending on their lifestyle, filamentous fungi obtain their nutrients from the environment. Filamentous fungi rely on external carbon sources because they are heterotrophic, but many can absorb inorganic nitrogen and other nutrients for growth and metabolism. They can make all the necessary amino acids from inorganic nitrogen [29]. Many protoplast isolation studies indicated that 0.6 M KCl acts as the best osmotic stabilizer [15, 18]. On the contrary, some protoplast isolation studies indicated that 0.4 M NaCl was effective in protoplast yield [14, 25]. Notably, the molecular weight of PEG is essential in the fusion process. A recent study indicated that the optimal osmotic stabilizer for the maximum interspecific fusion frequency was 40 % PEG with 10 mM $CaCl_2$,10 mM Tris-HCl (pH 7.5). Intraspecific fusion frequency was 6.2 and 7.2 % for *T. harzianum* and. *viride* [31]. Another investigation indicated that *T. reesei* and *T. harzianum* 40 % PEG with STC buffer weresuitable for intrafusion. When the protoplasts were exposed to the PEG solution, they were attracted and connected together as pairs, and the plasma membrane collapsed into merged protoplasmic contents, finally culminating in the fused protoplasts becoming single and enormous [34]. The present study used incubation with PEG (50% v/v),

10 mM $CaCl_2$, and 50 mM glycine for the protoplast fusion. The maximum level of fusion frequency was 7.1%.

The culture medium for regeneration is significant for the regeneration of fused protoplasts. PDA supplemented with 0.6 M KCl was used as culture for protoplast regeneration in the present study. The regeneration frequency was 71%. The resulting fusant grows faster than the parent strains. A report indicated that the frequency of regeneration of *G. putredinis* and *T. harzianum* was 51 and 61%, respectively [38]. A study reported that a good regeneration medium for *T. harzianum* protoplasts was Potato Dextrose Yeast Extract Agar medium supplemented with 0.6 M KCl, and the fusant showed a faster growth rate compared to the parents [10, 40]. The regenerated colonies were screened for the resistant character to confirm fusant. Colony resistance to nystatin and fluconazole is confirmed because the regenerated colonies obtained are fusant, which is also confirmed by the mycelial patterns of both the parent and fusant strains. It was also used to identify the colony morphology greatly differs in the fusion products [10, 40]. In *M. ruber*, hyphae - a thread-like tubular filamentous structure had septate in its cross walls with lipid droplets. In contrast, the fusant doesn't have any septate in its cross walls with lipid droplets. In the present study, the mycelia growth pattern and colony morphology were also used as a marker for fusant identification.

The extracellular and intracellular pigment production of *M.ruber* and Fusant were presented. The pigment level was increased in the fusant strain than in the parental strain. Fusion of protoplast from *M. ruber* and *P. ostreatus* may result in genetic inference at the metabolic level, which was the reason behind the elevation of pigment in fusant colonies. A recent study observed a variation or stimulation of pigment production during protoplast hybridization in other species of fungi [22]. The proximate composition of parental and fusant strains contains 7.28% of moisture, 4.87 % f ash content, 11.01% of cured protein, 7.16% of curde fat, 51.01% of carbohydrates, and 8.43% of crude fiber, respectively.

The freshness, flavor, and storage performance of edible fungus are affected by moisture content. Among the 23 edible fungi, there was a difference in the moisture content, nearly about 6.9～15.5 g/100 g. Ash content reflects the minerals, and minerals were the sole source of bones, hemoglobin, and acid-base balance of the body [47]. Torres *et al*., 2000 reported that the ash levels were compared to other nutritional sources. It was possible to observe much greater levels than those found in foods such as milk, eggs, and some beef cuts [41]. *Lentinus edodes (Berk.) Sing* had the lowest ash content of 1.27 g/100 g [47]. Carbohydrates are essential for structural compositions and energy release. Dietary fiber is known as the "gut scavenger." therefore, a higher intake of edible fungi rich in dietary fiber can help prevent various diseases and meet the requirements of changing the diet structure generally advocated at home and abroad [47]. Five kinds of edible fungi have more than 50% dietary fiber content, including *Ganoderma lucidum (Leyss. ex Fr.) Karst*. had the highest dietary fiber content, 70.2 g/100 g [47]. The fiber content in the mushrooms is good for essential compounds that exhibit different physiological and nutritional benefits [42]. The crude includes lipids, free fatty acids, mono, di, triglycerides, sterols, sterol esters, and phospholipids. Fatty acid compositions differ from each fungi species and are an essential component of organelles comprising about 30–70% of fungi [36]. *Pleurotus* species contain low lipids

and excellent sources of fatty acids like linoleic acid and oleic acid [36]. The earlier research evidence that *Pleurotus* species are good candidates for anti-inflammation and hypocholesterolemia in the human diet [36]. *Pleurotus* mushrooms are exceptionally high in folic acid (B9), also known as folic acid, a nutrient that cannot be produced in the body and must be supplied by the diet [36]. *Pleurotus* species are good sources of palatable proteins and are rich in minerals (Na, Ca, P, Fe, and K) and vitamins (vitamin C and B complex) [36]. A considerable difference was observed in the micronutrients and minerals present in the fusant than the parental strain in the present study, and it was confirmed that the hybrid has the characteristics of *M. ruber* and *P. ostreatus*.

In the present study, we have developed a new strain with genetic variance capable of producing an increased level of pigment with nutritional value through protoplast fusion. Protoplast fusion allows the generation of fusant with the characteristics of parent strains and elucidates the effectiveness of fusant for commercial applications.

Declarations

Ethics approval: Not applicable

Consent to Participate: Not applicable

Consent to Publish: Not applicable

Author contributions: All authors contributed to the study's conception and design. Suganya Kannan and Ayyakannu Usha Raja Nanthini performed material preparation, data collection, and analysis. The first draft of the manuscript was written by Suganya and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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References

1. AACC International (2009) AACC International approved methods of analysis. St. Paul, Minn.: AACC International.
2. Agunloye OM (2021) Effect of aqueous extracts of Pleurotus ostreatus and Lentinus subnudus on activity of adenosine deaminase, arginase, cholinergic enzyme, and angiotensin-1-converting enzyme. Journal of Food Biochemistry. doi: 10.1111/jfbc.13490

3. Agunloye OM, Oboh G, Falade AO (2021) Pleurotus ostreatus and Lentinus subnudus supplemented diets restore altered acetylcholinesterase and butyrylcholinesterase activities and improve antioxidant status in transgenic Drosophila melanogaster model. Journal of Dietary Supplements 18:372–386
4. Aloulou-Abdelkefi M, Trigui-Lahiani H, Gargouri A (2017) Autoclaved mycelium induces efficiently the production of hydrolytic enzymes for protoplast preparation of autologous fungus. Applied Biochemistry and Microbiology 53:230–236
5. Al-Turki AI, Khattab AA, Ihab AM (2008) Improvement of Glucoamylase Production by Aspergillus awamori Using Microbial Biotechnology Techniques. Biotechnology(Faisalabad) 7:456–462
6. Anne J, Peberdy JF (1975) Conditions for induced fusion of fungal protoplasts in polyethylene glycol solutions. Archives of Microbiology 105:201–205
7. Aravindan R, Viruthagiri T, Seenivasan A, Subhagar S (2008) Microbial production and biomedical applications of lovastatin. Indian Journal of Pharmaceutical Sciences 70:701
8. Association of official analytical chemists (AOAC) (1990) Official methods of analysis of the AOAC. Methods 932.06, 925.09, 985.29, 923.03. , 15th ed. Arlington, VA, USA.
9. Balasubramanian N, Juliet GA, Srikalaivani P, Lalithakumari D (2003) Release and regeneration of protoplasts from the fungus Trichothecium roseum. Canadian Journal of Microbiology 49:263–268
10. Balasubramanian N, Juliet GA, Srikalaivani P, Lalithakumari D (2003) Release and regeneration of protoplasts from the fungus Trichothecium roseum. Canadian Journal of Microbiology 49:263–268
11. Barh A, Sharma VP, Annepu SK, Kamal S, Sharma S, Bhatt P (2019) Genetic improvement in Pleurotus (oyster mushroom): a review. 3 Biotech 9:322
12. Bobek P, Galbavý S (1999) Hypocholesterolemic and antiatherogenic effect of oyster mushroom (Pleurotus ostreatus) in rabbits. Nahrung 43:339–42
13. Burchi F, Fanzo J, Frison E (2011) The Role of Food and Nutrition System Approaches in Tackling Hidden Hunger. International Journal of Environmental Research and Public Health 8:358–373
14. Cai Y, Christias C (2010) Isolation and fusion of protoplasts from the phytopathogenic fungus sclerotium rolfsii(sacc.). Braz J Microbiol 41:253–63
15. Cheng Y, Baclanger RR (2000) Protoplast preparation and regeneration from spores of the biocontrol fungus Pseudozyma flocculosa. FEMS Microbiology Letters 190:287–291
16. Dicks L, Ellinger S (2020) Effect of the Intake of Oyster Mushrooms (Pleurotus ostreatus) on Cardiometabolic Parameters—A Systematic Review of Clinical Trials. Nutrients 12:1134
17. Dikshit R, Tallapragada P (2013) Comparative study of Monascus sanguineus and Monascus purpureus for red pigment production under stress condition. International Food Research Journal 20:1235–1238
18. Eyini M, Rajkumar K, Balaji P (2006) Isolation, Regeneration and PEG-Induced Fusion of Protoplasts of Pleurotus pulmonarius and Pleurotus florida. Mycobiology 34:73

19. Glombik M, Bačovský V, Hobza R, Kopecký D (2020) Competition of Parental Genomes in Plant Hybrids. Frontiers in Plant Science. doi: 10.3389/fpls.2020.00200
20. Gunde-Cimerman N, Cimerman A (1995) Pleurotus Fruiting Bodies Contain the Inhibitor of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase—Lovastatin. Experimental Mycology 19:1–6
21. Haneef M, Ceseracciu L, Canale C, Bayer IS, Heredia-Guerrero JA, Athanassiou A (2017) Advanced Materials From Fungal Mycelium: Fabrication and Tuning of Physical Properties. Scientific Reports 7:41292
22. Hassan MM (2014) Influence of protoplast fusion between two Trichoderma spp. on extracellular enzymes production and antagonistic activity. Biotechnology & Biotechnological Equipment 28:1014–1023
23. Jayasuriya WJABN, Handunnetti SM, Wanigatunge CA, Fernando GH, Abeytunga DTU, Suresh TS (2020) Anti-Inflammatory Activity of Pleurotus ostreatus, a Culinary Medicinal Mushroom, in Wistar Rats. Evidence-Based Complementary and Alternative Medicine 2020:1–9
24. Khinsar KH, Abdul S, Hussain A, Ud Din R, Lei L, Cao J, Abbasi M, Ur Rehman A, Farooqui N, Yi X, Min H, Wang L, Mintao Z (2021) Anti-tumor effect of polysaccharide from Pleurotus ostreatus on H22 mouse Hepatoma ascites in-vivo and hepatocellular carcinoma in-vitro model. AMB Express 11:160
25. Kiełkowska A, Grzebelus E, Lis-Krzyścin A, Maćkowska K (2019) Application of the salt stress to the protoplast cultures of the carrot (Daucus carota L.) and evaluation of the response of regenerants to soil salinity. Plant Cell, Tissue and Organ Culture (PCTOC) 137:379–395
26. Kirn BK, Kang JH, Jin M, Kim HW, Shim MJ, Choi EC (2000) Mycelial protoplast isolation and regeneration of Lentinus lepideus. Life Sciences 66:1359–1367
27. Ku S, Park M, Ji G, You H (2016) Review on Bifidobacterium bifidum BGN4: Functionality and Nutraceutical Applications as a Probiotic Microorganism. International Journal of Molecular Sciences 17:1544
28. Llauradó Maury G, Morris-Quevedo HJ, Heykers A, Lanckacker E, Cappoen D, Delputte P, vanden Berghe W, Salgueiro Z, Cos P (2021) Differential Induction Pattern Towards Classically Activated Macrophages in Response to an Immunomodulatory Extract from Pleurotus ostreatus Mycelium. Journal of Fungi 7:206
29. Lübeck M, Lübeck PS (2022) Fungal Cell Factories for Efficient and Sustainable Production of Proteins and Peptides. Microorganisms 10:753
30. Muralidhar R v., Panda T (2000) Fungal protoplast fusion – a revisit. Bioprocess Engineering 22:429–431
31. Natesan B, Damodaran L (2008) Characteristics of protoplast inter, intra-fusant and regeneration of antagonistic fungi Trichoderma harzianum and Trichoderma viride. African Journal of Biotechnology 7:3235–3243
32. Patakova P (2013) Monascus secondary metabolites: production and biological activity. Journal of Industrial Microbiology and Biotechnology 40:169–181
33. Piperidis N (2021) GISH: Resolving Interspecific and Intergeneric Hybrids. pp 381–394

34. Prabavathy V, Mathivanan N, Sagadevan E, Murugesan K, Lalithakumari D (2006) Intra-strain protoplast fusion enhances carboxymethyl cellulase activity in Trichoderma reesei. Enzyme and Microbial Technology. . 38:719-723.
35. Raman J, Jang K-Y, Oh Y-L, Oh M, Im J-H, Lakshmanan H, Kong W-S (2021) Interspecific hybridization between Ganoderma lingzhi and G. applanatum through protoplast fusion. World Journal of Microbiology and Biotechnology 37:114
36. Raman J, Jang K-Y, Oh Y-L, Oh M, Im J-H, Lakshmanan H, Sabaratnam V (2021) Cultivation and Nutritional Value of Prominent Pleurotus spp.: An Overview. Mycobiology 49:1–14
37. Ren N, Liu J, Yang D, Liu X, Zhou J, Peng Y (2018) Preparation and Regeneration of Protoplasts from the Ethyl Vincamine Producing Fungus CH1 (Geomyces sp.). Natural Product Communications 13:1934578X1801300
38. Savitha S, Sadhasivam S, Swaminathan K (2009) Modification of paper properties by the pretreatment of wastepaper pulp with Graphium putredinis, Trichoderma harzianum and fusant xylanases. Bioresource Technology 100:883–889
39. Sekan AS, Myronycheva OS, Karlsson O, Gryganskyi AP, Blume YB (2019) Green potential of Pleurotus spp. in biotechnology. PeerJ 7:e6664
40. Sharma M, Sharma P, Singh R, Raja M, Sharma P (2016) Fast Isolation and Regeneration Method for Protoplast Production in Trichoderma harzianum. International Journal of Current Microbiology and Applied Sciences 5:891–897
41. Torres Agredo J, Mejía de Gutiérrez R, Escandón Giraldo CE, González Salcedo LO (2014) Characterization of sugar cane bagasse ash as supplementary material for Portland cement. Ingeniería e Investigación 34:5–10
42. Valverde ME, Hernández-Pérez T, Paredes-López O (2015) Edible Mushrooms: Improving Human Health and Promoting Quality Life. International Journal of Microbiology 2015:1–14
43. Velmurugan P, Lee YH, Venil CK, Lakshmanaperumalsamy P, Chae J-C, Oh B-T (2010) Effect of light on growth, intracellular and extracellular pigment production by five pigment-producing filamentous fungi in synthetic medium. Journal of Bioscience and Bioengineering 109:346–350
44. Wang T, Lin T (2007) Monascus Rice Products. pp 123–159
45. Wei W, Wu K, Qin Y, Xie Z, Zhu X (2001) Intergeneric protoplast fusion between Kluyveromyces and Saccharomyces cerevisiae – to produce sorbitol from Jerusalem artichokes. Biotechnology Letters 23:799–803
46. Younesi E, Ayseli MT (2015) An integrated systems-based model for substantiation of health claims in functional food development. Trends in Food Science & Technology 41:95–100
47. Yu Q, Guo M, Zhang B, Wu H, Zhang Y, Zhang L (2020) Analysis of Nutritional Composition in 23 Kinds of Edible Fungi. Journal of Food Quality 2020:1–9

Figures

Figure 1

Confirmation of fusant with nystatin and fluconozole

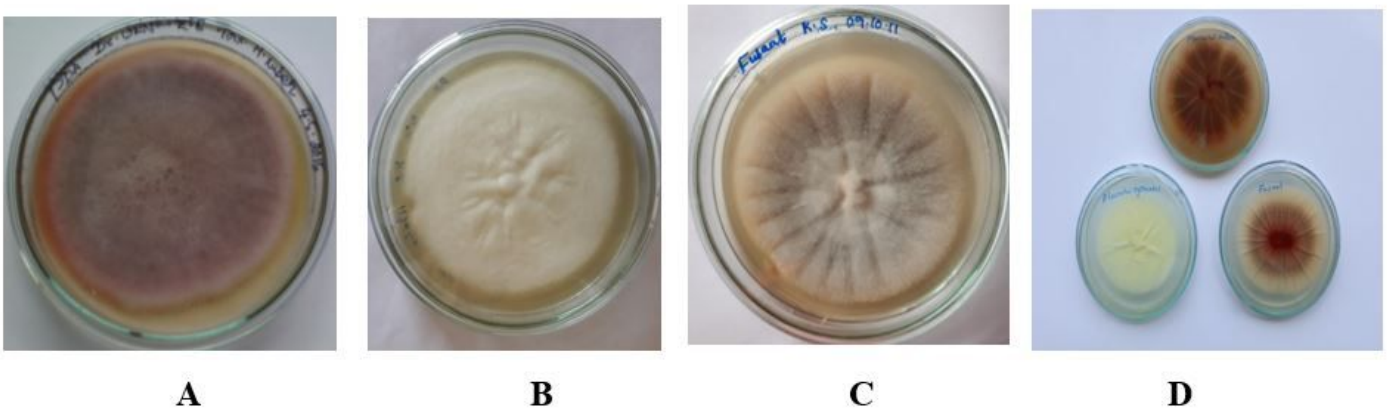

Figure 2

A and B - Parental strains of *Monascus ruber* and *Plerotus oystretus* and C- hybrid strain; D- reverse side of mycelia pattern of parental and fusant

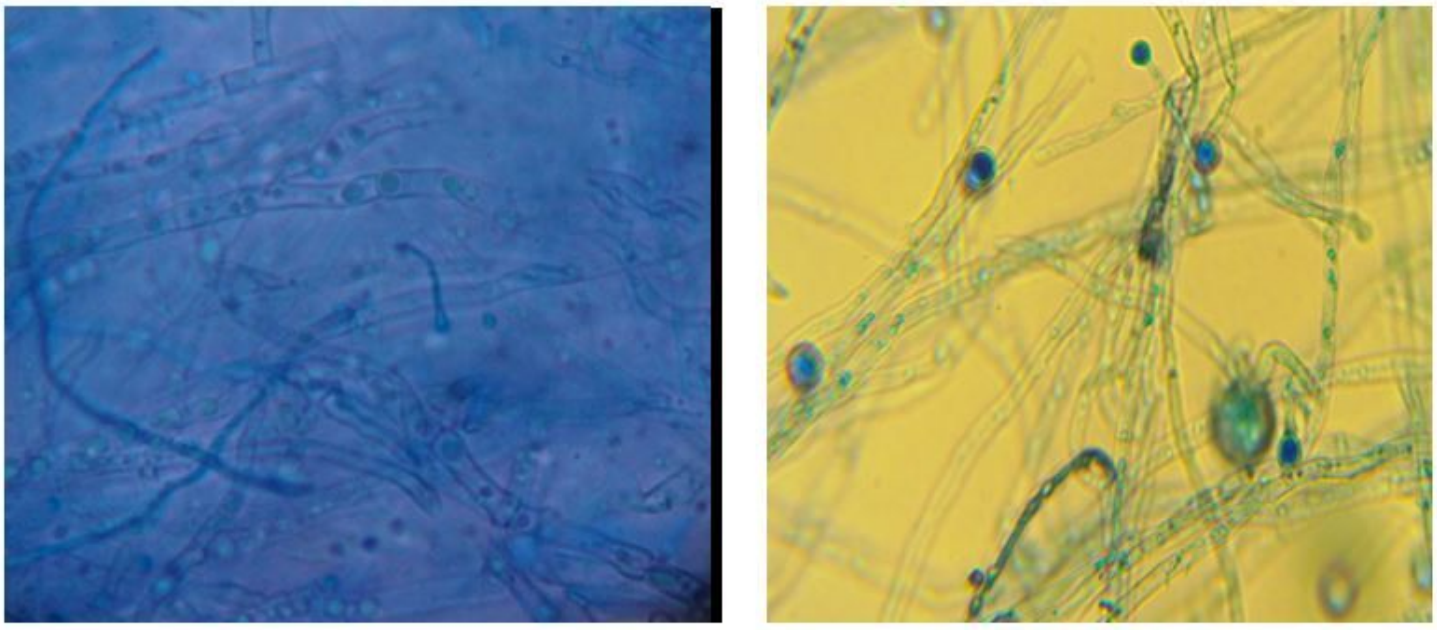

Figure 3

A- hyphae of *Monascus ruber* with septate, B- hyphae of fusant without septate

Figure 4

Extracellular and intracellular pigment yield of *Monascus ruber* and fusant