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Enhancement of Monacolin K Production by Intergeneric Hybridization between *Monascus purpureus* (Arg^-) and *Monascus ruber* (Thi^-, Met^-) Auxotrophs

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Abstract

This study aims to reconcile industrially suitable strains of higher red pigment producing *Monascus purpureus* MPA5 and higher Monacolin K producing *Monascus ruber* MRA_7, were tried to fuse for development of increased efficacy of targeted secondary metabolites production. The optimum concentration of protoplast mixture was standardized, before the fusion experiment. The effective fusion was observed with the solution of 30 % PEG 6000, $CaCl_2.2H_2O$ (0.01 M) and glycine (0.05 M). Colonies of the fusants were segregated using complete medium. The fusants were irradiated under UV- light in LD_{50} dose. On the basis of non-segregation of colony appearance after repeated subculturing in complete medium, 8 colonies were selected. Finally, one of the stable fusant MF_{11} grew more rapidly than one of the parental strain *M. purpureus* MPA_5, but not as fast as *M. ruber* MRA_7, were selected and grown on low grade rice as solid substrate for production of metabolites. Production of red pigments, alpha-amylase and acid protease were at intermediate stage between two parental strains but enhancement of Monacolin K was observed. High performance liquid chromatography (HPLC) analysis was revealed that Monacolin K content was increased as much as two and half times than the parents. No fluorescent yellow band was detected in TLC plate, indicating of citrinin negativity of the fusant MF_{11}. It is the first report of enhanced Monacolin K production by a stable fusant of *Monascus* sp.

Keywords: Protoplast Fusion, *Monascus purpureus*, *Monascus ruber,* Monacolin K

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INTRODUCTION

Protoplast fusion is a skilled technique which generates and induces genetic recombination and has been attempted in prokaryotic and eukaryotic cells including filamentous fungi.[1] Fusion can be done either between wild type or mutated strains, strains with incompatible heterokaryon, auxotrophic mutant and prototrophic strains.[2] Experimentally two protoplasts are tried to fuse to acquire parasexual hybrid protoplast, which contain heteroplasmic cytoplasm and fused nuclei of two parents.[3]

However, development of hybrid by protoplast fusion could be achieved either by closely related species or even distantly related.[4] The differentiation of the whole organism involves by the regeneration of protoplast into a normal cell.[5] Only a few reports exist on intergeneric fusion[6] but intra-specific and interspecific protoplast fusion are more common and includes *Mucor,*[7] *Rhizopus,*[5] *Aspergillus,*[8] *Penicillium*[9] and *Trichoderma*.[10] *Monascus* sp is a filamentous fungus which is important for production of pigments that are polyketide origin.[11] *Monascus* contain three major species (*M. purpureus, M. pilosus* and *M. ruber*) under the family Monascaceae within the class Ascomycetous.[12] The *Monascus* is massively used for coloring of rice, cheese, soybean and meat in different oriental countries like wise food colorant by using the red pigment.[13] Apart from this, in Asian countries the pigment gains a long-established food value though it is still forbidden in Europe and America. However, in recent years there are a number of patents registered in Japan, the United States, France and Germany reporting *Monascus* as food pigment. In spite of pigments, *Monascus* fermented red rice is also important for different metabolites like monacolin K and L (act as antihypercholesterolemic agents),γ-aminobutyric acid or GABA (act as hypotensive agent), citrinin (a mycotoxin) are found.[14]

Fermentative production of pigments and monacolins can be obtained using *Monascus* sp. in both solid state fermentation (SSF) and submerged fermentation (SmF).[11] However, solid state fermentation leads to higher yield than submerged.[15] An active methylated compound, Monacolin K was recovered from fermented broth of *M. ruber.* [16] Monacolin K (lovastatin, mevinolin and mevacor), a competitive inhibitor of HMG-CoA reductase, a very important enzyme for rate limiting action in biosynthesis of cholesterol, which is helpful in the catalysis of the reduction of 3-hydroxy-3-methylglutaryl coenzyme-A to mevalonate in biosynthesis of cholesterol.[17] Monacolin K was used as common medicine in the treatment of hypercholesterolemia.[18] FDA approved Monacolin K (MK) to use as hypocholesterolemic drug and this is the first fungal secondary metabolite to received FDA approval in August 1987.[19] In addition to its lipid-lowering activity, some studies have shown that MK prevents thrombosis,[20] reduces the incidence of atherosclerosis,[21] and stimulates bone formation,[22] induction of cancer cell death,[23] Parkinson's dsiseases[24] and Alzheimer's treatment of disease.[25] Therefore, RMR can be considered as a functional important compound and applied to the food industry. Though *Monascus* sp. is able to synthesize different useful secondary metabolites, but able to produce citrinin also, which is a mycotoxin having hepatotoxic and nephrotoxic effects in humans.[26] Therefore, citrinin becomes a very critical issue in consideration of red rice as a safe food supplement. Maximum permitted levels of citrinin in functional foods are approved at 0.2 mg/kg in Japan and 2 mg/kg in Taiwan.[27] However, some reports have been found where the citrinin concentrations in some commercially available red rice where were detected between 0.28 to 2,458.8 mg/kg.[12]

Traditionally *Monascus* was grown on rice to prepare red yeast rice, though different liquid media or solid media were applied to produce the secondary metabolites. The traditional method of fermentation is SSF but most of the industry used submerged or liquid fermentation for industrial fermentative processes.[15] Though it is possible in modification on cultural conditions in *Monascus* sp. positively have an effect on secondary metabolites constituents.[28] Variation of environmental factors may conclusively affect the production ability of the test organism, likewise in *Monascus* sp.

The experimental work emphasizes to standardize the protocol for the fusion technique to develop hyperactive hybrids, in terms of quality or quantity. In this study, attempts have been made to develop phenotypically stable fusant between

high pigment producing *M. purpureus* MPA_5 and higher Monacolin K producing strain of *M. ruber* MRA_7 and to select strain with dual traits.

MATERIALS AND METHODS

Organisms and Media

M. purpureus MTCC 410 and *M. ruber* MTCC 1880 were used as source organisms. MPA_5 and MRA_7 are the mutant strains developed from *M. purpureus* MTCC 410 and *M. ruber* MTCC 1880, respectively and used as parental strain for protoplast fusion. Cultures were grown and maintained in Malt Yeast (MY) medium (g^{-1}): glucose 10.0, yeast extract 3.0, malt extract 3.0 and peptone 5.0; pH 7.0, agar 2% used for solidification. Minimal medium (MM with50 µg ml^{-1} amino acids and 0.2% yeast extract was added, Czapek Dox (CD) medium was used to select fusants. For protoplast fusion and regeneration hypertonic (0.6 M KCl) Malt Yeast Agar (MYA) and Czapek Dox Agar (CDA) were used.

Mutagenesis of Parental Strains for Auxotroph Formation

Conidial suspension of *M. purpureus* MTCC 410 and *M. ruber* MTCC 1880 were prepared from 7-day-old culture in MYA and passed through filter paper to obtain 10 ml suspension of $1x10^8$ conidia ml^{-1}. This conidial suspension in watch glass was exposed to ultraviolet radiation in a laminar air flow for 5 to 30 minutes, with an increment of 5 minutes. To avoid photo reactivation after irradiation, the samples were kept at dark for 5 hours.[29] After dark treatment 0.1 ml of the irradiated suspension were plated in MYA and incubated at 30±2°C for 4 days. From the plate of 1% survival, colonies were selected visually and the isolates were transferred to plates containing MYA medium. Purity of the isolates verified by repeated streaking in MYA plate. Selected colonies were tested for their auxotrophic nature. Conidial suspension of the isolates was grown in Czapek Dox broth as minimal medium, incubated at 30±2°C for 4 days. Ungerminated spores (mutant) separated from developed germlings by glass wool filter. This process was repeated thrice to confirm that the remaining spores were not of the wild type. The selected isolates were repeatedly grown in Czapek Dox broth with amino acids(s) (50µg l^{-1}) supplementation and in combination to confirm their auxotrophic nature.

Protoplast Generation

For protoplast generation, 1 ml of spore suspension (~$2x10^8$ ml^{-1}) of the selected auxotrophs were inoculated into 100 ml MY broth medium and then incubated at 30±2°C for 48 h in rotary shaker (120 rpm). After incubation both the organisms were centrifuged in 10,000 rpm for 20 min and the suspended pellets were aseptically washed using suitable osmotic stabilizer (0.6 M KCl), at pH 6.0 after standardization.

Following the method of Klinsupa and co-researcher (2016), the germinated spores were suspended in hypertonic solution of KCl, NaCl, $MgSO_4$ and Sucrose (0.6 M) separately containing cell wall lytic enzymes of chitinase (1% - 3%) and cellulase (0.2% -0.5%), incubated at 30±2°C up to 5 h at 50 rpm. Samples from suspension were checked for protoplast formation at every 30 min intervals under microscope to determine the time required for the development of protoplasts. Then protoplast suspension was centrifuged (1000 rpm for 5 min) to remove enzymes and enumerated with a hemocytometer under a phase contrast microscope. The rate of protoplast formation (%) was estimated by the numbers of protoplast divided by the total cells.

Protoplast Fusion and Regeneration

Protoplast fusion was attempted with certain modification. Both the protoplasts were mixed (1:1) and maintained at 0.6 M KCl solution and centrifuged at 1000 rpm for 10 min. The pellets were suspended in the solution containing verify concentration of PEG with 0.01 M $CaCl_2.2H_2O$ and 0.05 M- glycine in 1:1:1 ratio, pH 6.0 and incubated at 30±2°C up to 15 min and observed under microscope. The fused protoplasts were centrifuged at 1000 rpm for 10 min. The pellet was washed with 0.6 M KCl and centrifuged twice. The pellet suspended in hypertonic Czapek Dox medium and incubated at 30±2°C for 24 h.

After incubation the culture (1 ml) was spread over minimal and complete medium, at hypertonic condition and covered with the same soft agar (0.5%) medium to make a sandwich

plate and incubated at 30±2°C for 7 days. The colonies developed in the minimal medium were selected the fusants that could grow without any requirement of amino acids. The protoplast formation rates were considered by the number of colonies which appeared on minimal medium as a percentage in number that grew on complete medium.

The isolated regenerants were grown onto MYA medium at 30±2° C for 7 days. The conidial mass (~1x10^8 conidia/ml) was exposed to UV for different time interval to find LD_{50}. For isolation of stable fusants, the conidial sample (~1x10^8 conidia/ml) was treated at LD_{50} dose and kept in dark for 5 hours. Then the sample was put on MYA medium plate and incubated at 30±2°C for 7 days. The appeared colonies were transferred to MYA slant and checked it for their stability.

Reversion of Protoplasts

Mycelial protoplasts of two isolates of *Monascus* sp. mutant (isolate MPA_5 and isolates MRA_7) were cultured in MY broth medium containing 1.2 M sorbitol and incubated at 30±2°C for 24 h. Different dilution at appropriate concentration were prepared, they were spreaded onto MY agar medium with 1.2 M sorbitol as supplementary. 0.5% agar was followed to cover and allowed to incubate at 30±2°C for 7 days. The following formula was used to calculate the reversion of protoplast onto mycelia and cell as percentage:

% protoplast reversion= Number of colonies grown on MY agar medium / Number of initial protoplasts'100

Measurement of Secondary Metabolites

Extracellular Red Pigment Production and Quantification

Parental cells, their auxotrophs and the isolated fusants were grown on MYA medium at 30±2°C for 7 days. Then spore solution was prepared (0.9% NaCl and 0.2% Tween 80).[30] Homogenous spore suspension (0.5 ml) was inoculated into 10 g of sterilized rice with 56% humidity and incubated at 30±2°C for 14 days. The rice was air dried in room temperature after fermentation and pigment was extracted by using 5 ml of ethanol in 95% concentration under shaking (110 rpm) for 60 min. The centrifuged of the extract was done at 10,000 rpm for 15 minutes, the supernatant was decanted and the extracellular red pigment concentration determined by spectrophotometer at 500 nm.[12]

Quantitative Estimation of Lovastatin (Monacolin K)

Slightly different method was applied for extraction and estimation of monacolin K from fermented solid materials, liquid chromatography procedure followed using ODS columns (150'4.6 nm ID) were used, and varied elution systems were attempted. Solid fermented products were dried and ground into powder. Powered sample (1 g) was mixed with 10 ml of orthophosphoric acid /water solution (75:25) and kept at 60°C under agitation for 10 minutes. Ethyl acetate (10 ml) was added to the extract solution, followed by 30 minutes treatment in a water bath at 60°C and centrifugation at 5000 rpm for 10 minutes. Vacuum concentrated supernatant (1 ml) supernatant was filtered through 0.45µM syringe membrane before infecting 20µL into column for HPLC. The mobile phase was used as a linear gradient of concentrated acetonitrile (ACN) (eluent A) and 0.1% TFA (eluent B) with the flow rate of 0.5 mL min^{-1}. The chromatogram was detected at 237 nm, and the temperature of column was set at 28°C.

Enzymatic Estimation

For detection of common hydrolyzing enzymes, amylase and acid protease estimation were carried out. After SSF, the fermented rice (10 gm) material was taken in a flask (100 ml), the enzymes extracted with 0.5% NaCl (100 ml) and filtered through Whatman number 1 filter paper. Then filtrate was analyzed for estimation of amylase,[31] and acid protease (Ichishima 1970) by starch and casein, respectively, as substrate. Amylase activity was assayed in terms of liberated reducing sugars (glucose equivalents) using dinitro-salicylic acid (DNSA). Acid protease was estimated in terms of released tyrosine equivalent using Folin-Ciocalteau Phenol reagent.

Qualitative Estimation of Citrinin

To detect citrinin, thin layer chromatography (TLC) was performed.[32] Fermented substrates was extracted with the presence of ethyl acetate and acidified to pH 5. Then the

aqueous layer was evaporated to dryness. The concentrated organic layer was loaded to TLC plate 60 F254 aluminum sheets (Merck, Germany). The mobile phase was used as ethyl acetate: acetone: water as 4:4:1. After completion of TLC run the plates were examined under UV light at 350 nm. A fluorescent pale yellow band was detected for presence of citrinin.

Biomass Estimation

After incubation the biomass of the fungal culture during growth was estimated by a gravimetric analysis method by using a digital balance. After filtration of the culture broth, the mycelia were separated from the broth on pre-weighed filter paper (Whatmann filter No. 1 filter paper). The culture was dried at 80°C and biomass was determine in grams per litre.

Statistical Analysis

Experiments carried out in triplicate and the graphical data were presented as mean ± standard error (SR) as required. The statistical analysis was performed using OriginPro 8.5 statistical software.

RESULTS AND DISCUSSION

Formation of Auxotrophic Mutants from *Monascus purpureus* and *Monascus ruber*

Spores of *M. purpureus* and *M. ruber*, that were UV irradiated for different time periods were plated in MYA medium and incubated for colony development. Primary selection of colonies was done from plates that showed 1% survival rate of the irradiated spores. UV exposure of 25 min and 30 min provided this survival rate for *M. purpureus* and *M. ruber*, respectively (Figure 1). Finally $1x10^5$ colonies were selected. Then the selected isolates were sub-cultured three times in the same medium and checked for stability of their physiological and morphological characters. To facilitate the process of selection during protoplast fusion, auxotrophic strains are preferred. To determine the auxotrophic nature of the isolates were allowed to grow in both minimal and complete media and three auxotrophic colonies of each parental strain were considered on the basis of their linear growth (mm). For identifying the amino acid requirements, auxotrophs were grown in Czapek Dox medium with supplementation of amino acid (s) either singly or in combination. On the basis of growth rate that measured as mycelial mass (g) on MY medium (100 ml) two strains were selected. The isolates MPA_5 (arg^-), mutant of *M. purpureus* and the isolate MRA_7 (thi^-, met^-), mutant of *M. ruber* were selected for further study (Table 1). Various treatments were found in literature for auxotrophic mutant formation like a combination of UV-light and sodium nitrate was used to induce mutation of *Trichoderma* sp.[33] Nitrosoguanidine ((N-methyl-N'-nitro-N-nitrosoguanidine) and UV-light separately used as mutagenic agent for auxotroph formation of *P. griseoroseum* and *P. expansum* respectively.[34]

Protoplast Formation from Mycelia of MPA_5 and MRA_7

For protoplast formation, 48 hr-old mycelia of MPA_5 and MRA_7 were incubated with enzymes of chitinase and cellulase (Sigma, USA) in different concentrations and combinations along with osmotic stabilizer (Figure 2). To find out a suitable osmotic stabilizer, the mycelia of MPA_5 and MRA_7 were treated with 1% chitinase + 0.5% cellulase enzymes in presence of various osmotic stabilizers in 0.6 M concentration and KClwas found most effective (Figure 3 A & B). Thereafter, the enzyme concentrations were varied to identify the optimum condition for protoplast formation. In case of MPA_5, 1% chitinase (10 mg/ml) and 0.5% cellulase (5 mg/ml) found to produce maximum number of protoplasts ($5.1x10^5$/ml) in 4 hrs (Figure 4A) wherein MRA_7 produced maximum number ($7.2x10^5$/ml), using 1.2% chitinase and 0.3% cellulase (Figure 4B). When the protoplast were incubated in minimal medium with 0.6 M KCl (regeneration media) the mycelia were found regenerating. The regeneration frequency of MPA_5 and MRA_7 were found 35.6% and 8.2%, respectively. KCl (0.6 M) was also found most effective osmotic stabilizer for protoplast formation in *Aspergillus awamori* and *A. oryzae*[35]and in *Monascus anka.*[36] Protoplast formation frequencies of *M. anka* MKA1 were optimized using 3% Usukizyme[8] and using 0.3% snailase + 0.3% cellulase[37]. Protoplast formation was induced with 2% Usukizyme+ 0.4% Novozyme 234 in *A.oryzae* AOK1[8] and additionally

0.5% lywallzyme in *A. terreus* CA99.[37] So from the previous report it showed that our result is in accordance with that reports.

Inter-specific Protoplast Fusion between MPA_5 and MRA_7

To study the frequency of formation of prototrophic colonies, the efficacy of fusogen (PEG 4000 and 6000) were tested. Figure 5 showing the representative photograph of protoplast fusion between isolate MPA_5 and MRA_7. After protoplast fusion, it found (Figure 6) that the prototrophic colonies appeared in all plates in the range of tested PEG concentration but PEG 6000 at 30% induced maximally ($1.24x10^3$ CFU/ml). Thus, the colonies developed in hypertonic minimal medium appeared as fusants between MPA_5 and MRA_7, (Figure 7 A, B) while no colonies appeared in non hypertonic medium. Also the fusants could found divided into white and red portions while they were allowed to grow in complete medium for 10 days (Figure 8A). Stability of the fusants were tested by irradiated with UV exposure in different time intervals and kept for dark treatment. After plating the UV exposed plates, LD_{50} dose was

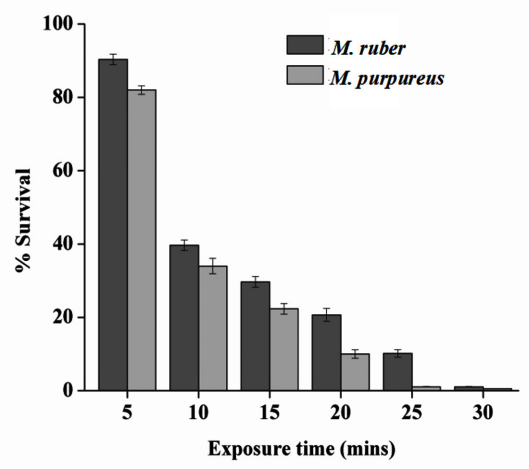

Figure 1. Determination of % survival of spores of *M. purpureus* and *M. ruber* during different time exposure (mins) of UV light. Values represent the mean±SR

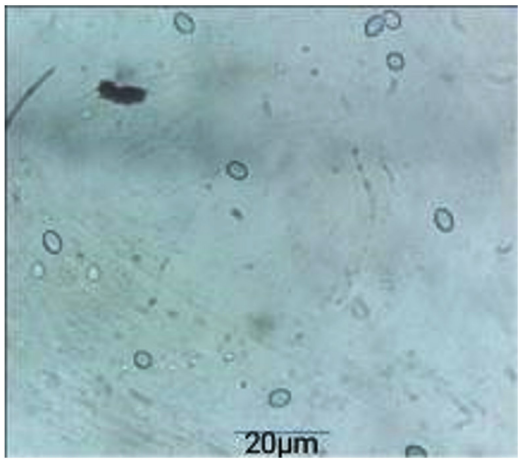

Figure 2. Microscopic photograph of protoplast formation mixture of MPA_5 and MRA_7

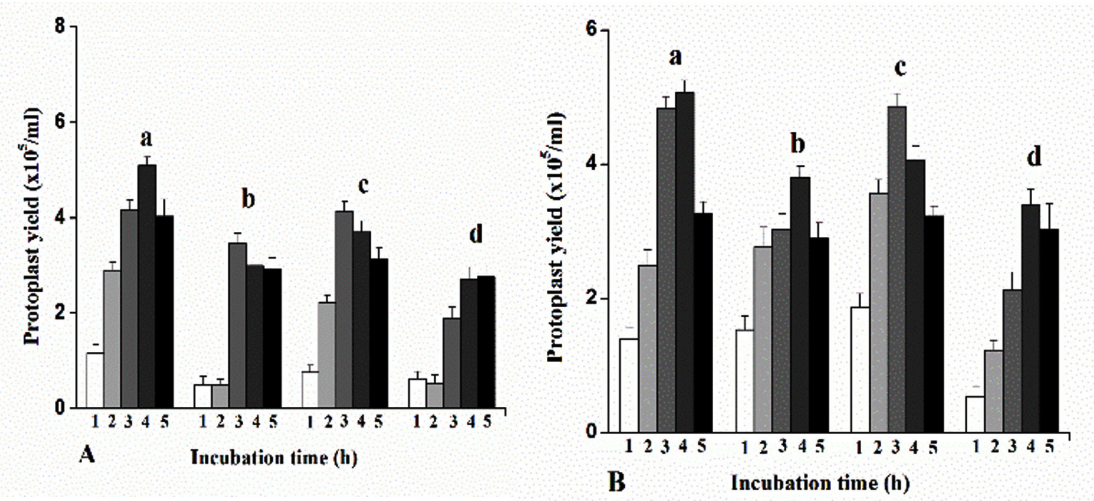

Figure 3. Effect of 0.6 M KCl (a), NaCl (b), $MgSO_4$(c) and Sucrose (d) during protoplast formation of MPA_5 (A) andMRA_7 (B) at different time interval using 1% chitinase + 0.5% cellulase enzyme treatment. Values represent the mean±SR

determined and found that $6x10^2$ colonies were grown in complete media. From them eight colonies were further selected because the hyphae were not segregated even after sub cultured several times in the complete media (Figure 8B). Finally, the stable fusant MF_{11} grew more rapidly than one of the parental strain *M. purpureus* MPA_5, but not as fast as *M. ruber* MRA_7. PEG-induced protoplasts fusion of fungus was influenced by the presence of cation and different concentration of them. PEG with 0.01 M $CaCl_2$ and 0.05 M glycine increased the number of fused colonies in *M.*

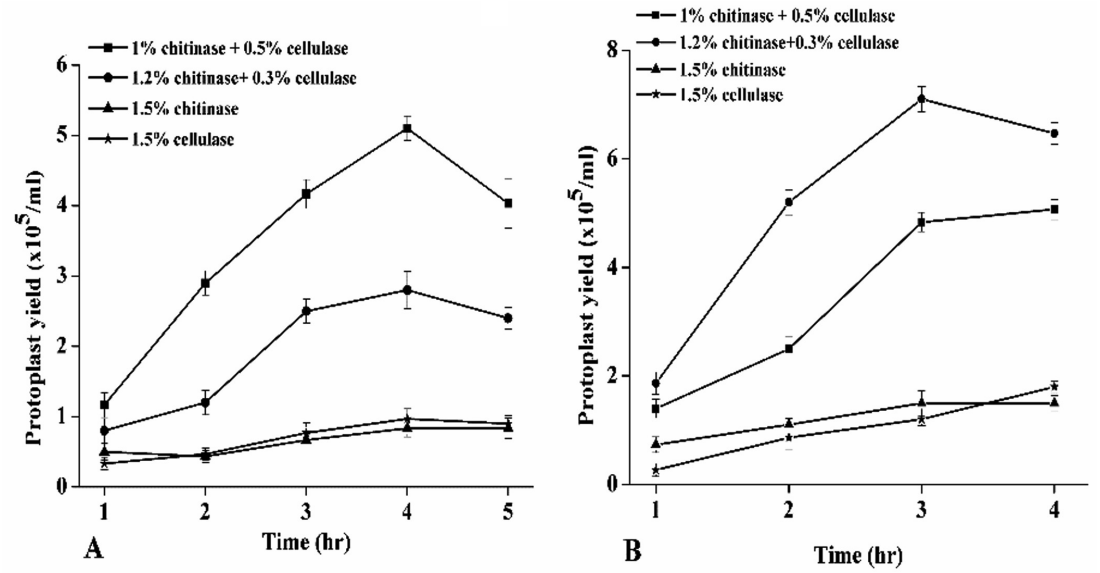

Figure 4. Effect of various concentration of chitinase and cellulase on the protoplast formation of MPA_5 (A) and MRA_7 (B) at different time interval using 0.6 M KCl osmotic stabilizer. Values represent the mean±SR

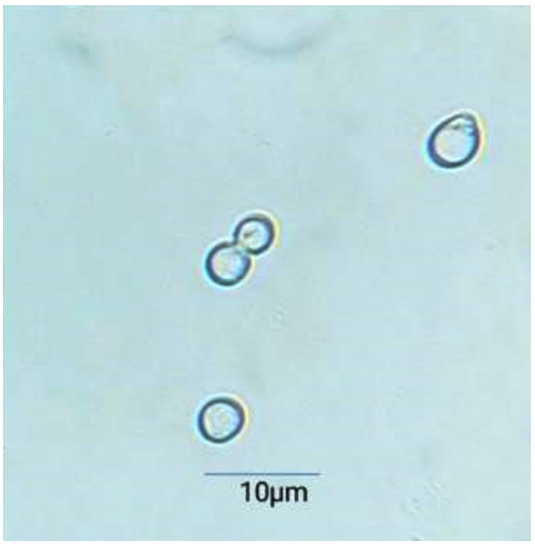

Figure 5. Microscopic photograph showing the protoplast fusion between MPA_5 and MRA_7

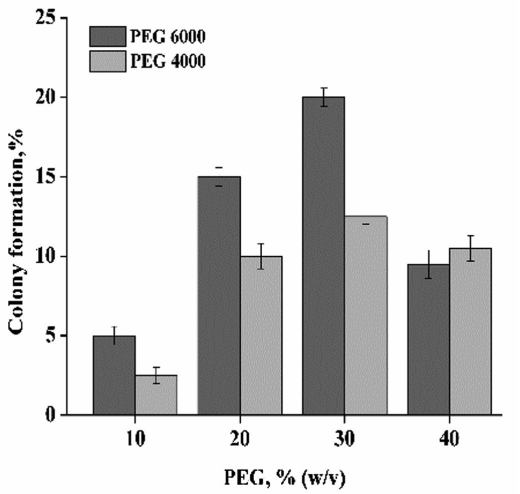

Figure 6. Effects of PEG concentration on colony formation by counting regenerating colonies after protoplast fusion between MPA_5 and MRA_7.Values represent the mean±SR

anka[36]. The fusion frequency of *M. purpureus* MPA_5 and *M. ruber* MRA_7 was ascertained as 0.3% (Table. 2). The protoplast fusion frequencies were observed as *Penicillium rouqefortii* and *Penicillium chrysogenum* as 0.1% to 0.7% (Leza et al., 2011), *M. anka* and *A. oryzae* as 0.14% [8]. In this present study, the protoplast fusion frequency is not less significant than reported once.

Extracellular Red Pigment Production by Fusants

All the fusants were grown in sterilized rice at 30±2°C up to 14 days. After fermentation, the pigment was extracted using ethanol as a solvent (after standardization) with agitation and centrifuged. The supernatant was tested for pigment production. The auxotroph MRA_7 reduces the production of pigments than its parental strain. The Figure 9 showed that all the fusants produced the red pigments but much less than the parents except the two fusantMF_8 and MF_{11}. The pigment production of fusant MF_{11} was observed since 72 h of fermentation and reached to its optimum on 11th day (35.5 U/gdfs) whereas the parental strain *M. purpureus* and its auxotroph MPA_5 produced much higher quantity of pigments were measured as 58.1 U/gdfs and 75.2 U/gdfs respectively. Many reports are available in the literature for the pigment production capabilities of *M. purpureus* like extracellular red pigment production of *M. purpureus* maximum yield was recorded in production media supplemented with 20g/L glucose and 0.3% monosodium glutamate (MSG).[38] The medium with fructose as a carbon source and low concentration of sodium nitrate promoted mycelium growth and production of red pigment in *M. purpureus*.[39] In solid state fermentation jackfruit seed was found as novel substrate for pigment production and adding of nitrogenous compound as an external source proved a betterment of pigment production of *M. purpureus* which is water soluble in nature.[40]

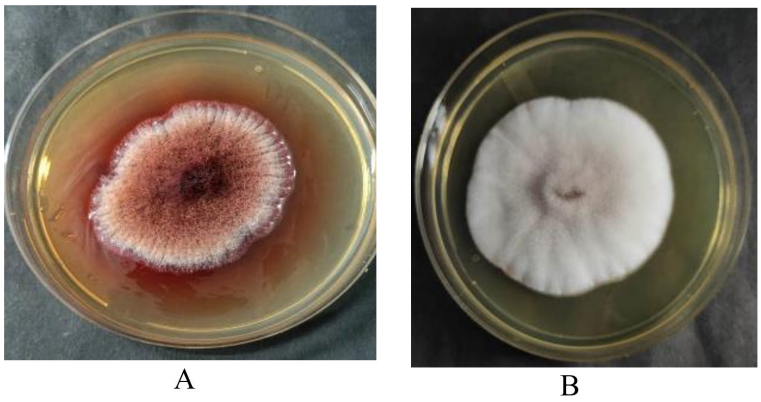

Figure 7. Colony morphology of isolate MPA_5 (A) and isolate MRA_7 (B) on malt yeast agar

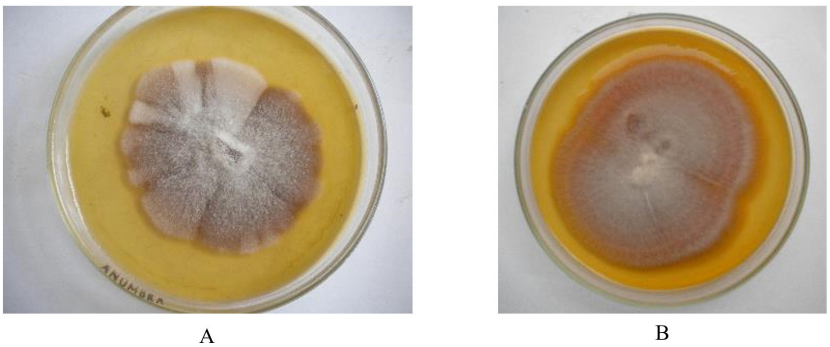

Figure 8. Growth pattern of segregated fusant (A) and non-segregated stable fusant (MF_{11}) (B) after UV exposure on malt yeast agar

Monacolin K Production by Fusants

Monacolin K content was measured from the solvent extracted supernatant of fermented rice and presented in Figure 10. All the fusants are capable to produce Monacolin K in SSF but the fusant MF_{11} produced maximally (2.22 mg/g) and found no less than 250% increased from the parents. Several reports were found on lovastatin production of *M. purpureus* and *M. ruber*.[17,41,42] In most cases the lovastatin yield is very less generally less than 20 mg/L. The production by the fusant may further increased after optimization.

Amylase and Acid Protease Activity of Fusants

The isolates, fusant (MF_{11}) and auxotrophs (MPA_5, MRA_7) were used for production of amylase and acid protease. From the Figure 11, it is found that production of alpha-amylase and acid protease of the fusant MF_{11} was 3015.5 U/g and 2200.5 U/g, respectively, much higher than the MPA_5 but less than the MRA_7. It could be correlated with the growth which was supported by the synthesis of hydrolyzing enzymes. It could be assumed that larger amount of enzymes production would probably assist them to grow fast. Generally,

Table 1. Mycelial mass and secondary metabolites estimation of *M. purpureus, M. ruber* and their stable selected mutants after incubation in MY medium at 30 ±2°C for 7 days

Strains	Auxotrophs	Red pigment assay (U/gdfs)	Mycelial mass (g/100 ml)
M. purpureus		58.12	9.0 ± 0.4
MPA_4	Thi^-, Arg^-	70.23	5.2 ± 0.3
MPA_5	Arg^-	75.15	8.5 ± 0.4
MPA_8	Leu^-	72.22	6.5 ± 0.2
Strains	Auxotrophs	Monacolin K assay (mg/g)	Mycelial mass (g/100 ml)
M.ruber		0.34	9.5 ± 0.2
MRA_3	Thi^-, Leu^-	0.71	8.5 ± 0.2
MRA_7	Thi^-, Met^-	0.84	10.5 ± 0.2
MRA_9	Lys^-	0.63	7.5 ± 0.2

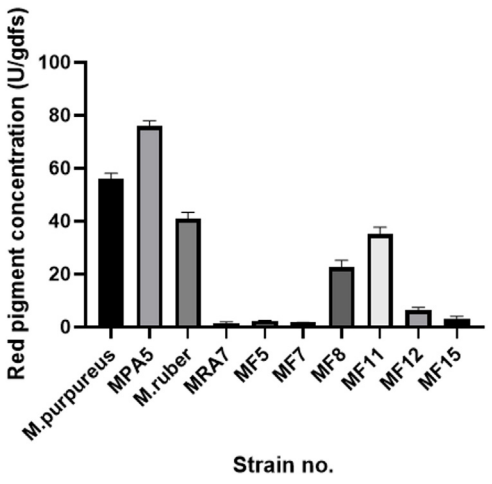

Figure 9. Red pigment producing capacity of *M. purpureus*, its auxotroph MPA_5 and all the fusants. Values represent the mean±SR, gdfs=gram per dry fermented substrate

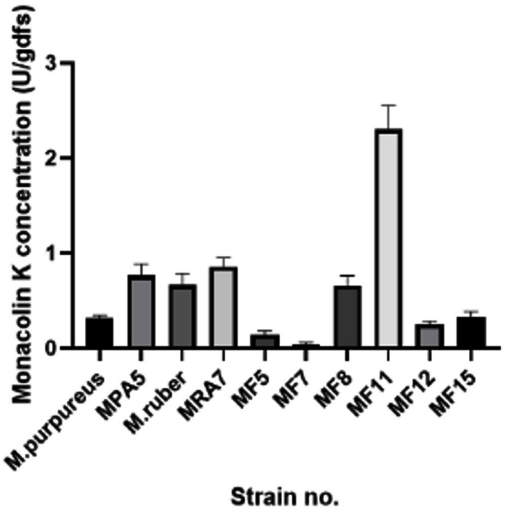

Figure 10. Monacolin K production rate of *M.purpureus*, *M.ruber*, their auxotrophs MPA_5, MRA_7 and all the fusants. Values represent the mean±SR.

Table 2. Protoplast fusion frequency, reversion and fusion formation estimation between MPA_5 and MRA_7

Strain Used	Strain Used	Reversion frequency onto medium (%)	No. of fusant (CFU/ml)
MPA_5	2.3 x 10^5	10.2	1.24 x10^3
MRA_7	5.5 x 10^5	12.6	

[a] Numbers of mycelial colonies on regeneration medium before protoplast fusion treatment

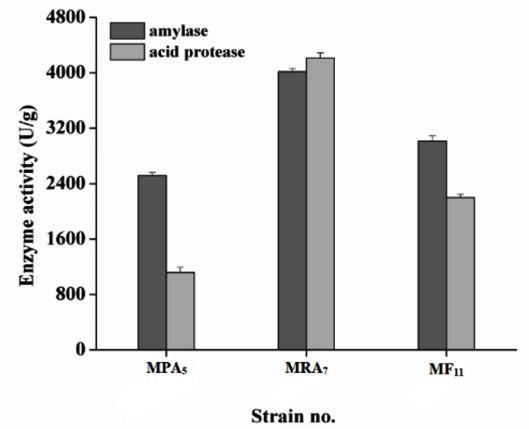

Figure 11. Enzyme profile of MPA_5, MRA_7 and their fusant MF_{11} during incubation in broken rice at 30 ± 2^0C, (a) amylase, (b) acid protease. Values represent the mean±SR

interspecific and intraspecific fusants obtained from mold showed higher enzyme productivity than their parental strains.[43,44] Though the enzyme production by the fusant MF_{11} much less than the parent MRA_7 but without optimization.

Citrinin Estimation

In TLC chromatogram no citrinin was detected in solid state culture (data not shown). Citrinin is a nephrotoxic and hepatotoxic agent, produced by *Monascus* sp. is related to serious health hazards.[45] But it is produced in very trace amount (nearer 0.1-500 mg kg^{-1}), whereas the content is allowed to consume below 200 mg/ml. TLC method was commonly used to separate citrinin from the *Monascus* sp.[32] During the time of rice fermentation, some risk of citrinin contamination may be defined in the fermentation process. Though this situation could be avoided either by detoxification of the mold or using any citrinin non-producer strain or by optimization of the fermentation process for reducing the amount of citrinin production. In this experiment, in case of fusant no citrinin was detected in comparison to the parents in the TLC plate, perhaps the citrinin pro was production was below of detection level by TLC.

This study suggested a protocol for protoplast fusion between the auxotrophs developed from *M. purpureus* and *M. ruber*. The fusants (MF_{11}) is found as stable in nature. Thus, the result show promise for its biotechnological application. The fusants (regenerants) could grow and produce successfully the pigments and Monacolin K in SSF using rice. But the fusant MF_{11} produces Monacolin K at significantly high amount than the parents but there is scope for further enhancement through production optimization. Apart from *Monascus*, some filamentous fungi like *Penicillum*, *Hypomyces*, *Doratomyces*, *Gymnoascus*, *Trichoderma*etc were found to produce Monacolin K but only *A. terreus* was used for large scale production.[46] MonacolinK containing *A. terreus* broth is not directly consumable because this organism is non edible and therefore its purification costs much**.** Wherever, *Monascus* species are nonpathogenic and used to make rice wine, soybean cheese, red rice etc in oriental countries. So enhanced rate of lovastatin from non toxic stain is scant. The production of hydrolyzing enzymes could be further correlated with the growth support. Till date there is no previous report of hybridization between *M. purpureus* and *M. ruber* by protoplast fusion.

CONCLUSION

Extensive efforts are being invested by researchers in harnessing new species of filamentous fungi for the manufacturing of economically important secondary metabolites. Monacolin K is a popular drug that effectively lowers blood cholesterol in human by blocking the enzyme that controls the rate of cholesterol synthesis and *Monascus* sp have the potential to synthesized monacolin K effectively. The protoplast fusion is one of the most acceptable

techniques for strain improvement. The aim of this study is to construct a stable fusant between two mutants of *M. purpureus* (Arg^-) and *M. ruber* (Thi^- , Met^-) auxotrophs. Though *M. purpureus* (Arg^-) mutant is a higher red pigment producer but low in Monacolin K production, whereas *M. ruber* (Thi^- , Met^-) mutant is a high Monacolin K producer. From this investigation, we have developed a stable fusant (MF_{11}) with enhanced Monacolin K producing capability, higher than both the parental mutants of *Monascus* sp. Shuffling of whole genome through protoplast generation and their fusion in various mutants of *Monascus* sp can be employed in industrial strain enhancement strategies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AS conceptualized the study, and performed experiments and formal analysis. KS supervised the study. AS and KS wrote, reviewed and edited the manuscript. Both authors approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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