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# Interspecific protoplast fusion of *Aspergillus terreus* and *Aspergillus flavus* for the enhancement of lovastatin production

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## ABSTRACT

A standard method for isolation, fusion and regeneration of protoplasts from Aspergillus flavus and Aspergillus terreus was developed. The protoplasts from A. flavus and A. terreus were isolated using Novozym 234 and cellulasse as lytic enzyme and potassium chloride as osmotic stabilizer. The interspecific fusion frequency was determined using 40% polyethylene glycol as fusogen. The regenerated fusants morphology, mycelia protein pattern, RAPD analysis and lovastatin analysis were compared with parental strains. Upon 7 days of fermentation, the stable fusant, produced lovastatin 0.92 mg/g which is higher than their parent wild type strains. 

## KEYWORDS

Lovastatin;
Protoplast fusion;
Regeneration;
Solid state fermentation.

## INTRODUCTION

Protoplasts, the cell devoid of cell wall with the cell components limited by the plasma membrane, is a wonderful versatile living system to understand the secrets of many biological systems of living organism and to manipulate and alter the genetic composition of an entire organism. Protoplasts are, by definition, the components of a live cell after experimental removal of the cell wall (Hawksworth *et al*., 1995). Protoplasts is an attractive model organism for studies of physiological and cytological processes related to cell wall formation, cell growth and differentiation and is used in basic research for enzyme synthesis and steroid transformation and strain improvement (Kelkar *et al*., 1990; Krautwig and Lorz, 1995). Protoplast represent an appropriate source for intact DNA protoplast fusion, electrophoretic karyotype or transformation (Gold *et al*., 1983) and also, for mutagenesis towards strain improvement (Kelkar *et al*., 1990; Azevedo, 1998; Varavella *et al*., 2004). Fungal protoplasts are an important tool in physiological and genetic research (Peberdy, 1989; Hamari *et al*., 1997, Prabhavathy *et al*., 2006) and genetic manipulation can successfully be achieved through fusion of protoplast in filamentous fungi that lack the capacity of sexual reproduction (Lalithakumari, 2000). The protoplast can be obtained in filamentous fungi from the mycelium, germinated conidia or intact conidia. For every fungus, enzymatic preparation, osmotic stabilizer and their concentrations must be assayed in order to establish ideal conditions for protoplast preservation, without internal material loss (Azevedo, 1998).

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## MATERIALS AND METHODS

### Preparation of spore suspension

To well sporulated PDA slants of *A. flavus* and *A. terreus*, 10 ml of spore suspension medium (0.9% NaCl, 0.1% Tween 80) was added. The surface was scrapped with a loop and suspension was collected. The suspension was agitated thoroughly using cyclomixer to suspend the spore and filtered in a glass wool. The concentration of the spore suspension was measured using a haemocytometer and adjusted to $1 \times 10^8$ spores/ml which was used as inoculum throughout the study.

### Preparation of mycelium

Conidia were collected from 5 days old cultures of *A. terreus* and *A. flavus* separately. The inoculum of *A. terreus* and *A. flavus* were prepared by adding 5 ml of sterile distilled water to 5 days old culture under aseptic conditions and centrifuged at 5000 rpm for 10 min. The conidial suspension of $1 \times 10^6$ conidia/ml was transferred aseptically into 100 ml potato dextrose broth. The flasks were incubated for 12-24 h at 27±2ºC on a rotary shaker at 150 rpm. The young germlings was harvested by filtration using millipore filter (0.45µm) and aseptically washed once with sterile distilled water followed by two washes with sterile osmotic stabilizer (KCl). This mycelia preparation of *A. terreus* and *A. flavus* was used for protoplast isolation.

### Isolation of protoplasts

Protoplasts from the mycelia preparation of *A. terreus* and *A. flavus* were isolated using mixtures of cell wall lytic enzymes. Novozyme (Sigma) and cellulase enzyme (crude prepared in our laboratory) were used for cell lysis. The novozyme was used individually and in combinations with cellulase to lyse the cell wall. Osmotic stabilizers are essential to provide osmotic support to the protoplast following removal of cell wall. In the present study KCl was used as the osmotic stabilizer. The mycelium (100 mg) was pretreated with the reducing agent β-mercaptoethanol in sodium phosphate buffer, pH 7.0 (0.5% v/v) for 2 min. Then transferred to a flask (25 ml) containing 5 ml of osmotic stabilizers, novozyme and cellulase. After incubation for 3h the contents were examined for presence of protoplast. The protoplast suspension was filtered through a millipore filter (0.45µm), diluted and then stained with crystal violet. The stained protoplasts were counted in a haemocytometer under a light microscope[1].

### Optimization of conditions for protoplast release

### Effect of lytic enzymes

The lytic enzymes Novozyme 234 (Sigma) and crude preparation of cellulase enzyme was from our laboratory from *T. harzianum*. Various concentration of Novozyme (2, 4, 6 mg/ml) was tested for analyzing the cell lysing efficiency. Cellulase enzyme (0.2 ml/ml) was also tested along with novozyme to improve the protoplast release.

### Effect of age of mycelium

Mycelium preparations were done from the two test fungal cultures of different age. 12 h, 24 h, and 48 h old fungal cultures were used for determining the optimum age for maximum protoplast release.

### Effect of osmotic stabilizers

Potassium chloride, the osmotic stabilizer, was used at different concentrations (0.4, 0.6, 0.8M) to determine the optimum concentration for maximum protoplast release.

### Effect of pH

pH of the mycelial preparations was varied from 4 to 6 to elucidate the optimum pH for protoplast release.

### Effect of incubation period

The effect of incubation period on protoplast release was tested by varying the incubation time from 2 to 4 (with 1h interval).

### Protoplast fusion

Protoplast fusion was performed according to the method of Anne and Pedeby[2]. After washing in 0.6M KCl, protoplasts obtained were centrifuged for 10 min at 700 rpm at 4ºC. The pelleted protoplasts from both fungi *A. terreus* and *A. flavus* were suspended in 1 ml of PEG 4000-6000(30%) w/v with 10 mM $CaCl_2$ and 50 mM glycine buffer (pH 5.8). Incubated at 36°C for 10 min and then transferred immediately to –20° C for 10 min. After incubation, the suspension was diluted with 5 ml of minimal media containing 0.6M KCl and finally resuspended in 5 ml of 0.6M KCl and plated on solid regeneration medium. Varied

concentration of PEG (30, 40, 50, and 60 %) was tested to determine the optimum condition for protoplast fusion. Optimum pH for increased fusion frequency was elucidated by varying the pH from 6 to 8 with 0.5 intervals.

### Protoplast regeneration

The protoplasts were assessed for their ability to regenerate into actively growing fungal colonies on agar medium. The protoplast suspension was diluted and 1 $\times 10^3$ protoplasts were plated in minimal potato dextrose agar (2%) containing 0.6M KCl stabilizer and were incubated at room temperature for 4 days.

### Fusant identification

The formation of fusant was confirmed by colony morphology, spore size and shape, mycelial protein pattern[3] and Random amplified polymorphic DNA (RAPD) analysis[12]. The color and growth pattern of parent and fusant strains were visually observed. Structure and shape of the conidiophores of the parents and fusant were observed microscopically. The production of lovastatin by the parent and fusant strains were compared.

### Mycelial protein pattern analysis

The mycelium from four day old fungal cultures of parents and fusent were filtered through Whatman No.1 filter paper under sterilized conditions and squeezed through sterilized filter paper[4]. The fungal mycelium was ground with 2 ml of Laemmli extraction buffer. The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was boiled for 15 min at 60°C and used as mycelial protein extract for evaluation of intragenetic variation of the electrophoretic karyotypes of parents and progeny on 10% SDS PAGE.

### RAPD analysis

Short oligonucleotides of random sequence (generally 10 bp length) will chance to complementary to numerous sequences within the genome. If two complementary sequence are present on opposite strands of a genomic region in the correct orientation and within a close enough distance with each other, the DNA between them can be amplified by PCR using decamers. Such amplified products are resolved by gel electrophoresis. RAPD polymorphisms may result from interspecific protoplast fusion (TABLE 1).

**TABLE 1 : RAPD-PCR reaction mix**

| S.No | Chemical/ enzymes | µL |
|---|---|---|
| 1 | DNA (10-25 ng) | 2.5 |
| 2 | 10 X PCR buffer (15 mM $MgCl_2$) | 2.5 |
| 3 | Oligonucleotide primer (2µm) | 2.5 |
| 4 | dNTPs (1 mM) | 2.5 |
| 5 | Tag DNA Polymerase | 0.5 |
| 6 | Sterile water | 14.8 |
| | Total | 25.3 |

The reaction mixture was overlaid with mineral oil and PCR was performed using short cycling protocol (Yu and Pauls, 1992). 1 cycle of 1 min at 97°C, 37 sec at 37°C and 1 min at 72°C, 35 cycles of 5 sec at 97°C, 15 sec at 37°C and 1 min at 72°C and the final extension at 72°C for 2 min.

### RAPD assay and PCR amplification

Amplification for RAPD analysis was carried out in a PTC—200TM thermal controller. The oligonucleotide primers CAG CGA GGC T, AGG GGT CTT G, CAG CAC CCA G and AGT CAG CCA G were used to produce RAPDs. The amplification products were analyzed on 1.5% agarose gel and viewed under UV after (ETBR) staining.

### Estimation of lovastatin production efficiency of parent and fusant

### Solid state fermentation

Solid substrates was dried in hot air oven at 60°C, accurately weighed to 8 g in Petri dishes (100mm×17mm), appropriately moistened with distilled water and autoclaved at 121°C for 20 min. After cooling, medium was inoculated with 0.8 ml (10% v/w) of spore suspension. Medium was thoroughly mixed and incubated at 30°C, in a humidity-controlled incubator for 7 days. After incubation the Petri dishes were harvested and analyzed for lovastatin content.

### Lovastatin extraction in solid state fermentation

Fermented material was dried at 40°C for 24 h, powdered and 2 g of the powdered material was extracted by 100 ml of methanol: water mixture (1: 1 v/v) at pH 7.7 in 250 ml Erlenmeyer's flasks keeping the flask at 30°C in rotary shaker at 200 rpm for 2 h. After 2 h, mixture was centrifuged at 10,000 rpm for

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10 min and supernatant was filtered through 0.45 μm membrane filter.

### Lovastatin estimation

Lovastatin in the clear extract was estimated by high performance liquid chromatography (HPLC) system (Agilent 1100 series) using C-18 column (250 mm length X 4.6 mm ID). A mixture of 0.02 M phosphate buffer (pH 7.7) and acetonitrile in ratio of 65:35 (v/v) was used as mobile phase. Flow rate was maintained at 1.0 ml/min and lovastatin was detected at 238 nm. The lovastatin peak was detected at a retention time of 9.2 min (Valera *et al*., 2005).

## RESULTS AND DISCUSSION

### Effect of lytic enzymes

In the present study protoplast was prepared from *A. flavus* and *A. terreus* by enzymatic digestion of the entire or part of the cell wall. Commercially available enzyme, Novozyme 234 (Sigma, USA) and the crude preparations of cellulase from *T. harzianum* prepared in our laboratory were used for cell lysis. The Novozyme was used individually and in combinations with cellulase (TABLE 2). The maximum protoplast yield of *A. flavus* and *A. terreus* were $24.19 \times 10^6$ and $26.21 \times 10^6$ when Novozyme 234 and cellulose were used in combinations (TABLE 2). In the present study for the successful release of viable protoplast from fungal mycelium, single and combinations of lytic enzymes used. Among the trials combination the mixture of lytic enzymes, Novozyme 234 (4 mg/ml) and cellulase (0.2 ml/ml) was found to be effective in the release of the viable protoplast.

**TABLE 2 : Effects of lytic enzymes on protoplast release from *A. terreus* and *A. flavus***

| Sr. No. | Novozyme (mg/ml) | Cellulase (ml/ml) | Protoplast released in *A. terreus* | Protoplast released in *A. flavus* |
|---|---|---|---|---|
| 1 | 2 | - | $16.3 \times 10^6$ | $15.3 \times 10^6$ |
| 2 | 4 | - | $18.4 \times 10^6$ | $17.5 \times 10^6$ |
| 3 | 6 | - | $20.2 \times 10^6$ | $19.8 \times 10^6$ |
| 4 | 2 | 0.2 | $22.3 \times 10^6$ | $20.2 \times 10^6$ |
| 5 | 4 | 0.2 | $26.21 \times 10^6$ | $24.19 \times 10^6$ |
| 6 | 6 | 0.2 | $24.2 \times 10^6$ | $23.4 \times 10^6$ |

### Effect of age of mycelium

The age of the mycelium is an important factor in protoplast release. Generally fungal protoplasts are prepared from young mycelium, which is more sensitive to the lytic action of the digestive enzymes than old mycelium[5]. In the present study, the yield was high in 24 h mycelium, the yield was about $26.2 \times 10^6$ and $24.1 \times 10^6$ in *A. terreus* and *A. flavus* (TABLE 3).

**TABLE 3 : Effect of age of mycelium on protoplast release from *A. terreus* and *A. flavus***

| Sr. No. | Age of mycelium (h) | Protoplast released in *A. terreus* | Protoplast released in *A. flavus* |
|---|---|---|---|
| 1 | 12 | $20.7 \times 10^6$ | $19.3 \times 10^6$ |
| 2 | 24 | $26.2 \times 10^6$ | $24.1 \times 10^6$ |
| 3 | 48 | $19.8 \times 10^6$ | $18.8 \times 10^6$ |

### Effect of osmotic stabilizers

Osmotic stabilizers play an important role in the release and maintenance of the integrability of the protoplast[6]. Osmotic stabilizers such as inorganic salts, sugars and sugar alcohols have been used to stabilize protoplast released from mycelium. The type and concentration of stabilizers will influence both yield and stability of protoplast.

**TABLE 4 : Effects of potassium chloride on protoplast release from *A. terreus* and *A. flavus***

| Sr. No. | Conc of KCl (M) | Protoplast released in *A. terreus* | Protoplast released in *A. flavus* |
|---|---|---|---|
| 1 | 0.4 | $21.7 \times 10^6$ | $18.3 \times 10^6$ |
| 2 | 0.6 | $28.1 \times 10^6$ | $25.4 \times 10^6$ |
| 3 | 0.8 | $25.6 \times 10^6$ | $19.5 \times 10^6$ |

The molarity of the osmotic stabilizers plays a vital role in the stability and regeneration frequency of the protoplast. In general, inorganic salts are more effective with filamentous fungi[7]. In the present study, osmotic stabilizer KCl was tried at different molar concentration. 0.6 M KCl served as the best osmotic stabilizer for release of protoplast (TABLE 4).

### Effect of pH

The effect of pH on the release of protoplast from *A. flavus* and *A. terreus* was checked by changing pH from 4 to 8 (TABLE 2.6). The result revealed that acidic pH yielded fairly good amounts of protoplast than neu-

tral and alkaline pH. The pH 5.0 was observed to be favorable for protoplast release. At pH 5, $26.4 \times 10^6$ and $24.5 \times 10^6$ protoplasts were released from *A. terreus* were obtained from *A. flavus* (TABLE 5).

**TABLE 5 : Effect of pH on protoplast release from *A. terreus* and *A. flavus***

| S.No. | pH | Protoplast released in *A. terreus* | Protoplast released in *A. flavus* |
|---|---|---|---|
| 1 | 4 | $20.7 \times 10^6$ | $19.3 \times 10^6$ |
| 2 | 5 | $26.4 \times 10^6$ | $24.5 \times 10^6$ |
| 3 | 6 | $19.3 \times 10^6$ | $18.4 \times 10^6$ |
| 4 | 7 | $17.3 \times 10^6$ | $15.5 \times 10^6$ |
| 5 | 8 | $15.4 \times 10^6$ | $14.3 \times 10^6$ |

## Effect of incubation period

The duration of incubation of the mycelium with lysing mixture for the release of protoplast was optimized. The result shows that after two hours of incubation the protoplasts started to release and at the third hour the release reached the maximum, and later it decreased because of the prolonged action of the lytic enzymes (TABLE 6).

**TABLE 6 : Effect of incubation period on protoplast release from *A. terreus* and *A. flavus***

| S.No. | Incubation period (h) | Protoplast released in *A. terreus* | Protoplast released in *A. flavus* |
|---|---|---|---|
| 1 | 2 | $16.7 \times 10^6$ | $16.3 \times 10^6$ |
| 2 | 3 | $26.2 \times 10^6$ | $24.1 \times 10^6$ |
| 3 | 4 | $22.8 \times 10^6$ | $21.8 \times 10^6$ |

The present study revealed that the optimal conditions for protoplast relase in *A. flavus* and *A. terreus* were mixture of Novozyme 234 (2 mg/ml) and cellulase (0.2 ml/ml) as lytic enzyme, mycelium age of 24 h, 0.6 M KCl as osmotic stabilizer, pH 5 and incubation period of 180 min.

## Polyethylene glycol (PEG) mediated fusion

The aggregation of two or more protoplast is not enough to start fusion. Protoplast surfaces bear strong negative charges due to which intact protoplasts in suspension repel each other. They can be linked and fused by the addition of calcium ions or PEG. It was found that different factors, concentration of fusogen and osmotic stabilizers, pH of fusogen, incubation mixture influence the aggregation, survival of protoplasts and subsequently the hybridization frequency. The introduction of PEG/$Ca^{2+}$ to induce fusion in fungal protoplasts was first developed by Anne and Peberdy[2]. PEG induced fusion results in reproducible high frequency formation, low toxicity to cells, reduced formation of binucleate heterokaryons and is non-specific. The molecular weight of PEG is critical to the fusion frequency and in most of the studies PEG concentration of 4000-6000 was used[8]. PEG concentration below 20% result in protoplasts lysis. In this present study 50% PEG with 10mM $Ca^{2+}$ was found to be effective in bringing effective protoplast fusion (TABLE 7).

**TABLE 7 : Influence of PEG concentration on interspecific protoplast fusion**

| PEG concentration frequency (%) | Fusion frequency (%) |
|---|---|
| 30 | 1.01 |
| 40 | 1.24 |
| 50 | 1.82 |
| 60 | 0.73 |

## Influence of pH on protoplast fusion

For an effective survival of the protoplasts during fusion treatment, pH of the fusion mixture is an important factor. Fusion occurred in PEG solution at pH levels lower than 7.0. Optimal procedures for fungal protoplast fusion were investigated by Anne and Peberdy[2] and it was found that a solution of 30 % (w/v) PEG containing 10mM $CaCl_2$ and 50 mM glycine at pH 7.5 was optimal. Kirimura *et al.*[9] have reported pH 4.5 as optimum for fusion between *Aspergillus terreus* and *Aspergillus usamii* protoplasts. In the present study the optimal fusion frequency was obtained at pH 7.0 in the presence of 10 mM $Ca^{2+}$ at 50 % PEG concentration (TABLE 8).

**TABLE 8 : Influence of pH on interspecific protoplast fusion**

| pH | Fusion frequency (%) |
|---|---|
| 6.0 | 0.34 |
| 6.5 | 0.93 |
| 7.0 | 1.82 |
| 7.5 | 0.69 |
| 8.0 | 0.52 |

## Protoplast regeneration

In the present study, PDA medium amended with

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the osmotic stabilizer has produced the substantial regeneration of protoplast. In the present study protoplast viability was assessed by direct regeneration in an osmotically stabilized medium. In this medium the regeneration frequency for *A. flavus* and *A. terreus* were 53% and 57% respectively. Usually when the fusion mixture is inoculated on selective media, mycelium from non fused or self fused protoplast will grow slowly, thereby helping in the isolation of the fusant progeny. The fusant culture of *A. flavus* and *A. terreus* showed faster growth compared to the parents. These observations coincide with the result observed on the fused protoplast of *Pichia stipitis* interspecific crosses[10].

**Fusant identification by colony morphology, spore shape and size**

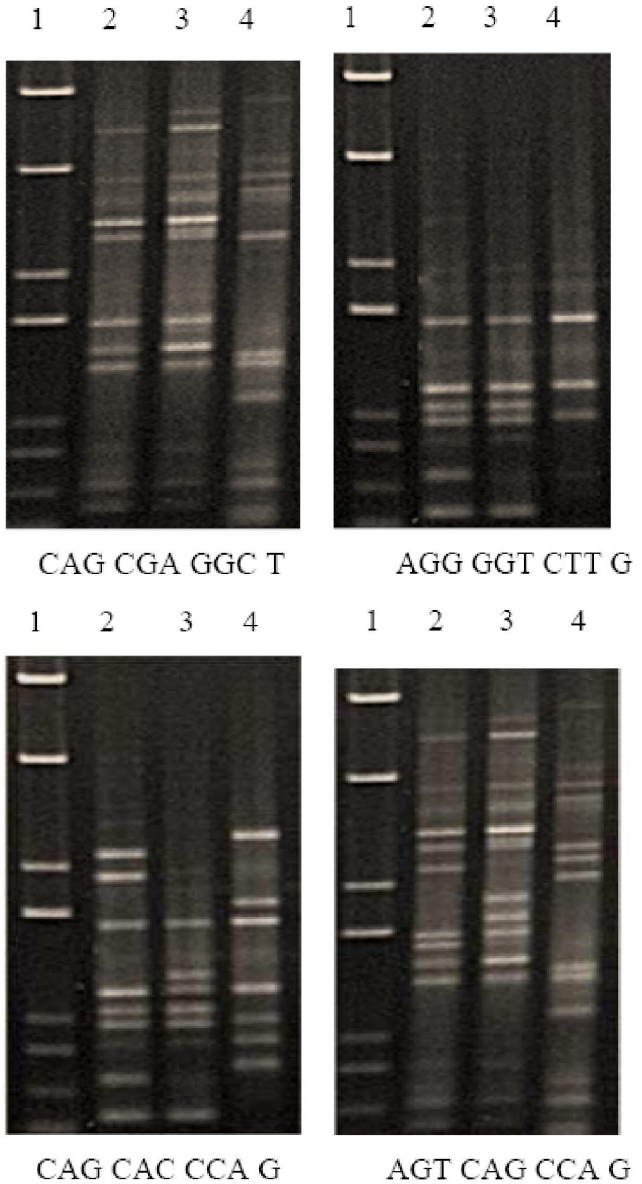
Colony morphology had been used to identify interspecific and intergeneric fusion products especially if the species differ greatly in colony morphology[11]. The parental colonies of *A. flavus* were green in colour and grew as small granular structures and *A. terreus* parental colonies were brown in color and colony varies from downy to powdery. The fusant strain colour was whitish brown. The spore morphology of *A. terreus*, the hyphae were septate and hyaline. Conidia are small, globose, and smooth. Conidiophores are smooth walled and hyaline terminating in mostly globose vesicles. In *A. flavus* conidia are typically globose to subglobose. Conidiophores are heavy walled, uncoloured, coarsely roughened. Vesicles are elongate when young, later becoming subglobose or globose. The fusant was more similar to the parental strain, *A. terreus*. The conidial sizes were 5.3624 µm, 4.4182 µm and 5.22 µm (dia) in *A. terreus, A. flavus* and fusant respectively.

**Fusant identification by mycelia protein pattern**

The protein polymorphism of the recombinant was identified by electrophoretic technique. The 66, 86, and 43 KDa proteins expressed in *A. terreus* were expressed in the fusant; the 38 and 52 KDa proteins present in *A. flavus* were strongly expressed in the fusant. This showed the relatedness of protein polymorphism between parents and fusant (Plate 2.3). This banding pattern confirms the hybrid formation. Proteins generally detect lower levels of variability and are restricted to a maximum of 20-40 stainable enzymes. In some cases, it showed high level of polymorphisms, but in few cases it was limited (Murphy *et al.*, 1990; Wayne *et al.*, 1991).

**Fusant identification by RAPD analysis**

In the present study four primers CAG CGA GGC T, AGG GGT CTT G, CAG CAC CCA G and AGT CAG CCA G were used in which CAG CGA GGC T and AGT CAG CCA G produced polymorphisms between the parents and fusant. When CAG CGA GGC T primer was used, a new band around 300-400 bp was present in fusant but absent in both parents. The 400- 500 bp band was well expressed in *A. terreus* and *A. flavus*




**Lane 1 : DNA markers (1000, 800, 600, 500, 300, 200, 100 bp); Lane 2 : *A. terreus*; Lane 3 : *A. flavus*; Lane 4 : Fusant strain**

**Figure 1 : RAPD analysis of *A. terreus, A. flavus* and fusant**

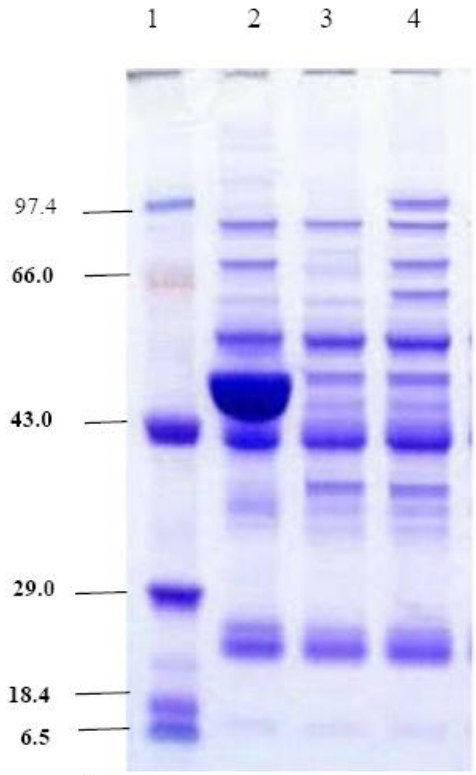
but absent in fusant. The 700- 800 bp band was present in *A. flavus* and fusant but absent in *A. terreus*. When the primer AGT CAG CCA G was used a new band around 400-500 bp was observed in fusant which was absent in both parents. The 7000 bp was expressed in *A. flavus* and fusant but slightly expressed in *A. terreus* (Figure 1). These observations further confirmed the successful formation of a protoplast fusant between *A. terreus* and *A. flavus*.

### Lovastatin yield

The production of lovastatin between the parent and the fusant was analyzed with HPLC. From the HPLC analysis, the concentration of lovastatin production from *A. flavus*, *A. terreus* and fusant was found to be 0.32 mg/g, 0.841 mg/g and 0.92 mg/g. (TABLE 9).

**TABLE 9 : Production of lovastatin in *A. terreus, A. flavus* and fusant**

| S.No | Strain | Lovastatin yield in mg/g |
|---|---|---|
| 1 | *Aspergillus flavus* | 0.32 |
| 2 | *Aspergillus terreus* | 0.741 |
| 3 | Fusant strain | 0.92 |




**Lane 1 : Marker; Lane 2 : *A. terreus*; Lane 3 : *A. flavus*; Lane 4 : fusant**

**Figure 2 : Protein profiling of *A. terreus, A. flavus* and fusant**

In 2005, Marlia Singgih *et al*. improved the production of lovastatin, by intraspecific protoplast fusion of two strains of *Aspergilus terreus*.

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