RESULTS

4.1 Isolation of fungal cultures

In this study, 219 fungal strains from different soil samples and endophytic isolates from the plant parts (stem, leaf) of visibly healthy plants were isolated on potato dextrose agar (PDA) and water agar (WA), respectively. Out of these, 134 were soil cultures and 85 were endophytic fungi. The soil samples were collected from Amritsar, Gurdaspur (Punjab, India) and Nanded (Maharashtra, India) and the plants (Bambusa arundinacea, Terminalia chebula, Ocimum sanctum, Aloe vera) were from the Guru Nanak Dev University campus, Amritsar.

4.2 Screening of the isolates for statin production

4.2.1 Bioassay of the cultures

The isolates were cultivated on the lactose based lovastatin production medium (LPM). The ethyl acetate extracts from fermented cultures were subjected to bioassay against Candida albicans and Rhodotorula rubra as test organisms. Seventy seven soil isolates showed bioactivity against both test organisms (Fig 4.1).

Whereas, thirteen soil isolates showed bioactivity against C. albicans and 9 against R. rubra alone. Amongst these soil isolates, maximal zone of inhibition against C. albicans was observed in extract of A. fumigatus (G) (3.5 cm) followed by A. terreus (GD13) (3.1 cm), Aspergillus sp. (49) (2.5 cm) whereas A. flavus (GD11) produced largest zone of inhibition (2.6 cm) against R. rubra followed by A. fumigatus (G) (2.4 cm), and (A. terreus) GD13 (1.7 cm) (Fig. 4.2, 4.3).

The data further revealed that of the eighty five endophytic fungal strains, isolated from the plants (B. arundinacea, T. chebula, O. sanctum, A. vera), thirty two endophytes were biologically active and twenty one showed zone of inhibition against both C. albicans and R. rubra. However, only six isolates were active against C. albicans and five inhibited R. rubra. An isolate from O. sanctum leaf, designated as TL-b, showed the highest zone of inhibition against C. albicans (3.7 cm) followed by TS-b and BAL-19 (2.0 cm). The extracts from isolates TL-b, OSL-8 and TCS-8 produced largest zones of inhibition (2 cm) against R. rubra. In addition TS-b, OSL-2, TCS-2, AA, TL-C, OCS-2, BAS-2, BAS-4, ASS, A4C (γ), AAc, AV-L1, TCS-4 and TCL-4 showed zones of inhibition ranging between 1.1-1.4 cm).
Fig. 4.1 Primary screening of soil and endophytic isolates

* Culture conditions: Working volume-250 ml flask containing 50 ml medium; Carbon source, lactose (5%); inoculum level, $6.6 \times 10^7$ spores/ml from 4 d old slants; initial medium pH, 6.5; incubation temperature, 30ºC; incubation period, 7 days; shaking conditions, 250 rpm.

Sample size:

Soil samples:
- Gurdaspur=62; Maharashtra=21; Amritsar=52

Plant samples:
- Ocimum sanctum (Tulsi): Leaf=16; Stem=12; Total=28
- Aloe vera (Aloe): Leaf=11; Stem=3; Total=14
- Terminalia chebula (Keekar): Leaf=14; Stem=8; Total=22
- Bambusa arundinacea (Bamboo): Leaf=13; Stem=6; Total=19
Results

*Left side bars depict zones of inhibition against *C. albicans*

*Right side bars depict zones of inhibition against *R. rubra*

\( \text{Fig 4.2} \) The zones of inhibition exhibited by the known soil isolates against *C. albicans* and *R. rubra* (a); *C. albicans* (b); *R. rubra* (c).
**Results**

**Fig. 4.3** Bioassay against *C. albicans* (left) and *R. rubra* (right) by agar diffusion method

**Fig. 4.4** TLC chromatogram observed under U.V. Lamp

Lane 1: lovastatin standard (Mevinolin); Lane 2: *Aspergillus terreus* MTCC 1251; Lane 3: Isolate G; Lane 4: Isolate M; Lane 5: Isolate 7; Lane 6: Isolate HF
4.2.2 TLC analysis

The bioassay positive culture extracts were resolved on TLC Silica gel 60 F$_{254}$ plates (Merck). The results in Fig. 4.4 showed that the metabolite spots resolved from extracts of *A. fumigatus* and *A. flavus* at (rf=0.82) were corresponding to spots shown by commercial lovastatin (mevinolin, Sigma). Many of bioassay positive cultures, however, did not show spots corresponding to lovastatin thus no discernible correlation between bioassay and lovastatin production could be established. The extracts were then subjected to quantitative and qualitative analysis using HPLC.

4.3 Quantification of statin using HPLC

The HPLC analysis of extracts from 219 isolates demonstrated that only 47 isolates including 9 endophytic cultures showed the peaks corresponding to hydroxy/lactone form of lovastatin (Fig.4.5).

Out of these, 18 isolates were identified as *A. terreus* on the basis of morphological and microscopic features. These *A. terreus* isolates (GD$_{13}$, GD$_{12}$, d, L, 9HH, HJ, S-11, u, Z, M, 7, J (H-3)-4, J(H-3)-14, J(H-3)-15, Hg-38, AT (MTCC), J (H-3)-12 and J (H-3)-18) produced lovastatin in the range of 1.36-190 mg/l (Fig.4.6). The largest numbers of *A. terreus* strains isolated from Gurdaspur soil samples produced lovastatin in the range of (1.6-83.76 mg/l). The isolates of *A.terreus* from Amritsar soil samples also produced low amounts of lovastatin. Besides bioassay positive cultures, two bioassay negative cultures from Gurdaspur soil samples and three from Amritsar were found to produce lovastatin. Two of the *A.terreus* isolates obtained from Deccan Plateau region of India (Maharashtra) were positive for bioassay and also showed HPLC peaks corresponding to lovastatin (mevinolin, Sigma). *A.terreus* strains (GD$_{13}$ and GD$_{12}$) isolated from the Maharashtra soil samples produced 190 mg/l and 153.94 mg/l of lovastatin, respectively. On the basis of above data, *A. terreus* GD$_{13}$, found to possess maximal lovastatin producing capability was selected for further studies.
Fig 4.5 HPLC chromatogram of GD₁₃ (*A. terreus*) showing \(\beta\)-hydroxy acid form (8.98 min) and lactone form (14.68 min) of lovastatin

* S.E @ 5% level

* Culture conditions: Working volume-250 ml flask containing 50 ml medium; Carbon source, lactose (5 %); inoculum level, \(6.6 \times 10^7\) spores/ml from 4 d old slants; initial medium pH, 6.5; incubation temperature, 30ºC; incubation period, 7 days; shaking conditions, 250 rpm.
Results

Besides *A. terreus* strains, other fungal isolates capable of lovastatin production were identified as *Aspergillus* sp. (24.96 mg/l), *A. fumigatus* (2.54 mg/l), *A. flavus* oryzae (0.68-28.25 mg/l), *A. nidulans* (2.14-19.22 mg/l), *A. niger* (0.14-1.38 mg/l), *Melanocarpus* (0.18-15.98), *Stemphylium* (0.42 mg/l), *Penicillium* (0.17-2.20 mg/l) and *Stachybotrys* (2.8 mg/l) (Fig.4.7). Amongst these, *A. flavus* oryzae, GD11, produced 28.25 mg/l lovastatin followed by 24.96 and 19.22 mg/l by *Aspergillus* sp. (49) and *A. nidulans* (GD6), respectively. The fungal strains of *Melanocarpus*, *Stemphylium* and *Stachybotrys* strains, though produced low levels of lovastatin, are being reported for the first time for statin producing capability.

From amongst endophytic fungal isolates, only nine showed lovastatin production in the range of 0.36-2.40 mg/l (Fig.4.8). Out of nine, four endophytes that produced lovastatin were isolated from *O. sanctum*, two each from *B. arundinacea* and *T. chebula*. The highest amount of lovastatin (2.4 mg/l) was produced by *B. arundinacea* stem isolate, BAS-2, followed by a stem isolate of *A. vera* (AV-c) (1.53 mg/l). The endophytic isolate, TI-b, that showed maximum zone of inhibition, however, produced lesser amount of lovastatin as compared to other *O. sanctum* isolates, JA and DDc that formed comparatively small zones of inhibition (0.9 cm). OCS-3 did not show any zone of inhibition and it also yielded very low amount of lovastatin (0.343 mg/l). Two of the positive isolates, TCS-2 (stem) and TCL-4 (leaf), were isolated from *T. chebula*. TCL-4 identified as *Alternaria* sp., did not show any zone of inhibition against *C. albicans* but produced 0.36 mg/l of lovastatin. The TS-b (*O. sanctum* stem), identified as *A. niger*, showed an appreciable zone of inhibition against *C. albicans* (2.0) and *R. rubra* (1.4 cm), however, did not show detectable amount of lovastatin by HPLC.

4.4 Identification of cultures

The cultures producing appreciable levels of lovastatin were identified using morphological, microscopic and molecular approaches employing sequencing of amplified (ITS I-5.8 S-ITS II) region (Table 4.1).

4.4.1 Microscopic examination of fungi

GD13 that produced maximal lovastatin and the other strains of *A. niger*, *A. flavus* oryzae, *A. fumigatus*, *A. nidulans*, *Melanocarpus albomyces*, *Stemphylium* sp, *Penicillium* sp., *Stachybotrys* sp. were identified on the basis of microscopic features employing slide culturing.
Results

Fig. 4.7 Lovastatin production by wild type fungal isolates other than *A. terreus*

* S.E @ 5% level
* Culture conditions: Same as Fig.4.6.

Fig. 4.8 Lovastatin yield of different endophytic isolates

* S.E @ 5% level
* Culture conditions: Same as Fig. 4.7.
### Table 4.1 Morphological features of the identified strains

<table>
<thead>
<tr>
<th>Culture name</th>
<th>Morphological/Microscopic features</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. terreus</em></td>
<td>Dull brown sporulation; yellow pigmentation. The culture showed cinnamon brown conidia with hyaline exudates on PDA after 5 days of incubation. Under phase contrast microscope, smooth walled, hyaline stipes were observed after 2 days of incubation. Vesicles were subglobose with hyaline and globose smooth walled conidia. In case of GD&lt;sub&gt;13&lt;/sub&gt;, the reverse of the plate was yellowish green due to diffusible pigment. (GD&lt;sub&gt;13&lt;/sub&gt;) KR149592 (M) KR149595 (7) KR149593</td>
<td></td>
</tr>
<tr>
<td><em>A. flavus oryzae</em></td>
<td>Green spores; brown fruiting bodies; black pigmentation -</td>
<td></td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>Beige cream spores; lemon orange. Uniseriate or biseriate phialides -</td>
<td></td>
</tr>
<tr>
<td><em>M. albomyces</em></td>
<td>Cottony white mycelium; no pigmentation The culture showed white cottony mycelia that grew profusely and touched the lid of plate within 2–3 days of incubation. The asporogenous hyphae coalesced afterwards and straw yellow exudates were observed -</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium</em> sp.*</td>
<td>Very dark green colony with white periphery and yellow exudates Colonies showed tough, closely interwoven felt of fine hyphae, with delicately floccose surface irregularly wrinkled in central portions and radially furrowed in marginal colony areas at first white turning to greenish pale grey. Chains of spherical spores from brush-like structures -</td>
<td></td>
</tr>
<tr>
<td><em>Stemphylium</em></td>
<td>White cottony mycelium. Spores contain many horizontal and transverse septa. -</td>
<td></td>
</tr>
<tr>
<td><em>Stachybotrys</em></td>
<td>Baby pink cottony growth. Bunch of elongated spores at the swollen tip of conidiophores -</td>
<td></td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>Black brown, cottony colony with little bit of sporulation; black pigmentation. Elliptical, solitary conidia with longitudinal septa bearing long filiform narrow, aseptate beak at tip present on medium sized conidiophores, -</td>
<td></td>
</tr>
</tbody>
</table>

*Lovastatin producing isolates*
Results

The culture GD$_{13}$ showed cinnamon brown conidia with hyaline exudates on PDA after 5 days of incubation. The reverse of the plate was yellowish green due to diffusible pigment. Under phase contrast microscope, smooth walled, hyaline stipes were observed on PDA after 2 days of incubation. Vesicles were sub-globose with hyaline and globose smooth walled conidia (Domsch et al., 1980) (Fig.4.9)

4.4.2 Molecular characterization of Fungi

The phylogenetic tree was constructed on the basis of ITS 1-5.8 S-ITS2 region (Fig 4.10). The NJ plot shows that the isolate GD$_{13}$, M and 7 are supported by high bootstrap values. These strains formed a distinct clade when compared to A. terreus sequences from NCBI indicating distinct phylogenetic origin and led to polyphyletic A. terreus strains. The dendrogram showed that the strain GD$_{6}$ identified as Emericella nidulans var.lata at the bottom of the tree. The other clades were also supported by high bootstrap values. The isolate 49 was identified as Aspergillus sp., G as Aspergillus fumigatus. A.terreus isolates M and 7 were closely related, however, GD$_{13}$ was phylogenetically distinct.

A.terreus strain GD$_{13}$ was taken up for further improvement in production by optimization and cyclic mutagenesis.

4.5 Growth pattern of lovastatin producing cultures in fermentation broth

When cultured on LPM medium, different fungal isolates formed filamentous growth or mycelial mat, other showed formed small to large pellet formation. The cultures which formed mycelial mat in broth were of velvety to cottony texture on PDA plate. Some isolates exhibiting similar morphology on plate, however, showed different morphologies in broth. The morphological differences were also apparent between different isolates of same genus as they formed pellets of different sizes and colours and some of them formed hyphal filaments. Some fungal species showed mixed morphology forming both biomass and pellets at the same time. Most of the A.terreus strains formed pellets ranging from 0.1 mm to 1.5 mm, A. nidulans (0.75-1.75 mm), A. niger (0.5-2 mm to macropellets), A. flavus oryzae (0.3-1.7 mm), A. fumigatus (0.7-1mm), Penicillium sp. (0.1-2.5 mm), Aspergillus sp. (0.75 mm) and A. nidulans (0.3-0.8 mm). Some A. niger and Penicillium strains formed large pellets of 7.5-10 mm and 2.0-2.5 mm, respectively. The latter differentiated into filamentous form.
Results

Fig. 4.9 Microscopic examination of isolate GD\textsubscript{13} \textit{(A. terreus)}

Fig. 4.10 Phylogenetic tree showing different lovastatin producing strains
Few \textit{A.niger} and \textit{A.fumigatus} strains formed macropellets with radiating spikes. The \textit{Stachybotrys} showed filamentous growth; however, the \textit{Stemphylium} sp. exhibited the mixture of clumped growth and pellets (0.3-0.5 mm). \textit{M. albomyces} formed mycelial mat. Most of the endophytes were of filamentous morphology. The final pH of culture broth of all the isolates was between 5-7.5. However, the final pH in case of \textit{A. niger} cultures was highly acidic to moderately acidic (pH range, 1-4.2).

### 4.6 Antiproliferative behaviour of lovastatin

The culture extracts fromLovastatin yielding isolates, \textit{A. terreus} (GD\textsubscript{13}), \textit{Aspergillus} sp. (49), \textit{A.flavus} (GD\textsubscript{11}) and \textit{A.nidulans} (GD\textsubscript{6}) were screened for antiproliferative activity against different cell lines derived from human HeLa (Hep-2), neuroblastoma (IMR-32), adenocarcinomic human alveolar basal epithelial cells (A-549) and human colorectal adenocarcinoma cells (HCT-15). The results in Table 4.2 show that the extracts from 49 \textit{Aspergillus} sp. (49) showed highest potential for cytotoxicity against all tested cell lines except Hep-2. \textit{A.terreus} (GD\textsubscript{13}) producing highest levels of lovastatin also showed high % of cytotoxicity against all cell lines tested in the experiment. The antiproliferative activity was upto 81-83 %. The \textit{A. terreus} (GD\textsubscript{13}) grown in presence of high level of yeast extract (@4.5 %) did not show anticancer activity against Hep-2 cell line. The culture GD\textsubscript{6} showed lesser potential for anticancer activity. However, in all the cases, the large zone of inhibition against test organisms and high lovastatin production correlated very well with the antiproliferative activity.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Isolate name</th>
<th>Conc. (μg/ml)</th>
<th>Hep-2 (%)</th>
<th>IMR-32 (%)</th>
<th>A-549 (%)</th>
<th>HCT-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GD\textsubscript{13} \textsuperscript{a}</td>
<td>100</td>
<td>81±4</td>
<td>81±4</td>
<td>82±4.1</td>
<td>75±3.75</td>
</tr>
<tr>
<td>2</td>
<td>GD\textsubscript{13} \textsuperscript{b}</td>
<td>100</td>
<td>71±3.6</td>
<td>64±3.2</td>
<td>82±4.1</td>
<td>83±4.2</td>
</tr>
<tr>
<td>3</td>
<td>GD\textsubscript{13} \textsuperscript{c}</td>
<td>100</td>
<td>0</td>
<td>44±2.2</td>
<td>40±2.0</td>
<td>29±1.45</td>
</tr>
<tr>
<td>4</td>
<td>GD\textsubscript{11} \textsuperscript{a}</td>
<td>100</td>
<td>47±2.35</td>
<td>60±3.0</td>
<td>67±3.35</td>
<td>64±3.2</td>
</tr>
<tr>
<td>5</td>
<td>GD\textsubscript{11} \textsuperscript{b}</td>
<td>100</td>
<td>18±0.9</td>
<td>34±1.7</td>
<td>56±2.8</td>
<td>32±1.6</td>
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<tr>
<td>6</td>
<td>GD\textsubscript{11} \textsuperscript{c}</td>
<td>100</td>
<td>11±0.55</td>
<td>61±3.05</td>
<td>51±2.55</td>
<td>53±2.65</td>
</tr>
<tr>
<td>7</td>
<td>GD\textsubscript{6} \textsuperscript{c}</td>
<td>100</td>
<td>17±0.85</td>
<td>22±1.10</td>
<td>33±1.65</td>
<td>38±1.90</td>
</tr>
<tr>
<td>8</td>
<td>49 \textsuperscript{c}</td>
<td>100</td>
<td>54±2.70</td>
<td>91±4.55</td>
<td>96±4.80</td>
<td>96±4.80</td>
</tr>
<tr>
<td>9</td>
<td>49 \textsuperscript{c}</td>
<td>100</td>
<td>53±2.65</td>
<td>76±3.80</td>
<td>72±3.60</td>
<td>45±2.25</td>
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<tr>
<td>10</td>
<td>5 FU</td>
<td>1x10\textsuperscript{-4} M</td>
<td>37</td>
<td>59</td>
<td>80</td>
<td>51</td>
</tr>
<tr>
<td>11</td>
<td>Mito-C</td>
<td>1x10\textsuperscript{-3} M</td>
<td>85</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Paclitaxel</td>
<td>1x10\textsuperscript{-5} M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Adriamycin</td>
<td>1x10\textsuperscript{-5} M</td>
<td>73</td>
<td>77</td>
<td>80</td>
<td>46</td>
</tr>
</tbody>
</table>

\textsuperscript{a}culture on lovastatin production medium containing soybean meal (@ 0.541 %)  
\textsuperscript{b} culture on lovastatin production medium containing yeast extract (@ 0.405 %)  
\textsuperscript{c} culture on lovastatin production medium containing yeast extract (@ 4.5 %)  
\textit{A.nidulans} (GD\textsubscript{6}); \textit{A.flavus} (GD\textsubscript{11}); \textit{A.terreus} (GD\textsubscript{13}); \textit{Aspergillus} sp. (49)
4.7 **Optimization of process parameters for lovastatin production by *A. terreus GD*<sub>13</sub> using one variable at a time approach**

*Aspergillus terreus* GD<sub>13</sub> strain, chosen after extensive screening, was optimized for lovastatin production using one factor at a time (OFAT) approach followed by statistical Box-Behnken design of experiments. Different parameters considered for optimization by OFAT were, effect of carbon sources and their concentration, nitrogen sources and their concentration, pH, inoculum level and age, etc.

4.7.1 **Effect of carbon sources**

The results in Figure 4.11 show that of the different carbon sources used, lactose supported maximal lovastatin (191.05 mg/l) production. Maltose, another disaccharide also supported good production (102.37 mg/l), followed by sucrose (57.066 mg/l), starch (47.34 mg/l) and glycerol (34.654 mg/l). However, complex carbohydrates such as corn flour, rice flour and wheat bran were poor carbon sources for lovastatin production.

4.7.2 **Effect of carbon source concentration**

The effect of varying lactose concentrations (1-15%) on lovastatin production was studied. Results in Figure 4.12 show that increase in lactose concentration from 1-11% resulted in steady increase lovastatin production. The observed lovastatin levels at 1% (87.39 mg/l) increased to (279.54 mg/l) at 11% registering an increase by 3.2 folds. Increasing concentration beyond 11% had marginal effect on lovastatin production.

4.7.3 **Effect of nitrogen sources**

The results in Figure 4.13 show that lovastatin yield was appreciably higher when either beef extract (310 mg/l) or soybean meal (290 mg/l) was used as a nitrogen source in production medium. Yeast extract and casein also supported high yields of lovastatin (218.68 mg/l and 217.09 mg/l), respectively. Inorganic nitrogen sources proved to be inefficient source of nitrogen as the lovastatin production with NH₄H₂PO₄ (39.109 mg/l), NH₄NO₃ (32.083 mg/l), NaNO₃ (24.25 mg/l) was observed. Soybean meal being less expensive when compared to beef extract and of plant based origin, was preferred over beef extract in further experiments.
Fig. 4.11 Effect of different carbon sources on lovastatin production by *A. terreus* GD\(_{13}\).

* Culture conditions: Working volume-250 ml flask containing 50 ml medium; Carbon sources (5% w/v); inoculum level, \(6.6 \times 10^7\) spores/ml from 4 d old slants; initial medium pH, 6.0; incubation temperature, 30ºC; incubation period, 7 days; shaking conditions, 250 rpm. (S.E @ 5% level)

Fig. 4.12 Effect of lactose concentration on lovastatin production by *A. terreus* GD\(_{13}\).

* Culture conditions: Same as Fig. 4.11 except lactose concentration (1-15 %) (S.E @ 5% level)
**Results**

![Graph showing effect of different nitrogen sources on lovastatin production by *A. terreus* GD13.](image)

**Fig. 4.13** Effect of different nitrogen sources on lovastatin production by *A. terreus* GD13.

* Culture conditions same as Fig 4.12 except for nitrogen sources. (S.E @ 5% level)

* CSL (Corn steep liquor); S.M (Soybean meal); Y.E (Yeast extract); B.E (Beef extract); M.E (Malt extract)

![Graph showing effect of soybean meal concentration on lovastatin production by *A. terreus* GD13.](image)

**Fig. 4.14** Effect of soybean meal concentration on lovastatin production by *A. terreus* GD13.

* Culture conditions: Same as Fig 4.13 except for soybean meal concentration (S.E @ 5% level)
4.7.4 Effect of Soybean meal concentration

The results in Figure 4.14 shows that the soybean meal when tested at different concentrations (0.135-1.08 %), yielded highest lovastatin (400.568 mg/l) production at 0.945 %. A 4.69-folds increase in level of lovastatin was observed at 0.945% when compared to 0.135%. Further increase in soybean meal concentration resulted in decline in lovastatin production.

4.7.5 Effect of pH

The results in Figure 4.15 show the effect of initial pH of the medium on lovastatin production. Maximal level of lovastatin (397.32 mg/l) was observed when the pH of the medium was in the range of 5.0 to 6.0. A drastic decline in the lovastatin production was observed under alkaline conditions at medium pH of 8.5 resulting in 3.52 folds lower production as compared to pH 6.0. Similar trends were observed under acidic conditions where the lovastatin production declined significantly at pH of 4.5 or below.

4.7.6 Effect of inoculum level

When the inoculum level was tested in the range of 1-7% (v/v) it was found that an inoculum level of 6% supported maximal lovastatin production (468.99 mg/l). The lowest inoculum level (0.5% v/v) employed in this study failed to support lovastatin production beyond 41.88 (mg/l). However 1% (v/v) resulted in sharp increase in the lovastatin production (269.4 mg/l) indicating a threshold level of inoculum required for lovastatin production. Increase in inoculum thereafter resulted in gradual increase in lovastatin yield upto 6 % (v/v) (Fig.4.16).

4.7.7 Effect of different activation media

The effect of different activation media was assessed on lovastatin production by A.terreus GD13 in lactose-based lovastatin production medium. The maximal lovastatin production (475.244 mg/l) was achieved when PDA used for propagating the cultures. The LPM inoculated from the PDB activated culture produced almost the same amount of lovastatin as the culture inoculated from the spore suspension prepared from the 4 d old slants (470.704 mg/l).
Fig. 4.15 Effect of pH for lovastatin production by *A. terreus* GD13.

* Culture conditions same as Fig 4.14 except for varying initial pH of the medium (S.E @ 5% level.).

Fig. 4.16 Effect of inoculum level on lovastatin production by *A. terreus* GD13.

* Culture conditions: Same as Fig 4.15 except for varying inoculum levels (S.E @ 5% level.)
The fermentation broth inoculated from YME activated culture showed 1.27-folds (372.204 mg/l) decrease in lovastatin production as compared to that on PDA. The fermentation broth inoculated with spores developed on other activation media resulted in drastic decrease in lovastatin production, namely, GYE (244.69 mg/l), Czapek dox agar (115.232 mg/l), MYE (90.558 mg/l), YPS (75.873 mg/l), LA (60.177 mg/l) and SDA (47.763 mg/l), respectively (Fig. 4.17).

4.7.8 Effect of inoculum age

Inoculum age had a very pronounced effect on lovastatin yield. Maximal statin was obtained with spore suspension prepared from 4 d old slant as inoculum. However PDB activated cultures (24-96 h old) showed decreased levels of statin, except for 84 h old activated culture that recorded lovastatin (465.588 mg/l) production was comparable to that obtained with spore suspension (467.577 mg/l) (Fig. 4.18).

4.7.9 Effect of inorganic components/salts on the production of lovastatin

This experiment was conducted to study the significance of components of basal salt solution in the production medium. The study was carried out by preparing medium devoid of one of the component of basal salt solution. The results in Fig 4.19 shows that medium without MgSO$_4$ resulted in drastic decrease lovastatin (45.1 mg/l) production when compared to control (470.98 mg/l). The removal of NaCl, ZnSO$_4$ and TES also yielded lower lovastatin corresponding to 180.02, 260.68 and 280.7 mg/l, respectively. However, addition of KH$_2$PO$_4$ (@ 0.02 %) and KH$_2$PO$_4$ (@ 0.08 %) into the medium yielded 260 and 350.1 mg/l of lovastatin.

4.8 Optimization of lovastatin production using response surface methodology

On the basis of one factor at a time experiments, four process parameters namely lactose, soybean meal concentration, inoculum level (spore count/ml) and inoculum age, found earlier to be crucial for lovastatin production, were chosen as independent variables to study their interactive effect on lovastatin production.
Results

**Fig. 4.17** Effect of activation medium for propagating spores on lovastatin production by *A. terreus* GD$_{13}$.

*Culture conditions same as Fig 4.16 except for different activation media (S.E @ 5% level.).

**Fig. 4.18** Effect of inoculum age on lovastatin production by *A. terreus* GD$_{13}$.

* Culture conditions same as Fig 4.17 except for varying inoculum age (S.E @ 5% level.).
**Fig. 4.19** Effect of media devoid of inorganic components from the production medium on lovastatin production by *A. terreus* GD13.

*Culture conditions same as Fig. 4.18 except the absence of inorganic components from the medium and two flasks in duplicate containing varying levels of KH₂PO₄ (S.E @ 5 %)

*MgSO₄: Medium without MgSO₄; * NaCl: Medium without NaCl; * ZnSO₄: Medium without ZnSO₄; * TES: Medium without TES; * KH₂PO₄ (0.02 %): Medium containing KH₂PO₄ (@ 0.02 %); * KH₂PO₄ (0.05 %): Medium containing KH₂PO₄ (@ 0.05 %)
The study employed Box-Behnken design of experiments and response surface methodology (RSM) using 29-flask experiment that had five central points. The interactive effect of four process parameters i.e. lactose and soybean meal, inoculum size (spore concentration) and age of the spore culture, on the production of lovastatin was evaluated employing response surface methodology (RSM). The experimental data for lovastatin production showed that the quadratic regression coefficient $R^2 = 95.9\%$ to produce second-order model was significant as revealed by p-value ($P < 0.001$). The data suggested that only 4.1% of the total variation was not explained by the model. In addition, the model F-value = 23.5 indicated that the model is significant and accurately represented the data in experimental region. The analysis of variance showed insignificant lack of fit for the model, further indicating the robustness of the experimental data to explain the relationships between the chosen variables and lovastatin production. The equation of the model fitted was as follows:

$$
\text{Lovastatin (mg/l) (Predicted)} = -5758 + 171X_1 - 772X_2 + 1365X_3 + 109X_4 + 5X_1X_2 - 12X_1X_3 + 204X_2X_3 - 5X_2X_4 - 3X_3X_4 - 4X_{12} - 199X_{22} - 89X_{32} - 5X_{42}.
$$

The magnitude of p-values (Table 4.3) indicated that in linear terms, inoculum level and lactose were significant at $p < 0.001$, while soybean meal was significant at $p < 0.01$ level. On the other hand, the quadratic interaction between all the parameters was significant at $p < 0.001$ level. Two way interactions between soybean meal and inoculum level was the most significant with highest $\beta$-coefficient of 204, indicating that this interaction influenced the production of lovastatin positively.
Table 4.3 Regression coefficients of the variables affecting lovastatin production

<table>
<thead>
<tr>
<th>Term</th>
<th>Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-5758</td>
</tr>
<tr>
<td>% lactose***</td>
<td>171</td>
</tr>
<tr>
<td>% soybean meal**</td>
<td>-772</td>
</tr>
<tr>
<td>Inoculum level***</td>
<td>1365</td>
</tr>
<tr>
<td>Inoculum age*</td>
<td>109</td>
</tr>
<tr>
<td>% lactose* % lactose***</td>
<td>-4</td>
</tr>
<tr>
<td>% soybean meal* % soybean meal***</td>
<td>-199</td>
</tr>
<tr>
<td>Inoculum level* Inoculum level***</td>
<td>-89</td>
</tr>
<tr>
<td>Inoculum age* Inoculum age***</td>
<td>-5</td>
</tr>
<tr>
<td>% lactose*% soybean meal</td>
<td>5</td>
</tr>
<tr>
<td>% lactose* Inoculum level**</td>
<td>-12</td>
</tr>
<tr>
<td>% lactose* Inoculum age</td>
<td>0</td>
</tr>
<tr>
<td>% soybean meal<em>Inoculum level</em>**</td>
<td>204</td>
</tr>
<tr>
<td>% soybean meal*Inoculum age</td>
<td>-5</td>
</tr>
<tr>
<td>Inoculum level* Inoculum age</td>
<td>-3</td>
</tr>
<tr>
<td>$R^2$</td>
<td>95.9</td>
</tr>
<tr>
<td>$R^2$ (adjusted)</td>
<td>91.5</td>
</tr>
</tbody>
</table>

***p<0.001; **p<0.01; *p<0.1

The results in Table 4.4 show that using this experimental design lovastatin production ranging from 537–1342 mg/l could be achieved.
Table 4.4 Box Behnken design of experiment for production of lovastatin by *A. terreus* GD$_{13}$

<table>
<thead>
<tr>
<th>Flask</th>
<th>Lactose (%)</th>
<th>S.M. (%)</th>
<th>Inoculum level (spores/ml)</th>
<th>Inoculum age (days)</th>
<th>Lovastatin (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual</td>
<td>Predicted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11 (0)</td>
<td>1.582 (+1)</td>
<td>10$^6$ (-1)</td>
<td>8 (0)</td>
<td>706.000</td>
</tr>
<tr>
<td>2</td>
<td>6 (-1)</td>
<td>0.941 (0)</td>
<td>10$^7$ (0)</td>
<td>12 (+1)</td>
<td>885.000</td>
</tr>
<tr>
<td>3</td>
<td>16 (+1)</td>
<td>0.941 (0)</td>
<td>10$^8$ (+1)</td>
<td>8 (0)</td>
<td>796.000</td>
</tr>
<tr>
<td>4</td>
<td>11 (0)</td>
<td>0.941 (0)</td>
<td>10$^7$ (0)</td>
<td>8 (0)</td>
<td>949.000</td>
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<tr>
<td>5</td>
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<td>0.941 (0)</td>
<td>10$^6$ (-1)</td>
<td>8 (0)</td>
<td>689.000</td>
</tr>
<tr>
<td>6</td>
<td>11 (0)</td>
<td>0.3 (-1)</td>
<td>10$^8$ (+1)</td>
<td>8 (0)</td>
<td>689.000</td>
</tr>
<tr>
<td>7</td>
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<td>10$^7$ (0)</td>
<td>4 (-1)</td>
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</tr>
<tr>
<td>8</td>
<td>11 (0)</td>
<td>1.582 (+1)</td>
<td>10$^8$ (+1)</td>
<td>8 (0)</td>
<td>1342.000</td>
</tr>
<tr>
<td>9</td>
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<td>0.941 (0)</td>
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<td>4 (-1)</td>
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</tr>
<tr>
<td>10</td>
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<td>10$^6$ (-1)</td>
<td>8 (0)</td>
<td>621.000</td>
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<td>10$^7$ (0)</td>
<td>8 (0)</td>
<td>942.000</td>
</tr>
<tr>
<td>12</td>
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<td>10$^7$ (0)</td>
<td>4 (-1)</td>
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<td>13</td>
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<tr>
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<tr>
<td>15</td>
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<td>622.000</td>
</tr>
<tr>
<td>16</td>
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<td>19</td>
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<td>4 (-1)</td>
<td>634.000</td>
</tr>
<tr>
<td>20</td>
<td>11 (0)</td>
<td>0.941 (0)</td>
<td>10$^7$ (0)</td>
<td>8 (0)</td>
<td>998.000</td>
</tr>
<tr>
<td>21</td>
<td>6 (-1)</td>
<td>0.3 (-1)</td>
<td>10$^7$ (0)</td>
<td>8 (0)</td>
<td>597.000</td>
</tr>
<tr>
<td>22</td>
<td>16 (+1)</td>
<td>0.3 (-1)</td>
<td>10$^7$ (0)</td>
<td>8 (0)</td>
<td>537.000</td>
</tr>
<tr>
<td>23</td>
<td>6 (-1)</td>
<td>1.582 (+1)</td>
<td>10$^7$ (0)</td>
<td>8 (0)</td>
<td>985.000</td>
</tr>
<tr>
<td>24</td>
<td>11 (0)</td>
<td>1.582 (+1)</td>
<td>10$^7$ (0)</td>
<td>12 (+1)</td>
<td>947.000</td>
</tr>
<tr>
<td>25</td>
<td>11 (0)</td>
<td>0.941 (0)</td>
<td>10$^8$ (+1)</td>
<td>12 (+1)</td>
<td>945.000</td>
</tr>
<tr>
<td>26</td>
<td>11 (0)</td>
<td>0.3 (-1)</td>
<td>10$^8$ (+1)</td>
<td>12 (+1)</td>
<td>959.000</td>
</tr>
<tr>
<td>27</td>
<td>11 (0)</td>
<td>0.941 (0)</td>
<td>10$^6$ (-1)</td>
<td>4 (-1)</td>
<td>626.000</td>
</tr>
<tr>
<td>28</td>
<td>11 (0)</td>
<td>0.3 (-1)</td>
<td>10$^6$ (-1)</td>
<td>8 (0)</td>
<td>575.000</td>
</tr>
<tr>
<td>29</td>
<td>11 (0)</td>
<td>1.582 (+1)</td>
<td>10$^7$ (0)</td>
<td>4 (-1)</td>
<td>969.000</td>
</tr>
</tbody>
</table>
The results in Table 4.4 show that using this experimental design lovastatin production ranging from 537–1342 mg/l could be achieved. The response surface contour plots (Figure 4.20 a-c) highlight the interactive effect of soybean meal and inoculum level on the production of lovastatin, which was found to be highly significant (Table 4.3). The results in contour plot (Figure 4.20 a) show that the production medium inoculated with a spore concentration of $1 \times 10^8$ spores/ml yielded 650 mg/l of lovastatin in the presence of 0.3% w/v soybean meal as a nitrogen source. The level of lovastatin increased to 1050 mg/l (Figure 4.20 b) when soybean meal concentration was adjusted at 0.941% (w/v). However, maximal level of lovastatin ($1280 \pm 42.426$ mg/l) was achieved when soybean meal (1.582%; w/v) and inoculum level ($1 \times 10^8$ spores/ml) were employed for production (Figure 4.20 c).

The observed values were achieved at lactose concentration ranging between 9–11% (w/v). The results obtained under optimal conditions were validated by conducting experiments in triplicate, and repeatedly showed production of $1320 \pm 42.426$ mg/l lovastatin confirming the above observations.

### 4.9 Production profile of lovastatin by *A. terreus* GD$_{13}$

The production profile (Figure 4.21) showed that a maximal level of biomass (35.1 g/l) was achieved after 4 days of incubation, whereas peak value for lovastatin ($1342 \pm 50$ mg/l) was achieved after 7 days of fermentation. The lovastatin titre of 55 mg/l gradually increased from day 1 to 1342 mg/l at day 7. Thereafter, it started to decrease to 1053 mg/l by the day 12. Efficient utilization of lactose was seen right from the day 1 with the gradual increase in biomass and lovastatin yield. However, biomass growth started to decline after day 4 but continuous rise in lovastatin titre was observed till the day 7 after which lovastatin production also started decreasing. The lactose was almost completely utilized by the day 7.
Results

4.20 (a)

4.20 (b)

4.20 (c)

Fig. 4.20 (a-c) Contour plots showing effect of increased levels of soybean meal concentration on lovastatin (mg/l) production by *A. terreus* GD<sub>13</sub>.

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Fig. 4.21 The profile of lovastatin production by *A. terreus* GD13 under optimized conditions. The Y axis (left) shows residual lactose (g/l) and biomass (g/l). The right Y axis shows the lovastatin level (mg/l). The X axis shows the incubation period in days.

*Culture conditions: working volume 50 ml medium containing lactose, 11% (w/v); soybean meal, 1.58% (w/v); inoculum level 1 x 10^8 spores/ml from 8 d old slants; initial medium pH, 6.0; incubation temperature, 30°C; shaking conditions, 250 rpm (S.E @ 5% level)
4.10 **Lovastatin yield exhibited by GD$_{13}$ at higher soybean meal concentrations**

When the strain GD$_{13}$ was subjected to RSM, it produced maximal lovastatin in the LPM using 1.582 % nitrogen. As the nitrogen concentration was increased from 1.620 % to 2.295 %, the lovastatin yield rose up to 1648 mg/l at 1.620 %. However, further increase in soybean meal concentration led to decrease in lovastatin production. In fact, the biomass increased with the increase in soybean meal concentration but the lovastatin production declined (Table 4.5). When the *A. terreus* strain was cultivated at the fermentor level, 1500 mg/l of lovastatin was obtained.

**Table 4.5** Lovastatin yield exhibited by GD$_{13}$ at higher soybean meal concentrations

<table>
<thead>
<tr>
<th>Concentration of soybean meal (%)</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.620</td>
<td>1648±24</td>
<td>38.66±0.026</td>
</tr>
<tr>
<td>1.755</td>
<td>1103±20</td>
<td>38.46±0.130</td>
</tr>
<tr>
<td>1.890</td>
<td>1080±20</td>
<td>43.76±0.034</td>
</tr>
<tr>
<td>2.025</td>
<td>1102±9</td>
<td>42.36±0.027</td>
</tr>
<tr>
<td>2.160</td>
<td>1147±38</td>
<td>43.98±0.129</td>
</tr>
<tr>
<td>2.295</td>
<td>1115±50</td>
<td>43.94±0.017</td>
</tr>
</tbody>
</table>

*Culture conditions: Same as Fig. 4.21 except for varying soybean meal concentration (S.E. @ 5 % level).

4.11 **Fed batch experiment**

The culture GD$_{13}$ was also subjected to fed batch experiment. It was found that the culture fed only once at 48 h (@ 6 % lactose) produced maximal levels of the lovastatin (934.407 mg/l), though the initial lactose used in the experiment was only 5 % (Fig. 4.22). That means, the initial C:N ratio was 26.25. When the lactose (@ 6 %) was fed at 48 h, the carbon content rose by 2.394 %, however, no nitrogen source was supplemented into the medium. This implies that C:N ratio was raised further. In other cases, where the lactose was added (@ 6 %) in divided doses at different hours, there was slight increase in C:N ratio, that did not prove beneficial for increasing lovastatin yield. The flask fed continuously from 48-144 h produced very low levels of lovastatin (237.458 mg/l). Even the feeding started at 72 or 96 h did not prove beneficial for the lovastatin production. Batch fermentation (620 mg/l) was much better than any of the feeding profiles except that fed at 48 h.
**Results**

**Fig. 4.22** Feeding profile of *A. terreus* GD₁₃ in fed batch experiment. The Y axis shows lovastatin yield (mg/l), biomass (g/l), residual lactose (g/l) and protein (mg/ml). The X-axis shows the feeding time.

* S.E @ 5% level

*Culture conditions: Working volume-250 ml flask containing 50 ml medium; Carbon source, lactose (5 %); Nitrogen source, soybean meal (0.945 %); inoculum level, 3ml of 6.6x10⁷ spores/ml from 4 d old slants; initial medium pH, 6.0; incubation temperature, 30ºC; incubation period, 7 days; shaking conditions, 250 rpm.

**Fig. 4.23** Effect of carbon sources on the production of lovastatin by *A. terreus* GD₁₃ under solid state fermentation

CF: Corn flour; PP: Pea Pod; CC: Corn cob; OP: Orange Peel; B: Bagasse; RS: Rice straw; WB: Wheat bran; WS: Wheat straw; SS: Sorghum straw; SM: Soybean meal; GS: Groundnut shell; RF: Rice flour; CP: Cucumber peel

*Culture conditions: Carbon source (5 g); distilled water (15 ml); Inoculum (2 ml) containing 6.6x10⁷ spores/ml from 4 d old slants; pH, 6.0; incubation temperature, 30 ºC; incubation period, 7 d (S.E @ 5 %)
4.12 Lovastatin production on solidified culture medium

A cheap medium comprising of vegetable waste/lignocellulosic residues as carbon source devoid of any other component of culture medium was tested for lovastatin production using solid state fermentation. Maximal statin production (6 g/kg) was observed, when pea pod was used as a carbon source, followed by sorghum straw, wheat bran, rice straw and orange peel (Fig. 4.23). These carbon sources supported high levels of statin production in the presence of distilled water only. However, when the basal lovastatin production medium was added in place of water, the lovastatin production declined. Groundnut shell, rice flour and cucumber powder were found to be poor sources for statin production.

4.13 Strain improvement programme

4.13.1 Mutagenesis and screening

_A. terreus_ GD\textsubscript{13} strain that produced highest levels of lovastatin was also taken up for strain improvement employing mutation-selection scheme (Fig. 4.24) to isolate hyper-producing mutants.

The first run of mutagenesis involved the selection of the mutants (UV+ NTG) of GD\textsubscript{13} were depending upon their resistance to lovastatin resistant property. Seventeen mutants were isolated onto the medium containing 2000 µg/ml of lovastatin. The mutants that were resistant to lovastatin exhibited a broad range of lovastatin titres (Fig. 4.25). Out of the seventeen mutants obtained, 1.01 to 1.96 fold increments in the lovastatin yield was observed in ten mutants, however, seven of them produced reduced levels of lovastatin in comparison to wild type GD\textsubscript{13} strain (Fig. 4.25). One of the mutants, named as ‘2-P’, producing 258.4 mg/l of lovastatin, showed 1.36 fold enhancement in the production of lovastatin. This mutant was chosen for strain improvement as it showed stability with regard to the lovastatin titres when subcultured repeatedly. However, LR\textsubscript{8} was an unstable lovastatin-resistant mutant that could not produce same levels of lovastatin (372 mg/l) on repeated subculturing though it produced maximal levels of lovastatin showing 1.96 fold increase in lovastatin production as compared to GD\textsubscript{13}. 

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Results

GD_{13} (Wild strain) → NTG (30 min)
2-P (Lovastatin-resistant mutant) → NTG (15 min), UV (15 min)
IA_{169} (Iodoacetamide-resistant mutant) → NTG (15 min), UV (15 min)
EM_{19} (N-ethylmaleimide-resistant mutant)

Fig. 4.24 Genealogy of the hyper-producing mutant EM_{19}

Fig. 4.25 Lovastatin yield exhibited by lovastatin-resistant mutants
It was further observed that 2-P yielded 600 mg/l of lovastatin on replacing the nitrogen source in the medium, from soybean meal to beef extract. (Table 4.6).

**Table 4.6** Lovastatin yields of selected wild and mutant strains of each stage**

<table>
<thead>
<tr>
<th>Stage of Mutagenesis</th>
<th>Name of the mutant</th>
<th>Lovastatin yield (mg/L)</th>
<th>Biomass (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>GD&lt;sub&gt;13&lt;/sub&gt;</td>
<td>320±10.0 (190±10.0)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.74</td>
</tr>
<tr>
<td>I</td>
<td>2-P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>600±20.0</td>
<td>17.86</td>
</tr>
<tr>
<td>II</td>
<td>IA&lt;sub&gt;169&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>736±20.0</td>
<td>18.20</td>
</tr>
<tr>
<td>III</td>
<td>EM&lt;sub&gt;19&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1424±20.0</td>
<td>19.30</td>
</tr>
</tbody>
</table>

<sup>a</sup> lovastatin-resistant mutant; <sup>b</sup> iodoacetamide-resistant mutant; <sup>c</sup> N-ethylmaleimide-resistant mutant; <sup>d</sup> Grown on medium containing soybean meal

* SE @ 5% level

** Soybean meal replaced with beef extract in the production medium.

Further, in the second and the third stage of mutagenesis, resistance to iodoacetamide and N-ethylmaleimide, respectively, were used as the selection markers, to boost the lovastatin titres. The second stage of mutagenesis was marked by the isolation of thirty-two mutants resistant to iodoacetamide and produced 1.12-2.85 folds higher lovastatin yields (Fig. 4.26) in comparison to the parent strain (2-P). The best mutant, IA<sub>169</sub>, showed resistance to 110 μg/ml iodoacetamide, producing lovastatin (@736 mg/l). The other iodoacetamide-resistant mutants were resistant to lower levels of iodoacetamide. IA<sub>169</sub>, producing highest lovastatin titres, was preferred over other mutants for the next round of mutation (Table 4.8).

The third stage of mutagenesis gave rise to 36 mutants that were resistant to N-ethylmaleimide. When screened on lovastatin production medium, the different mutants displayed raised as well as lowered levels of lovastatin. Of the thirty-six mutants obtained, some yielded very high amount of lovastatin whereas others showed lovastatin titres almost equivalent to IA<sub>169</sub> parent selected in the second stage of mutagenesis. Nineteen mutants manifested 1.003- 1.93 fold increment in the titres of lovastatin in comparison to the parent IA<sub>169</sub> while few showed undetectable titres of lovastatin (Fig. 4.27). The maximum lovastatin of 1424 mg/l was exhibited by mutant EM<sub>19</sub> after 7 days seventh day of fermentation on a medium that contained beef extract as a nitrogen source (Table 4.8). EM<sub>29</sub> (N-ethylmaleimide-resistant) mutant presenting
Results

Fig. 4.26 Lovastatin yield exhibited by iodoacetamide-resistant mutants

Fig. 4.27 Relative lovastatin yield of N-ethylmaleimide-resistant mutants

Fig. 4.28 (a) Compact Pellet of GD\textsubscript{13} (wild) in comparison to (b) fluffy pellet of EM\textsubscript{19} (mutant)
a negligible lovastatin production was actually a morphological mutant that showed altered spore colour and diminished sporulation on PDA plate as well as reduced biomass production in the fermentation medium. However, appreciable amounts of biomass were formed by the mutants that exhibiting high titres of lovastatin (Table 4.8). The lovastatin titre was influenced by the amount of biomass, however, a significant correlation was missing between the size of the pellet and titre of the lovastatin. The mutants did not show much variation in the pellet size. Nevertheless, lovastatin production, in majority of the cases, was affected by the morphology of the pellet, as in case of mutant EM$_{19}$, that produced highest amount of lovastatin. It formed a compact colony on PDA and produced a fluffy pellet on LPM during shake flask culture in comparison to wild type ancestral strain GD$_{13}$ (Fig. 4.28).

The mutagenesis and the screening programme followed in this work for the selection and development of the hyper-producing mutant (1424 mg/l) proved very efficient. It could produce 7.5-fold higher lovastatin in comparison to the wild type isolate (190 mg/l). Moreover, the improved strain (EM$_{19}$) exhibited higher lovastatin as well as higher productivity (g/l/h) of lovastatin.

4.13.2 Protoplast fusion

4.13.2.1 The production of lovastatin by parental and heterokaryon strains obtained by intraspecific protoplast fusion

Intraspecific protoplast fusion between strains of the fungus A. terreus for the breeding of lovastatin hyper-producing recombinant strains was carried out to further improve lovastatin yield. The GD$_{13}$ and EM$_{34}$ were chosen as parental strains. GD$_{13}$ was characterized as (Caf$^{\text{res}}$/Acri$^{\text{sen}}$) and EM$_{34}$ as (Caf$^{\text{sen}}$/Acri$^{\text{res}}$) on the basis of resistance/sensitivity to acriflavine (900 µg/ml) and caffeine (1250 µg/ml). The intraspecific hybridization between protoplast of these strains resulted in 19 heterokaryotic fusants. When plated on medium containing acriflavine as well as caffeine (Fig. 4.29), some of the fusants exhibited constricted growth and also differed in texture and pigmentation.
Fig. 4.29 Images of heterokaryons resulting from protoplast fusion of wild (GD$_{13}$) and mutant (EM$_{34}$) parent
Some of the fusants were white in colour and non-sporulating on PDA (Fig. 4.30).

Out of the 19 heterokaryons obtained, some produced low while some exhibited high levels of lovastatin production (Table 4.10). Four heterokaryons, namely, 2b, 5b, 9b and 13 yielded appreciable lovastatin titres showing a significant increase of 2.8-6.0-folds in lovastatin production as compared to the parental strain GD13 that could produce 300.186 (mg/l) of lovastatin when cultured on LPM. In some of fusants, lovastatin production potential declined drastically as compared to the parental strains. Interestingly, the fusants that were white in colour and non-sporulating produced low titres of lovastatin. Selected fusants 2b, 5b, 9b and 13 were further subjected to diploidization.
**Table 4.7** Lovastatin production of the heterokaryons obtained from intraspecific protoplast fusion of *A. terreus* GD<sub>13</sub> (wild parent) and EM<sub>34</sub> (mutant parent)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the heterokaryon</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GD&lt;sub&gt;13&lt;/sub&gt;</td>
<td>300.186±22.48</td>
<td>28.76</td>
<td>22.576</td>
</tr>
<tr>
<td>2</td>
<td>EM&lt;sub&gt;34&lt;/sub&gt;</td>
<td>850.232±32.5</td>
<td>30.57</td>
<td>27.698</td>
</tr>
<tr>
<td>3</td>
<td>2b</td>
<td>1800±48.987</td>
<td>30.986</td>
<td>23.012</td>
</tr>
<tr>
<td>4</td>
<td>5b</td>
<td>912.56±35.908</td>
<td>29.402</td>
<td>30.67</td>
</tr>
<tr>
<td>5</td>
<td>9b</td>
<td>1132.13±37.786</td>
<td>32.246</td>
<td>16.79</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>830.908±25.681</td>
<td>31.95</td>
<td>20.591</td>
</tr>
<tr>
<td>7</td>
<td>1b</td>
<td>520.897±15.089</td>
<td>30.96</td>
<td>22.761</td>
</tr>
<tr>
<td>8</td>
<td>1s</td>
<td>35.277±2.45</td>
<td>31.24</td>
<td>17.454</td>
</tr>
<tr>
<td>9</td>
<td>10bs</td>
<td>256.333±10.34</td>
<td>25.86</td>
<td>16.278</td>
</tr>
<tr>
<td>10</td>
<td>11bs</td>
<td>119.559±7.232</td>
<td>31.06</td>
<td>32.40</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>165.706±9.214</td>
<td>32.08</td>
<td>35.97</td>
</tr>
<tr>
<td>12</td>
<td>3s</td>
<td>78.651±7.86</td>
<td>27.90</td>
<td>14.355</td>
</tr>
<tr>
<td>13</td>
<td>4b</td>
<td>725.783±34.25</td>
<td>30.39</td>
<td>22.74</td>
</tr>
<tr>
<td>14</td>
<td>4s</td>
<td>31.437±2.52</td>
<td>15.09</td>
<td>23.011</td>
</tr>
<tr>
<td>15</td>
<td>14</td>
<td>165.142±9.21</td>
<td>41.66</td>
<td>35.94</td>
</tr>
<tr>
<td>16</td>
<td>5s</td>
<td>37.838±2.67</td>
<td>15.43</td>
<td>16.100</td>
</tr>
<tr>
<td>17</td>
<td>3b</td>
<td>437.266±7.98</td>
<td>30.3</td>
<td>32.36</td>
</tr>
<tr>
<td>18</td>
<td>6s</td>
<td>121.49±10.28</td>
<td>17.97</td>
<td>36.190</td>
</tr>
<tr>
<td>19</td>
<td>7b</td>
<td>378.514±15.54</td>
<td>22.34</td>
<td>36.154</td>
</tr>
<tr>
<td>20</td>
<td>7s</td>
<td>198.133±5.24</td>
<td>31.06</td>
<td>36.34</td>
</tr>
<tr>
<td>21</td>
<td>12</td>
<td>504.568±9.98</td>
<td>29.60</td>
<td>27.641</td>
</tr>
</tbody>
</table>
4.13.2.2 Diploidization of the best haterokaryons and the screening of the resultant diploids for lovastatin production

The diploidization of the fusants (2b, 5b, 9b and 13) was performed by exposing heterokaryons to 0.2 % camphor. The diploidization was indicated by sector formation. The spores picked from these sectors (diploids) showed 1.6-1.8-folds larger spore size when observed microscopically using micrometer, as compared to their respective heterokaryons. Similarly the larger nuclear size was also observed (Fig. 4.31).

![Spore and nucleus size of diploid 2b VIII.](image)

**Fig. 4.31** Spore and nucleus size of diploid 2b VIII, (b) is larger than the heterokaryon 2b, (a)

70 diploids were picked on plate and 39 were screened for lovastatin production (Table 4.11). Diploid producing higher and lower levels of lovastatin when compared to the heterokaryon from which they were derived, were observed. Some diploids produced lovastatin titres even lower than the parental strains, GD_{13} and EM_{34}. The low lovastatin producing diploids discharged wine brownish colour in LPM broth. The diploids obtained from heterokaryon 2b were found to be the high lovastatin producers showing 3.3-7.3 folds increase than the parental strain GD_{13} (Table 4.8).
Table 4.8 Lovastatin production exhibited by the diploids derived from the hyperproducing heterokaryons

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of diploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Specific yield (mg/g bm)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5b</td>
<td>900.165</td>
<td>29.14</td>
<td>30.89</td>
<td>21.550</td>
</tr>
<tr>
<td>2.</td>
<td>5b-b</td>
<td>955.408</td>
<td>30</td>
<td>31.85</td>
<td>23.766</td>
</tr>
<tr>
<td>3.</td>
<td>5b-Hd</td>
<td>968.596</td>
<td>32.78</td>
<td>29.548</td>
<td>11.968</td>
</tr>
<tr>
<td>4.</td>
<td>5b-1 (d)</td>
<td>844.17</td>
<td>28.88</td>
<td>29.23</td>
<td>21.942</td>
</tr>
<tr>
<td>5.</td>
<td>5b-1 (L)</td>
<td>1250.136</td>
<td>31.5</td>
<td>39.69</td>
<td>16.207</td>
</tr>
<tr>
<td>6.</td>
<td>5b-1 (D)</td>
<td>33.023</td>
<td>28.42</td>
<td>1.161</td>
<td>22.483</td>
</tr>
<tr>
<td>7.</td>
<td>5b-1 (D)</td>
<td>1131.178</td>
<td>31.36</td>
<td>36.07</td>
<td>7.309</td>
</tr>
</tbody>
</table>

**Diploids derived from heterokaryon 5b**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of diploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Specific yield (mg/g bm)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2b</td>
<td>1819.924</td>
<td>31.04</td>
<td>58.632</td>
<td>22.968</td>
</tr>
<tr>
<td>2.</td>
<td>2b XIII L</td>
<td>1894.372</td>
<td>33.56</td>
<td>56.447</td>
<td>12.196</td>
</tr>
<tr>
<td>3.</td>
<td>2b XIII d</td>
<td>985.654</td>
<td>25.92</td>
<td>38.027</td>
<td>24.756</td>
</tr>
<tr>
<td>4.</td>
<td>2b XIII la</td>
<td>1730.636</td>
<td>33.08</td>
<td>52.317</td>
<td>26.159</td>
</tr>
<tr>
<td>5.</td>
<td>2b VIII</td>
<td>2150.896</td>
<td>35.08</td>
<td>61.314</td>
<td>4.196</td>
</tr>
<tr>
<td>6.</td>
<td>2b HD</td>
<td>1458.516</td>
<td>32.82</td>
<td>44.43</td>
<td>20.873</td>
</tr>
<tr>
<td>7.</td>
<td>2b III</td>
<td>1768.208</td>
<td>30.88</td>
<td>57.261</td>
<td>19.605</td>
</tr>
</tbody>
</table>

**Diploids derived from heterokaryon 2b**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of diploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Specific yield (mg/g bm)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>9b</td>
<td>1141.458</td>
<td>32.62</td>
<td>34.993</td>
<td>15.758</td>
</tr>
<tr>
<td>2.</td>
<td>9b I s</td>
<td>1220.908</td>
<td>29.32</td>
<td>41.640</td>
<td>13.963</td>
</tr>
<tr>
<td>3.</td>
<td>9b V L</td>
<td>1021.535</td>
<td>37.68</td>
<td>27.111</td>
<td>27.869</td>
</tr>
<tr>
<td>4.</td>
<td>9b II</td>
<td>519.239</td>
<td>30.26</td>
<td>17.159</td>
<td>23.509</td>
</tr>
<tr>
<td>5.</td>
<td>9b V d</td>
<td>840.216</td>
<td>32.04</td>
<td>26.223</td>
<td>28.354</td>
</tr>
<tr>
<td>6.</td>
<td>9b IV d</td>
<td>1649.132</td>
<td>37.22</td>
<td>44.307</td>
<td>16.328</td>
</tr>
<tr>
<td>7.</td>
<td>9b VI</td>
<td>938.309</td>
<td>34.52</td>
<td>27.182</td>
<td>4.830</td>
</tr>
<tr>
<td>8.</td>
<td>9b I us</td>
<td>960.208</td>
<td>32.02</td>
<td>29.988</td>
<td>23.880</td>
</tr>
</tbody>
</table>

**Diploids derived from heterokaryon 9b**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of diploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Specific yield (mg/g bm)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>13</td>
<td>824.808</td>
<td>31.56</td>
<td>26.135</td>
<td>20.973</td>
</tr>
<tr>
<td>2.</td>
<td>13 (10a) d</td>
<td>1001.923</td>
<td>32.50</td>
<td>30.828</td>
<td>5.892</td>
</tr>
<tr>
<td>3.</td>
<td>13-2(d)</td>
<td>1216.268</td>
<td>32.60</td>
<td>37.308</td>
<td>3.647</td>
</tr>
<tr>
<td>4.</td>
<td>13-6’ (l)</td>
<td>300.578</td>
<td>25.05</td>
<td>11.999</td>
<td>3.769</td>
</tr>
<tr>
<td>5.</td>
<td>13-6 (l)</td>
<td>395.695</td>
<td>25.52</td>
<td>15.505</td>
<td>38.042</td>
</tr>
<tr>
<td>6.</td>
<td>13-2’-L</td>
<td>1168.387</td>
<td>32.49</td>
<td>35.961</td>
<td>2.337</td>
</tr>
<tr>
<td>7.</td>
<td>13-4’(d)</td>
<td>847.518</td>
<td>31.69</td>
<td>26.744</td>
<td>14.78</td>
</tr>
<tr>
<td>8.</td>
<td>13-0(d)</td>
<td>784.243</td>
<td>31.20</td>
<td>25.135</td>
<td>25.618</td>
</tr>
<tr>
<td>9.</td>
<td>13-4’L</td>
<td>558.967</td>
<td>27.79</td>
<td>20.114</td>
<td>25.219</td>
</tr>
<tr>
<td>10.</td>
<td>13-7’</td>
<td>496.869</td>
<td>27.11</td>
<td>18.321</td>
<td>17.846</td>
</tr>
<tr>
<td>11.</td>
<td>13-8’(d)</td>
<td>862.276</td>
<td>31.10</td>
<td>27.726</td>
<td>27.242</td>
</tr>
<tr>
<td>12.</td>
<td>13-3</td>
<td>1277.006</td>
<td>28.76</td>
<td>44.402</td>
<td>3.840</td>
</tr>
<tr>
<td>13.</td>
<td>13 (6d)</td>
<td>330.445</td>
<td>25.20</td>
<td>13.113</td>
<td>4.403</td>
</tr>
<tr>
<td>14.</td>
<td>13-2’/d</td>
<td>889.569</td>
<td>31.78</td>
<td>27.991</td>
<td>23.823</td>
</tr>
<tr>
<td>15.</td>
<td>13 (12d)</td>
<td>925.741</td>
<td>31.20</td>
<td>29.671</td>
<td>15.901</td>
</tr>
<tr>
<td>16.</td>
<td>13-5’</td>
<td>1451.351</td>
<td>30.94</td>
<td>46.910</td>
<td>3.897</td>
</tr>
<tr>
<td>17.</td>
<td>13-6’/d</td>
<td>1053.208</td>
<td>33.42</td>
<td>31.514</td>
<td>19.223</td>
</tr>
<tr>
<td>18.</td>
<td>13-4’/m</td>
<td>533.624</td>
<td>27.77</td>
<td>19.216</td>
<td>20.018</td>
</tr>
<tr>
<td>19.</td>
<td>13-(10a)j</td>
<td>912.169</td>
<td>32.042</td>
<td>28.468</td>
<td>20.588</td>
</tr>
<tr>
<td>20.</td>
<td>13-8’L</td>
<td>95.01</td>
<td>31.60</td>
<td>3.01</td>
<td>11.911</td>
</tr>
<tr>
<td>21.</td>
<td>13-L</td>
<td>933.924</td>
<td>32.05</td>
<td>29.140</td>
<td>22.907</td>
</tr>
</tbody>
</table>

**Diploids derived from heterokaryon 13**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of diploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Specific yield (mg/g bm)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.</td>
<td>GD13</td>
<td>295.186</td>
<td>28.84</td>
<td>10.409</td>
<td>22.455</td>
</tr>
<tr>
<td>46.</td>
<td>EM14</td>
<td>869.294</td>
<td>30.66</td>
<td>28.353</td>
<td>17.838</td>
</tr>
</tbody>
</table>

*Specific lovastatin yield = Lovastatin yield/ g biomass*
Results

Two of the diplods derived from 2b, i.e, 2b XIII L and 2b VIII, produced 1.04 and 1.18 folds higher lovastatin than the immediate heterokaryon (2b) from which they were derived. All the diploids derived from the heterokaryon 2b shared morphology with parental strains (Fig. 4.32).

Fig. 4.32 Images of the heterokaryon 2b, (a) and the diploids, (b-g) obtained through diploidization using 0.2 % camphor
The diploids derived from 5b showed lovastatin titres comparable to the parental strain EM$_{34}$ (844.17-1250.136 mg/l) except one diploid, 5b-1(L) which was the low producer of lovastatin (33.023 mg/l). This low lovastatin producing strain showed a slight difference in morphology. It formed a white cottony diploid sector (Fig. 4.33 a) though it did not show a very low biomass or lactose utilization. Specific lovastatin yield, i.e., mg of lovastatin produced per g of biomass was just 1.16 mg/g. In fact, the highest producer of lovastatin (5b-a(d)) amongst the diploids, derived from 5b, showed the formation of light and dark brown concentric rings in its colony (Fig. 4.33 b).

The diploids derived from the heterokaryon 9b also produced the lovastatin in the range comparable to that of heterokaryon itself and the one of the parental strains, EM$_{34}$, except the one (9b II) that exhibited 1.76-folds increase in lovastatin as compared to the wild parent GD$_{13}$. The 9b II showed appreciable utilization of lactose and amount of biomass. In fact, the morphology of this diploid was almost same as that of the heterokaryon.

Diploids derived from heterokaryon 13 showed a wide range of lovastatin titres. One of the diploids, 13-8’ L, yielded 95.01 mg/l ofLovastatin, however, producing appreciable amount of biomass and showing efficient sugar utilization (Fig. 4.33 d). This diploid also showed altered morphology with white velvety texture. Some of the diploids derived from fusant 13 produced lovastatin titres comparable to that of the wild parent GD$_{13}$ and others showed a range of lovastatin production above than that with highest being 1451.351 mg/l. One of the diploids, 13-7’, though showed a great variability in the morphology, however, produced moderate titres of Lovastatin (496.869 mg/l) (Fig. 4.33 c). Surprisingly, wide scale variations in Lovastatin production, lactose utilization and morphology were observed in the diploids derived from the heterokaryon 13.

Conclusively, the high Lovastatin producing diploids also formed higher biomass and showed efficient lactose utilization. On examining macroscopically, they looked like their parents, however, the trend varied amongst the low producers of Lovastatin. The diploid strain 2bVIII derived from heterokaryon 2b showed maximal Lovastatin production (2150 mg/l) with biomass of 35.08g/l after 7 d of fermentation when compared to other diploids and parent strains (Table 4.8). This diploid showed 2.47-fold increase in Lovastatin production as compared to one of the parents (EM$_{34}$) strain.
Results

Fig. 4.33 Images of the diploids with altered morphology
4.13.2.3 Haploidization of the best diploids and the screening of the resultant haploids for lovastatin production

Diploids producing appreciable levels of lovastatin were subjected to haploidization in presence of 0.5 μg/ml of benomyl in PDA medium. Most of the haploids did not show much increase in lovastatin production. Morphology of the haploid sectors was almost similar to the respective diploids. The haploids derived from the diploid sector 2b-IIId, namely, 2b-IIId (o) and 2b-IIId (i) showed 1.01 and 1.033 folds increase in lovastatin production as compared to the respective diploid (Fig. 4.34, Table 4.9). In the haploids derived from 2bIII, lovastatin production was almost similar in diploid as well as their respective haploids.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of haploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haploids derived from diploid 2b-IIId</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Diploid)</td>
<td>2b-IIId</td>
<td>1489.516</td>
<td>32.509</td>
<td>22.987</td>
</tr>
<tr>
<td>1</td>
<td>2b-IIId (o)</td>
<td>1504.41</td>
<td>30.92</td>
<td>27.214</td>
</tr>
<tr>
<td>2</td>
<td>2b-IIId (i)</td>
<td>1538.67</td>
<td>31.42</td>
<td>32.200</td>
</tr>
<tr>
<td></td>
<td>Haploids derived from diploid 2bIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Diploid)</td>
<td>2bIII</td>
<td>1750.009</td>
<td>30.243</td>
<td>20.043</td>
</tr>
<tr>
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<td>2bIII (o)</td>
<td>1676.065</td>
<td>31.409</td>
<td>27.285</td>
</tr>
<tr>
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<td>31.564</td>
<td>9.0119</td>
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<td>Haploids derived from diploid 2bVIII</td>
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<td></td>
<td></td>
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<tr>
<td>Control (Diploid)</td>
<td>2bVIII</td>
<td>2140.976</td>
<td>34.94</td>
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<td>2288.703</td>
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<td>2bVIII (1)</td>
<td>2161.4</td>
<td>32.459</td>
<td>10.900</td>
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<tr>
<td>3</td>
<td>2bVIII (2)</td>
<td>2247.001</td>
<td>31.463</td>
<td>8.367</td>
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<tr>
<td>4</td>
<td>2bVIII (3)</td>
<td>1216.663</td>
<td>30.967</td>
<td>22.013</td>
</tr>
</tbody>
</table>
### Results

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of haploid</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2b VIII (4)</td>
<td>1736.855</td>
<td>30.452</td>
<td>15.423</td>
</tr>
<tr>
<td>6</td>
<td>2b VIII (s)</td>
<td>1580.583</td>
<td>30.289</td>
<td>22.227</td>
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**Haploids derived from diploid 5b-a (d)**

<table>
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<th>5b-a(d)</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Diploid)</td>
<td></td>
<td>1257.980</td>
<td>30.134</td>
<td>17.237</td>
</tr>
<tr>
<td>1</td>
<td>5b-a (d) o</td>
<td>1008.605</td>
<td>30.09</td>
<td>26.038</td>
</tr>
<tr>
<td>2</td>
<td>5b-a (d) i</td>
<td>357.863</td>
<td>28.708</td>
<td>23.901</td>
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**Haploids derived from diploid 9b I s**

<table>
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<tr>
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<th>9b I s</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Diploid)</td>
<td></td>
<td>1215.989</td>
<td>29.546</td>
<td>14.564</td>
</tr>
<tr>
<td>1</td>
<td>9b I s(2)</td>
<td>590.945</td>
<td>33.76</td>
<td>29.636</td>
</tr>
<tr>
<td>2</td>
<td>9b I s (1)</td>
<td>408.694</td>
<td>31.18</td>
<td>23.082</td>
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</table>

**Haploids derived from diploid 9b IV d**

<table>
<thead>
<tr>
<th></th>
<th>9b IV d</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Diploid)</td>
<td></td>
<td>1600.135</td>
<td>30.54</td>
<td>30.740</td>
</tr>
<tr>
<td>T</td>
<td>9b IV d (1)</td>
<td>1736.855</td>
<td>30.786</td>
<td>27.548</td>
</tr>
<tr>
<td>U</td>
<td>9b IV d (2)</td>
<td>1099.205</td>
<td>30.243</td>
<td>14.747</td>
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</tbody>
</table>

**Haploids derived from diploid 13-2 d**

<table>
<thead>
<tr>
<th></th>
<th>13-2 d</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Diploid)</td>
<td></td>
<td>1222.788</td>
<td>32.79</td>
<td>35.98</td>
</tr>
<tr>
<td>1</td>
<td>13-2d (L)</td>
<td>1238.84</td>
<td>28.8</td>
<td>33.091</td>
</tr>
<tr>
<td>2</td>
<td>13-2d (D)</td>
<td>988.885</td>
<td>27.02</td>
<td>35.050</td>
</tr>
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</table>

**Haploids derived from diploid 13-2’-L**

<table>
<thead>
<tr>
<th></th>
<th>13-2’-L</th>
<th>Lovastatin yield (mg/l)</th>
<th>Biomass (g/l)</th>
<th>Residual lactose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Diploid)</td>
<td></td>
<td>1184.579</td>
<td>32.76</td>
<td>2.599</td>
</tr>
<tr>
<td>1</td>
<td>13-2’-L (1)</td>
<td>440.685</td>
<td>27.48</td>
<td>29.885</td>
</tr>
<tr>
<td>2</td>
<td>13-2’-L (2)</td>
<td>495.536</td>
<td>29.14</td>
<td>31.951</td>
</tr>
</tbody>
</table>
Out of six haploid sectors derived from the diploid 2b VIII, three stable haploid sectors were obtained which showed almost 1.01-1.069 folds increase in lovastatin production as compared to the diploid sector (2b VIII). Other three showed declined lovastatin production.

![Graph showing lovastatin production by parental, diploid, and haploid strains](image)

**Fig. 4.34** Lovastatin production by parental, diploid and haploid strains of *A. terreus*

Out of two haploid sectors derived from 5b-a (d), one showed many-folds reduced lovastatin production and the other showed lovastatin titre close to the diploid. The haploid derived from 9b Is also showed declined lovastatin production. Out of two haploid sectors derived from 9b IV d, one showed 1.085 folds increase in lovastatin production. In case of haploids of 13-2’-L, production decreased to almost half and the haploids of 13-2d showed almost comparable levels of lovastatin production to the diploids from which they were derived.

4.14 Screening of parental, heterokaryon, diploid and haploid strains for lovastatin production under SSF

The parental, heterokaryon, diploid and haploid strains were screened for their lovastatin production potential under SSF. Though, initially pea pod, yielded very high levels of lovastatin (6g/Kg of substrate) under SSF but the results with pea pod could not be reproduced. Therefore, sorghum straw, wheat bran and rice straw in addition to
pea pod were used as carbon source. pea pod as a control. The wild strain GD$_{13}$, EM$_{34}$ (mutant parent) and 2b (heterokaryon), respectively, yielded lovastatin titre of 5.3, 7.2 and 7.6 g/Kg of substrate when sorghum straw was used as a carbon source (4.10). However, the optimal carbon source for the diploids 2b III and 2b VIII was found to be wheat bran that supported 7.3 and 7.5g/Kg of substrate of lovastatin. The haploid 2b VIII (o) produced highest lovastatin titre of 8 g/Kg of substrate when rice straw was used as a carbon source. Using any of the carbon source, the production in wild, mutant, diploid and the haploid strains were comparable. However, the sorghum straw did not prove to be a very good carbon source in case of diploid 2b VIII.

Table 4.10 Lovastatin yield of parents, heterokaryon, diploid and haploid using different carbon sources under SSF

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lovastatin yield (g/Kg of substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pea Pod (control)</td>
</tr>
<tr>
<td>1. GD$_{13}$</td>
<td>1.837</td>
</tr>
<tr>
<td>2. EM$_{34}$</td>
<td>1.271</td>
</tr>
<tr>
<td>3. 2b</td>
<td>2.853</td>
</tr>
<tr>
<td>4. 2b III</td>
<td>1.095</td>
</tr>
<tr>
<td>5. 2b VIII</td>
<td>1.664</td>
</tr>
<tr>
<td>6 2b VIII (o)</td>
<td>1.839</td>
</tr>
</tbody>
</table>

When sorghum straw was used as a carbon source and the distilled water was replaced with basal medium (LPM) under SSF, the lovastatin production decreased in case of GD$_{13}$, EM$_{34}$ and the heterokaryon 2b (Table 4.11). However, it showed an increase of 1.6-folds in diploid 2b III (9.08 g/Kg of substrate), 3-folds in diploid 2b VIII (6.6 g/Kg of substrate), 1.83-folds in haploid 2b VIII (o) (9.1 g/Kg of substrate), respectively.
Table 4.11 Lovastatin yield of parents, heterokaryon, diploids and haploid using sorghum straw as a carbon source under SSF

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Culture name</th>
<th>Lovastatin yield (g/Kg of substrate) using distilled water</th>
<th>Lovastatin yield (g/Kg of substrate) using lovastatin production medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GD₁₃ (wild parent)</td>
<td>5.340</td>
<td>4.910</td>
</tr>
<tr>
<td>2.</td>
<td>EM₃₄ (mutant parent)</td>
<td>7.210</td>
<td>4.080</td>
</tr>
<tr>
<td>3.</td>
<td>2b (heterokaryon)</td>
<td>7.520</td>
<td>6.950</td>
</tr>
<tr>
<td>4.</td>
<td>2b III (diploid)</td>
<td>5.790</td>
<td>9.080</td>
</tr>
<tr>
<td>5.</td>
<td>2b VIII (diploid)</td>
<td>2.200</td>
<td>6.590</td>
</tr>
<tr>
<td>6.</td>
<td>2b VIII (o) (haploid derived from diploid 2b VIII)</td>
<td>4.950</td>
<td>9.1</td>
</tr>
</tbody>
</table>

4.15 Secretome analysis of the intracellular proteins of the parental strains, diploid and haploid strains

The results in fig 4.35 show protein expression profile of parent, heterokaryon and diploids. The diploid 2b VIII seemingly produced high levels of proteins.

The peptide mass fingerprinting of selected protein bands 1-10 was performed. The maximally expressed protein was vacuolar protein transportase (band 9) followed by short chain dehydrogenase (band 6). The other identified proteins were GGPP synthase (band 3), FAD synthase (band 1) and elongation factor gamma (band 2). Secretome analysis of the protein bands was followed by the RNA expression profiling to find out the genes that upregulated and resulted in enhanced production of lovastatin.

4.16 RNA expression analysis of *pks* genes involved in the production of lovastatin

To study the expression of *pks* genes involved in the production of lovastatin, cDNA was synthesized using RNA from parental and recombinant strain. The cDNA was further subjected to the PCR using 26 s gene as a control.
**Fig. 4.35** SDS-PAGE gel of parental (GD$_{13}$ and EM$_{34}$) and recombinant strains (2b and 2b VIII).

*FAD synthase, band 1; Elongation factor-gamma, band 2; GGPP-synthase, band 3; Thioredoxin reductase, band 4; Hypothetical protein, band 5; Short chain dehydrogenase, band 6; Hypothetical protein, band 7; Hypothetical protein, band 8; Vacuolar protein transportase; Transelongation factor, α*

**Fig. 4.36** RNA of the wild parent strain (GD$_{13}$), mutant strain (EM$_{34}$), heterokaryon (2b) and diploid (2b VIII)
The RNA was isolated in the form of 28 s, 18 s and 5.8 s bands on the agarose gel containing ethidium bromide (Fig 4.36). The first strand of cDNA of parental (GD₁₃ and EM₃₄), and heterokaryon (2b) and diploid (2b VIII) was amplified using primers for ketosynthase (ks) and methyltransferase (mt) domain of lovastatin nonaketide synthase (lovB) and lovastatin diketide synthase (lovF) genes. The amount of cDNA was normalized using primers corresponding to 26 S rRNA. The results in Fig. 4.37 and 4.38 show expression profile in case of ks and mt. The normalized cDNA was used to study differential expression of lovB ks, lovF ks as well as lovB mt and lovF mt genes. The results (Fig. 4.37 a) shows that expression of gene lovB ks was low in GD₁₃ but was 1.23-folds high in ethylmaleimide-resistant mutant EM₃₄ and 1.38-folds higher in the heterokaryon 2b increased to 1.61-folds higher expression in diploid when compared to GD₁₃.

In case of lovF ks, the expression was 1.49- folds higher in the heterokaryon followed by the diploid 2bVIII (1.41 folds) and the mutant parental strain (1.01-folds) as compared to the wild parent strain GD₁₃ (Fig. 4.37 b).

It was observed that the expression of lovB mt in case of heterokaryon was 2.936 folds higher than the wild parent, however, it was the highest in the diploid derived from heterokaryon 2b (Fig. 4.38 a).

Expression of lovF mt was the highest in the mutant EM₃₄ and was 2.075-folds higher as compared to the wild parent GD₁₃ (Fig. 4.38 b). The heterokaryon 2b also showed 1.825-folds higher expression, however, in diploid expression levels were almost similar to the wild type strain GD₁₃.
Fig. 4.37 The semi-RT PCR products of \textit{lovB ks} gene expressed by GD$_{13}$ (wild parent), EM$_{34}$ (mutant parent), 2b (heterokaryon) and 2bVIII (diploid).

Fig. 4.38 The semi-RT PCR products of \textit{lovB MT} gene expressed by GD$_{13}$ (wild parent), EM$_{34}$ (mutant parent), 2b (heterokaryon) and 2bVIII (diploid).