CHAPTER 3
MATERIALS AND METHODS

3.1 Microorganisms and culture maintenance

The fungal culture of *Aspergillus terreus* MTCC 279, *Monascus purpureus* MTCC 369, *Monascus ruber* MTCC 1880, *Penicillium citrinum* MTCC 1751, *Penicillium brevicompactum* MTCC 549, *Mucor hiemalis* MTCC 157 were obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained on the potato dextrose agar (PDA) slants at 4°C and sub-cultured every 30 days.

3.2 Inoculum preparation

Actively growing slants were used to prepare the spore suspension of *A. terreus* in sterile water. 10% (v/v) spore suspension was inoculated to conical flasks containing the basal medium: 100 g dextrose, 10 g peptone, 2 g KNO₃, 2 g NH₄H₂PO₄, 0.5 g MgSO₄.7H₂O and 0.1 g CaCl₂ in 1000 mL of distilled water, adjusted to pH 6. These cultures were incubated at 30°C for 48 h in a shaking incubator at 120 rpm. 5 per cent of this preculture was used to inoculate the production medium. Fermentation was carried out at 30°C for 7 days using *A. terreus*, 10 days for *M. Purpureus*, 5 days for *M. ruber*, 10 days for *P. citrinum*, 8 days for *P. brevicompactum* and 2 days for *M. hiemalis*.

3.3 Submerged fermentation

Submerged fermentation was conducted in 250 mL Erlenmeyer flasks containing 100mL of different production medium as per the statistical experimental designs. The fermentation period varies according microorganism, temperature, initial pH of the fermentation medium 30 °C, 6.0 respectively. Inoculum volume and agitation speed was maintained constant at 5% v/v and 120 rpm respectively for all fermentation runs except optimization of fermentation conditions. Various carbon, nitrogen sources and mineral salts were tested for the production of compactin, lovastatin and pravastatin using various microorganisms under submerged fermentation.
3.4 Solid state fermentation

The screening of various solid substrates and agriculture waste were tested for compactin, lovastatin production. Agricultural waste such as wheat bran (Triticum spp bran), cotton seed powder (Gossypium spp seed), bagasse, ground nut cake (Arachis hypogaea cake), coffee husk powder (coffeae arabica husk) and tamarind seed powder (Tamarindus indica seed) and solid substrates such as besan flour (Cicar aritinum), ragi (Eleusine coracana), millet (Pennisetum Typhoides), rice flour, black gram (Vigna mungo), green gram (Vigna radiata), green peas (Pisum sativum), yellow peas (Lathyrus aphaca), white beans (Phaseolus vulgaris), maize (Zea mays), rice bran (Oryza Sativa Francois), long grain rice (Oryza sativa indica), sweet potato powder (Ipomoes batatas) and sago starch (Manihot esculanta), were tested. Experiments were carried out in 250 mL Erlenmeyer flasks containing 5 g of dry substrate, supplemented with mineral solution to have an initial moisture content of 66% (w/w). 5 mL of nutrient supplement solution consisting of glucose (5%, w/v), malt extract (1%, w/v), MgSO₄ (0.07%, w/v) and MnSO₄ (0.2%, w/v) was used to adjust the initial moisture content. The contents of the flasks were mixed and autoclaved at 121°C at 15 psi for 20 min. The seed medium was inoculated with 10⁶ spores mL⁻¹ and incubated at 25°C for 72 h.

3.5 Mixed substrate solid state fermentation

The substrates that yielded higher compactin and lovastatin production were selected for the mixed substrate SSF experiments. The concentration of the substrates in various combinations was determined by central composite design (CCD) for compactin and lovastatin production using A. terreus, M. Purpureus, M. ruber, P. citrinum, P. brevicompactum. The contents of the flasks were mixed, autoclaved at 15 psi and the fermentation was carried out at 30°C for 7 days with pre-optimized initial moisture content of 66.66% and inoculum size of 5 % active culture.

3.6 Mixed culture solid state fermentation

The high yielding strains A. terreus, M. Purpureus, M. ruber, P. citrinum, P. brevicompactum were taken for the production of compactin and lovastatin in co-culture fermentation. Actively growing slants were taken and spore suspension of two
strains was prepared using sterile water. 5% v/v spore suspension was inoculated to separate conical flask containing the basal medium: 100 g dextrose, 10 g peptone, 2 g KNO₃, 2 g NH₄H₂PO₄, 0.5 g MgSO₄·7H₂O and 0.1 g CaCl₂ in 1000 mL of distilled water, adjusted to pH 6. These cultures were incubated at 30 °C for 48h in a shaking incubator at 120 rpm. 5 g of substrate was taken in a 250 mL Erlenmeyer flask and 5 mL of basal medium containing 38.9 g dextrose, 9.68 g malt extract, 1.96 g MnSO₄·H₂O, 0.73 g MgSO₄·7H₂O was added to 1000 mL. The contents of the flask were mixed, autoclaved and cooled. The flask which contains the substrate was inoculated with two different cultures into the same fermentation flask. The fermentation was carried out at 30 °C for the maximum period of the two strains taken, with inoculum size of 2.5 mL of each two cultures total of 5 mL were inoculated into the fermentation medium.

3.7 Mixed substrate- mixed culture solid state fermentation

The three high yielding compactin and lovastatin substrates at various combinations as determined by CCD were chosen for mixed substrate mixed culture SSF. The substrates for various mixed culture fermentation used in this study was shown below:

i. Green peas, long grain and barley with *A. terreus* and *M. Purpureus*

ii. Green peas, long grain and barley with *M. Purpureus* and *M. ruber*

iii. Green peas, millet and ragi with *A. terreus* and *M. ruber*

The optimized fermentation condition such as incubation temperature, initial moisture and inoculum size was explained in co-culture fermentation. Various combinations of substrates were optimized using RSM with different co-culture for highest compactin and lovastatin production.

3.8 Extraction of compactin and lovastatin

After fermentation, the harvested samples were homogenized to recover the intracellular product. An equal volume of ethanol was added to fermentation broth and the suspension was kept in an incubated rotary shaker for 1 h at 200 rpm and 40°C. The suspension was filtered through a whatman filter paper and then through a micro filter
(Millipore) of 0.22 mm pore diameter. 20 μL of the filtrate was analyzed for compactin and lovastatin by HPLC.

3.9 Recovery of pravastatin

After bioconversion of compactin to pravastatin, an equal volume of ethanol was added to fermentation broth and the suspension was kept in an incubated rotary shaker for 1 h at 200 rpm and 40°C. The suspension was filtered through a whatman filter paper and then through a micro filter (Millipore) of 0.22 mm pore diameter. 20 μL of the filtrate was analyzed for pravastatin in HPLC.

3.10 Analysis of compactin, lovastatin and pravastatin

Analysis of compactin was carried out in Shimadzu HPLC (LC20 AT prominence) at 238 nm in Luna C18 column of particle size 5 μm and (250 x 4.6) mm I.D, UV detector (SPD 20 A) and the column oven (CTO-10 AS vp) at 45°C. Binary gradient system was used and the samples were injected manually using Rheodyne injector of 20 μL. The mobile phase used was ACN and 0.1% orthophosphoric acid in the ratio of 60:40 respectively. The eluent was pumped at a flow rate of 1.5 mL min⁻¹. Standard compactin containing 5 mg were obtained from Sigma-Aldrich. Various concentrations of compactin dissolved in ACN was prepared and analyzed in HPLC. The equation of the standard curve for the various concentrations of compactin (Y) versus peak area (X) is 

\[ Y = 49870 \times X \text{ with } R^2 = 0.9952. \]

The retention time of compactin elutes at 9.5 min of a fermented sample. Pharmaceutical grade lovastatin (lactone form) tablets containing 40 mg lovastatin per tablet were obtained from Merck Laboratories. Various concentrations of lovastatin dissolved in ACN was prepared and analyzed in HPLC. The equation of the standard curve for the various concentrations of lovastatin (Y) versus peak area (X) is 

\[ Y = 44250 \times X \text{ with } R^2 = 0.993. \]

As lovastatin is produced as a mixture of lactone and free β-hydroxyacid form, the standards were prepared in both the forms. The retention time of lovastatin in its beta hydroxyacid form elutes at 6.5 min and the chromatography of a fermented sample. Various concentrations of pravastatin dissolved in ACN was prepared and analyzed in HPLC. The equation of the standard curve for the various concentrations of pravastatin (Y) versus peak area (X) is 

\[ Y = 24685 \times X \text{ with } R^2 = 0.998. \]
As pravastatin is produced as a mixture of lactone and free β-hydroxyacid form, the standards were prepared in both the forms. The retention time of pravastatin in its beta hydroxyacid form elutes at 2.5 min and the chromatography of a fermented sample.

3.11 Plackett-Burman design

Biochemical systems are multivariable processes, in which numerous potentially influential factors are involved, it is necessary to analyze the process with an initial screening prior to optimization (Montgomery, 2001; Box et al., 1978). The PB experimental design was used to evaluate the relative importance of various nutrients for the production of compactin and lovastatin by various microbes such as strains A. terreus, M. Purpureus, M. ruber, P. citrinum and P. brevicompactum in submerge fermentation. The effect of each variable was determined with the following equation.

\[ E_{xi} = 2 \left( \Sigma H_{xi} - \Sigma L_{xi} \right) / N \]  
Eq 1

Where, \( E_{xi} \) is the concentration effect of the tested variable, \( H_{xi} \) and \( L_{xi} \) are the concentration of compactin or lovastatin at high level and low level of the same variable. The main effect of the components is negative, it indicates that the concentration required for enhancing compactin or lovastatin production is lower than the concentration used in the PB design. Similarly if the effects is positive, the amount of required for the production of compactin or lovastatin was higher than the concentration used in the design.

3.12 Response surface methodology

Statistical methods provide an efficient alternative methodology for traditional one factor at a time approach to optimize a particular process by considering the mutual interactions among the variables and to give an estimate of the combined effects of these variables (Lim et al., 2005). An orthogonal \( 2^3 \) factorial central composite experimental design with six star points (\( \alpha =1.682 \)) and six replicates at the centre with a total of 20 experiments \( N = 2^k + 2k + n_0 = 20 \) were used to optimize the various combination of mixed substrates. The total run number for CCD with respect to the concentration of the components is determined by full factorial points \( 2^k \), where \( k \) is the number of variables, at centre points and two axial points for each variable \( (a=2^k/4, \)
which is =2 for k=3). For statistical calculation, the test factors were coded by the following equation:

\[ x_i = \frac{(X_i - X_0)}{\Delta X_i}, \quad i = 1, 2, 3, \ldots, k \]

Eq 2

Where \( x_i \) in Eqn (1) is the dimensionless value of an independent variable, \( X_i \) is the real value of an independent variable; \( X_0 \) is the real value of the independent variable at the centre point; \( \Delta X_i \) is the step change value. These components were tested at five coded levels namely -1.682, -1, 0, +1 and +1.682. The experimental data obtained was fitted to the following quadratic polynomial equation:

\[ Y = X_0 + \sum x_i + \sum x_i^2 + \sum x_i x_j \]

Eq 3

where yield (Y) is the predicted response variable in Eqn (2), \( i \) and \( j \) are the linear and quadratic coefficients respectively, \( \beta \) is the regression coefficient of the model and \( x_i, x_j \) \((i = 1, 3; j = 1, 3, l = j)\) represent the independent variables (media components) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination \( R^2 \). Design expert software (version 6.0.5; Stat-Ease. Inc., MN, USA) was used for the regression and graphical analysis of the experimental data. The optimum levels of the selected variables were obtained by solving the regression equation using MATLAB software and by analyzing the response surface and contour plots.