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A Novel Approach for Enhancement of Lovastatin Production using *Aspergillus* Species

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Abstract

Submerged cultures (SmF) of filamentous fungi (*Aspergillus terreus, Aspergillus flavus*) are widely used to produce commercially important metabolite lovastatin. Limited investigations have been made on optimization of the different nutrients and process parameters using standard optimization methods**.** No previous work has used statistical analysis in documenting the interactions between nutritional and process parameters in lovastatin production. With this lacunae**,** Plackett-Burman experimental design was used for the first time to screen and investigate the effects of the nine factors —i.e. Source (lactose, glycerol and honey) and concentrations of carbon (40–80 g/L), source (mycological peptone and yeast extract) and concentrations of Nitrogen (5–25 g/L); p^H of the fermentation process (6.0-7.6); Temperature of the fermentation process (24 ^oC –32 ^oC) ; Agitation (120 – 200 rpm) and Fermentation time (5–13 days) —on the concentrations of lovastatin produced in batch cultures (SmF) of *Aspergillus terreus* (newly discovered strains (nhceup) MTCC-11045, *Aspergillus terreus* (NHCEUPBT) MTCC-11395, *Aspergillus flavus* (NHCEUPBTE) MTCC-11396 and *Aspergillus terreus* MTCC-1782. Lovastatin in the sample was confirmed and estimated by UV Spectrophotometry, HPLC and FTIR analysis; Plackett-Burman design identified the "source and concentrations of C, N, pH and incubation period" were the principal factor influencing the production of lovastatin. Temperature and agitation were found to have least impact on lovastatin production. Botha a limitation and excess of carbon and nitrogen reduced lovastatin titers. A medium containing 70 g/L carbon supplied as lactose, 20 g/L nitrogen supplied Yeast extract, 6.8p^H, and 10 days of incubation period were shown to support high titers (2990 mg/L) of lovastatin production in submerged fermentation process. The above optimized fermentation conditions raised the lovastatin titer by 1.7-fold compared with the yield (1761.6 mg/L) of lovastatins by using Dox-rice medium as a carbon. Optimization by using Response Surface Methodology is under study.

Highliights

Keywords: *Aspergillus terreus*, Lovastatin, Plackett-Burman design, RP-HPLC, FTIR, Lactose.

Introduction

Lovastatin is a competitive inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A reductase, the rate limiting enzyme of cholesterol biosynthesis pathway (Alberts *et al.*, 1980). Both in vitro and in vivo studies of lovastatin in humans and animals exhibit potent inhibitory activity on cholesterol biosynthesis and plasma cholesterol level (Kaneko *et al.*,

1978). HMG-CoA reductase inhibition has beneficial pleiotropic effects (Camelia *et al.,* 2008). Lovastatin have been shown to inhibit cellular proliferation and induce apoptosis and necrosis in several experimental settings including that of breast cancer, thus making them potential anticancer agents (Kalwitter *et al.,*2010). Some preclinical studies were suggested that lovastatin administered in a nanobead preparation may be therapeutically useful in hastening repair of human fractures (Olivera *et al.,* 2010). Lovastatin inhibits brain endothelial cell Rho-mediated lymphocyte migration and attenuates experimental autoimmune encephalomyelitis (Greenwood *et al.,* 2003). Fermentation derived lovastatin is a precursor for Simvastatin, a powerful semi-synthetic statin commercially available as Zocor ™.

Aspergillus terreus, Aspergillus oryzae are known to produce cholesterol reducing drug- Lovastatin **(**Novak *et al*., 1997). Lovastatin isolated from *Aspergillus terreus* was the first statin to be approved by FDA in 1987 for therapeutic use (Devi *et al.,* 2011). Submerged cultures of filamentous fungi (*Aspergillus terreus, Aspergillus flavus*) are widely used to produce commercially important metabolites such as lovastatin (C24H36O5), Mevinolin, Monacolin K and Mevacor (Lopez *et al.,* 2004). In earlier period, lovastatin was produced by liquid surface fermentation technique (Gabriela *et al.,* 1999) but currently submerged state fermentation (SmF) techniques (Jaivel *et al*., 2010) are employed throughout the world. Optimization of the different parameters of submerged fermentation process (Smf) has been done by the various researchers (Atalla *et al*., 1999, Atalla *et al*., 2008, Osman *et al*., 2011,). Limited investigations have been made on optimization of the different nutrients and process parameters using standard optimization methods (Lopez *et al.,* 2004, Sayyad *et al*., 2007). No previous work has used statistical analysis in documenting the interactions between nutrient concentrations and process parameters in lovastatin production.

Therefore in this present investigation, Plackett-Burman experimental design was used for the first time to screen and investigate the effects of the nine factors —i.e. Source and concentrations of Carbon, source and concentrations of Nitrogen; p^H of the fermentation process; Temperature of the fermentation process; Agitation and fermentation time —on the concentrations of lovastatin produced in batch cultures (Smf) of *Aspergillus terreus* (newly discovered strains (nhceup) MTCC-11045, *Aspergillus terreus* (NHCEUPBT) MTCC-11395, *Aspergillus flavus* (NHCEUPBTE) MTCC-11396 and *Aspergillus terreus* MTCC-1782. The values of the various factors in the experiment ranged widely, as follows: Three different carbon sources namely lactose, glycerol and honey were tested at different concentration ranging from 40–80 g/L. Two different nitrogen sources: mycological peptone and yeast extract were tested at different concentration ranging from 5–25 g/L, with pH of the fermentation process varying in 6.0-7.6 and temperature being 24^{o}C –32^{o}C. Agitation of the media was set in between 120 – 200 rpm and incubation period being 5–13 days. Lovastatin in the sample was confirmed and estimated by UV Spec, HPLC and FTIR analysis; The Plackett-Burman design identified the "source and concentrations of C, N, pH and incubation period" were the principal factor influencing the production of lovastatin. Temperature and agitation were found to have least impact on lovastatin production. Both a limitation and excess of carbon and nitrogen reduced lovastatin titers. A medium containing 70 g/L carbon supplied as lactose, 20 g/L nitrogen supplied Yeast extract, 6.8p^H and 10 days of incubation period was shown to support high titers (2990 mg/L) of lovastatin production in submerged fermentation process.

Material and methods

All the chemicals and reagents used in this study were of high purity (Analytical grade, Merck & Qualigens brand).

Microorganisms: The cultures of *Aspergillus terreus* (newly discovered strains (nhceup**)** MTCC-11045, *Aspergillus terreus* MTCC 1782 obtained from Microbial Technology (IMTECH), *Aspergillus terreus* (NHCEUPBT) MTCC-11395, *Aspergillus flavus* (NHCEUPBTE) MTCC-11396 were employed in the present study. Newly Isolated strains of *Aspergillus terreus* (SSM3) and *Aspergillus flavus* (SSM8) were sequenced by Internal Transcribed Regions (ITS) and deposited with Microbial Type Culture Collection & Gene Bank of the Institute of Microbial Technology (IMTECH), Chandigarh, India. All the cultures were initially screened for contamination and the pure cultures were maintained on potato dextrose agar (PDA) medium slants at 4°Cand subcultured in 30-day intervals (Sayyad *et al.,* 2007, Panda *et al.,* 2010).

Plackett-Burman experimental design: Lactose, Glycerol, Honey, Mycological peptone, Yeast extract were the five medium constituents, p^H of the fermentation process; Temperature of the fermentation process; Agitation and Fermentation time were the four process parameters total nine parameters were selected for the study. The

Plackett-Burman experimental design for eleven variables (Tables 1and 2), i.e. five nutritional components, four process parameters and two dummy variables, were used to evaluate the impact on the enhanced production of lovastatin. The values of the various factors in the experiment ranged widely, as follows: Three different carbon sources namely lactose, glycerol and honey were tested at different concentration ranging from 40–80 g/L. Two different nitrogen sources: mycological peptone and yeast extract were tested at different concentration ranging from 5–25 g/L, with pH of the fermentation process varying in 6.0-7.6 and temperature being 24°C –32°C. Agitation of the media was set in between 120 – 200 rpm and incubation period being 5–13 days (Table-1). Data analysis was carried out by the standard procedure of Plackett-Burman experimental design **(**Plackett & Burman, 1946; Chauhan *et al.,* 2006, Abdel-Fattah *et al.*, 2007).

Preparation of inoculum cultures: Spore suspensions of *Aspergillus terreus* MTCC-11045, *Aspergillus terreus* MTCC-1782, *Aspergillus terreus* (NHCEUPBT) MTCC-11395, *Aspergillus flavus* (NHCEUPBTE) MTCC-11396 were prepared from actively growing slants by single spore isolation technique using 2% Tween 20 solution (Wang *et al.*, 1997) and diluted to a concentration of 5.7 x 10^6 spores per ml. Spore counting was carried out using a hemocytometer. A total of 7.5 ml (15%) of spore suspension was inoculated into conical flasks containing 50 ml of the basal medium (Composition per litre: corn steep liquor 5g, tomato paste 40g, oat meal 10g, glucose 10g, trace element stock solution 10ml (Composition per litre:$FeSO_4.7H_2O$ 1g, $MnSO_4.4H_2O$ 1g, $CuCl_2.2H_2O$ 25mg, $CaCl_2.2H_2O$ 100mg, H_3BO_3 56mg, $(NH_4)_6Mo_7O_{24}.2H_2O$ 19mg, $ZnSO_4.7H_2O$ 200mg and 1 litre of distilled water ,p^H adjusted to 6.8). The seed cultures were incubated in a rotary shaker-incubator at 180 rpm at 28°C for 24 hours (Siamak *et al.,* 2003).

Submerged fermentation process (SmF): Ten percent of the seed broth was used as inoculums for the production medium (Lactose 70g, Yeast extract 20g, Mycological peptone 5g, Tomato paste 30g,Oat meal 20g, Sodium acetate 10g Ammonium sulphate 5g Potassium dihydrogen phosphate 2g, Trace element stock solution 10ml (Composition per litre:$FeSO_4.7H_2O$ 1g, $MnSO_4.4H_2O$ 1g, $CuCl_2.2H_2O$ 25mg, $CaCl_2.2H_2O$ 100mg, H_3BO_3 56mg, $(NH_4)_6Mo_7O_{24}.2H_2O$ 19mg, $ZnSO_4.7H_2O$ 200mg and 1 litre of distilled water ,p^H adjusted to 6.8) (adjust using 1 M NaOH).The 250 ml culture flasks each containing 50 ml of production medium were incubated in a rotary shaker-incubator at 180 rpm at 28°C for 10 days.

Down Stream Processing of Lovastatin from SmF samples: After 10 days of fermentation process, p^H of the final fermentation broth was measured and adjusted to 2.1 using dilute H_3PO_4 (2N) or dilute sulphuric acid (2N). Acidified broth was heated to about 50°C and stir at 50–60 °C for about 24 hours. (90% of the acid form was converted to lactone form) and filtered using Whatman no 1filter paper. Mycelial cake and filtered broth was extracted separately with equal quantity (50 ml) of toluene in a shaker incubator (180 rpm) at 35^0 C for 2 hours. Solvent layer (organic phase) was separated by cold centrifugation at 3000 g (8300 rpm) for 8 min and toluene extract was concentrated at about 60° C. The slurry was dissolved in 5 ml of acetonitrile and taken for the analysis (Kumar *et al*., 2006).

Table 1: Concentrations of variables of liquid medium in Plackett-Burman design for submerged-state fermentation.

Designation	Variables	Low Level (-) Per Liter	High Level (-) Per Liter
X_1	Glycerol	40 g	80g
X_2	Lactose	40 g	80g
X_3	Honey	40 g	80g
X_4	Temperature	24°C	28°C
X_5	P^H	6.0	7.6
D_1	Dummy 1	-	-
X_6	Agitation speed(rpm)	120	200
X_7	Incubation period(days)	5	13
X_8	Yeast extract	5 g	25 g
X_9	Mycological peptone	5 g	25 g
D_2	Dummy 2	-	-

UV Spectrophotometric analysis of lovastatin: Prepared sample were analyzed qualitatively for the presence of lovastatin at different nm (210 nm – 350nm), subsequently lovastatin was detected and estimated at 238 nm, using pure lovastatin (Biocon laboratories, Bangalore, India) as a standard in UV/Visible spectrophotometer. (Shimadzu, Model no UV-2450 and Software UV-probe 2.21) (Lingappa *et al*., 2004).

HPLC analysis of lovastatin: The prepared sample extracts and lovastatin standard (Biocon laboratories, Bangalore, India) were quantitatively analyzed for the presence of lovastatin in the fungal extracts. HPLC analysis was carried out at 238 nm by using reverse phased 250 x 4.6 mm inertsil ODS -3 C 18 column of 5 um particle size, 20 µl loop injector, Shimadzu instrument model: Ao6SM7696M, version 5.032 software. The eluent was a mixture of acetonitrile and 0.1 per cent phosphoric acid (60:40 v/v), flow rate was 1.2 ml min-1 and temperature of column was at 25 °C (Morovjan *et al.*, 1997; Sayyad *et al*., 2007). Lovastatin was quantified as its lactone form and ß hydroxy acid form, by HPLC. As the open hydroxy acid form of lovastatin is unstable, it was prepared freshly from lactone form, whenever necessary. The lovastatin lactone form was suspended in 0.1M NaOH and heated at 50°C for at least one hour in a shaking water bath. Subsequently, the suspension was adjusted to p^H 7.7 with 1 M HCl, filtered through 0.45 µm filters and diluted to the concentration of 5 µg /ml (Friedrich *et al.*, 1995).

ATR-FTIR analysis of lovastatin: Final confirmation of lovastatin in the prepared sample was done by using FTIR/ Diamond ATR, Model: FTIR-8400S, Brand Name: Shimadzu. ATR was fitted with a single bounce diamond at 45° internally reflected incident light providing a sampling area of 1 mm in diameter with a sampling depth of several microns. A small amount of the sample was directly placed on the diamond disk and liquid sample kept in liquid sample holder. Sample was scanned for absorbance over the range from 4000 to 400 wave numbers (cm-1) at solution of 1 cm-1 (Nazzal *et al*., 2002; Patel *et al.*, 2010).

Results and Discussion

Plackett-Burman experimental design: Maximum lovastatin production was found in the experimental trial 4, whereas, minimum in trial 11(Table 2) under submerged state fermentation by *Aspergillus terreus* (newly discovered strain- nhceup) MTCC-11045. The Plackett Burman design identified the "source and concentrations of C, N, p^H and incubation period" were the principal factor influencing the production of lovastatin. Temperature and agitation were found to have least impact on lovastatin production (Table – 2). Both a limitation and excess of carbon and nitrogen reduced lovastatin titers. A medium containing 70 g/L carbon supplied as lactose, 20 g/L nitrogen supplied as Yeast extract, 6.8 pH and 10 days of incubation period were shown to support high titers (3.065 mg/L) of lovastatin production in submerged fermentation process. The effects of various nutritional factors and process parameters on lovastatin production based on the observations of Plackett- Burman design experiments were shown in (Table 3). Results showed that the main parameters affecting the production of the lovastatin were determined as Lactose, Yeast extract, P^H and Incubation period with Mean Square of 6.953, 8.793, 9.020 and 6.954 respectively and very high *F* values (41.970, 3.951,2.230 and 41.960, resp.).

Table 2: Plackett-Burman experimental design of 12 trials for eleven variables (5 nutrients + 4 processes + 2 dummy) (+ High level, - Low level) along with observed concentration of Lovastatin in fermented broth

Trail	X_1	X_2	X_3	X_4	X_5	D_1	X_6	X_7	X_8	X_9	D_2	Lovastatin mg/L
1	+	+	-	+	+	+	-	-	-	+	-	1.328
2	-	+	+	-	+	+	+	-	-	-	+	1.533
3	+	-	+	+	-	+	+	+	-	-	-	1.430
4	-	+	-	+	+	-	+	+	+	-	-	3.065
5	-	-	+	-	+	+	-	+	+	+	-	1.653
6	-	-	-	+	-	+	+	-	+	+	+	0.817
7	+	-	-	-	+	-	+	+	-	+	+	1.532
8	+	+	-	-	-	+	-	+	+	-	+	2.452
9	+	+	+	-	-	-	+	-	+	+	-	1.737
10	-	+	+	+	-	-	-	+	-	+	+	2.146
11	+	-	+	+	+	-	-	-	+	-	+	0.715
12	-	-	-	-	-	-	-	-	-	-	-	0.624

Table 3: Influence of medium variables on lovastatin production

Designation	Variables	ÓH	ÓL	Mean square	Effects	F-Value	% of contribution
X_1	Glycerol	9.194	10.020	0.062	-0.150	1.016	0.0102
X_2	Lactose	12.261	6.953	2.560	0.965	41.970	0.4252
X_3	Honey	9.196	10.018	0.061	-0.140	1.000	0.0101
X_4	Temperature	10.101	9.013	0.108	0.197	1.770	0.0179
X_5	P^H	10.250	9.020	0.136	0.220	2.230	0.0225
D_1	Dummy 1	9.156	10.019	0.016	-0.148	1.000	0.0101
X_6	Agitation speed(rpm)	10.114	9.100	0.093	0.184	1.525	0.0154
X_7	Incubation period	12.260	6.954	2.560	0.964	41.960	0.4251
X_8	Yeast extract	10.412	8.793	0.214	0.296	3.951	0.0400
X_9	Mycological peptone	9.195	10.019	0.061	-0.150	1.000	0.0101
D_2	Dummy 2	9.200	10.120	0.077	-0.148	1.262	0.0127

UV Spectrophotometric analysis of lovastatin SmF samples: The qualitative confirmation and quantitative estimation of lovastatin was done spectrophotometrically. The samples and the standard exhibited a peak at 238 nm in the spectrophotometer scanning in Fig 1. Yields of lovastatin of different culture were found to be (newly discovered strains) *Aspergillus terreus* (nhceup) MTCC-11045 (2990 mg/L), *Aspergillus terreus* (nhceupbt) MTCC-11395 (2250 mg/L), *Aspergillus flavus* (nhceupbte) (1650 mg/L) and *Aspergillus terreus MTCC-1782* (900 mg/L). From the results, it was deduced that among four cultures *Aspergillus terreus* (newly discovered strain- nhceup) MTCC-11045 had shown highest titer of lovastatin Production in submerged fermentation process screened and optimized by Plackett-Burman experimental design. Submerged fermentation process yield of lovastatin (2990 mg/L) was almost similar with Experimental depicted values of lovastatin (3065 mg/L).

HPLC analysis of lovastatin: HPLC analysis also confirmed quantitatively the amount of lovastatin in the fungal extract. Retention time of standard lovastatin and sample (*Aspergillus terreus* MTCC-11045) for β hydroxy acid form was 9.947 and 9.977 min respectively and for lactone form 17.225 and 17.175 respectively (Fig. 2- A, B,

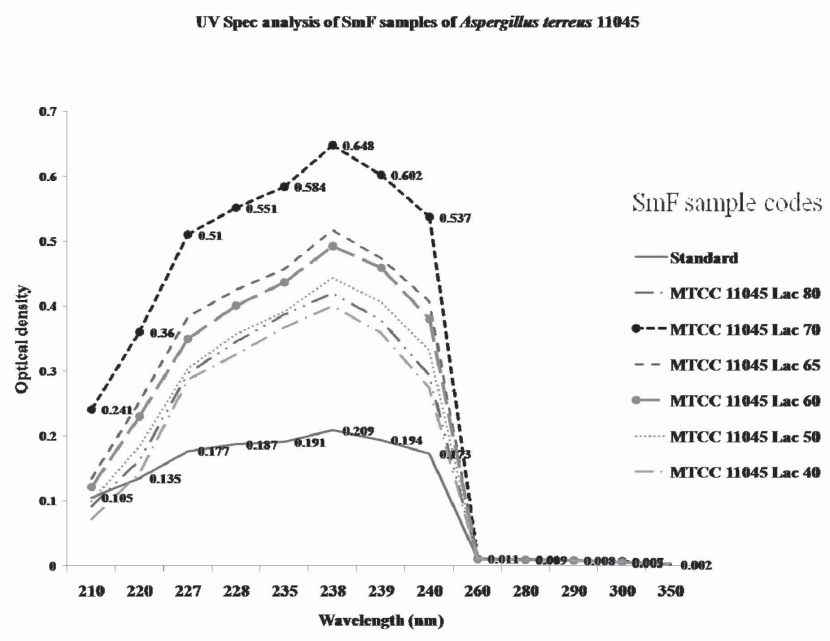

Fig 1: UV Spectrophotometric analysis of lovastatin (SmF sample)

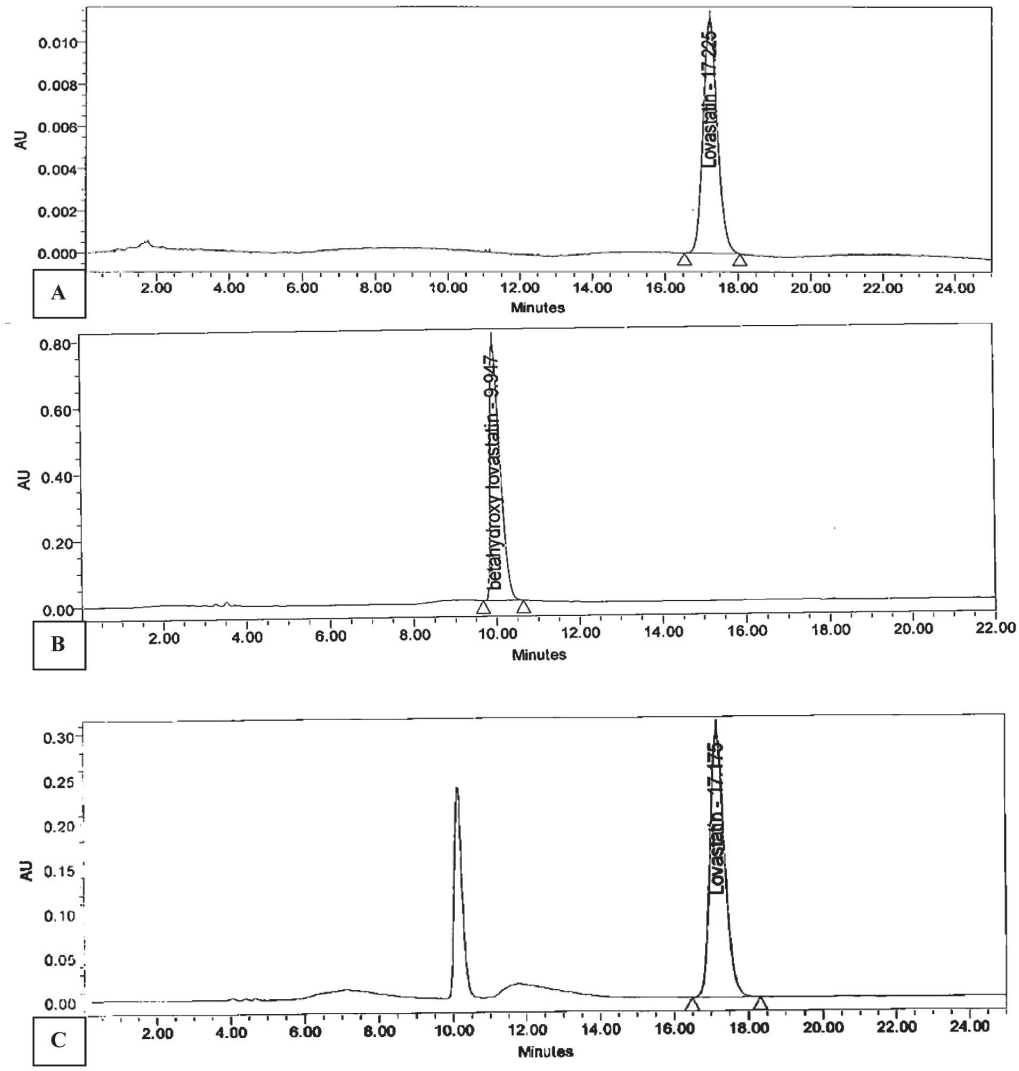

Fig. 2: Reverse Phase HPLC analysis of Lovastatin

A. Lactone form of standard lovastatin .B. β-hydroxy acid form of standard lovastatin C. *Aspergillus terreus* MTCC-11045(SmF extract).

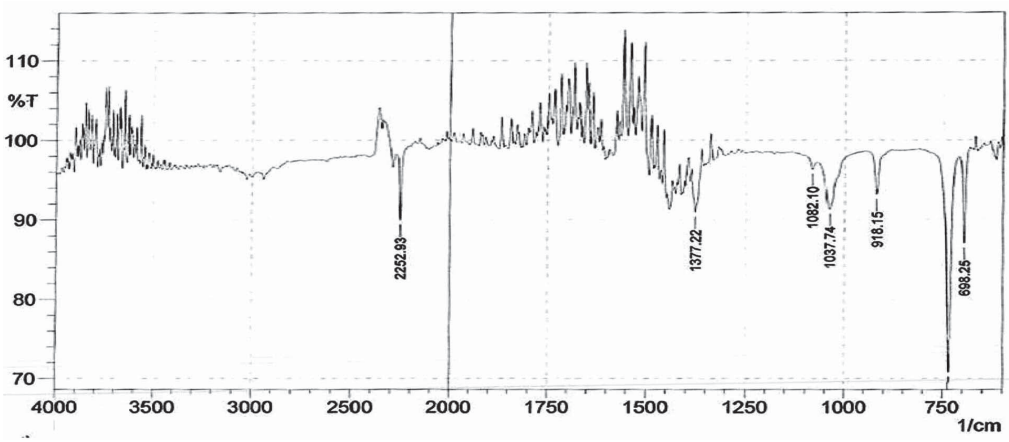

Fig 3: ATR-FTIR analysis of *Aspergillus terreus* MTCC-11045SmF extracts (Lovastatin)

C). Qualitative analysis and quantification of lovastatin in HPLC was carried out by considering both forms of lovastatin (lactone and β-hydroxy acid form) β-hydroxy acid form elutes earlier in the chromatographic column followed by lactone form (Friedrich *et al.,* 1995) and also the fermentation extract was lactonized which contains principally consists lovastatin in the form of lactone form.

ATR-FTIR analysis of lovastatin: The ATR-FTIR spectra of *Aspergillus terreus* MTCC-11045 smf extracts (Lovastatin) were shown in (Fig. 3). The spectrum presented characteristic peaks at 3538 cm-1 (alcohol O-H stretching), 3011 cm-1 (olefinic C-H stretching), 2920 cm-1 (Methylene C-H asymmetric stretching), 2252.93 cm-1 (disturbed alkynes) 1728, 1710, 1700 cm-1 (lactone and ester carbonyl stretch), 1377.2 cm-1 (methyl symmetric bend), 1359 cm-1 (methyl symmetric bend respectively), 1260 cm-1 (lactone C-O-C asymmetric bend), 1082.10 cm-1(lactone CC symmetric bend), 1037.74 cm-1 (ester C-O-C symmetric bend), 918.15 cm-1 (alcohol C-OH stretch) and 750 cm-1 (benzene strong) and 698.25 cm-1(C-H, cis-disubstituted alkenes strong)and confirm the presence of lovastatin in the samples. Lovastatin containing the lactone ring gives characteristic peak at 1725, 1711, 1700 cm-1 (Patel *et al.,* 2010, Wikipedia).

Conclusion

In the present study, attempts were made to Screening and investigating the effects of the nine factors —i.e. Source (lactose, glycerol and honey) and concentrations of carbon (40–80 g/L), source (mycological peptone and yeast extract) and concentrations of Nitrogen (5–25 g/L); p^H of the fermentation process (6.0-7.6); Temperature of the fermentation process (24 ^oC –32 ^oC); Agitation (120 – 200 rpm) and Fermentation time (5–13 days) —on the concentrations of lovastatin produced in batch cultures (Smf) of (newly discovered strains) *Aspergillus terreus* (NHCEUP) MTCC-11045 SmF extracts, *Aspergillus terreus* (NHCEUPBT) MTCC-11395 and *Aspergillus flavus* (NHCEUPBTE) and *Aspergillus terreus* MTCC-1782. Lovastatin in the sample was confirmed and estimated by UV Spec, HPLC and FTIR analysis. The above optimized fermentation conditions raised the lovastatin titer by 1.7-fold compared with the yield (1761.6 mg/L) of lovastatins by using Dox-rice medium as a carbon source (Atalla *et al.,* 2008), 15.3 – fold compared with the yield (188.3 mg/L) using oat meal as a carbon source (Osman *et al.,* 2011), 14.3 –fold compared with the yield (202.8mgdm^{-3}) using lactose as a carbon source (Lopez *et al.*, 2004). Optimization of Solid State Fermentation (SSF) process by using Response Surface Methodology is under study.

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