ISSN 1330-9862
(FTB-2143)

original scientific paper

Supercritical Fluid Extraction of Lovastatin from the Wheat Bran Obtained after Solid-State Fermentation

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Received: August 6, 2008
Accepted: February 4, 2009

Summary

The objective of the present work is to extract lovastatin with minimum impurity by using supercritical carbon dioxide (SC-CO_2). A strain of *Aspergillus terreus* UV 1617 was used to produce lovastatin by solid-state fermentation (SSF) on wheat bran as a solid substrate. Extraction of lovastatin and its hydroxy acid form was initially carried out using organic solvents. Among the different screened solvents, acetonitrile was found to be the most efficient. SC-CO_2 was used for extraction of lovastatin from the dry fermented matter. The effect of supercritical extraction parameters such as the amount of an *in situ* pretreatment solvent, temperature, pressure, flow rate and contact time were investigated. The maximum recovery of lovastatin was obtained with 5 mL of methanol as an *in situ* pretreatment solvent for 1.5 g of solid matrix, flow rate of the supercritical solvent 2 L/min, temperature 50 °C, and contact time 155 min at a pressure 300 bar. The lovastatin extract obtained after optimizing the conditions of supercritical fluid extraction was found to have 5-fold more HPLC purity than the organic solvent extract.

Key words: supercritical CO_2 extraction, solid-state fermentation, lovastatin, *Aspergillus terreus*

Introduction

Compelling regulations on the use of hazardous, carcinogenic, or toxic solvents, as well as high energy costs for solvent regeneration have curtailed the growth of the natural extract industries. One of the alternative extraction methodologies is the supercritical fluid extraction (SFE) technique that complies with both the consumer preference and regulatory control. SFE uses clean, safe, nonflammable, noncorrosive, nontoxic and environmentally friendly and nonpolluting solvents that do not leave behind any harmful residues. Its near-ambient critical temperature (31.1 °C), good solvation power, low viscosity and high diffusivity make it ideally suitable for thermolabile natural products especially in food and pharmaceutical applications. SFE is suitable for extraction of the non-polar compounds having a relative molecular mass lower than 500 Da (*1*). There are few reports on SC-CO_2 extraction of microbial metabolites directly from the fermented biomass such as microbial lipids from the alga *Scenedesmus obliquus* (*2*). Freeze-drying prior to SFE has been shown to be advantageous for extraction of lipids from several microbial biomasses. These include eicosapentaenoic acids (EPA) from the fungus *Saprolegnia costatum* (*3*); from the microalga *Skeletonema costatum*, a marine diatom; from *Ochromonas danica*, a fresh water phytoflagellate (*4*); and also from the fungi of the genus *Mortierella* (*5*). There are also reports on the isolation of carotenoids and chlorophyll a from *Nannochloropsis gaditana* and from *Synechococcus* sp. (*6,7*). Recently, griseofulvin extraction from the solid matrix obtained after solid-state fermentation (SSF) has also been reported (*8*).

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Lovastatin, a potent drug for lowering blood cholesterol, acts by competitively inhibiting the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A reductase (HMG--CoA), which catalyzes the rate limiting step of cholesterol biosynthesis (*9*). It has also been reported as a potential therapeutic agent for suppressing tumor growth through the inhibition of nonsterol isoprenoid synthesis (*10*). Lovastatin is produced by various filamentous fungi such as *Aspergillus terreus* (*9,11*), *Penicillium citrinum* (*12*) and *Monascus rubber* (*13*). Commercial production of lovastatin on media optimized by response surface methodology (RSM) is based on batch fermentation using *A. terreus*, and most literature deals with this species (*14–18*). Szakács *et al.* (*19*) reported lovastatin production both by submerged as well as SSF.

In the fermentation broth, lovastatin is mostly present in its hydroxy acid form (lovastatin hydroxy acid or mevinolinic acid). Lovastatin, a β-hydroxy lactone, is sparingly soluble in water and soluble in organic solvents, while β-hydroxy acid is water-soluble. There are few reports on the purification of lovastatin, with most of available literature being patented. Kumar *et al.* (*20*) patented a purification process for isolation of lovastatin on large scale (6 to 8500 L) extraction, wherein the acidified fermentation broth was extracted with toluene. In another patent (*21*) the acidified fermentation broth was extracted with butyl acetate and *n*-octanol (49:51), or ethyl acetate and cyclohexane (65:35). Lovastatin is a slightly non-polar compound with a molecular mass of 404.55 Da, making it amenable to SC-CO_2 extraction. The solubility of the lovastatin in pure CO_2 and that modified with various solvents was investigated by Larson and King (*22*). Taylor *et al.* (*23*) investigated primary and secondary modifiers for the subcritical extraction of lovastatin from Mevacor tablets with carbon dioxide.

The present work investigates the potential of SC-CO_2 for extraction of lovastatin and its hydroxy acid form directly from the solid matrix obtained after SSF, and its comparison with organic solvent extraction.

Materials and Methods

Microorganism

A. terreus UV 1718 is a UV mutant of *A. terreus* ATCC 20542, and was a gift from an Indian pharmaceutical company. This strain is one of the mutants from the strain improvement program of the pharmaceutical company.

Supercritical CO_2 equipment

Laboratory scale supercritical CO_2 equipment (Speed SFE, Applied Separation, USA) was used in the present study with working conditions identical to those described by Saykhedkar and Singhal (*8*).

SSF for production of lovastatin

Initially, several agro-industrial waste substrates such as wheat bran, corn hull, rice husk, sugarcane bagasse, orange peel, orange pulp, cotton seed oil cake and groundnut oil were screened for the production of lovastatin (results not shown). Wheat bran supported the highest production of lovastatin, and was therefore chosen for further study. Fermentation was carried out with 5 g of wheat bran (0.25–0.45 mm) supplemented with 1.5 mL of K_2HPO_4 (1 g/L) and 1.5 mL of a trace ion solution ($MgSO_4 \cdot 7H_2O$ 0.5 g/L, $ZnSO_4 \cdot 4H_2O$ 3.4 mg/L, NaCl 0.5 g/L, $FeSO_4 \cdot 7H_2O$ 5 mg/L, $CoCl_2 \cdot 6H_2O$ 2 mg/L and $MnSO_4$ 1.6 mg/L). The inoculum size used was 1 mL ($2.5 \cdot 10^7$ spores/mL) (20 % by volume per mass) and adjusted to final moisture content of 66.8 % with distilled water (pH=6). The medium was thoroughly mixed with sterile glass rod and incubated at 28 °C and 80 % relative humidity in humidity controlled chamber for 3 days. At the end of the fermentation period, the solid matrix (comprising the SSF substrate, biomass and the metabolites under study) was dried in a hot air oven at 50 °C for 48 h. It was then ground in mortar, passed through a sieve to obtain an average particle size of 263 μm, and stored in a deep freezer (–20 °C) until analysis of lovastatin.

Organic solvent extraction

For the organic solvent extraction (OSE) of lovastatin, initially various solvents were screened such as acetonitrile, methanol, ethyl acetate, butyl acetate, toluene and chloroform for maximum recovery of lovastatin. Acetonitrile gave maximum extraction of both hydroxy acid and lactone lovastatin. Extraction was carried out in two stages. In the first step, 40 mL of each organic solvent were mixed with 1.5 g of solid matrix and sonicated for 5 min, followed by incubation at 28 °C, 200 rpm for 2 h. In the second step, the extract was filtered to separate the biomass, and then centrifuged at 10 000 rpm for 10 min to separate the spores from the extract. This was confirmed further by five individual extractions, each carried out in different conical flask with a fresh sample of solid matrix. Clear extract obtained was stored in glass bottles at –5 °C until analysis.

Supercritical carbon dioxide extraction

Moisture may inhibit the contact between the extraction fluid and the sample. Thus the removal of moisture by freeze-drying or oven drying is recommended prior to SFE. CO_2 cylinders were supplied by Bombay Carbon Dioxide Gas Corporation, India. In each experiment, 1.5 g of dried biomass was loaded into a 10- or 100-mL extraction column and 0.5-μm spare filters were placed at both ends of the extraction column to prevent the transfer of the particles. The extraction column was put into the temperature controlled chamber of the supercritical fluid extractor and equilibrated to a pre-set extraction temperature. The high pressure pump compressed the CO_2 to the desired pre-set pressure. The supercritical phase at the outlet of the supercritical fluid extractor was passed though two automatic valves in which the pressure was reduced slowly *via* collection bottle. The temperature of the restrictor valve was kept 10 °C higher than in the extractor chamber. The percentage recovery of lovastatin by SFE was optimized by varying the extraction parameters such as the volume of an *in situ* pretreatment solvent (1, 2.5 and 5 mL of methanol), temperatures (40, 50 and 60 °C), pressure (200, 300

and 400 bar), the flow rate of supercritical solvent (1, 1.5 and 2 L/min) and contact time.

For the SFE extraction without any pretreatment with methanol, 10-mL extraction vessel was used. The conditions of extraction were 40 °C, 300 bar at a flow rate of 2 L/min for 60 min. The size of the sample was 1.5 g, having an average particle size of 263 μm. For the *in situ* pretreatment study, a 100-mL vessel was used, the downstream glass wool of solid matrix was moistened with 1, 2.5 and 5 mL of methanol and the extraction was carried out using the same operational conditions.

HPLC analysis

Lovastatin was identified by comparison with the original standard kindly provided by Biocon Ltd, India. Lovastatin was quantified on an HPLC system (Jasko, Japan) equipped with a UV detector and a Hamilton C_{18} column (250×4.6 mm, 5 μm i.d.), and an eluent comprising acetonitrile and 0.1 % phosphoric acid (60:40). The flow rate used was 1 mL/min and the injection volume was 20 μL. The chromatogram was recorded at 238 nm. Data acquisition and analysis were done on PC based software. For conversion of lovastatin to lovastatin hydroxy acid, 20 mg of lovastatin powder were suspended in 25 mL of methanol and 0.025 M NaOH, and incubated in orbital incubator shaker at 45 °C and 100 rpm for 30 min. After completion of the reaction, pH of the solution was adjusted to 7.7 using 0.1 M HCl. A standard plot was prepared by diluting the above solution.

Lovastatin and the corresponding lovastatin hydroxy acid were identified by their retention time. The mass fractions of lovastatin and lovastatin hydroxy acid were added and reported as yield. The percentage recovery of lovastatin was defined as the mass of lovastatin obtained by SC-CO_2 extraction per gram of solid matrix divided by the mass of lovastatin obtained by OSE per gram of solid matrix multiplied by hundred.

Results and Discussion

Organic solvent extraction

In most patents, the acid form of lovastatin has been converted to the lactone form, and then extracted with organic solvents. In our work, an attempt was made to select a solvent that would extract both hydroxy acid and lactone lovastatin. It was found that acetonitrile could extract both compounds. This had not been reported earlier. The advantage of this approach is handling smaller volumes for the conversion of acid to lactone, as compared to that of doing the same in the fermentation broth. Secondly, being an SSF, the use of acid catalyst for conversion of acid to lactone would be very difficult.

Initially, the attempt was made to extract the hydroxy acid and lactone form of lovastatin by using various solvents, both polar and non-polar. It was seen that the extraction of lovastatin hydroxy acid decreased with a decrease in the polarity of the organic solvent. Hence, polar organic solvents like methanol, acetonitrile and ethyl acetate were found to be more suitable than the non-polar solvents to extract both the lactone and lovastatin hydroxy acid. Among the polar solvents, acetonitrile was found to be the most efficient (results not shown). An average lovastatin content of (1722±50) μg/g of dried fermented matter was obtained.

Supercritical carbon dioxide extraction

Effect of methanol as an *in situ* pretreatment solvent

In the initial study, the SC-CO_2 was used in SFE without any pretreatment with methanol in a 10-mL sample vessel under the conditions indicated in the Materials and Methods section. The percentage recovery of lovastatin was very low, at 17.5 %. This result was in accordance with Larson and King (22), who reported the solubility of lovastatin to be very low in SC-CO_2 as compared to the methanol modified SC-CO_2.

According to Larson and King (22), the co-solvent can be introduced into the system in two ways. In the first method, glass wool placed up-steam of the solid sample in the extractor is moistened with another solvent (methanol) so that the SC-CO_2 is modified as it passes through the glass wool, and the modified SC-CO_2 then passes through the solute bed. This type of solvent is also called entrained solvent. In the second method, CO_2 and the solvent are pre-mixed at a specific concentration. Due to the lower percentage recovery of lovastatin by SC-CO_2 in the absence of any solvent, methanol was used as an *in situ* pretreatment solvent with pure CO_2. For the addition of methanol to the extraction vessel, the methodology of filling the sample and glass wool in the extraction vessel was changed, as shown in Fig. 1.

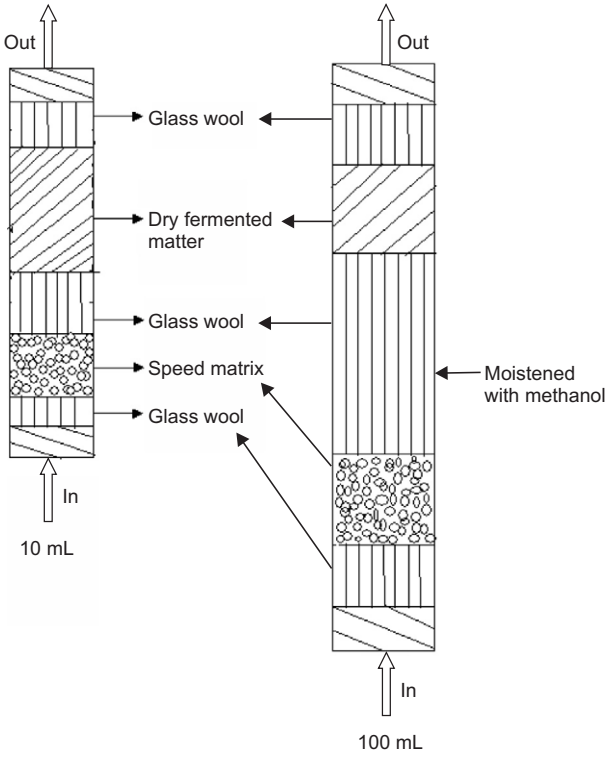

Fig. 1. Modification of the filling strategy of SC-CO_2 extraction vessel

The percentage recovery of lovastatin increased with an increase in the volume of methanol (Fig. 2). The highest percentage recovery of 58.11 % was obtained using 5 mL of methanol with a batch size of 1.5 g of solid matrix. A further increase in methanol was difficult to handle, and hence all further studies were done with 5 mL of methanol as an *in situ* pretreatment solvent.

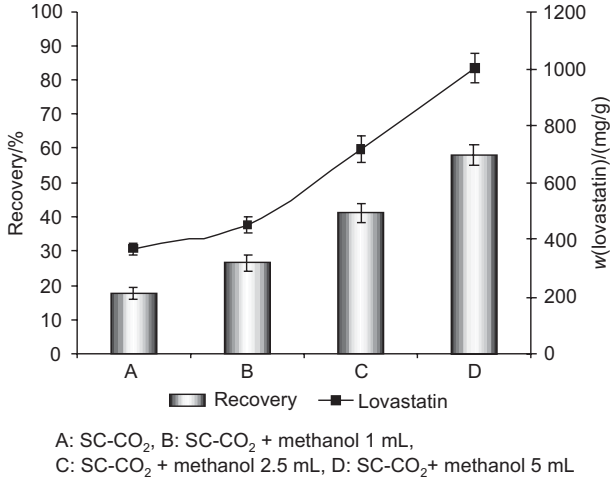

Fig. 2. Effect of methanol as an *in situ* pretreatment solvent on the percentage recovery of lovastatin from the solid matrix obtained after SSF

Effect of temperature

Using SC-CO_2 modified with 5 mL of methanol as an *in situ* pretreatment solvent, the effect of temperature with respect to pressure on percentage recovery was investigated. All experiments were conducted at constant flow rate of 2 L/min using dried solid matrix with average particle diameter of 263 μm. The results are shown in Figs. 3a-c. It is evident that the effect of the changes in temperature and pressure on the percentage recovery was not uniform. The mass fractions of lovastatin obtained at 200 bar after 55 min of extraction were 648.99, 1373.58 and 659.24 μg/g at 40, 50 and 60 °C, respectively. The percentage recovery increased from 37.69 to 79.78 % when temperature increased from 40 to 50 °C, and it again decreased from 79.78 to 38.29 % with further increase in temperature from 50 to 60 °C.

The percentage recovery of lovastatin at 300 bar and temperature of 40–60 °C is shown in Fig. 3b. The pattern of extraction was similar as at 200 bar. The mass fractions of the obtained lovastatin were 948.69, 1472.67 and 949.11 μg/g at 40, 50 and 60 °C, respectively after 55 min of extraction. Here, the percentage recovery increased from 55.1 to 85.53 % when temperature was increased from 40 to 50 °C, and it again decreased from 85.53 to 55.12 % when temperature was further increased from 50 to 60 °C. The initial percentage recovery up to 35 min at 50 °C was 70.28 %. Further increase in time to 55 min increased it to 85.53 %. This might be due to sufficient amount of methanol available with SC-CO_2 for extraction of lovastatin initially, but after some time methanol may have been exhausted from the glass wool, and during that period only CO_2 might have been available for extraction.

The percentage recovery of lovastatin as a function of temperature at 400 bar is shown in Fig. 3c. The mass fractions of the obtained lovastatin were 1245.32, 1136.45 and 1064.86 μg/g at 40, 50 and 60 °C, respectively after 55 min of contact time. The percentage recovery increased from 61.84 to 72.33 % when temperature was decreased from 60 to 40 °C. This could be due to an increase in the solvent density with the decrease of temperature. At 400 bar, the highest percentage recovery obtained was 72.33 % at 40 °C.

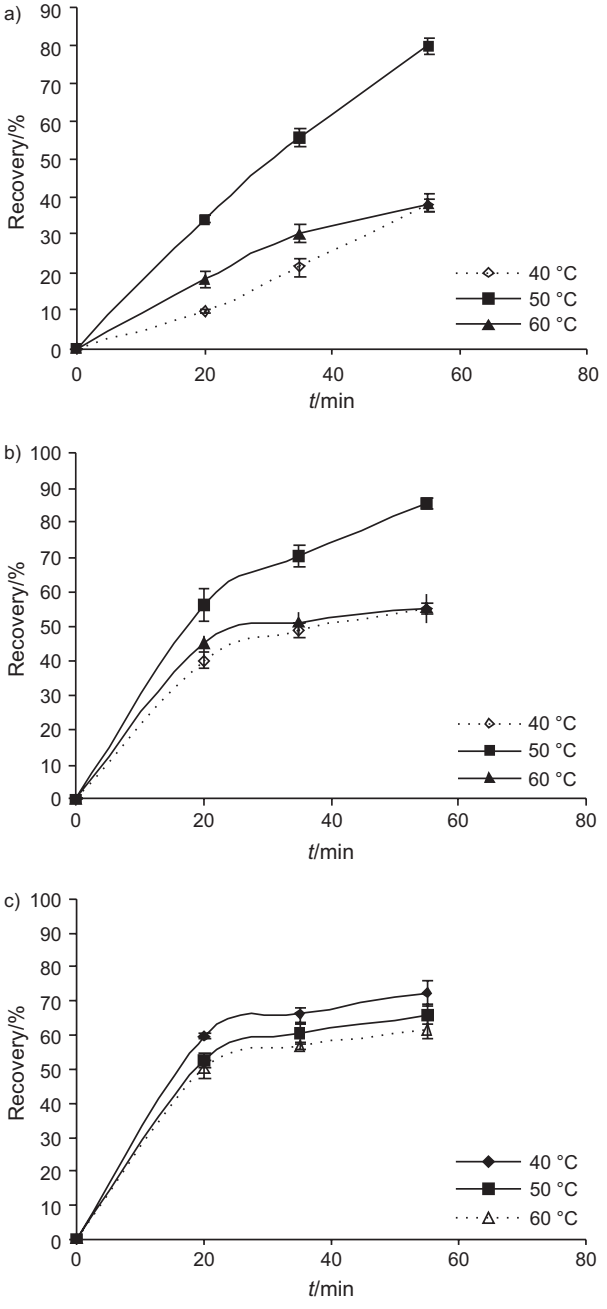

Fig. 3. Effect of temperature on the percentage recovery of lovastatin from solid matrix after SSF at a) 200 bar, b) 300 bar and c) 400 bar

In general, the extractability of the compounds with SC-CO_2 depends on the occurrence of individual functional groups in these compounds, the molecular mass, and polarity. The solubility of the compounds therein is strongly influenced by temperature, pressure and entrainer (22). In this study, percentage recovery of lovastatin was higher at 300 bar and 50 °C as compared to other conditions of extraction used in the study.

Effect of pressure

The second stage of SC-CO_2 experiments was carried out at constant temperature of 50 °C, constant flow rate of 2 L/min and pressures of 200, 300 and 400 bar for varying time periods ranging from 20 to 55 min using dried solid matrix with average particle diameter of 263 μm. The results indicate the percentage recovery to increase with an increase in pressure from 200 to 300 bar. The obtained mass fractions of lovastatin were 1373.58 and 1472.67 μg/g at 200 and 300 bar, respectively after 55 min of contact time (Fig. 4). This phenomenon is well known and explained by the fact that an increase in pressure increases the density of supercritical CO_2, resulting in an increase in the solvation power. The percentage recovery ranged from 79.78 to 85.53 with an increase in pressure from 200 to 300 bar, which could be attributed to a corresponding increase in the density of the supercritical fluid. Fig. 4 also shows that an increase in pressure from 300 to 400 bar decreased the percentage recovery, indicating an optimum extraction pressure to exist around 300 bar. The obtained mass fractions of lovastatin were 1472.67 and 1136.45 μg/g at 300 and 400 bar, respectively. This could be explained by the fact that an increase in the pressure decreases the diffusivity. In addition, an increase in the pressure causes the solid matrix to become more packed, and thereby decreases the void fraction.

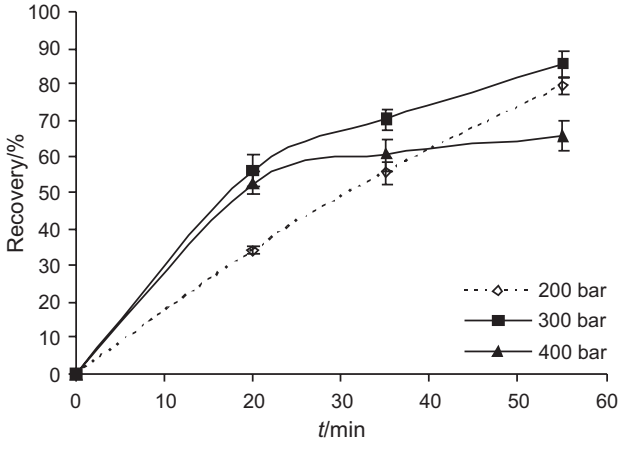

Fig. 4. Effect of pressure on the percentage recovery of lovastatin from solid matrix after SSF

Effect of supercritical CO_2 flow rate

These experiments were carried out with 5 mL of methanol as an *in situ* pretreatment solvent, constant temperature of 50 °C, constant pressure of 300 bar at SC-CO_2 flow rates of 1, 1.5 and 2 L/min, using dried solid matrix with average particle diameter of 263 μm. Fig. 5 shows the effect of SC-CO_2 flow rate on the percentage recovery of lovastatin as a function of flow rate and contact time varying from 20 to 55 min. The obtained mass fractions of lovastatin were 1403.66, 1434.33 and 1472.66 μg/g at flow rates of 1, 1.5 and 2 L/min, respectively after 55 min. The increase in flow rate from 1 to 2 L/min increased the percentage recovery only marginally from 81.52 to 85.53 %. This could be due to the fact that all experiments were done at a flow rate that was already high. Hence, further significant increase in extractability was not observed with an increase in the flow rate.

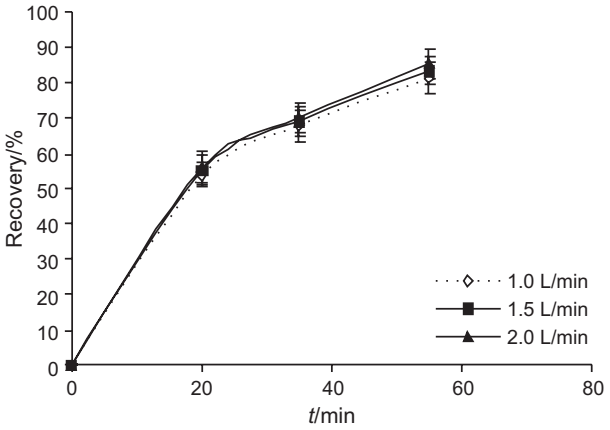

Fig. 5. Effect of the flow rate of SC-CO_2 on the percentage recovery of lovastatin from solid matrix after SSF

Effect of contact time

Final stages of SC-CO_2 experiments were carried out for maximum lovastatin recovery at optimum SC-CO_2 conditions, which were established as a temperature of 50 °C, a pressure of 300 bar, a flow rate of 2 L/min and average particle diameter of 263 μm. Fig. 6 shows the effect of contact time on the percentage recovery of lovastatin from the SSF matrix powder. Recovery of 97.38 % was obtained after 155 min of contact time. It was also observed that almost 90.8 % of lovastatin were recovered in the first 75 min. The small percentage recovery at a later stage indicates that there might be no extractable lovastatin available for SC-CO_2, possibly due to strong interaction between the matrix and lovastatin. A

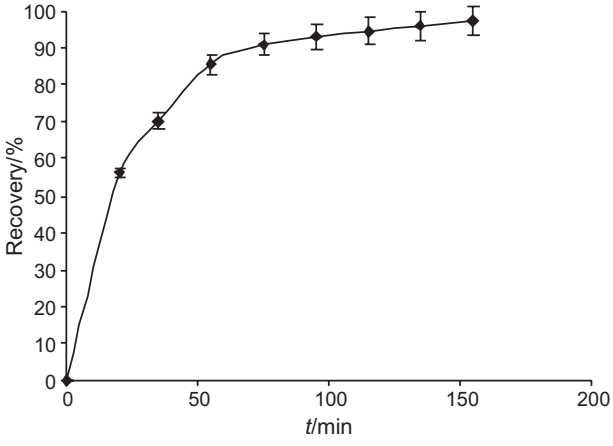

Fig. 6. Effect of contact time on the percentage recovery of lovastatin from solid matrix after SSF

report by Manzoni *et al*. (*15*) suggests that 83 % lovastatin is associated with the mycelium, and 17 % is free in the culture filtrate.

The extract obtained after SC-CO_2 was compared with that obtained by OSE. The SFE extract had 45–50 % HPLC purity of lovastatin as compared to the OSE extract, which had 10–15 % HPLC purity. The OSE extract was dark yellowish and brown in colour, while that of SC-CO_2 was pale yellow. This indicated the selectivity of the SC-CO_2 towards the non-polar compounds. Fig. 7 shows the superimposed chromatogram of the OSE extract and that of SFE extract obtained under the optimized conditions of SFE. The higher purity of SFE extract eases further purification of lovastatin, and in addition requires far less solvents as compared to conventional method of extraction and purification.

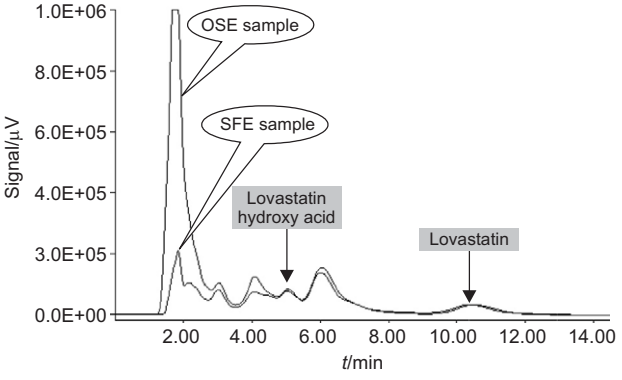

Fig. 7. Superimposed chromatogram of OSE and the best extract conditions of SFE

This study is the first of its kind for extraction of lovastatin directly from the solid matrix obtained after SSF that contains the biomass as well as the metabolite. There are reports on SFE of pure lovastatin to compute the solubility parameters (*23*), and SFE of lovastatin from Mevacor tablets (*22*), but none on downstream processing or on the solid matrix obtained after SSF.

Conclusions

SSF for the production of lovastatin is a viable commercial alternative to submerged fermentation, but it requires a different strategy for downstream processing. This work brings the potential of supercritical carbon dioxide extraction for isolation of lovastatin with lesser impurity directly from the solid matrix obtained after SSF.

Acknowledgements

The authors acknowledge the Department of Biotechnology, Government of India for funding this project. We are also thankful to Biocon Ltd., India for the gift of lovastatin standard.

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