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ORIGINAL ARTICLE

Lovastatin Production from Agrowastes using *Aspergillus terreus* by Solid state fermentation

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ABSTRACT
Lovastatin (C24H36O5) is a blood cholesterol lowering drug. It acts by competitively inhibiting the enzyme HMG-CoA which catalyzes the rate limiting step of cholesterol biosynthesis in eukaryotic cells. The major drawback in the submerged fermentation is that its yield is proportional to the biomass, with the high cell density causing the increase of the fermentation broth viscosity and the complexity in stirring and oxygen mass transfer. An alternative strategy for submerged fermentation is the SSF (solid state fermentation) process, which offers a good environment for fungi to grow, therefore high mycelia density and high lovastatin production can be expected. Ability of two strains of Aspergillus terreus (isolate 18 and 76) for production of lovastatin in solid state fermentation (SSF) using wheat bran has been investigated using one-factor approach. pH 6, 30°C incubation temperature, 1g wheat bran, 70% initial moisture content, and 5 days fermentation time, without addition of any other nutrient sources yielded maximum production of lovastatin of 2.58 mg/g dry substrate and 2.5mg/g dry substrate for A. terreus 18 and 76, respectively, using wheat bran as substrate. All the 14 different agrowastes for lovastatin production by the two isolates showed that all the substrates supported significantly varied levels of lovastatin production by the two isolates (P<0.05). Wheat husk supported lovastatin production of 2.91 mg/g and 3.33 mg/g dry substrate by isolate 18 and 7, respectively, without any addition of nutrient supplements.
Key words: *Solid substrate fermentation, wheat bran, agrowastes, optimization, Lovastatin, A.terreus, one-factor approach*

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INTRODUCTION

Lovastatin, a potent drug for lowering the blood cholesterol, was the first statin accepted by United States Food and Drug Administration (USFDA) in 1987 as a hypercholesterolemic drug [1]. It is a competitive inhibitor of HMG-CoA reductase, a key enzyme in the cholesterol production pathway [2]. Lovastatin is a cholesterol lowering agent produced as a secondary metabolite by a number of molds [3]. Though this product can be produced by cultures of *Penicillium* species [4], *A. terreus* [5-7], *Monascus* species [8,9], *Hypomyces*, *Doratomyces*, *Phoma*, *Eupenicillium*, *Gymnoascus*, and *Trichoderma* [10], only production of this compound by *A. terreus* was commercialized [11]. Microorganisms are able to produce lovastatin in SSF or submerged culture [5,7,12-15]. Lovastatin is an intracellular product and mostly accumulated in the mycelia [16], hence, fungi are suitable for lovastatin production under SSF system.
The United States Food and Drug Administration (US FDA) has approved SSF for generating clinical drugs of fungal Origin [4]. SSF process offers a better opportunity for the biosynthesis of low-volume high cost products [17].
Currently, lovastatin is mainly produced by submerged fermentation (SmF), very few reports are there on solid state fermentation (SSF). In recent years, researchers have shown an increasing interest in SSF as a potential alternative of submerged fermentation because it uses economical substrates (agricultural

residues), requires fewer processing and down-streaming stages, utilizes lesser power and generates lesser effluent. It provides an opportunity to exploit agro-industrial residues for the production of value added products [18]. Moreover, SSF has higher product yield and offers better product stability [19].
In the present investigation, we evaluate the feasibility of solid state fermentation process for the production of lovastatin by two fungal strains of *Aspergillus terreus*, isolate 18 and 76, respectively, using wheat bran as the substrate and also to optimize the process parameters (both physico-chemical and nutritional) that maximize the lovastatin yield in a step-wise manner**.** The study also evaluates the efficacy of the two *A.terreus* strains for producing lovastatin by using other agrowastes.

MATERIALS AND METHODS

Microorganism maintenance

Two *Aspergillus terreus* isolates No.18 and 76, isolated in the previous study from soil were used as inoculum. Both the fungal isolates were maintained on Potato dextrose agar (PDA) slants. Slants were maintained at 4ºC and sub cultured routinely.

Identification and characterization lovastatin producing strain

Microscopically identification of the fungal isolate with maximum lovastatin producing strain was carried out. Genomic identification of the strain was done using 18S rDNA technique. Genomic DNA [20] was isolated from the pure culture pellet. The ~1.5 kb 18S rDNA fragment was amplified using the primers; 27f (50-AGA GTT TGA TCC TGG CTC AG-30) as forward and 1492r (50-TAC GGT TAC CTT 10 GTT ACG ACT T-30) as reverse primer. Consensus sequence of 706 bp of 18S region was generated from forward and reverse sequence data using aligner software. The 18S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score Fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5. Sequence data was aligned and analyzed for finding the closest homologs for the microbe.

Inoculum Preparation

Inoculum of *Aspergillus terreus* was prepared by adding 10 ml of sterilized water along with 0.1% (v/v) Tween 80 solution in pure culture plate. The spore surface was scrapped with an inoculating loop to suspend the spores in the solution and the obtained spore suspension was used as the inoculum for the fermentation process.

Solid state fermentation (SSF)

Lovastatin production by *Aspergillus terreus* isolates, 18 and 76, was optimized under SSF. Wheat bran was used as a substrate in the present study for the process of fermentation. To remove moisture, wheat bran was kept in hot air oven at a temperature of 40ºC for 30 min. Various parameters analyzed included incubation temperature (25 – 40 ±2 °C), pH (4.0 – 8.0), incubation period (1 to 8 days), initial moisture content of the substrate (40 –80 % v/w), substrate concentration (1g to 7.5 g, v/w), inoculum density (0.5 -2.5 ml). Once a given parameter was optimized, it was kept constant at that level while varying the other parameters individually. A set of conical flask of 250 ml were used to carry out the process of fermentation. A set of conical flask each containing 5g of wheat bran, 80% of moisture and 2 ml of spore suspension containing 10^7- 10^8 spores/ ml were incubated for 11 days, unless otherwise stated.

Evaluation of agrowastes for lovastatin production

Fourteen different solid agrowaste substrates such as rice bran, oil cakes- rape seed , yellow mustard, black mustard, coconut, Onion seed (*Nigella sativa*) , Mahua (*Madhuca longifolia*), Flax seed (*Linum usitatissimum*); orange peel, wheat husk, ragi husk, corn cob, rice straw and groundnut shell were used to perform SSF for lovastatin production by the two isolates under the optimized conditions of production standardized with wheat bran, to evaluate the most suitable substrate.

Lovastatin extraction and estimation

Screening medium was filtered using filter paper in order to get the culture filtrate containing lovastatin. pH of the broth was adjusted to 2 then equal volume of ethylacetate was added in the broth and it was kept in the rotatory shaker at 100 rpm for 2 h. Using separating funnel broth was separated from ethylacetate (organic phase) containing lovastatin. Ethylacetate was allowed for drying. 1 ml of ethanol was added in the residue. This residue was used as an extract for further analysis. Lovastatin was estimated colorimetrically by the method of mohiesharaf El- Din *et al*. 2010 [21].

Statistical analysis

All the experiments were done twice with 3 replicates. Individual culture flasks were considered as experimental units. Data was analysed by One Way ANOVA and all multiple comparisons among means were performed using Duncan's new multiple range test (α = 0.05).

RESULTS

Identification of isolates by microscopy and phylogenetic analysis

The two fungal isolates 18 and 76 grew fast on PDA medium after 5-7 days of incubation. Macroscopic characters included colony characters of examined isolates with difference in their colors from pale-yellow to dark yellow, while the reverse pigments ranged from yellow to dark gray color. The colonies texture raised surfaces velvety, tough, yellowish, powdery. Microscopic characters did not reveal variations among the two isolates under interest; conidiophores were typically long, hyaline and smooth giving rise to sub-spherical vesicles that were biseriate. Conidia was smooth walled and slightly elliptical. 18S rDNA sequence analysis identified both the isolates 18 and 76 as *Aspergillus terreus*. The culture, isolate 18 was similar to *Aspergillus terreus* isolate ATE1 (GenBank Accession Number: KM462826.1) while isolate 76 was similar to *Aspergillus terreus* strain BIOS PTK 5 (GenBank Accession Number: JX985737.1) based on nucleotide homology and phylogenetic analysis.

Effect of different fermntation parameters on lovastatin production by A.terreus 18 and 76

Wheat bran has been used for this study because it is one of the cheapest easily available agrowastes and it serves as a good source for fungal mycelia growth on its surface. This study has been carried out only on wheat bran without the use of any other nutrient source, unless otherwise stated.

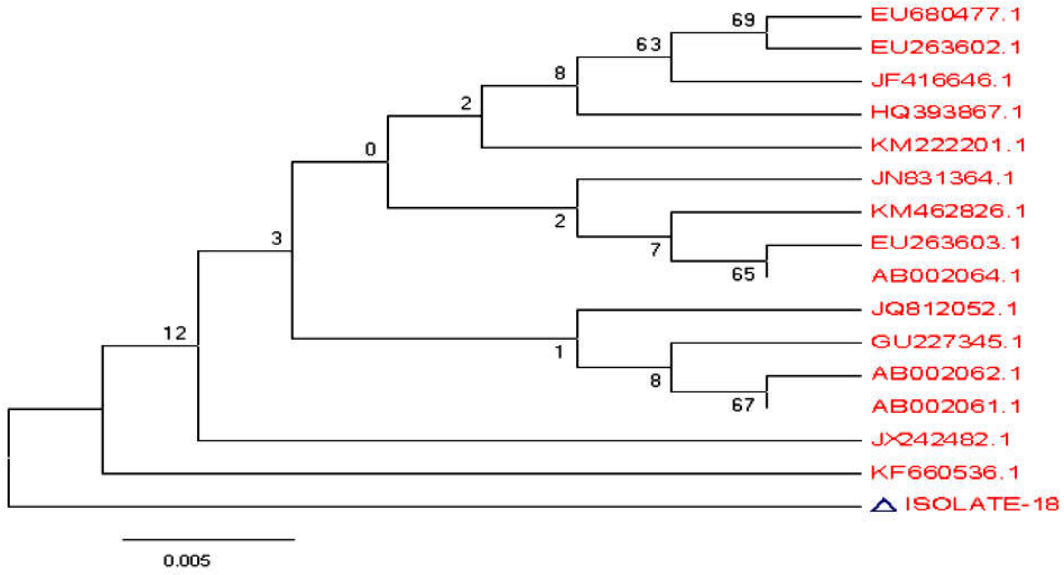

Fig.1a

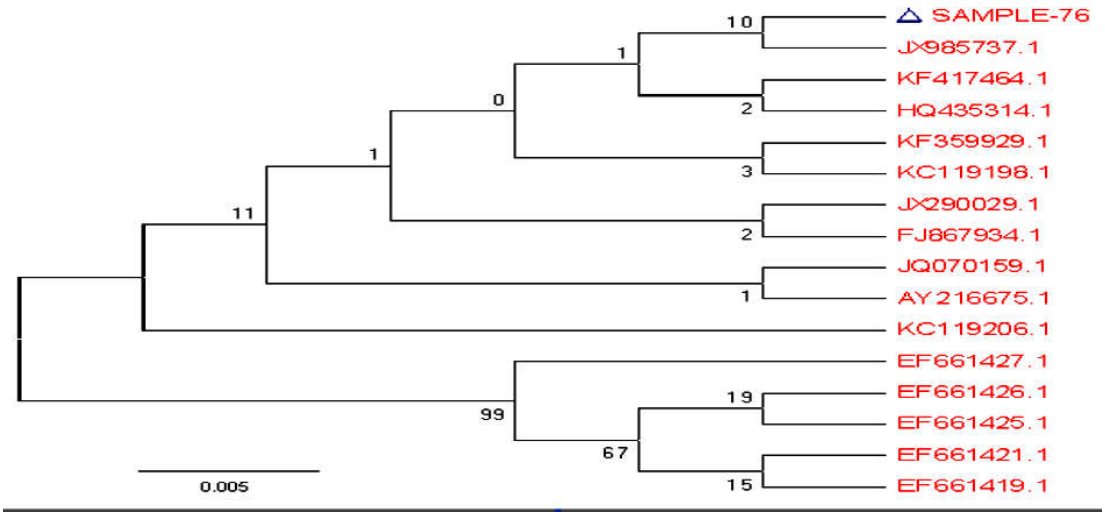

Fig.1b

Fig. 1 a and b. Phylogenetic analysis of isolate a. 18 and b. 76.

The effect of fermentation time for maximum production of lovastatin was studied by incubating 250 ml of flask containing 5g of wheat substrate for various time periods varying from 3 to 8 days. Incubation time had significant effect on lovastatin production ($P<0.5$) by both the isolates. The maximum lovastatin yield obtained was 0.55 mg/g dry substrate and 0.61 mg/g dry substrate in the case of isolate 18 and 76, respectively, by day 5. Temperature had significant effect on lovastatin production by both the isolates ($P<0.5$). Results indicated that maximum lovastatin production (0.55 mg/g dry substrate by isolate 18 and 0.61 mg/g dry substrate by isolate 76) was obtained when SSF was carried out at 30°C. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms. At temperatures lower or higher than that of optimum, less lovastatin production was observed. Moisture content had significant effect on lovastatin production by both the isolates ($P<0.5$). Enhanced lovastatin production with increase in the substrate moisture content up to 70 % was observed beyond which it declined. The highest production of lovastatin (0.63mg/g by isolate 18 and 0.66 mg/g dry substrate by isolate 76) was obtained at a moisture level of 70% and it declined sharply at lower and higher levels of moisture content. To confirm the role of oxygen in lovastatin production, different bed depths created by different amounts of the wheat bran (1g, 2.5g, 5g and 7.5g) were studied. Production of lovastatin gets influenced by the amount of substrate used. In this study, substrate amount had significant effect on lovastatin production by both the isolates ($P<0.5$). Maximum yield (2.33 mg/g by isolate 18 and 2.33 mg/g dry substrate by isolate 76) with 1g wheat bran which gradually decreased as the amount of substrate increased. Reducing the wheat bran concentration from 5 g to 1g brought about a significant increase in lovastatin production. Similar observations were made [22] with average lovastatin content of 0.91 mg/g of dry solid in case of 3cm bed depth after which the yield decreased with increasing bed depth. As the bed depth increased due to increasing substrate concentration, lovastatin content decreased drastically. Inoculum volume had a significant effect on lovastatin production by both the isolates ($P<0.5$). The maximum yield of lovastatin (2.33 mg/g and 2.33 mg/gdry substrate) was obtained with 2 ml of *A.terreus* isolates 18 and 76, respectively, as shown in Table 1. With the further increase in inoculum volume, the yield of lovastatin decreased. Initial pH of the fermentation had a profound effect on lovastatin production by both the isolates ($P<0.5$). Maximum lovastatin yield (isolate no. 18 produced 2.58 mg/g and isolate 76 produced 2.5 mg/g dry substrate) was recorded at pH 6.0. A further increase in pH resulted in gradual decrease of lovastatin.

Table 1 : Yield of lovastatin by *Aspergillus terreus* isolates 18 and 76 under various fermentation conditions.

Parameters Fermentation Period (days)	Lovastatin yield (mg/gdry substrate) by isolate 18	Lovastatin yield (mg/g dry substrate) by isolate 76
3	0.15 ^{d}	0.15^{e}
4	0 .23^{c}	0.22^{de}
5	0.55^{a}	0.62^{a}
6	0.42^{b}	0.43^{b}
7	0.37^{b}	0.33^{c}
8	0.25^{c}	0.28^{cd}
Temperature (˚C)		
25	0. 42^{ab}	0.35^{b}
30	0. 55^{a}	0.62^{a}
35	0. 33^{bc}	0.40^{b}
40	0. 22^{c}	0.15^{c}
Moisture content (%)		
40	0.27^{c}	0.22^{c}
50	0.28^{bc}	0.28^{c}
60	0.35^{b}	0.38^{b}
70	0.63^{a}	0.67^{a}
80	0.57^{a}	0.58^{a}
Substrate (g)		

1	2.33[a]	2.33[a]
2.5	0.90[b]	0.93[b]
5	0 .65[bc]	0.61[c]
7.5	0.52[c]	0.58[c]
Inoculum (mL, v/v)		
0.5	0.83[c]	0.58[d]
1	0.92[c]	0.83[d]
1.5	1.17[c]	1.17[c]
2	2.33[a]	2.33[a]
2.5	1.92[b]	1.83[b]
pH		
4	0.58[c]	0.58[c]
5	1.17[b]	1.00[c]
6	2.58[a]	2.50[a]
7	2.25[a]	2.25[a]
8	1.50[b]	1.58[b]

[a,b,c] indicating that means within column are significantly different.

Effect of different carbon and nitrogen supplements on lovastatin production

Of the 5 different carbon sources added to the wheat husk, it was observed that in case of isolate 18 maltose supported maximum lovastatin production (3.75 mg/g dry substrate) and in case of isolate 76 lactose supported maximum lovastatin production (3.33 mg/g dry substrate as compared to wheat husk without addition of any carbon source as a control. Of the 8 different nitrogen sources that were added to wheat husk, peptone supported maximum yield (3.17 mg/g dry substrate) in case of isolate 18 and sodium nitrate supported maximum yield (3.67 mg/g dry substrate) in case of isolate 76 in comparison to wheat husk (Table 2).

Table 2 :Lovastatin production from different agrowastes *A. terreus* isolates 18 and 76 by SSF.

Agrowastes	**Lovastatin yield (mg/g)by isolate 18**	**Lovastatin yield (mg/g)by isolate 76**
Wheat bran(control)	2.83[a]	2.50[b]
Ragi husk	1.83[b]	1.75[cd]
Corn cob	1.00[def]	1.00[f]
Rice straw	1.42[c]	1.50[de]
Rice bran	0.33[h]	0.25[i]
Mahua oil cake	2.08[b]	1.92[c]
Orange peel	1.83[b]	1.75[cd]
Groundnut shell	1.25[cd]	1.33[e]
Linum usitatissimum (Alsi)oilcake	1.17[cde]	1.08[f]
Nigella sativa (Kalonji)oil cake	0.92[ef]	0.83[fg]
Rapeseed oil cake	0.75[fg]	0.67[gh]
Yellow musturd oil cake	0.75[fg]	0.67[gh]
Black musturd oil cake	.5833[gh]	0.50[h]
Coconut oil cake	1.42[c]	1.50[de]
Wheat husk	2.92[a]	3.33[a]

[a,b,c...] indicating that means within column are significantly different.

Table 3. Effect of different carbon and nitrogen supplements to wheat husk on lovastatin production by *A.terreus* 18 and 76.

Parameter	Lovastatin yield (mg/g) by isolate 18	Lovastatin yield (mg/g)by isolate 76
Carbon sources		
Control	3.00^{bc}	3.17^{abc}
Maltose	3.75^{a}	2.67^{bc}
Lactose	3.41^{ab}	2.67^{bc}
Glucose	2.67^{cd}	3.33^{ab}
Sucrose	2.67^{cd}	3.00^{abc}
Xylose	2.17^{d}	2.50^{c}
Nitrogen sources		
Control	3.00 ^{a}	3.25^{a}
Beef extract	3.08^{a}	2.42^{b}
Ammonium Sulphate	1.42^{cd}	2.25^{b}
Sodium nitrate	1.67^{c}	3.67^{a}
Urea	1.17^{d}	1.25^{c}
Peptone	3.17^{a}	2.25^{b}
Trypton	1.75^{c}	1.33^{c}
Soyabean meal	1.08^{d}	1.00^{c}
Yeast extract	2.67^{b}	1.08^{c}

*Significantly different at 5% level.
^{a,b,c} indicating that means within column are significantly different.

Evaluation of agrowastes for lovastatin production by SSF

The efficacy of the two strains on the production of lovastatin in SSF using 14 different agrowastes under wheat bran optimized SSF conditions was investigated. All the 14 agrowastes supported lovastatin production in significantly varied levels ($P<0.05$) (Table 3). Isolate 18 produced lovastatin ranging from 0.33 mg/g dry substrate using rice bran to 2.91 mg/g dry substrate using wheat husk while isolate 76 produced as low as 0.25 mg/g dry substrate using rice bran to as high as 3.33 mg/g dry substrate on wheat husk.

Wheat husk supported 1.15 fold higher level of lovastatin production by isolate 76 as compared to wheat bran. However in case isolate 18, the yield obtained with wheat husk was comparable to wheat bran.

DISCUSSION

The lovastatin yield increased steadily from third day to fifth day which explained that lovastatin is a kind of secondary metabolite and its accumulation in mycelia seems growth related and slightly decreased from day 5 onwards, which may be due to the onset of death phase of microorganism and nutrient depletion. Parvatham *et.al.,* (2012) observed highest production of lovastatin by *Aspergillus flavipes* on the 7th day of fermentation (14.77 mg/g dry substrate), after which there was a decline in the yield of lovastatin [22]. In a similar study 8 day fermentation period supported maximum lovastatin yield (0.98mg/g dry substrate) by an endophyte *A.niger* and decreased production with further extension of the incubation period to 11 days [23]. Similar observations were reported by Siamak *et al.* (2003) in case of *Aspergillus terreus*, they investigated the production of lovastatin at 7 days of incubation with a level of 55 mg lovastatin per liter of screening production medium [24]. Most reports indicated incubation periods of 6-10 days to be optimal for lovastatin production by various fungi [25-28, 19].

Decline in lovastatin production at higher temperatures might be due to denaturation of lovastatin or its inactivation at higher temperatures. These results are coinciding with those previously reported for lovastatin production by *Monascus ruber, Monascus purpureus* and *Aspergillus terreus* [7,15,23, 29,30].

Lower yield at higher moisture content may be presumably due to poor oxygen availability caused by excessive replacement of air by water in the void volume. Likewise, moisture level below optimum leads to reduced solubility of the nutrients of the solid substrate and lower degree of swelling of substrate, the available oxygen is sufficient but the water content is not enough to support good metabolic activity and dissipation of heat generated and may account for lowerlovastatin production 14. Moisture content of 60% (v/w) was observed for both *Aspergillus flavipes* and *Aspergillus terreus* under SSF [7,31] Maximum lovastatin yield (8.04 mg/g dry substrate) was achieved at 60% (v/w) initial moisture content by SSF of *Aspergillus fischeri* using *coconut oil cake* [22]. In a study by Prabhakar *et.al.* (2012) wheat bran with 65% moisture content was found to be optimum moisture content [32]. The same 60% (v/w) moisture content was also observed for both *Aspergillus flavipes* and *Aspergillus terreus* under SSF [19, 33].

Less amount of substrate does not allow the proper growth of organism because of less surface area and limitation of nutrient and high substrate affects the flow of oxygen and leads the accumulation of heat which may affect the yield of lovastatin. Substrate concentration in fixed volume of flask influences the bed depth for fermentation, thus influencing the growth and yield. Optimal substrate concentration gives optimal bed depth and presumably better molecular diffusion of air (O_2) from the stagnant gas phase of the head space through the interparticle voids into the biomass film on the surface of particles [25]. Increasing substrate concentration increases the bed depth above the critical value (the depth below which the oxygen concentration falls to zero during the fermentation), there will be little or no growth in that region [34]. The critical bed depth is mainly a function of substrate particle size, substrate packing and vessel geometry.

With low inoculum volume, the yield is also low due to insufficient microbes to form mycelia and accumulate lovastatin [15]. Increase of incoulum beyond optimum decreased lovastatin production which might be due to the depletion of the available nutrients in the production medium, yielding poor mycelia growth, thus promoting less product formation. With low inoculum volume, the yield is also low due to the insufficient microbial culture to form mycelia and produce lovastatin. They also reported 20% v/w to be optimum for lovastatin production [15]. Panda *et al*., (2008) reported highest yield of lovastatin with inoculum volume 5.10 mL using co-culture of *Monascus purpureus* and *Monascus ruber* under SSF [35].

Higher pH influenced lovastatin production due to the denaturation or inactivation of the microbial strain because pH strongly influences the transport of various components across the cell membrane which in turn supports the cell growth and product formation and most of the fungi are active in the pH range of 3.5-7.0 and also lower pH avoids the contamination by other microbes. Attalla *et al* reported the maximum production of mevinolin (96.22 mg/l) at pH 6.5 by using *Aspergillus terreus.* Similarly, Panda *et al*., [35] reported maximum lovastatin production at pH-6.0 using co-culture of *Monascus purpureus* and *Monascus ruber* under SSF where as Valera *et al*., [19] reported highest yield of lovastatin production with *Aspergillus flavipes* at pH 5.0 under SSF.

The study of lovastatin productivity in case of solid state fermentation as well as submerged fermentation has shown that in many cases yield obtained was much higher in case of SSF as compared to submerged fermentation [36]. In solid state fermentation few strains produced secondary metabolites 17 times more than submerged fermentation. In contrast it was also observed that few strains which are giving high yield in case of submerged fermentation will not give good yield in case of solid state fermentation. (Praveen *et.al.,* 2012) studied that the higher lovastatin production in SSF is primarily related to enhanced transcriptional rates of biosynthetic genes *lov*E and *lov*F resulting in yield increase by 4.6 fold and 2 fold respectively [37]. The SSF fermentation for lovastatin production was studied with *Aspergillus flavipes* [19], *Monascus ruber* [33] and solid-liquid fermentation with *Monascus ruber* [30]. The lovastatin yields were 4~6 mg/g of dry solid, 16.78mg/g of dry solid and 131 mg/L respectively. *Aspergillus fischeri* showed maximum lovastatin yield (9.71 mg/g dry substrate) at pH 5.0 on SSF using coconut oil cake [22].

Lovastatin can be produced from 9 molecules of acetyl-CoA during the fermentation [38]. Since acetate production by microbial activity is correlated with carbohydrate fermentation, source of carbohydrate is important for production of this product. Lignocelluloses including cellulose, hemicelluloses, and lignin are the main components of agricultural biomass, therefore, for production of acetate, these macromolecules must first be hydrolyzed into their subunits such as glucose, xylose, in the presence of the appropriate enzymes, such as cellulase, hemicellulase, pectinase and cellulobiase. The resulted monomers can then be used during the fermentation process of fungi and production of acetyl-CoA which can be used as substrate for lovastatin production. Therefore, the microorganisms selected for SSF must be able to degrade the lignocelluloses by producing sufficient amount of the appropriate enzymes to hydrolyze the respective lignocelluloses fractions. Production of cellulase (such as betaglucosidase, endoglucanase, and cellobiohydrolase) and hemicellulases (mainly xylanase) enzymes by *A. terreus* and their effects on lignocelluloses degradation have been well documented [39–41]. However, there is no known data on production of lignin degradation enzyme by *A. terreus.*

CONCLUSION

The current investigation was mainly focused on the evaluation of the potentiality of two *Aspergillus terreus* isolates no.18 and 76 for utilization of wheat bran as the substrate for the production of lovastatin under standardized solid state fermentation. It has proved the feasibility of solid state fermentation as a promising technique in exploiting cheaply available agro-residual wastes as substrates for the large-scale production of microbial metabolites of biotechnological importance ultimately leading to an effective solid waste management.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

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