

Research Article

Lovastatin Production by *Aspergillus terreus* Using Agro-Biomass as Substrate in Solid State Fermentation

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Ability of two strains of *Aspergillus terreus* (ATCC 74135 and ATCC 20542) for production of lovastatin in solid state fermentation (SSF) using rice straw (RS) and oil palm frond (OPF) was investigated. Results showed that RS is a better substrate for production of lovastatin in SSF. Maximum production of lovastatin has been obtained using *A. terreus* ATCC 74135 and RS as substrate without additional nitrogen source (157.07 mg/kg dry matter (DM)). Although additional nitrogen source has no benefit effect on enhancing the lovastatin production using RS substrate, it improved the lovastatin production using OPF with maximum production of 70.17 and 63.76 mg/kg DM for *A. terreus* ATCC 20542 and *A. terreus* ATCC 74135, respectively (soybean meal as nitrogen source). Incubation temperature, moisture content, and particle size had shown significant effect on lovastatin production ($P < 0.01$) and inoculums size and pH had no significant effect on lovastatin production ($P > 0.05$). Results also have shown that pH 6, 25°C incubation temperature, 1.4 to 2 mm particle size, 50% initial moisture content, and 8 days fermentation time are the best conditions for lovastatin production in SSF. Maximum production of lovastatin using optimized condition was 175.85 and 260.85 mg/kg DM for *A. terreus* ATCC 20542 and ATCC 74135, respectively, using RS as substrate.

1. Introduction

Lovastatin is a potent drug for lowering the blood cholesterol and it 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, which is a key enzyme in the cholesterol production pathway [2]. Lovastatin is a secondary metabolite during the secondary phase (idiophase) of fungi growth [3]. 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]. Although the ability of different groups of fungi for production of lovastatin was reported in many studies, only production of this compound by *A. terreus* was

commercialized (for manufacture of high quantity of lovastatin for used as anticholesterol drug) [11]. Microorganisms are able to produce lovastatin in SSF or submerged culture [5, 7, 12–15]. Experiment showed that quantity of lovastatin production in SSF is significantly higher than submerged culture [5]. Different substrates were used for lovastatin production in SSF, including sorghum grain, wheat bran, rice, and corn [5, 7]. These substrate materials are normally expensive and are competing with food or feed ingredients for human and livestock. On the other hand, large quantity of agro-industrial biomass such as RS and OPF are produced globally particularly in the tropical countries. These agro-biomass are often burned away for disposal, causing huge environmental concerns, with only some remaining being used as roughage feed for ruminant livestock. These

biomasses are, however, potential substrates for growth of microorganisms and production of biomaterials.

Over the last 250 years, the concentration of atmospheric methane (CH₄) increased by approximately 150% [16], with agricultural activities contributing 40% of the total anthropogenic source, of which 15 to 20% is from enteric fermentation in ruminants [17]. On the other hand, ruminal CH₄ production accounts for between 2 to 15% of dietary energy loss for the host animals [18]. Because of the negative effects on environment and the host animal nutrition, mitigation of enteric CH₄ emission in ruminant livestock had been extensively researched, including the use of various mitigating agents such as ionophores [19], organic acids [20], fatty acids [21], methyl coenzyme M reductase inhibitors [22], vaccine [23], and oil [24]. However, these technologies have limited application primarily because they, besides suppressing CH₄ also, decrease nutrients digestibility (such as oil and fatty acids), have negative effect on human and animal health (antibiotics), or are not economically acceptable (methyl coenzyme M reductase inhibitors and vaccine).

Wolin and Miller [25] showed significantly reduction in growth and activity of methanogenic Archaea using lovastatin without any negative effect on cellulolytic bacteria that was due to the effect of this drug on inhibition the activity of HMG-CoA reductases in the archaeal microorganisms. It is uneconomical to use pure lovastatin as a feed additive for the mitigation of CH₄ production in ruminants. Production of this component using low-cost substrate and process for being used as animal feed additive were the main objective of present study.

Thus, the primary objective of this study was to investigate the efficacy of two strains of *A. terreus* (ATCC 20542 and ATCC 74135) for production of lovastatin using RS and OPF as substrates. In addition, the effects on nitrogen source, mineral solution, moisture, incubation time, pH, inoculum size, particle size, and incubation time on lovastatin production were investigated.

2. Materials and Methods

2.1. Substrate. RS and OPF were collected from the local fields in the state of Selangor, Malaysia. The materials were ground and sieved through mesh size 6 to obtain particles size of about 3.4 mm and dried in oven at 60°C for 48 h and used in SSF studies.

2.2. Microorganism and Preparation of Spore Suspension. *A. terreus*, ATCC 20542 and ATCC 74135, used in this study was obtained from the American Type Culture Collection (ATCC). They were maintained on potato dextrose agar (PDA) slants at 32°C for 7 days, stored at 4°C, and sub-cultured every two weeks. For the preparation of spore suspension, 10 mL of sterilized 0.1% Tween-80 solution was added to the 7-day old culture slants of the fungi at the end of incubation, surface of the culture was scratched with sterilized loop, and the Tween-80 solution containing spores was transferred into 100 mL Schott bottle containing the same solution and agitated thoroughly using a shaker to suspend the spores. The number of spores was measured

using a hemocytometer and adjusted to approximately 10⁷ spores/mL for use as inoculum throughout the study.

2.3. Solid State Fermentation. This study consisted of two subexperiments. In the first, the efficacy of lovastatin production by two strains of *A. terreus* (ATCC 20542 and ATCC 74135) using two types of agro-biomass (RS and OPF) as substrate was examined. In addition, soybean meal, urea and ammonium sulphate were used as nitrogen sources and the need to supplement mineral to enhance the fermentation process was studied. In the next subexperiment, fermentation conditions were optimized for maximum lovastatin production in SSF. The procedure of SSF for each subexperiments was described below. Both subexperiments were conducted in triplicate. Presented data are Mean ± Standard Deviation.

Subexperiment 1: Effect of Substrate, Nitrogen, and Mineral Solution. Solid state fermentation was carried out in 500 mL Erlenmeyer flasks containing 20 g of the respective substrate (RS or OPF). The moisture content of the substrate were adjusted with mineral solution (KH₂PO₄: 2.1 g/L, MgSO₄: 0.3 g/L, CaCl₂: 0.3 g/L, FeSO₄: 0.11 g/L, ZnSO₄: 0.3 g/L) or distilled water to produce a moisture content of approximately 75%. pH of all the solutions were adjusted to 6 before adding into the solid substrate. For the study on effect of nitrogen source on lovastatin production, 1% urea, 1% ammonium sulphate, or 10% of soybean meal was added in to the solid culture. The contents in the flasks were autoclaved for 15 min at 121°C and after cooling the flasks to room temperature, 10% inoculum were added and the contents of the flasks were thoroughly mixed. The flasks were incubated at 32°C for ten days.

Subexperiment 2: Optimization the Fermentation Condition. Since the production of lovastatin using RS as substrate was significantly higher than that for OPF, a follow-up experiment was conducted to optimize several factors (pH, temperature, particle size, inoculums size, and initial moisture content) known to affect SSF process for production of lovastatin by both strains of *A. terreus* using only RS as substrate. The second experiment consisted of five subexperiments, each evaluating the effect of one of the above five factors on the SSF process with the remaining factors being constant. For study on the effect of pH, because of the difficulty of adjusting the pH of solid sample in SSF, pH of solution was adjusted using 1 M Sodium hydroxide and 1 M hydrogen chloride to pH 5, 6, 7, and 8 (before adding in the substrate). Incubation temperatures between 25 to 42°C, five different particle sizes: <425 μm (mesh no. 40), 425–600 μm (mesh no. 30), 600–1400 μm (mesh no. 14), 1.4–2 mm (mesh no. 10), and 2–3.35 mm (mesh no. 6), inoculums size of 5, 10, and 15% and initial moisture contents at 50, 66, and 75% were investigated.

2.4. Extraction and Determination of Lovastatin. At the end of fermentation, the solid culture was dried at 60°C for 48 h and 0.5 g of the dry culture was extracted with 15 mL methanol and shaking in a shaker for 60 min at 220 rpm [5].

TABLE 1: Effect of nitrogen source and mineral solution on lovastatin production in solid state fermentation by *A. terreus* using rice straw (RS) and oil palm frond (OPF) as substrates.

Treatments	Lovastatin production (mg/kg DM)	
	ATCC 20542	ATCC 74135
RS	154.48 ± 22.88 ^a	157.07 ± 1.92
RS plus mineral	119.35 ± 16.59 ^{ab}	146.09 ± 7.49
RS plus mineral and urea	96.3 ± 3.02 ^{bc}	118.26 ± 6.38
RS plus mineral and soybean meal	126.36 ± 22.84 ^{ab}	139.63 ± 25.45
RS plus mineral and ammonium sulphate	66.19 ± 3.39 ^c	129.39 ± 21.90
Significant	**	NS
OPF	7.84 ± 0.14 ^b	13.09 ± 2.22 ^b
OPF plus mineral	9.66 ± 0.03 ^b	7.2 ± 1.55 ^b
OPF plus mineral and urea	21.25 ± 4.66 ^b	17.58 ± 3.03 ^b
OPF plus mineral and soybean meal	70.17 ± 4.84 ^a	63.76 ± 14.00 ^a
OPF plus mineral and ammonium sulphate	10.06 ± 0.31 ^b	11.16 ± 3.41 ^b
Significant	**	**

NS: not significantly different.

** Significantly different at 1% level.

^{a,b,c} indicating that means for each substrate within column are significantly different.

After filtration with membrane filter (0.2 μm), the concentration of lovastatin in the filtrate was assayed using HPLC (Waters, USA, 2690) attached with an ODS column (Agilent, 250 \times 4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile and water (70:30 by volume) contained 0.5% acetic acid. The flow rate was 1 mL/min. The photo diode array (PDA) detection range was set from 210 to 400 nm and lovastatin was detected at 237 nm. The sample injection volume was 20 μL , and the run time was 12 min. Since two forms of lovastatin (lactone and β -hydroxyl) are normally present in the fermented culture, they were separately determined in the HPLC. Commercial lovastatin (mevinolin K, 98%, HPLC grade, M2147, Sigma, USA) used as standard is in the lactone form. And β -hydroxyl lovastatin was produced from the lactone form using the method of Friedrich et al. [26]. Briefly, to prepare β -hydroxyl acid, lactone lovastatin was suspended in 0.1 M NaOH and heated at 50°C for 1 h in a shaking incubator. Subsequently, the mixture was adjusted to pH 7.7 with 1 M HCl, filtered through 0.2 μm filters and used as standard for HPLC. The retention times of the hydroxyl and lactone forms of lovastatin were 6.668 and 10.898 min, respectively. Different concentrations ranged from 0.5 to 500 ppm of lovastatin were used as standard and standard curve of lovastatin.

2.5. Scanning Electron Microscope. Microscopy analysis was conducted using a Scanning Electron Microscope (SEM) to determine the morphological growth of *A. terreus* on the surface of RS. The samples were dried (60°C for 48 h), cut into 1 mm size, and affixed to a metal SEM stub and sputter coated in gold using SEM coating unit (BAL-TEC SCD 005 Spotter coater). The coated specimens were viewed using Environmental Scanning Electron Microscope (Philips XL30, Germany) at accelerating voltage of 15–25 KV.

2.6. Chemical Analysis. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the detergent

system [27]. Acid detergent lignin (ADL), crude protein (CP), and ash were determined using the method described by AOAC [28]. Hemicelluloses content was estimated as the difference between NDF and ADF, while cellulose content was the difference between ADF and ADL.

2.7. Statistical Analysis. All of the experiments were done with 3 replicate. Individual culture flasks were considered as experimental units. Data were analyzed as a completely randomized design (CRD) using the general linear model (GLM) procedure of SAS 9.2 [29] with a model that included treatment effects and experimental error. All multiple comparisons among means were performed using Duncan's new multiple range test ($\alpha = 0.05$).

3. Results and Discussion

3.1. Lovastatin Determination. Lovastatin was quantified as its β -hydroxyl and lactone forms (Figure 1). The β -hydroxyl of lovastatin is the more active form of this drug but is unstable [5], thus preparation for its standard solution was prepared freshly from the lactone form according to Friedrich et al. [26]. Because the hydroxyl form of lovastatin is not stable, lactone form is normally the primary lovastatin detected in the fermented products. The quantities of lovastatin reported in Table 1 were the combination of the two forms, but results of this study show that β -hydroxyl form is the dominant lovastatin in the solid cultures.

It was reported that the conditions needed for the conversion of lactone into the β -hydroxyl form are high pH (e.g., by addition of NaOH), heating to 50°C, and naturalization by acid. Since none of the above conditions was applied in this study, the β -hydroxyl form of lovastatin present in the extract in this study is believed to be a direct product of SSF and not due to conversion from the lactone form. Hydroxyl lovastatin has been reported to be the more active form of this drug [26], and its efficacy for inhibition of HMG-CoA reductase will be validated in subsequent experiment.

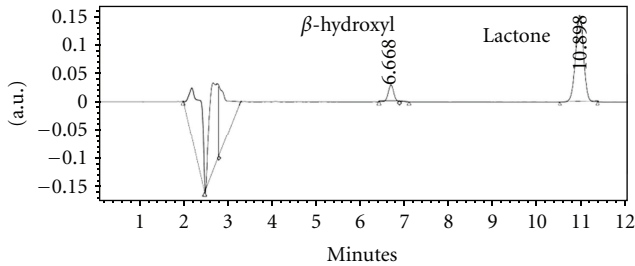


FIGURE 1: HPLC chromatogram of lovastatin in β -hydroxyl and lactone forms.

3.2. Effect of Substrate. In the first experiment, the efficacy of two strains of *A. terreus* on the production of lovastatin in SSF using RS and OPF as substrate was investigated. Lovastatin productions by both strains using RS and OPF as substrates are shown in Table 1. Quantity of lovastatin production by both strains of *A. terreus* in the RS samples was higher than OPF. The highest lovastatin production was 154.48 and 157.07 mg/kg DM, respectively, for *A. terreus* ATCC 20542 and ATCC 74135 using RS as substrate without additional nitrogen and mineral supplementation.

Although the two strains of *A. terreus* tested were capable to produce lovastatin from RS and OPF, results of this study showed that RS is a more suitable substrate, producing significantly higher lovastatin than OPF.

Lovastatin can be produced from 9 molecules of acetyl-CoA during the fermentation (Figure 2) [30]. 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 (Figure 2). 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 β -glucosidase, endoglucanase, and cellobiohydrolase) and hemicellulases (mainly xylanase) enzymes by *A. terreus* and their effects on lignocelluloses degradation have been well documented [31–33]. However, there is no known data on production of lignin degradation enzyme by *A. terreus*.

RS has two main advantages over OPF as substrate for *A. terreus* in SSF. Lignin content of RS is half that of OPF and its hemicelluloses content is higher than OPF (Figure 3). The lower production of lovastatin recorded for OPF using the two *A. terreus* could be partly due to the absence of enzyme to degrade the lignin component of the biomass. Reports also showed that *A. terreus* has high ability to produce hemicellulase enzymes such as xylanase [34], making RS

which contains high hemicelluloses more appropriate as substrate for lovastatin production.

3.3. Effect of Mineral Solution and Nitrogen Source. Effects of mineral solution and nitrogen on lovastatin production by *A. terreus* are shown in Table 1. Addition of mineral solution has no significant effect on quantity of lovastatin production by both strains of *A. terreus*. Although addition of nitrogen source has no significant effect on lovastatin production by *A. terreus* ATCC 74135 ($P > 0.05$), it negatively affected lovastatin production by *A. terreus* ATCC 20542 ($P < 0.01$) mainly in the ammonium sulphate treatment. Supplementation of minerals and nitrogen sources (especially soybean meal) increased the total lovastatin to about 64 mg/kg DM in *A. terreus* ATCC 74135 using OPF, but it is still half of that compared to RS (Table 1).

Minerals and nitrogen are essential growth nutrients for microorganisms [15]. In submerged fermentation, these nutrients must be added into the medium to sustain the growth of the microorganisms [34]. Agricultural biomass such as RS contain mineral components [35, 36]. Ash content of RS and OPF which is indicator of mineral content of these by products was shown in Figure 3. This quantity can supply the request of *A. terreus* for growth and lovastatin production and additional mineral solution has no effect on enhancement of the lovastatin production by this fungus.

Although nitrogen source is important for fermentation, but carbon:nitrogen (C:N) ratio is more important for lovastatin production. Negative effect of additional nitrogen source on lovastatin production was reported in previous study [37]. In the study of growth of *A. terreus*, and lovastatin production, the above authors reported that type of carbon and nitrogen sources and C:N ratio in the medium has important effect on lovastatin production and by increased the C:N ratio (from 14.4 to 23.4 and 41.3) the ability of *A. terreus* for lovastatin production was increased. Use of a slowly metabolized carbon source (lactose) in combination with either soybean meal or yeast extract under nitrogen-limited conditions gave the highest lovastatin production. Effect of C:N ratio on lovastatin production was also studied by Bizukojc and Ledakowicz [6] who reported that higher C:N ratio can provide better fermentation condition for lovastatin (mevinolinic acid) production (Figure 4). Results of the chemical analysis showed that the availability of N in RS (CP = 4.2%) is higher than OPF (CP = 3.2%) (Figure 3). On the other hand, stranger bands in the cell wall polymers of OPF in comparison to the RS due to the higher lignin (Figure 3) can suppress the availability of nitrogen for fungal activity in OPF. It can be concluded that RS without additional nitrogen has better C:N ratio in comparison to the samples that contain additional nitrogen sources. In contrast, to achieve the suitable C:N ratio in the OPF culture, additional nitrogen source is requested.

3.4. Effect on Lignocellulose Reduction. Although the main objective of this study was to investigate the ability of *A. terreus* to produce lovastatin using biomass as substrate, ability of this fungus to reduce the lignocelluloses content

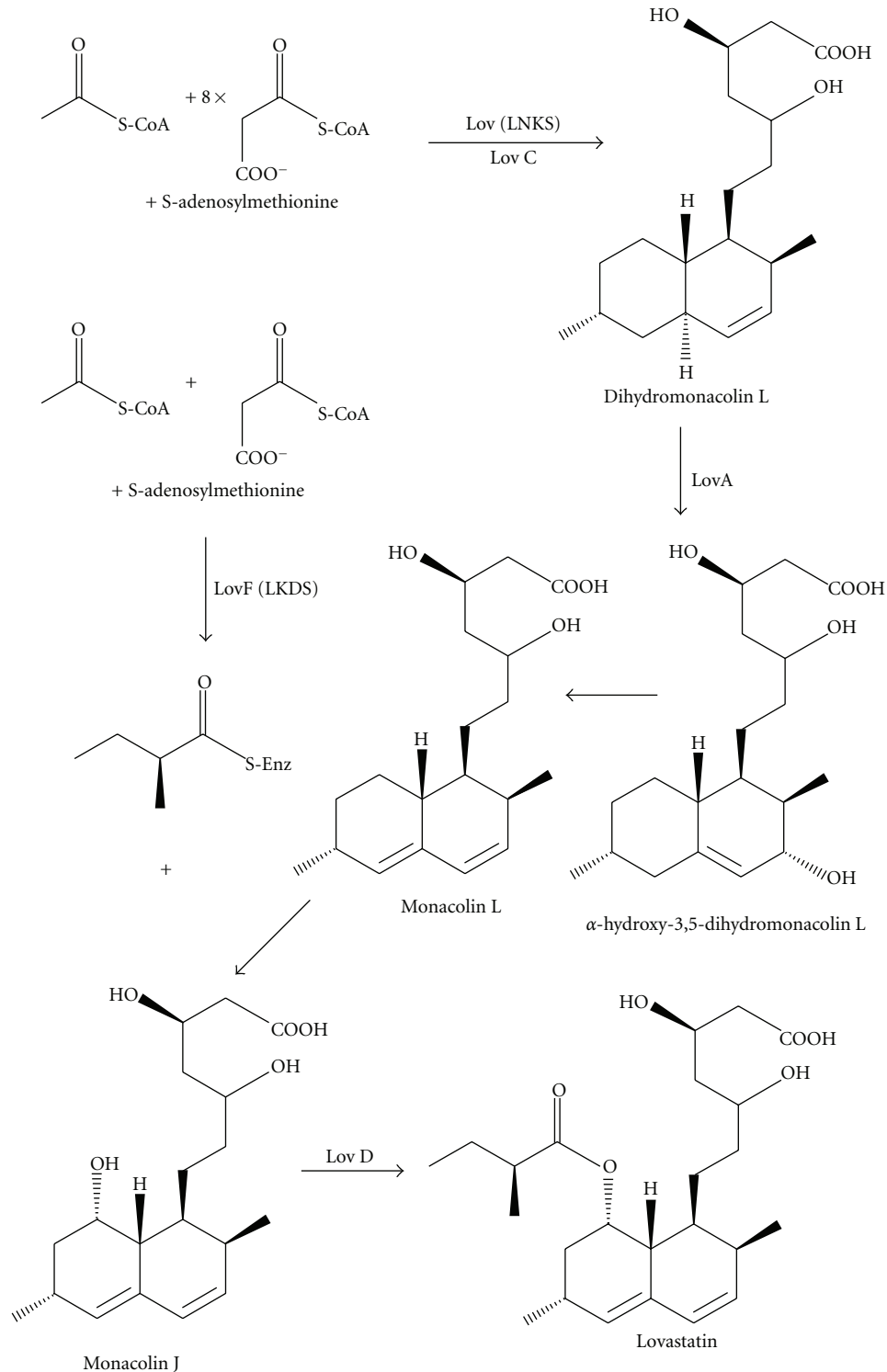


FIGURE 2: Lovastatin production pathway [30].

of RS and OPF was also important, particularly when these materials would be considered as feed for ruminants. Effect of the two strains of *A. terreus* on reduction of lignocelluloses content of RS and OPF is shown in Table 2. Results show that (i) additional mineral solution has no effect ($P > 0.05$) to enhancing the ability of *A. terreus*

to reduce lignocelluloses content of RS and OPF but (ii) nitrogen supplement increases the ability of the fungi to degrade cellulosic materials. Both nitrogen solution, urea and ammonium sulphate significantly increased lignocellulosic components reduction. *A. terreus* has higher ability for reduction of lignocelluloses content of RS compared to OPF.

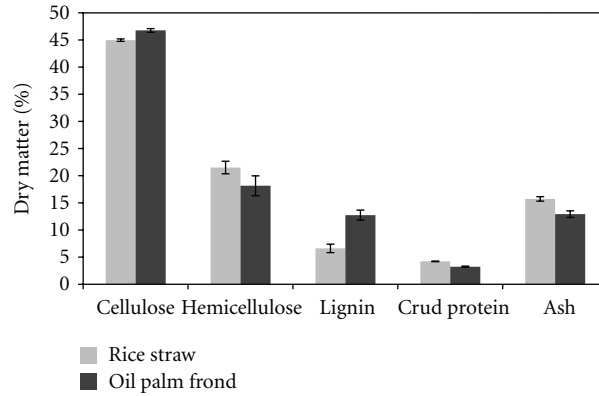


FIGURE 3: Lignocellulose, crude protein, and ash content of RS and OPF.

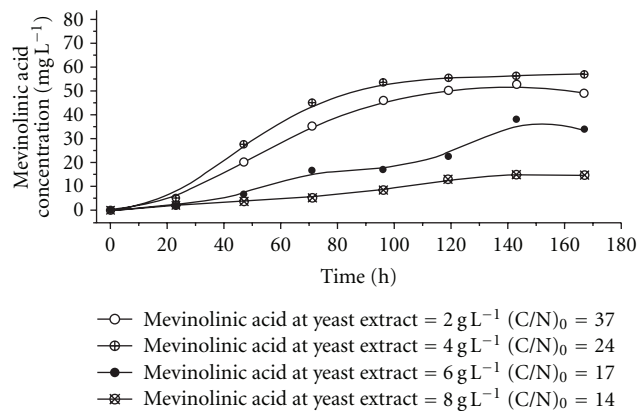


FIGURE 4: The evolution of mevinolinic acid (lovastatin) production in the batch culture at different initial yeast extract concentrations and C:N ratio content [6].

Although the effect of ammonium sulphate on lignocelluloses content reduction was higher than urea, this difference was not significant (Table 2). Because of its affordable price, urea is the most widely used nonprotein nitrogen in ruminant feed [38]. Thus, it is used as the nitrogen source in the following experiment for *A. terreus*.

Based on the higher yield of lovastatin using RS as substrate, the follow-up study for optimization of influencing factors (pH, temperature, particular size, inoculum size and initial moisture content and incubation time) was examined using the two strains of fungi on RS alone with addition of urea.

3.5. Effect of Initial pH. Study on initial pH (Figure 5) indicated that this factor has no significant effect on lovastatin production by both strains of *A. Terreus* ($P > 0.05$); however, pH 6 produced the highest lovastatin; 67.88 and 85.49 mg/kg DM by *A. terreus* ATCC 20542 and *A. terreus* ATCC 74135, respectively. There was no significant difference in lovastatin production for pH of 5 to 8; however, pH higher than 7 or lower than 6 had negative effect on lovastatin production. Kumar et al. [39] showed that pH 5.8–6.3 provided optimum conditions for lovastatin production by *A. terreus* in the batch process. Other researchers [40–42] also reported

optimum pH of within the range of 5–7 using various fungi for statin production. The above information thus suggested that optimum initial pH for lovastatin production in SSF is near neutral pH with small variations which could be due to the types of substrate and microorganism used in the fermentation process. On the other hand, [43] reported that by controlling pH and slowly adding the carbon source lovastatin yield could increase five folds.

3.6. Effect of Temperature. One of the important factors effecting microbial activity and thus biomaterial production is incubation temperature. Results of this study suggest that 25°C is optimum temperature for lovastatin production in SSF by the two strains of *A. terreus* with maximum production of 171.61 and 202.93 mg/kg DM for *A. terreus* ATCC 20542 and *A. terreus* ATCC 74135, respectively (Figure 6). Increasing the incubation temperature to higher than 25°C has negative effect on lovastatin production and *A. terreus* ATCC 74135 being more sensitive to the temperature change. Similar incubation temperature (25°C) was also reported previously [43, 44] for lovastatin production. Optimum temperature of 29.5°C for lovastatin production had been reported by Panda et al. [40] using *Monascus purpureus* and *Monascus Ruber* while Kumar et al. [39] showed that 28°C is

TABLE 2: Effect of SSF on cellulose and hemicellulose contents of RS and OPF (% of dry mater).

Treatments	<i>A. terreus</i> ATCC 20542		<i>A. terreus</i> ATCC 74135	
	Cellulose	H-cellulose	Cellulose	H-cellulose
Nonfermented RS	44.97 ± 0.22 ^{ab}	21.50 ± 1.16 ^a	44.97 ± 0.22 ^a	21.50 ± 1.16 ^a
Fermented RS (FRS)	46.83 ± 2.12 ^a	18.06 ± 1.20 ^{bc}	45.12 ± 0.23 ^a	13.13 ± 0.79 ^b
FRS plus mineral	45.80 ± 0.67 ^{ab}	19.63 ± 0.80 ^{ab}	44.91 ± 0.72 ^a	12.38 ± 0.76 ^b
FRS plus mineral and urea	40.70 ± 2.52 ^c	16.96 ± 1.78 ^{dc}	41.97 ± 0.61 ^b	9.21 ± 1.05 ^c
FRS plus mineral and ammonium sulphate	42.72 ± 3.69 ^{bc}	15.11 ± 1.65 ^d	40.97 ± 0.51 ^c	7.79 ± 2.50 ^c
Significant	*	**	**	**
Nonfermented OPF	46.76 ± 0.34 ^a	18.13 ± 1.83	46.76 ± 0.34 ^a	18.13 ± 1.83
Fermented OPF (FOPF)	43.22 ± 2.59 ^b	19.36 ± 0.31	46.23 ± 1.17 ^a	15.66 ± 1.05
FOPF plus mineral	43.04 ± 1.28 ^b	15.82 ± 2.92	45.34 ± 0.95 ^{ab}	15.01 ± 0.82
FOPF plus mineral and urea	42.37 ± 1.92 ^b	16.90 ± 0.15	43.40 ± 0.89 ^b	15.40 ± 0.62
FOPF plus mineral and ammonium sulphate	44.66 ± 0.54 ^{ab}	18.30 ± 1.80	43.59 ± 1.69 ^b	15.00 ± 1.51
Significant	*	NS	*	NS

NS: not significantly different.

* Significantly different at 5% level.

** Significantly different at 1% level.

^{a,b,c} indicating that means within column are significantly different.

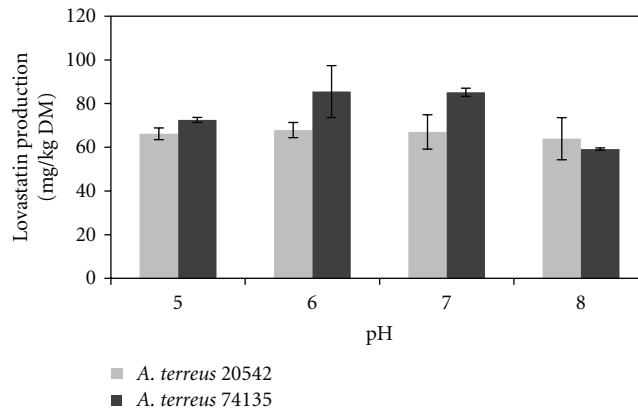


FIGURE 5: Effect of pH on lovastatin production by two strains of *A. terreus* ($P > 0.05$).

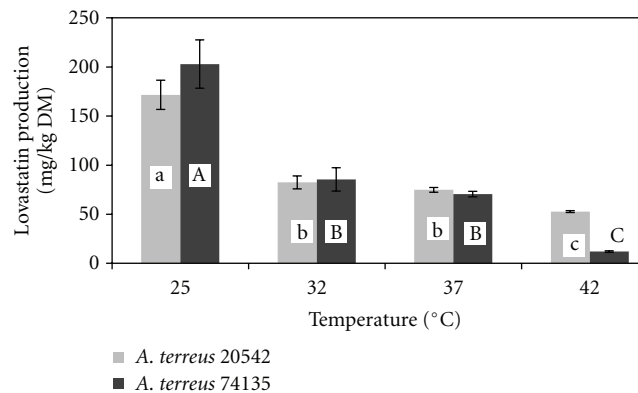


FIGURE 6: Effect of incubation temperature on lovastatin production by *A. terreus* ($P < 0.01$). (a, b, and c) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, and C) indicate differences among means between samples for *A. terreus* ATCC 74135.

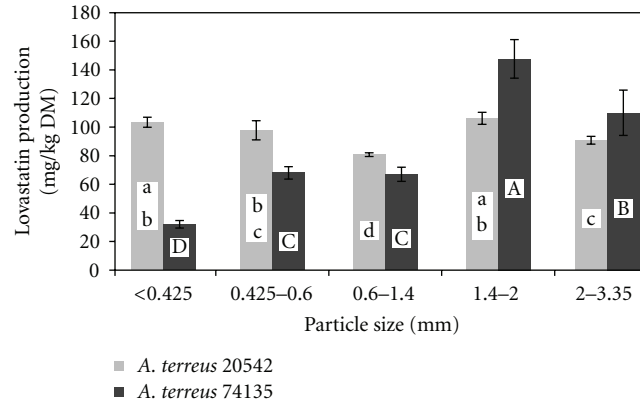


FIGURE 7: Effect of particle size on lovastatin production by two strains of *A. terreus* ($P < 0.01$). (a, b, c, and d) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, C, and D) indicate differences among means between samples for *A. terreus* ATCC 20542.

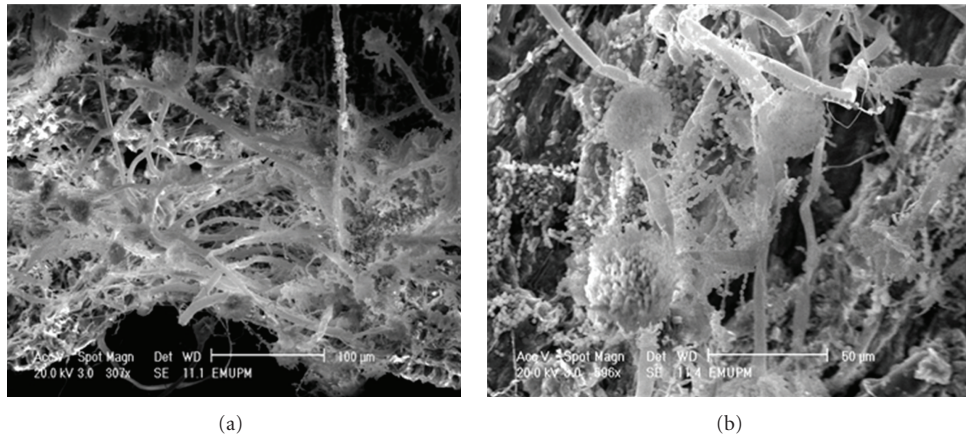


FIGURE 8: Scanning electron micrographs of *A. Terreus* ATCC 20542 (a) and *A. Terreus* ATCC 75135 (b) on the surface of RS. High concentration of mycelium and spore could have negative effects on air flow in the solid culture.

optimum incubation temperature for lovastatin production by *A. terreus*.

3.7. Effect of Particle Size. Five different particle sizes of RS were used to study the effect of this factor on lovastatin production. The results suggest optimum particle size of RS for lovastatin production was between 1.4 to 2 mm and *A. terreus* ATCC 74135 is more sensitive to changes in particle size (Figure 7). The above results are differed with that of Valera et al. [41], who found increasing particle size of wheat bran as substrate (from 0.4 to 1.1 mm) in SSF resulted in reduction of lovastatin production. They further reported an interaction effect between particle size and moisture content of solid material on lovastatin production.

There are two opposing effects of particle size on SSF process at any given moisture content. The first is small particle size that increases surface area of solid materials for the attachment and growth of the fungi. The second is smaller particle size that reduces interspace between particles and gas phase oxygen transfer and thus reduces the growth potential of the aerobic microorganisms. In addition, growth

and multiplication of microorganisms on the surface of solid materials further reduce the gas phase space and make it even more difficult for air transfer between solid particles. Effect of high micellium content on reduction of the gas phase space of RS culture are shown in Figure 8. High concentration of mycelium and spores present between the solid particles reduce the flow of air in the culture and could reduce the available oxygen for growth of *A. terreus*.

On the other hand, some reports that indicate reduction of particle size enhanced production of lovastatin in SSF [15]. Wei et al. [7] reported that grinding rice through 20 mesh size ($840 \mu\text{m}$) has positive effect but further reduction using 40 mesh size ($420 \mu\text{m}$) reduced lovastatin production compared with the natural size of rice grain. The formation of lovastatin is strictly dependent on the oxygen supplied; however, Bizukojc and Ledakowicz [6] reported that aeration rate up to 0.308 vvm is preferred for lovastatin biosynthesis as higher aeration has negative effect on lovastatin production.

3.8. Effect of Moisture. Moisture content had significant effect on lovastatin production ($P < 0.01$) (Figure 9). Results

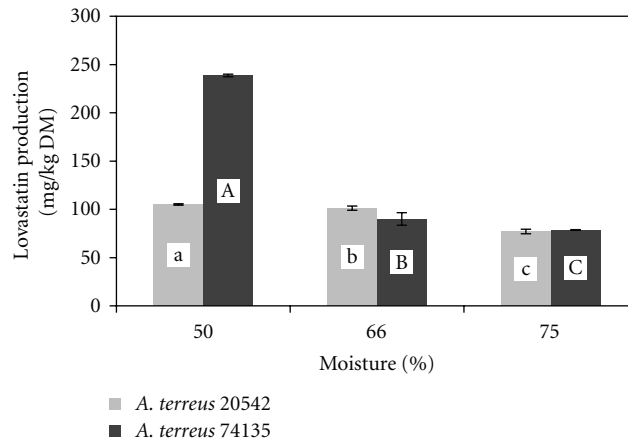


FIGURE 9: effect of moisture content on lovastatin production by two strains of *A. terreus* ($P < 0.01$). (a, b, and c) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, and C) indicate differences among means between samples for *A. terreus* ATCC 20542.

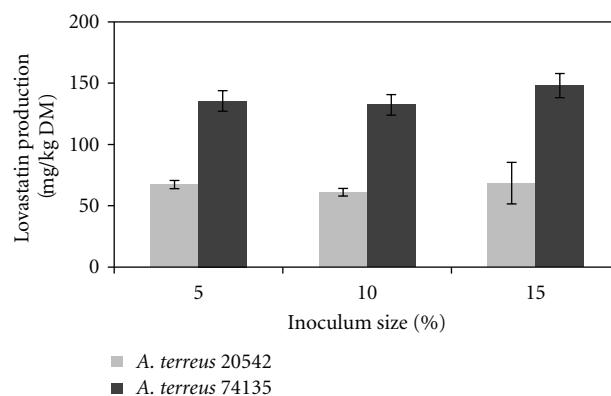


FIGURE 10: Effect of inoculum size on lovastatin production by *A. terreus* ($P > 0.05$).

of this study suggest that 50% moisture content is optimum for lovastatin production by two strains of *A. Terreus*. Results also showed that *A. Terreus* ATCC 74135 is more sensitive to the moisture and at 50% moisture production of lovastatin by this strain can be up to 238.74 mg/kg DM. Other studies [41, 42] reported that slightly higher (58 to 60%) initial moisture produced maximum yield of statin using other fungi. High moisture content resulted in aggregation of substrate particles, reduction of aeration and leading to anaerobic conditions [45].

3.9. Effect of Inoculum Size. Figure 10 shows that different inoculum sizes did not affect ($P > 0.05$) lovastatin production by the two strains of *A. Terreus*. Previous study showed no significant difference in lovastatin production using 5×10^7 to 10×10^7 spores/mL (within the range used in this study), but use of lower than 5×10^7 spores/mL can depress the production of lovastatin [15].

3.10. Effect of Incubation Time. To study the effect of incubation time on lovastatin production, optimal conditions for all the other factors (pH = 6, temperature = 25°C, inoculum size = 10% and moisture = 50%) obtained

earlier were applied. Results of the study suggested that maximum production of lovastatin was achieved on day 12 with lovastatin production of 175.85 mg/kg DM for *A. terreus* ATCC 20542 but day 8 with lovastatin production of 260.85 mg/kg DM for *A. terreus* ATCC 74135 (Figure 11). In both fungi cultures, the concentration of lovastatin increased until day 8 with no significant effect on lovastatin production thereafter. Pansuriya and Singhal [15] reported that lovastatin production by *A. terreus* in SSF using wheat bran increased until day 3 of fermentation with no further enhancement thereafter. The shorter duration of the above study compared to the present study could be due to higher quality of substrate (rice bran versus RS). The yields of lovastatin obtained in this study were about 20% of those reported [5, 42] using wheat bran and groundnuts oil cake as substrates. The lower lovastatin yield recorded in this study, using agro-biomass as compared to those using high-energy grains and oil seeds, was acceptable.

4. Conclusion

Results of this study suggest that RS is the better substrate than OPF for lovastatin production in SSF. Although

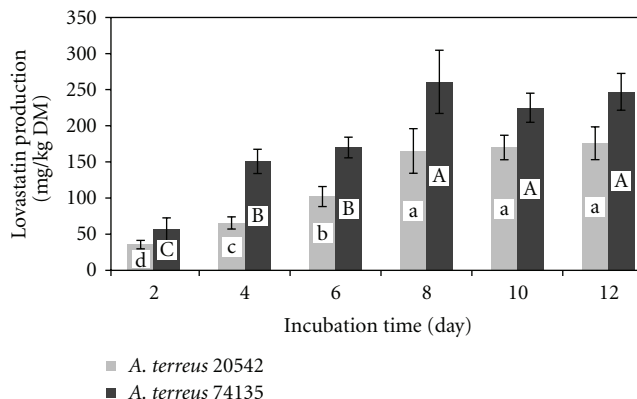


FIGURE 11: Lovastatin production by *A. terreus* in optimum condition at different time of incubation ($P < 0.01$). (a, b, c, and d) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, and C) indicate differences among means between samples for *A. terreus* ATCC 20542.

additional nitrogen source has no benefit for improvement the lovastatin production, it enhances lignocelluloses degradation by the fungi. Since one of the main objectives of this study was to evaluate the use of the fermented RS as ruminant feed, urea was supplemented as nitrogen source in the fermentation process. Results of optimization experiment indicate that pH 6, 25°C incubation temperature, 10% inoculum size, 50% moisture content, and 8 days fermentation are the best conditions for maximum lovastatin production in SSF using RS as substrate with *A. terreus* ATCC 74135 recorded higher lovastatin production of 260.85 mg/kg DM after 8 days fermentation.

The present study provides a new insight for production of lovastatin and/or other similar biomaterials of high value from agro-biomass, which otherwise may be sources of pollutant to the environment. Furthermore, the lovastatin enriched fermented RS has the potential to be used as antimethanogenesis feed supplement for reduction of enteric methane production in the ruminant animals. The above suggestion needs further investigations.

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