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ORIGINAL ARTICLE

Optimization of lovastatin production from *Aspergillus fumigatus*

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Abstract The present investigation, focused on screening of various fungal species for Lovastatin production using different agro-based wastes, also, for maximizing lovastatin productivity by isolated *Aspergillus fumigatus* using response surface methodology (RSM). The following substrates (Olive cake; Pea pods; sugarcane bagasse; wheat bran; rice hulls; beet peel; Potato peel and groundnut shells) were screened to evaluate their effectiveness for lovastatin production, using different fungal species, (*Aspergillus niger; Rhizopus oligosporus; Penicillium citrinum* and isolated *Aspergillus fumigatus*) under solid state fermentation (SSF). Wheat bran was the most suitable substrate for lovastatin production with all fungal species. Optimum conditions of lovastatin production by wheat bran have been attained efficiently by response surface methodology (RSM) using isolated *Aspergillus fumigatus* under solid state fermentation (SSF). The lovastatin yield of (3.353 mg/g DFM) was obtained at an optimum temperature of 28 °C; pH of 5.00; initial moisture content of 70% and incubation period of 12 days. This Lovastatin has the possibility to use in different therapeutic applications.

1. Introduction

Lovastatin is produced as a secondary metabolite of the polyketide pathway by various fungi including *Penicillium* spp. [5]; *Monascus* spp. [12]; *Trichoderma* spp. [4] and *Aspergillus terreus* [8]. *A. terreus* is known to be the best lovastatin-producing species [23]. Statins (e.g. lovastatin) are fungal secondary metabolites, also considered a group of medically important inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase), which catalyzes the rate limiting step of cholesterol biosynthesis [19,22]. Clinically, statins are used as lipid-lowering drugs that effectively lower LDL-cholesterol levels and reduce the risk of cardiovascular events in dyslipidemic patients [3,10]. It decreases LDL level more than other cholesterol lowering drugs, [13]. Lovastatin does not only find a role as anti-cholesterol agent but also plays a key role as an anti-inflammatory agent; cancer cell apoptosis; renal function restoration; treatment for bone disorders and suppressed production of tumor necrosis factor [19]. There is also an increased interest in statins non-lipid activities such

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as, protection of host cellular damage [27]. In addition, lovastatin has been used in the biomedical applications such as treating coronary heart diseases; Alzheimer's disease [25]. For statin production an alternative strategy for submerged fermentation is the solid state fermentation process (SSF), where solid state fermentation is more advantageous, which offers a good environment for fungi to grow, therefore high mycelia density and high lovastatin production can be expected [19]. According to Pandey et al. [16] solid state fermentation is a process where a wide range of agricultural wastes can be used for growing fungal species and to minimize the overall valuable product cost. Also, it eases optimization parameters, as it involves lower media cost; stability of the product; increased yield and better substrate porosity [2,21]. The present study aims to optimize and demonstrate the effect of different factors and factor–factor interactions on Lovastatin production by isolated *Aspergillus fumigatus* using response surface methodology (RSM) under solid state fermentation.

2. Materials and methods

2.1. Organisms

Different fungal species were selected for the production of Lovastatin using solid state fermentation (*Aspergillus niger* NRRL 595; *Rhizopus oligosporus* NRRL 2710 and *Penicillium citrinum*). The three strains were purchased from NRRL: Northern Regional Research Laboratory, United States Department of Agriculture, Peoria Illinois, USA. In addition, twenty-two fungal isolates were purified from mangrove tree sediments grown along the shores of Red sea, Makadi village, Hurghada region, Egypt.

2.2. Identification of the most efficient lovastatin producer isolate

The most potent fungal strain, isolated from (mangrove trees sediments grown along shores of Red sea, Makadi village, Hurghada region, Egypt) was identified by its morphological and conidial features in the culture growth and DNA partial sequencing. DNA sequencing of the most potent fungal strain was carried out with PCR amplicon. The 28S r DNA sequence D1/D2 region was amplified by PCR from fungal genomic DNA using PCR universal primers:

DR-5′-GGTCCGTGTTTCAAGACGG-3′ and DF-5′-ACCCGCTGAACTTAAGC-3′ respectively, where it has been identified as *Aspergillus fumigatus* (Fig. 1). Identification has been performed at Macrogen Company, Korea.

2.3. Culture maintenance and inoculum preparation

Cultures of all tested fungi were maintained on potato dextrose agar (PDA) at 28 °C for 10 days. Spores that formed were then scrapped and suspended in sterile dist. Water with 0.1% (v/v) Tween 80 and vigorously shaken for 1 min, 2 mL spore suspensions were used as the inoculums for the present investigation [20].

Figure 1 Screening for lovastatin production using different agro-based waste.

2.4. Culture preparations and conditions

Eight agro-wastes (Olive cake; Pea pods; sugarcane bagasse; wheat bran; rice hulls; beet peel; Potato peel and groundnut shells) were dried at 70 °C for 24 h., cooled and grounded. Ten grams of each solid substrates was taken separately in 250 mL Erlenmeyer's flasks and was moistened with distilled water containing ($MgSO4{\cdot}7H_2O$ (0.15 g/l); (NH4)2HPO4 (0.25 g/l); NaCl (1 g/l) to maintain the moisture content of 70% (v/w) [9]. Then, substrates were autoclaved at 121 °C for 20 min, cooled to room temperature and flasks were inoculated with 2 mL of fungal spore suspension. The contents in the flask were mixed thoroughly to ensure uniform distribution of the inoculum and flasks were incubated at 28 °C for 10 days [6].

2.5. Extraction of lovastatin

At the end of SSF, lovastatin concentration was analyzed, where the fermented material was dried at 60 °C for 24 h, powdered, and 4 g of the powdered material was extracted with ethyl acetate (pH 3.0) in 250 mL Erlenmeyer's flasks. It was then, incubated at 28 °C in rotary shaker at 200 rpm for 2 h. Then the mixture was centrifuged at 1500 rpm for 20 min. Filtration was done using Whatman filter paper No. 1 for separation of fungal cell biomass from the filtrate. The supernatant was stored in glass bottles at 4 °C until used for further analysis [20]. To 1 mL of the supernatant; 1 mL of acetic acid (1%) was added and incubated for 10 min. From the above solution, 1 mL was taken and diluted 10 times with methanol, and its absorbance was read at 238 nm, using UV–Visible spectrophotometer [11].

$$\text{Concentration of Lovastatin (mg/g)} = \frac{(\text{Concentration of Lovastatin (mg/ml)})}{\text{Amoun t of substrate (Wheat bran) taken (g)}} * \text{Dilution factor}$$

2.6. Design of experiment (DOE)

Response surface method; central composite design (CCD) model (Table 1); based on four factors and five levels was used to study the effect and interactions between temperature (A) in the range between 24 and 40 °C; pH (B) in the range between 4 and 8; moisture content (C) in the range between 40% and 80%, and incubation period (D) in the range between 3 and 15 days for maximum lovastatin production by *Aspergillus fumigatus*. Experimental designs were performed using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, *ver* 7.0.0). Experiments were performed in triplicates with six central points. A total of 78 runs were employed in CCD to estimate curvature and interaction effects of selected variables, and finally, significance of the obtained model was checked by *F*-test (calculated *p*-value) and goodness of fit by multiple correlation *R* as well as determination R^2 coefficients. Lovastatin concentration (mg/g) was the measured experimental response.

Statistical Analysis: Analysis of variance (ANOVA) was used to estimate the statistical parameters for optimization of culture conditions. A probability value of *P* value < 0.05 was used as the criterion for statistical significance.

3. Results and discussion

3.1. Screening of different agro based wastes for lovastatin production using several fungal species by solid state fermentation

Table 2 and Fig. 1 showed that, among all screened fermented substrates with different fungal strains, wheat bran has topped the list with a maximum lovastatin yield, where the yield was 2.84; 2.69; 2.53 and 1.84 mg/g of dried fermented matter (DFM) by *A. fumigatus*; *A. niger*; *Penicillium citrinum* and *Rhizopus oligosporus*. This result is in accordance with [18,22,14,7] and Raghunath et al. [20] who, also found wheat bran to be a suitable substrate for lovastatin production. On other hand, sugar cane bagasse; olive cake and potato peel also yielded good amount of lovastatin by all different fungal strains. 2.41, 2.28 and 2.25 mg/g dry fermented matter, by *A. niger*

Table 1 Central composite design (CCD) used for optimization of lovastatin production (organized in triplicate runs).

Run number	Temperature (°C)	pH	Moisture Content (%)	Incubation period (Days)
1–3	28	5	50	6
4–6	36	5	50	6
7–9	28	7	50	6
10–12	36	7	50	6
13–15	28	5	70	6
16–18	36	5	70	6
19–21	28	7	70	6
22–24	36	7	70	6
25–27	28	5	50	12
28–30	36	5	50	12
31–33	28	7	50	12
34–36	36	7	50	12
37–39	28	5	70	12
40–42	36	5	70	12
43–45	28	7	70	12
46–48	36	7	70	12
49–51	24	6	60	9
52–54	40	6	60	9
55–57	32	4	60	9
58–60	32	8	60	9
61–63	32	6	40	9
64–66	32	6	80	9
67–69	32	6	60	3
70–72	32	6	60	15
73–75	32	6	60	9
76–78	32	6	60	9

Table 2 Screening for lovastatin production using different agro-based waste.

Lovastatin concentration (mg/g)	*Aspergillus fumigatus*	*Aspergillus niger*	*Penicillium citrinum*	*Rhizopus oligosporus*
Wheat bran	2.84	2.69	2.53	1.84
Olive cake	2.28	2.12	1.94	1.78
Beet peel	1.75	1.63	1.28	1.81
Groundnut shell	1.94	1.38	1.31	0.69
Pea pods	1.13	1.59	1.22	1.03
Potato peel	2.25	2.03	1.38	0.78
Bagasse	2.41	2	1.56	0.88
Rice hulls	1.75	1.19	0.78	0.56

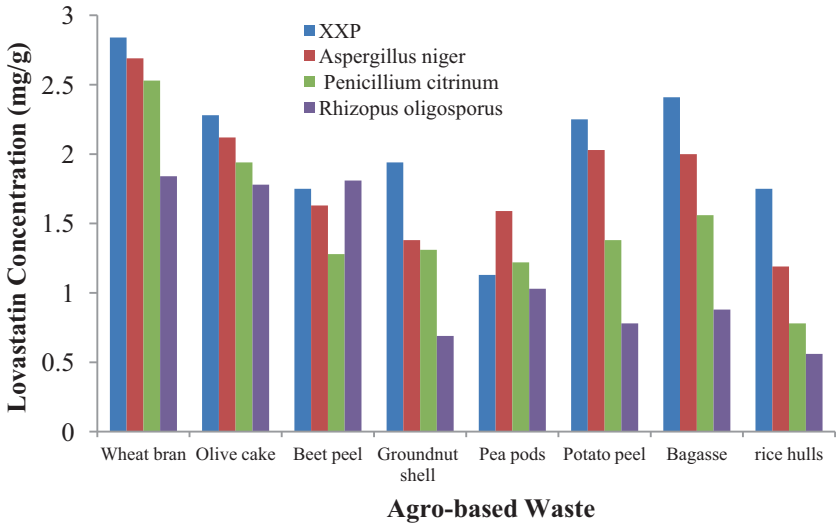

Figure 2 Phylogenetic tree based on rDNA gene sequencing, showing the phylogenetic relationship of *Aspergillus fumigatus* within representative species of the genus *Aspergillus*.

Table 3 Analysis of variance (ANOVA) results for Lovastatin central composite design.

Source	Sum of Squares	df	Mean square	*F*-Value	*p*-value (Prob > F)*
Model	56.97	12	4.747	36.479	< 0.0001
A-Temperature	19.66	1	19.662	151.080	< 0.0001
B-pH	7.58	1	7.576	58.212	< 0.0001
C-Moisture content	0.20	1	0.203	1.561	0.2159
D-Incubation period	0.01	1	0.010	0.078	0.7814
AB	3.09	1	3.088	23.729	< 0.0001
AC	0.79	1	0.795	6.108	0.0161
BC	0.49	1	0.487	3.742	0.0574
BD	0.37	1	0.368	2.828	0.0974
CD	0.47	1	0.474	3.644	0.0607
A^2	20.28	1	20.280	155.834	< 0.0001
B^2	6.81	1	6.813	52.350	< 0.0001
C^2	9.79	1	9.793	75.250	< 0.0001
Residual	8.46	65	0.130		
Lack of fit	8.13	12	0.678	109.375	< 0.0001
Pure error	0.33	53	0.006		
Cor total	65.43	77			

* Values of "Prob > F" less than 0.05 indicate model terms are significant.

Table 4 Central composite design runs with actual and predicted response values.

Run number	Lovastatin mg/g			Run number	Lovastatin mg/g		
	Actual value	Predicted value	Residuals		Actual value	Predicted value	Residuals
1	2.956	2.499	0.457	40	0.950	1.050	−0.100
2	2.975	2.499	0.476	41	0.943	1.050	−0.107
3	2.944	2.499	0.445	42	0.931	1.050	−0.119
4	1.169	1.204	−0.035	43	1.723	1.730	−0.007
5	1.069	1.204	−0.135	44	1.775	1.730	0.045
6	1.175	1.204	−0.029	45	1.784	1.730	0.054
7	1.506	1.317	0.189	46	1.125	0.935	0.190
8	1.536	1.317	0.219	47	1.131	0.935	0.196
9	1.544	1.317	0.227	48	1.119	0.935	0.184
10	1.144	1.036	0.108	49	0.769	1.767	−0.998
11	1.125	1.036	0.089	50	0.845	1.767	−0.922
12	1.103	1.036	0.067	51	0.875	1.767	−0.892
13	2.972	2.860	0.112	52	0.000	−0.323	0.323
14	3.018	2.860	0.158	53	0.000	−0.323	0.323
15	2.806	2.860	−0.054	54	0.000	−0.323	0.323
16	1.191	1.050	0.141	55	1.931	2.303	−0.372
17	1.141	1.050	0.091	56	2.034	2.303	−0.269
18	1.163	1.050	0.113	57	2.063	2.303	−0.240
19	2.750	2.080	0.670	58	0.886	1.005	−0.119
20	2.688	2.080	0.608	59	0.288	1.005	−0.717
21	2.662	2.080	0.582	60	0.880	1.005	−0.125
22	0.919	1.285	−0.366	61	0.884	1.292	−0.408
23	1.031	1.285	−0.254	62	0.891	1.292	−0.401
24	0.969	1.285	−0.316	63	0.882	1.292	−0.410
25	3.188	2.897	0.291	64	1.288	1.504	−0.216
26	3.156	2.897	0.259	65	1.306	1.504	−0.198
27	3.175	2.897	0.278	66	1.297	1.504	−0.207
28	1.675	1.601	0.074	67	2.731	2.915	−0.184
29	1.625	1.601	0.024	68	2.756	2.915	−0.159
30	1.594	1.601	−0.007	69	2.746	2.915	−0.169
31	1.753	1.364	0.389	70	2.719	2.963	−0.244
32	1.731	1.364	0.367	71	2.756	2.963	−0.207
33	1.794	1.364	0.430	72	2.781	2.963	−0.182
34	1.150	1.083	0.067	73	2.750	2.939	−0.189
35	1.125	1.083	0.042	74	2.875	2.939	−0.064
36	1.069	1.083	−0.014	75	2.843	2.939	−0.096
37	3.256	2.860	0.396	76	2.844	2.939	−0.095
38	3.238	2.860	0.378	77	2.813	2.939	−0.126
39	3.353	2.860	0.493	78	2.813	2.939	−0.126

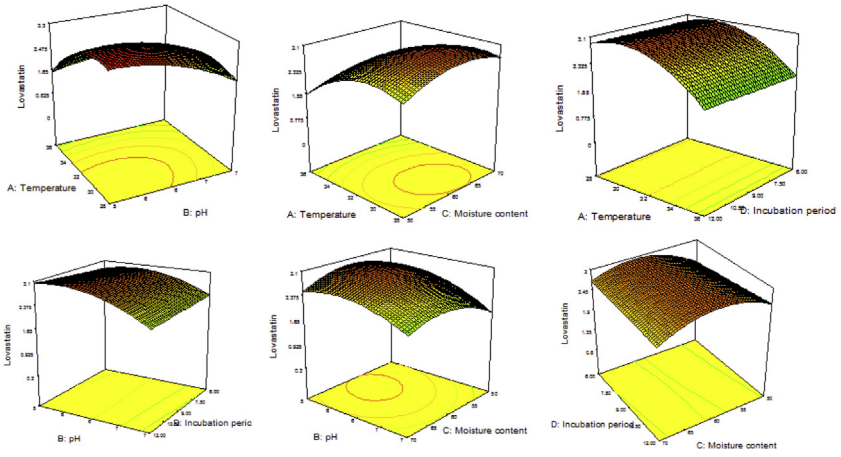

Figure 3 3D plots of the effect of different factors and factors interactions on lovastatin production by *Aspergillus fumigatus* under SSF.

respectively. While, groundnut shells; beet peel; rice hulls and pea pods showed low production of lovastatin 1.94; 1.75; 1.75 and1.13 mg/g dry fermented matter, respectively by *Rhizopus oligosporus*. These results are in accordance with [17]. Based on the results of the screening study, it is clear that, *Aspergillus fumigatus* has the greatest affinity to produce lovastatin using wheat bran (2.84 mg/g). As wheat bran fermentation with *A. fumigatus* produces maximum yield of lovastatin, wheat bran was selected as suitable substrate for further optimization study with *A. fumigatus* under solid state fermentation using response surface methodology.

3.2. Identification of the potent lovastatin producing fungus isolate

Fungal colonies produce thousands of minute gray-green conidia (2–3 μm). The fungus can grow at 37 °C, up to 50 °C temperatures. The morphological and conidial features in the culture growth and DNA partial sequencing showed that the most potent lovastatin producing fungal isolate is *Aspergillus fumigatus*. The phylogenetic relationship of *Aspergillus fumigatus* within representative species of the genus *Aspergillus* is shown in Fig. 2.

After running the CCD, the experimental results were statistically analyzed using analysis of variance (ANOVA). The results of ANOVA are shown in Table 3.

The Model *F*-value of 36.48 implies the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The model showed that A, B, AB, AC, A^2, B^2, C^2 are significant model terms. The "Lack of Fit F-value" of 109.37 implies the Lack of Fit is significant and there is only a 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise.

The "Predicted R-Squared" of 0.8072 is in reasonable agreement with the "Adjusted R-Squared" of 0.8468. The adequate Precision – measures the signal to noise ratio and a ratio greater than 4 is desirable – equals to 22.313 which indicates an adequate signal. The R^2 value of 0.8707 indicates that the model is reliable. Accordingly, this model can be used to navigate the lovastatin design space. The interactions between factors are shown in the following figures. The actual and predicted results of the model runs are shown in Table 4 based on the final equation of the model shown below (see Fig. 3).

3.3. Final equation in terms of actual factors

$$\text{Lovastatin (mg/g)} = -45.94002 + (1.89923 * \text{Temperature}) + (1.16020 * \text{pH}) + (0.53989 * \text{Moisture content}) + (0.37787 * \text{Incubation period}) + (0.063411 * \text{Temperature} * \text{pH}) - (3.21719E{-}003 * \text{Temperature} * \text{Moisture content}) + (0.010073 * \text{pH} * \text{Moisture content}) - (0.029188 * \text{pH} * \text{Incubation period}) - (3.31319E{-}003 * \text{Moisture content} * \text{Incubation period}) - (0.034645 * (\text{Temperature})^2) - (0.32129 * (\text{pH})^2) - (3.85203E{-}003 * (\text{Moisture content})^2).$$

Response Surface Methodology (RSM) approach has been well adopted to assess lovastatin yield and determine the optimum fermentation parameters [24]. Maximum lovastatin yield (3.353 mg/g) was recorded at pH 5.0. This result is in accordance with [2] also, Valera et al. [26] and Atalla [1] indicated that, increase in pH resulted in a gradual decrease in 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 support the cell growth and product formation. Maximum lovastatin yield was at a temperature of 28 °C this result falls in line with [17,15] where, further increase in temperature, more heat is accumulated, leads to poor heat dissipation and reduces the oxygen level, thereby reducing the growth of microorganism. The maximum lovastatin yield was achieved at 12 days of fermentation period, this result is in accordance with [20]. On the other hand, maximum lovastatin yield was achieved at initial moisture content (70 v/w). As the moisture content increases, the air in fermentation medium decreases, resulting in poor oxygen availability [26,17] Also, an increase or a decrease in moisture content affected the oxygen and water balance [14], thereby decreasing lovastatin yield. The higher lovastatin yield associated with solid state fermentation (SSF) [25] is due to increased mycelial density [21]; and increased porosity [18]. Also, it involves lower media and product cost.

4. Conclusion

The current investigation was mainly focused on the screening of various fungal species for lovastatin production utilizing different agro-based wastes under solid state fermentation. The isolated fungus *Aspergillus fumigatus* showed the maximum yield of lovastatin. As optimum conditions of lovastatin production by *Aspergillus fumigatus* using wheat bran have been attained efficiently by response surface methodology (RSM), the Lovastatin yield (3.353 mg/g dry fermented matter) was achieved with the following optimized culture conditions (temp. of 28 °C; pH of 5.00; initial moisture content of 70% and incubation period of 12 days). The feasibility of solid state fermentation (SSF) as a promising technique in exploiting cheaply available agro-residual and employing response surface methodology (RSM) as an optimization technique not only increases the yield but also results in economic lovastatin production.

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