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Biotransformation of Agricultural Wastes into Lovastatin and Optimization of a Fermentation Process Using Response Surface Methodology (RSM)

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Abstract: Lovastatin is a competitive inhibitor of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA Reductase). The HMG-CoA reductase is responsible for the production of mevalonate by the reduction of HMG-CoA. It is a rate-limiting step in the production of cholesterol. The current study demonstrates the production of lovastatin from an ethidium bromide mutated strain of *Aspergillus terreus* ATE-120 (saprophytic fungus) that is grown on 1–3% NaOH pretreated substrate of sugar cane bagasse (*Saccharum officinarum* L.). For the hyperproduction of lovastatin, different optimization parameters such as temperature, pH, inoculum size, fermentation period, and inoculum age were mentioned and analyzed via response surface methodology. The RSM results indicate that the maximum lovastatin yield (156.43 mg/L) was predicted at a 5.5 pH, 35 °C temperature, 4 mL inoculum size, 36 h inoculum age, and 48 h fermentation via solid state fermentation. According to these results, the effect of pH had a significant effect on lovastatin production, while other parameters had an insignificant effect, and coefficients of determination (R^2) having a value of 77.24% indicates the goodness of the proposed model. The structure of the obtained drug was confirmed by nuclear magnetic resonance. Moreover, an X-ray diffraction analysis of the sample was carried out to characterize the physical form of the lovastatin. It can be concluded from the above study that the maximum yield of the drug can be found via RSM and that the selected strain (*Aspergillus terreus* ATE-120) has good potential for lovastatin production through solid-state fermentation.

Keywords: lovastatin; agricultural wastes; sugarcane bagasse; *Aspergillus terreus* mutant; nuclear magnetic resonance (NMR); X-ray diffraction (XRD); response surface methodology (RSM)

Citation: Javed, S.; Azeem, M.; Mahmood, S.; Al-Anazi, K.M.; Farah, M.A.; Ali, S.; Ali, B. Biotransformation of Agricultural Wastes into Lovastatin and Optimization of a Fermentation Process Using Response Surface Methodology (RSM). *Agronomy* **2022**, *12*, 2848. https://doi.org/10.3390/agronomy12112848

Academic Editors: David Correa-Galeote and Antonio Serrano

Received: 19 October 2022
Accepted: 6 November 2022
Published: 15 November 2022

1. Introduction

Lovastatin is a drug, which is used to reduce cholesterol in the blood. It is considered the first cholesterol-lowering drug that acts as a competitive inhibitor of the HMG-CoA for enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) and does not allow the production of mevalonate (an important step for cholesterol production) [1,2]. In 1987, the Food and Drug Administration (FDA) of the United States of America recommended it as the most effective drug against high cholesterol levels [3]. It is a whitish crystalline powder, non-hygroscopic in nature, and usually insoluble in water but, it is soluble in acetonitrile, methanol, ethanol, and some other organic solutes and it is also named mevinolin. It is used in the form of free acids as well as in lactone, therapeutically. More technically, lovastatin is soluble in the form of a lactone ring while insoluble in the form of β-hydroxic acid. Invivo and invitro studies suggest that lovastatin plays an inhibitory role on the plasma low-density lipid (LDL) cholesterol level in patients that

suffer from dyslipidemia and normal biological production of cholesterol in the body of normal humans and other animals [4,5]. So, it is an important drug for the treatment of high cholesterol levels in the blood; a condition called hypercholesterolemia, associated with various myocardial infarctions and cardiovascular diseases (CVDs) [6]. More applications of lovastatin have been observed therapeutically in the inhibition of induced apoptosis, and cellular proliferation, and in various experimental settings, such as necrosis in the case of blood cancers, thus lovastatin also acts as an agent against cancerous cells [7]. It is also used for the treatment of Alzheimer's disease, the treatment of coronary heart diseases [8], the treatment of certain disorders related to bones, and the low production of TNFs (Tumor necrosis factors) [9].

It has been observed that lovastatin can be produced by various species of fungi in the polyketide pathway as a secondary metabolite. These species include; *Aspergillus terreus* [10], Trichoderma species [11], Monascus species [12], and Penicillium species [13]. The screening analysis of various species has been studied and it was observed that among all the above-mentioned species; the best lovastatin-producing specie may be *Aspergillus terreus* [14]. It is not only the fungal secondary metabolite but it is also among the group of all those chemicals that act as competitive inhibitors of enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) medically, which produces a product of mevalonate; the ultimate rate-limiting step of cholesterol biosynthesis [10–16].

To produce lovastatin by fermentation experimentally, instead of liquid-state fermentation (LSF) and substrate-level fermentation (SmF), solid-state fermentation (SSF) is a more significant technique. It provides the fungi with an improved environment to grow and produce. As SSF provides the fungi with more mycelial density, lovastatin is more concentrated [9]. In the process of solid-state fermentation, various types of agricultural wastes can be used as substrates, which provide the medium of growth for different strains. These agricultural wastes are; barley, bagasse, wheat bran, gram bran, soybean meal, fruit wastes, etc. The proper maintenance of the fermentation parameters such as temperature, pH, inoculum age, inoculum size and fermentation period, etc. are optimized to produce significant products with a low cost of media, more substrate porosity, high yield, and more stability of product [16–18].

This research-based study aimed to elaborate on the effect of the optimization of fermentation parameters such as temperature, pH, inoculum size, fermentation period, and inoculum age. The interaction between the different fermentation parameters is also studied as well as other factors that affect fermentation and the interactions between factors for lovastatin production that takes place through *Aspergillus terreus* ATE-120, which was isolated. Afterward, a response surface methodology (RSM) was used under SSF (solid-state fermentation). The RSM technique is used to establish the functional relationships between different variables and shows the central composite design (CCD), Analysis of variance (ANOVA), and Contour graphs (CG) to show the inter-relationships among different variables. Moreover, Nuclear Magnetic Resonance (NMR) and X-ray Diffraction (XRD) have been carried out to characterize the fermented drug lovastatin.

2. Materials and Methods

2.1. Induction of Mutation

The induction of chemical mutation in the strain of *Aspergillus terreus* ATE-120 (already published) was reported by Azeem et al. [19]. A stock solution was prepared that contained 0.5 mg/mL of ethidium bromide (*w*/*v*). EB (Sigma-Aldrich, Gillingham, UK) stock solution was prepared in the laboratory. 1×10^7 spores mL^{-1} of *Aspergillus terreus* ATE-120 was added in Vogel medium to make a 9 mL solution kept at 37 °C in a water bath (Eyela, Tokyo, Japan). After intervals of a specific time, one milliliter (mL) sample is withdrawn and by using normal saline solution, washing of cell pellet takes place thrice. For one minute and at 12,000 rpm, the sample was centrifuged using a centrifuge machine (Mikro 20 Hettich, Tuttlingen, Germany), to eliminate the chemical mutagen from the sample specimen [19].

2.2. Substrate Pretreatment

Sugarcane bagasse was used as a substrate for fermentation. It was first obtained from Shakarganj Sugars Mills Jhang, Pakistan. It can also be acquired from the local market. For the removal of dust and other dirt particles, the sugarcane bagasse was washed with cold water. This washing process was thorough. The substrate was ground to 40 mm and a total of 30 mL of NaOH sample obtained, which possessed 1% to 3% concentration of solute NaOH. 2 g of the bagasse powder was added to each flask of the concentration. The prepared samples were kept at the temperature of 80 °C, neutral pH, and placed in the microwave for incubation for 10 min. After proper filtration, the prepared samples were rinsed with tap water for the completion of pretreatment, and the samples were then dried at 65 °C [19].

2.3. Preparation of Inoculum and Solid-State Fermentation

The production of the drug lovastatin occurred through the solid-state fermentation method of Azeem et al. [19]. To conduct this experiment, a temperature-controlled incubator (SLI-220, Eyela, Shangai was used. The triplicated 500 mL Erlenmeyer flasks were moistened with 7 mL Vogel medium that contained 2 g of pre-treated sugarcane bagasse (carrier substrate). By using M NaOH/M HCl solutions, the pH of the flasks was maintained at 6 and the media flasks were autoclaved at 121 °C for 15 min. After that, the flasks were cooled at room temperature, and then these triplicated flasks were inoculated with the 5 mL of homogeneous suspension that contained a strain of *Aspergillus terreus* ATE-120 and then were placed for 72 h for the sake of SSF under these conditions [19].

2.4. Lovastatin Extraction

In the fermentation broth that was fermented for 3 days, 10% 1N HCl was added. In this acidified broth the ethyl acetate was added in equal amounts to the solution at the temperature of 70 °C and then centrifuged at 180 rpm for 2 h. For the filtration of broth, the filter paper named Whatman paper No. 1 was used. Meanwhile for 10 min, at 3000 rpm the filtrate was again centrifuged. At this time the organic phase was collected and from this organic phase of filtrate, one milliliter was added with 10 milliliters of the 1% trifluoroacetic acid for lactonization. For the evaporation of the moisture, the temperature of the extract increased to 80 °C, then acetonitrile was used to dilute it and the solution was then filtered for HPLC analysis [20].

2.5. Lovastatin Analysis

The HPLC method was used for the quantification and identification of lovastatin by the method of [21]. For this purpose, the obtained samples were prepared, as the filtered broth was 10-fold diluted with acetonitrile-water (1:1 by volume) and the analysis took place by using HPLC (Hitachi, Ibaraki, Japan) fully equipped with a UV detector (Hitachi L-2400) at 238 nm and a Hitachi L-2130 (C-18) column. In this solution, the solvent was prepared by mixing 0.1% phosphoric acid (60:40 by volume) along with acetonitrile. With a flow rate of 1.5–1.6 mL/min, a 20 µL sample was injected into the column.

2.6. Design of Experiment (DOE)

For the central composite design (CCD) model of the response surface method (RSM), Design Expert 11 Software was used for optimization parameter analysis. The obtained values are mentioned in Table 1, which is based on five factors to study the significance and interrelationships between pH (A) present in the fixed range from 3 to 10; temperature (B) in the range between 25 °C to 45 °C; inoculum size (C) in the range between 2 mL to 5 mL; inoculum age (D) in the range between 12 h to 72 h and fermentation time (E) in the range between 24 h to 96 h for the maximum lovastatin production by *Aspergillus terreus* Strain ATE-120. Experimental designs were performed using Design-Expert software (stat-Ease, Inc.1300 Godward Street Northwest, Suite 6400 Minneapolis, MN 55413, USA, version 11.0.0). To estimate the effect of interactions between different factors in the form

of selected variables and curvatures, at the end, an F-Test (Calculated *p*-values) was also checked to attain the effectiveness of the model, and the significance of fit by R^2 coefficients determination along with multiple correlation R by total 50 runs in the central composite design (CCD). Experimental response of the concentration of lovastatin was measured in mg/L. Lastly, to gauge the fermentation parameters statistically, the statistical technique ANOVA (Analysis of variance) was used against the optimization of the culture conditions. For the statistical significance of the experiment, the *p*-value or probability value was obtained up to <0.05 as a criterion [22].

Table 1. Runs report and Central Composite Design (CCD) that compares the predicted and actual response values.

Run	A: pH	B: Temp. (°C)	C: Inoculum Size (mL)	D: Inoculum Age (h)	E: Fermentation Time (h)	Lovastatin mg/L	
						Actual	Predicted
1	10	25	2	12	96	58.65	34.08
2	3	25	2	72	96	45.67	50.98
3	10	45	5	72	96	19.76	12.40
4	3	45	5	72	96	41.76	40.06
5	5.5	35	4	42	36	122.65	103.12
6	5.5	35	4	36	48	156.43	88.00
7	3	25	3	12	60	74.23	62.06
8	6.5	35	4	42	60	86.44	84.40
9	3	25	2.5	72	24	34.62	38.10
10	10	25	2.5	72	96	15.43	15.98
11	10	25	3	12	24	17.89	18.62
12	6.5	35	3	42	60	92.34	85.42
13	3	25	2	12	96	45.62	45.15
14	3	25	2	72	24	52.65	40.76
15	6.5	35	4	27	60	81.72	92.71
16	6.5	35	4	42	60	86.98	84.40
17	10	45	5	12	24	25.12	24.95
18	10	45	5	12	24	22.27	24.95
19	6.5	35	4	42	60	47.23	84.40
20	3	25	2	72	96	54.28	50.98
21	8.25	35	3	42	60	36.87	62.70
22	6.5	35	3	42	78	92.65	97.23
23	10	45	5	72	96	16.87	12.40
24	6.5	35	4	42	60	91.45	84.40
25	10	25	2	72	24	24.23	19.63
26	10	25	2	12	24	21.31	19.55
27	10	25	2	72	24	19.65	19.63
28	6.5	35	3.5	42	60	89.23	76.81
29	3	45	5	72	24	35.14	44.93
30	3	45	5	12	96	33.24	38.22
31	10	45	5	72	24	27.25	21.03
32	6.5	30	3.5	42	60	83.26	92.21
33	3	25	2.5	12	24	28.25	52.99
34	6.5	35	3	42	60	87.21	85.42
35	10	25	2	12	96	21.98	34.08
36	10	45	5	12	96	19.42	24.39
37	3	25	2	12	24	25.64	26.86
38	10	25	2	72	96	19.87	26.08
39	10	45	5	72	24	15.32	21.03
40	10	45	5	12	96	26.43	24.39
41	3	45	5	72	24	36.32	44.93
42	3	45	5	12	96	34.24	38.22
43	6.5	35	4	42	60	45.24	84.40
44	3	45	4.5	12	24	36.31	25.95
45	4.75	35	3.5	42	60	51.23	54.16
46	6.5	40	4	42	60	84.34	75.43
47	6.5	35	3	42	42	96.54	108.44
48	3	45	5	72	96	44.23	40.06
49	6.5	35	3	42	60	97.24	85.42
50	3	45	5	12	24	34.78	35.02

2.7. NMR Spectroscopy

All 1HNMR measuring was carried out using a Bruker-Advance 400 Ultrashield spectrometer (BrukerBioSpin, Rheinstetten, Germany) equipped with a 5-mm SEI probe with Z-gradient coils and an Automatic Sample Changer B-ACS 120 (BrukerBioSpin, Rheinstetten, Germany). NMR spectra were acquired at 293.2 K.

2.8. X-ray Diffraction (XRD)

X-ray diffraction analysis of samples was carried out to characterize the physical form i.e., amorphous or crystalline nature of LOV in samples of the optimized batch in an X-ray diffractometer (D8 Advance, Bruker, Billerica, MA, USA) with Cu Kα radiation (λ = 1.54060A°). The scanning rate was 10°/min and the diffraction angle 2θ was 10–80°.

3. Results and Discussion

The improvement of the strain *Aspergillus terreus* by mutagenesis through ethidium bromide and pretreatment with alkali (1–3% NaOH) of sugar cane bagasse (showed better results in a screening experiment over corncobs, wheat straw, and banana stalk) for the hyper-production of the cholesterol-lowering drug lovastatin by solid-state fermentation is related to the results in Azeem et al. [19]. Experimental evidence has shown that the maximum lovastatin yield (91 ± 1.77 mg/L) with dry cell mass (4.49 ± 0.81 mg/L) was recorded by a mutant ATE-120 before the optimization parameters. However, after implementation of fermentation parameters such as pH, temperature, inoculum size, fermentation period, and inoculum age for the synthesis of the cholesterol-lowering drug lovastatin by *Aspergillus terreus* ATE-120 at the optimized level and treated bagasse used as a substrate; a significant production of lovastatin is observed with the maximum yield (115.43 mg/L) taking place at a pH value of 5.5. The outcome is quite similar to Chanakya et al. [16], Valera et al. [23], and Atalla et al. [24]. These results indicate that a gradual change in pH causes the inactivation or denaturation of the fungal strain thus influencing the reaction and causing a decrease in the production of lovastatin. It is also due to the factor that the transportation of various chemicals by active transport or by passive transport and solid bulky material by phagocytosis etc. across the cell membrane is strongly influenced by pH, which is responsible for supporting the cell growth and product formation. The effect of pH is shown in Table 1. The optimum level of lovastatin yield was gained at a temperature of 35 °C and these results are related to the conclusions drawn by Wei P-l et al. [25], and Panda et al. [26]. It also indicates that an increase in temperature leads to no significant dissipation of heat and causes a decrease in the oxygen level of the system, thus reducing the growth of the fungal strain. The maximum yield of lovastatin takes place with the inoculum size of almost 4 mL, at 48 h of the fermentation period along with the inoculum age of almost 36 h. These outcomes are also related to the results of Raghunath et al. [27]. Various researchers suggested that solid-state fermentation (SSF) is more valuable for the higher yield of lovastatin [8]. This high yield causes an increase in the mycelial density of fungus [17] and increased porosity [28]. It also decreases product and media costs.

3.1. The Outcome of the Design of the Experiment (DOE) for Lovastatin Production

Response surface methodology (RSM) is an efficient mechanism of analysis that critically and efficiently explores the explanatory variables through optimized values. The interactions between different variables in terms of the fermentation parameters are very significantly understandable by contour plots and three-dimensional (3D) response surface graphs obtained through RSM optimization. The regression equations are quite easily examined and described in a visual manner that shows the required response by experimental variables [29].

In this study, the effect of basic optimization parameters applied on solid-state fermentation (SSF) including pH, temperature, inoculum size, inoculum age, and fermentation time (represented by symbols A, B, C, D, and E, respectively) on lovastatin production from a mutated strain of *Aspergillus terreus* ATE-120 by RSM was studied. Central Composite

Design (CCD) demonstrates the analysis of the combination of different parameters that were derived experimentally. It depends on different combinations of the five fermentation parameters as represented in Table 1. The result of the production of lovastatin was given as input values to the Design Expert 11 software (RSM analysis software) for the prediction of results and it analyzed the continuous effects. From the values of Table 1, it has been shown that in run 39 there is the least amount of lovastatin produced, whereas on the other hand, in run 06 a maximum amount has been shown, which were 15.32 mg/L and 156.43 mg/L, respectively, as also indicated by Chanakya et al. [16], Valera et al. [23] and Atalla et al. [24]. The differences in both conditions were the former pH of 10, the temperature of 45 °C, the inoculum size of 5 mL, inoculum age of 72 h, and fermentation time of about 24 h while the latter was with pH of 5.5, and the temperature of 35 °C. These results have accordance with Wei P-l et al. [25], and Panda et al. [26], while inoculum size of 4 mL, inoculum age of 36 h, and fermentation time of 48 h are results in accordance with Raghunath et al. [27]. From the results obtained, it showed that a great change in the amount of pH, a difference in temperature values, and fermentation time of lovastatin were obtained. This is consistent with Miyake et al. [30].

3.2. Statistical Analysis of Lovastatin Production

ANOVA (Analysis of Variance) results show the statistical analysis of the experimental to the predicted values. This all has been accomplished by running CCD (Figure 1). All the results of ANOVA are mentioned in Table 2. Observing the F-values of the model indicates that it is significant and has a value of 7.99 with the chance of only 0.01% that the large "Model F-value" may be due to noise. The significance of the model terms is determined by *p*-values that are not more than 0.05. The current experiment shows that among all interactive variables, A, A^2, and E^2 model terms are significant. It is indicated that the non-significance of the model term will be if the values are more than 0.1000. Lack of Fit F-value of 3.58 implies the Lack of Fit is not significant relative to the pure error. There is only a 0.90% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant Lack of Fit is good. The "Predicted R-Squared" of 0.0652 is in reasonable agreement with the "Adjusted R-Squared" of 0.6154. Adequate Precision—measures the signal-to-noise ratio and a ratio greater than 4 is desirable—which equals 7.4397 which indicates an adequate signal. The R^2 value of 0.7724 indicates that the model is reliable. Accordingly, this model can be used to navigate the lovastatin design space as mentioned in Table 2.

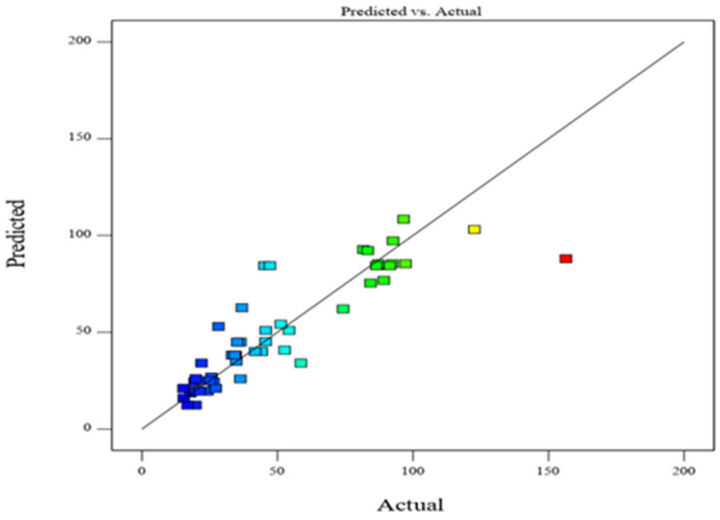

Figure 1. Central Composite Design (CCD) model shows the Graphical representation of Predicted vs. Actual response where different colors are indicated by the value 15.32 156.43 of Lovastatin (mg/L).

Table 2. Model Summary Statistics.

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS
Linear	32.96	0.0775	−0.0274	−0.1209	58,070.93
2FI	22.05	0.6810	0.5403	−0.0546	54,635.52
Quadratic	20.16	0.7724	0.6154	0.0652	48,429.31
Cubic	13.35	0.9415	0.8314		*

Table 2 shows the model statistics of the experiment and represents that the substrate (sugar cane bagasse), which was pre-treated with NaOH, yields lovastatin in good quantity and quality using the fungus *Aspergillus terreus* ATE-120 strain. The significance of the results by using Design Expert 11 software along with Analysis of variance (ANOVA) is also mentioned in Table 3 below.

Table 3. Central Composite Design (CCD) model of lovastatin by Analysis of variance (ANOVA) results.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	40,014.02	20	2000.70	4.92	<0.0001
A-pH	2075.87	1	2075.87	5.11	0.0315
B-Temp.	0.0385	1	0.0385	0.0001	0.9923
C-Inoculum size	3.85	1	3.85	0.0095	0.9232
D-Inoculum age	6.09	1	6.09	0.0150	0.9034
E-Fermentation time	160.32	1	160.32	0.3943	0.5350
AB	1615.27	1	1615.27	3.97	0.0557
AC	1518.02	1	1518.02	3.73	0.0632
AD	313.81	1	313.81	0.7718	0.3869
AE	21.98	1	21.98	0.0540	0.8178
BC	352.49	1	352.49	0.8669	0.3595
BD	499.79	1	499.79	1.23	0.2767
BE	497.40	1	497.40	1.22	0.2778
CD	495.23	1	495.23	1.22	0.2788
CE	398.65	1	398.65	0.9804	0.3303
DE	104.51	1	104.51	0.2570	0.6160
A^2	1724.85	1	1724.85	4.24	0.0485
B^2	235.83	1	235.83	0.5800	0.4525
C^2	198.20	1	198.20	0.4875	0.4906
D^2	2.90	1	2.90	0.0071	0.9333
E^2	1335.99	1	1335.99	3.29	0.0802
Residual	11,791.56	29	406.61		
Lack of Fit	8773.69	13	674.90	3.58	0.0090
Pure Error	3017.88	16	188.62		
Cor Total	51,805.58	49			

* Values of "*p*-values" less than 0.05 indicate model terms are significant. Here A, B, C, D and E represents the fermentation parameters/factors such as pH, temperature; Inoculum size, inoculum age and fermentation time respectively AB. AC, AD, AE, BC, BD, BE, CD, CE, DE, A^2, B^2, C^2, D^2 and E^2 are the interaction between these factors; Cor total, Corrected total

The interactions between the different fermentation parameters that are applied for the optimization of the fermentation of the fungus *Aspergillus terreus* strain ATE-120 are shown in the following Figure 2a–j. Table 1 shows all the predicted and actual results of the model runs, which entirely depend on the final equation as described below in detail.

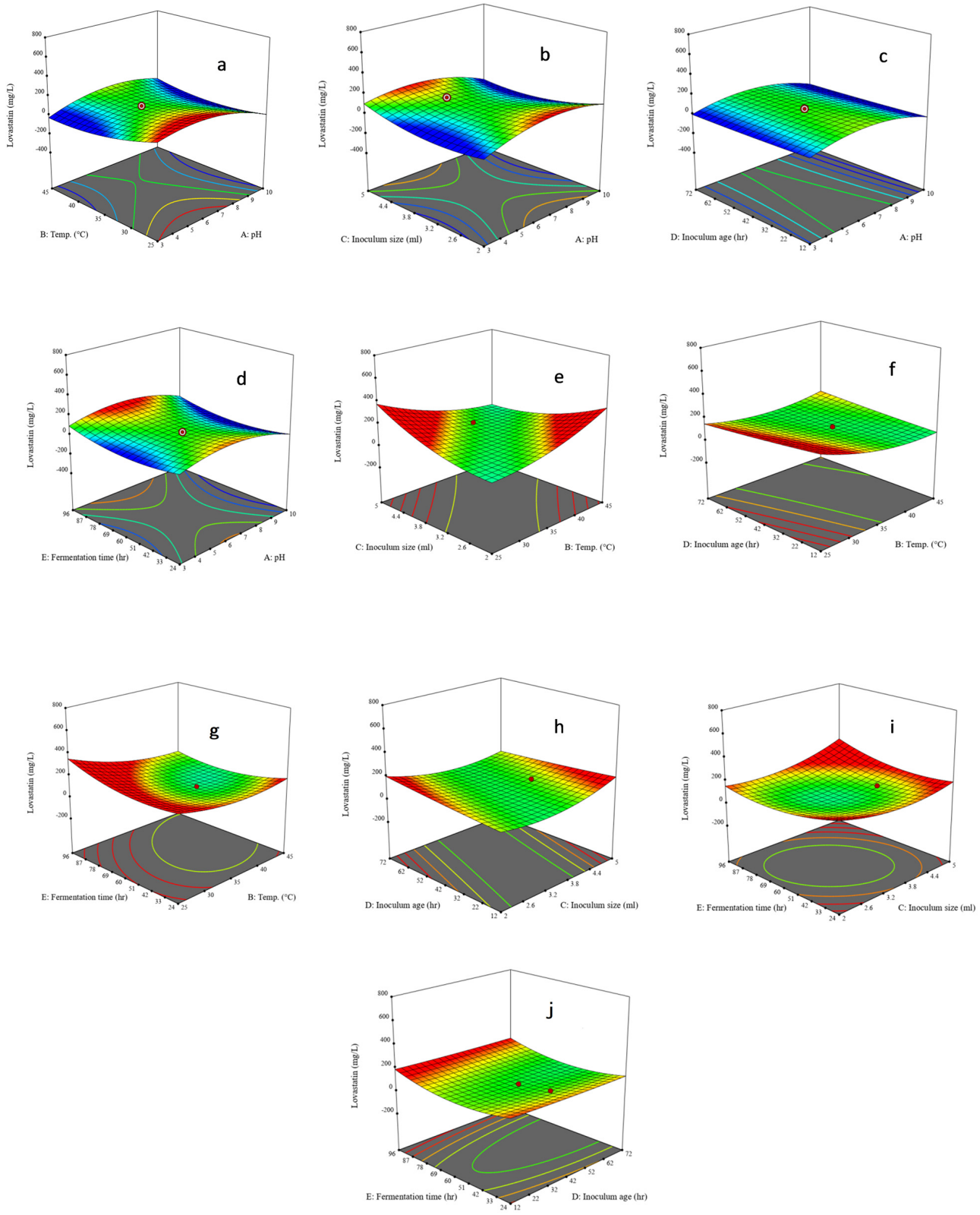

Figure 2. Three Dimensional plots show the effect of (**a**) factor AB (**b**) factor AC (**c**) factor AD (**d**) factor AE (**e**) factor BC (**f**) factor BD (**g**) factor BE (**h**) factor CD (**i**) factor CE (**j**) factor DE of various fermentation parameters for optimization and factors to factor inter-relationship for the synthesis of lovastatin through *Aspergillus terreus* ATE-120 by SSF.

3.3. In Terms of Actual Factors, the Final Equation of the Experiment

Lovastatin = −67.55226 + (97.37397 pH) − (16.98928 Temp.) + (174.66212 Inoculum size) − (1.72509 Inoculum age) − (4.56437 Fermentation time) + (1.46696 pH × Temp.) − (9.91122 pH × Inoculum size) − (0.032913 pH × Inoculum age) − (0.007461 pH × Fermentation time) − (9.80732 Temp. × Inoculum size) + (0.140621 Temp. × Inoculum age) − (0.124044 Temp. × Fermentation time) − (0.959645 Inoculum size × Inoculum age) + (0.757125 Inoculum size × Fermentation time) − (0.001869 Inoculum age × Fermentation time) − (8.82280 pH^2) + (0.619025 $Temp.^2$) + (32.41020 $Inoculum\ size^2$) + (0.006001 $Inoculum\ age^2$) + (0.053750 $Fermentation\ time^2$).

The above equation can generate the predictions in terms of the effect of provided stages of every variable value. To identify the relative impact of the variables, the coded equation that is obtained by comparing the variable coefficients is useful.

It indicates the mathematically calculated plot (predicted) and experimental plot (actual) in the form of a graphical representation of the model to produce lovastatin. The experimental values of lovastatin are quite like the predicted values. It also indicates that there is a strong correlation between experimental (Actual) and prediction model results. Thus, it shows that the regression model that was developed by the values, is reliable and through it, the production of lovastatin can be predicted [31,32].

3.4. Analysis of Parameters on Production of Lovastatin by Response Surface Methodology

In Figure 2a–j, some interactions are mentioned between different fermentation parameters. The 3D response surface plots indicate the production of lovastatin. These plots are based on the interrelationship between different fermentation parameters. The interrelationship among parameters AB (pH and temperature), AC (pH and inoculum size), AD (pH and inoculum age), AE (pH and fermentation time), BC (temperature and inoculum size), BD (temperature and inoculum age), BE (temperature and fermentation time), CD (inoculum size and inoculum age), CE (inoculum size and fermentation time) and DE (inoculum age and fermentation time) peaks show the quantity of produced lovastatin is demonstrated in Figure 2a–j, respectively. In these interactions, the effect of any two factors can be studied by keeping the other three factors constant. The production of lovastatin was observed to be maximum at 156.43 mg/L, 122.65 mg/L, 97.24 mg/L, and 156.43 mg/L concerning interactive parameters of AB, AC, AD, and AE, respectively. Similar findings have also been reported by Kumar et al. [10], as it is already understood that pH and temperature are critical optimization parameters that are involved in controlling enzyme activity as well as the growth of living organisms. Among all interactions, AB (temperature and pH) and AE (pH and fermentation time) have shown the best combination along with AC (pH and inoculum size) and AD (pH and inoculum age) as shown in Figure 2a–d while BD (temperature and inoculum Age), CE (inoculum size and fermentation time) and DE (inoculum age and fermentation time) have shown better interactions in terms of the maintenance of fermentation parameters while BC (temperature and inoculum size), BE (temperature and fermentation time) and CD (inoculum size and inoculum age) show adverse effects in terms of the maintenance of fermentation parameters for the production of lovastatin. Their negative impact regarding interactions indicates that these interactions have no direct linage on the biosynthetic pathway of lovastatin production.

The NMR spectra for the confirmation of purified fermented lovastatin produced by the mutated strain of *Aspergillus terreus* (ATE-120) under optimized conditions are shown in Figure 3. The NMR measurements were performed on a Bruker Advance 400 MHz instrument. The ^{1}H-NMR spectrum of the isolated impurity was further confirmed by comparing ^{1}H-NMR signals with those of lovastatin (Figure 3) and found that it is structurally close to lovastatin except for some structural variations. The ^{1}H-NMR spectrum of the isolated impurity suggests the presence of other groups; the presence of these groups can be confirmed by locating a singlet of a few protons and a multiple of one proton. NMR Spectroscopy is used more often than 13C NMR, partly because proton spectra are much easier to obtain than carbon spectra. The 13C isotope is only present in about 1% of carbon

atoms, and that makes it difficult to detect. The fungal lovastatin obtained from ^{1}H NMR was in accord with data reported earlier [33,34]. The NMR analysis of the standard was not shown. Since the hydroxyl form of lovastatin is not stable, the lactone form is normally the primary lovastatin detected in fermented products [35]. The ^{1}H spectra of the fungal sample yielded a molecular formula $C_{24}H_{36}O_5$ (Figure 4).

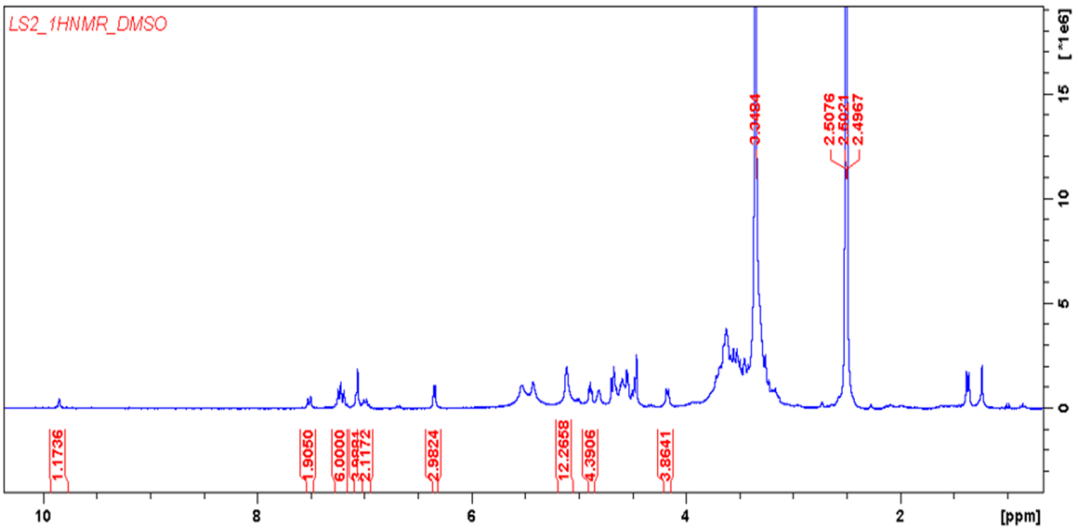

Figure 3. The ^{1}H NMR Spectra for the confirmation of lovastatin. NMR spectra suggests the following peaks on (LS_2); 985 (1H, S, COOH), 7.51 (2H, d, 2 × CH, J = 5.5 mHz), 7.22 (6H, m, 3 × CH_2), 7.06 (4H, S, 4 × CH), 6.99 (2H, m, 1 × CH_2), 6.34 (3H, d, J = 3.34 mHz), 5.11 (12H, d, 4 × CH_3), 4.90 (4H, t), 4.17 (4H, d, J = 3.6 mHz).

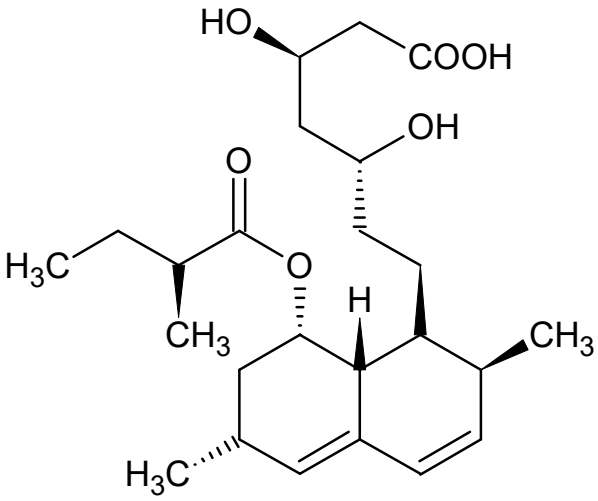

Figure 4. Structure of lovastatin produced by *Aspergillus terreus* ATE-120.

After the structural confirmation of fermented lovastatin by Nuclear magnetic resonance NMR presented in Figure 3, the physical form of the drug was determined by the X-ray diffraction method (XRD) as shown in Figure 5. The diffraction spectra of fermented lovastatin showed sharp and intense peaks of crystallinity. Moreover, the fermented lovastatin indicates a decrease in crystallinity or the slightly amorphous nature of the drug. In Figure 5, data given from XRD analysis shows that the fermented drug is 84.2% crystalline. Verma et al. [36] reported that the diffraction spectra of pure LOV showed sharp and intense peaks of crystallinity and X-RD analysis of the pure lovastatin shows that the pure drug is 89.4% crystalline which is in close agreement with our findings.

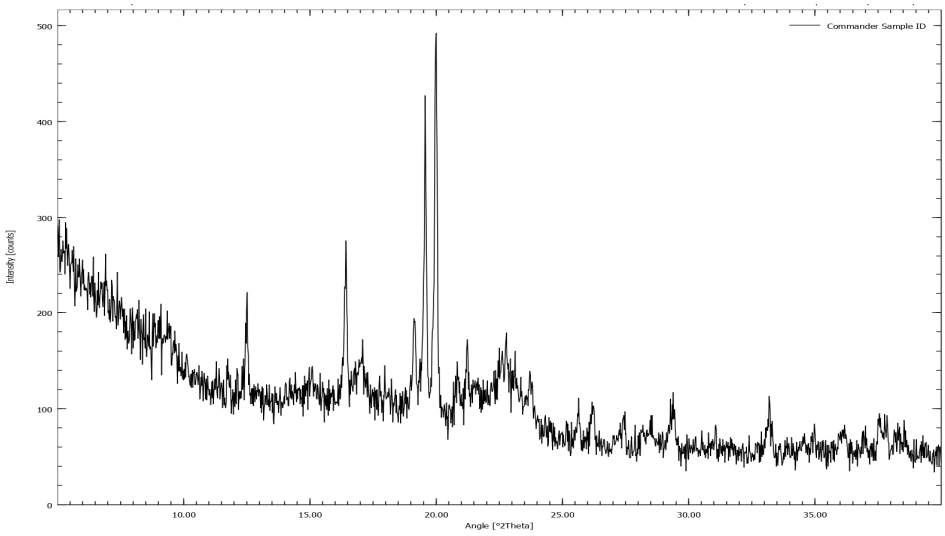

Figure 5. Shows the X-ray Diffraction (XRD) Spectra of the fermented lovastatin.

4. Conclusions

The strain improvement of *Aspergillus terreus* ATE-120 (saprophytic fungus) using ethidium bromide and pretreatment of sugar cane bagasse (*Saccharum officinarum* L.) with NaOH along with the response surface methodology technique were excellent strategies for the hyper-production of lovastatin through solid state fermentation. The structure of the obtained drug was confirmed by nuclear magnetic resonance (NMR). Moreover, an X-ray diffraction analysis of the sample was carried out to characterize the physical form of the lovastatin. It can be inferred from the above study that after a pharmacokinetic study of fermented lovastatin, this drug can be used as a natural substitute to treat hypercholesterolemia.

Author Contributions: Conceptualization, S.J.; methodology, S.J.; software, S.J., S.M., M.A.F. and K.M.A.-A.; validation, S.J.; formal analysis, M.A. and S.M.; investigation, M.A., S.A. and B.A.; resources, S.J., B.A., S.A. and K.M.A.-A.; data curation, M.A.; writing—M.A., S.J. and M.A.F.; writing—review and editing, S.J., S.A., K.M.A.-A. and B.A.; visualization, S.J.; supervision, S.J.; project administration, S.J. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Researchers Supporting Project number (RSP-2021/154), King Saud University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All strains, fermented products, and chemicals are available from the authors.

Acknowledgments: The authors would like to extend their sincere appreciation to the Researchers Supporting Project number (RSP-2021/154), King Saud University, Riyadh, Saudi Arabia.

Conflicts of Interest: The authors declare no conflict of interest.

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