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Optimization of Lovastatin Production from A Local Isolate of *Aspergillus terreus* A50 in Solid State Fermentation by Classical and Statistical Methods

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Abstract

Lovastatin is one of the most important compounds that is produced from some filamentous fungi, being employed in the reduction of hypocholesterolemia. The results of screening, after the collection of seventy-three local fungal isolates from different areas, demonstrated that the local isolate *Aspergillus terreus* A50 was the best isolate for lovastatin production, with a concentration of 12.66 µg/ml, through the submerged fermentation. Lovastatin produced from *A. terreus* A50 showed antimicrobial activities against a *Candida albicans* isolate. Solid state fermentation (SSF) was the best system to produce the highest yield of lovastatin by *A. terreus* A50 as compared to the submerged fermentation (SmF) system, with and without agitation. The optimum conditions for lovastatin production by SSF were also determined. The parameters included carbon sources (wastes), carbon sources mixture, incubation temperature, and moisturizing solution, which are commonly used in classical procedures. The results showed that a higher lovastatin production of 102.321 µg/gm substrate was obtained in the culture containing wheat bran and oat bran (1:1 w:w), sodium acetate, moisture ratio of 1.2 v:w, pH 7, incubation temperature of 30 °C and incubation period of 6 days. Some of these parameters, including pH, incubation period, and moisture ratio were determined by utilizing the Response Surface Method (RSM) as a statistical approach.

Keywords: Lovastatin, *Aspergillus terreus*, Optimization, Solid state fermentation, Central composite design.

تحديد الظروف المثلى لإنتاج اللوفاستاتين من العزلة المحلية لفطر *Aspergillus terreus* A50 باستخدام تخمرات الحالة الصلب بطرق تقليدية واحصائية

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الخلاصه

يعد اللوفاستاتين أحد أهم المركبات التي تنتجها بعض الفطريات الخيطية ، والتي تستخدم في تخفيض نسبة الكوليسترول في الدم. أظهرت نتائج الغربلة بعد جمع ثلاثة وسبعين عزلة فطرية محلية من مناطق مختلفة ، أن العزلة المحلية لفطر *Aspergillus terreus* A50 كانت أفضل عزلة لإنتاج اللوفاستاتين وبتركيز 12,66 ميكروغرام / مليلتر باستخدام تخمرات الحالة المغمورة. كما أظهر اللوفاستاتين المنتج من

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العزلة المحلية لفطر *A. terreus* A50 فعالية مضادة للميكروبات ضد عزلة خميرة *Candida albicans*. أظهرت النتائج بان افضل نظام تخمير لإنتاج اللوفاستاتين من العزلة المحلية لفطر *A. terreus* A50 كان باستخدام تخمرات الحالة الصلبة , مع أعلى تركيز مقارنة بالوفاستاتين المنتج باستخدام نظام تخمرات الحالة المغمورة مع وبدون تحريك. تم تحديد الظروف المثلى لإنتاج اللوفاستاتين من العزلة المحلية لفطر *A. terreus* A50 بواسطة تخمرات الحالة الصلبة. وشملت هذه الظروف المصدر الكربوني ، الخليط من المصادر الكربونية ، درجة حرارة الحضن ، ومحلول الترطيب ، حيث حددت بطرق تقليدية. أظهرت نتائج تحديد الظروف المثلى بان أعلى إنتاج للوفاستاتين كان 102,321 ميكروغرام/غرام من المادة الاساس في وسط يحتوي على نخالة القمح ونخالة الشوفان (1: 1 وزن:وزن) ، صوديوم اسيتيت ، نسبة الرطوبة 1,2 (حجم:وزن) ، الرقم الهيدروجيني 7,0 ، درجة حرارة الحضن 30 درجة مئوية وفترة الحضن 6 أيام. تم تحديد بعض هذه الظروف بما في ذلك الرقم الهيدروجيني (7) ، فترة الحضن (6 أيام) ، ونسبة الترطيب (1,2 حجم:وزن) باستخدام طريقة الاستجابة السطحية (RSM) كطريقة إحصائية.

Introduction

Lovastatin is a polyketide component produced by certain fungi during their secondary metabolism. Lovastatin, also known as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) inhibitor, is a class of lipid-lowering medication that reduces illness and mortality in patients at high risk of cardiovascular disease. Whereas statins act as competitive inhibitors for HMG-CoA reductase, a rate-constraining enzyme of cholesterol biosynthesis [1]. Statins hinder the change of HMG-CoA to mevalonic acid in the mevalonate pathway [2]. The first reported production of statin was from the fungus *Penicillium citrinum* by the Japanese microbiologist Akira Endo in 1970. He was screening organisms for discovering antimicrobial materials, which prompted the discovery of the first statin named mevastatin [3]. Statin was also isolated from another organisms, *Aspergillus terreus*, and named as lovastatin [4]. There are two methods of fermentation that are used to produce lovastatin, namely the submerged fermentation and solid-state fermentation (SSF). Production of lovastatin by these two approaches has been widely investigated, with the filamentous fungi exhibiting a tremendous potentiality. The production of lovastatin by SSF has gained much attention in biotechnology studies for the its anti-hypercholesterolemic properties. Some of the main advantages of SSF for lovastatin production from *A. terreus* include low cost residues, higher productivity, low energy requirements, lower waste water production, extended stability of products, and low production costs [5]. Lovastatin is produced as a secondary metabolite by various organisms, including *A. niger, A. terreus, A. flavus, Penicillium purpurogenum, Monascus spp,* and *Trichoderma viride*. The strains that are mostly utilized on industrial scales are *Penicillium* spp, *A. terreus* and *M. ruber*. *Aspergillus terreus* is a filamentous ascomycota with a significant provenance for the generation of lovastatin [6]. The commercial success of a fermentation process depends a lot on the continuous improvement in productivity by strain improvement programs. The success of any strain improvement program in turn depends on efficient screening techniques. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in suboptimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement the substrates externally with these nutrients. It has also been a practice to pretreat, chemically or mechanically, some of the substrates before utilizing them in SSF processes, thereby making them more easily accessible for microbial growth. Among the several factors that are important for microbial growth and enzyme production, utilizing a particular substrate, particle size, and moisture level/water activity (aW) are the most critical [7]. In this work the aim was to determine the optimum conditions for lovastatin production from the local isolate of *A. terreus* A50 by utilizing solid state fermentation**.**

MATERIALS AND METHODS

Collection of isolates

Seventy three fungal isolates were collected from different areas. Forty one isolates were from the Biotechnology Department\ Collage of Science\ Baghdad University, three were from the Collage of Biotechnology\ Al-Nahrain University, seventeen were from the Biology Department\ College of Science\ Baghdad University, eight were from the Tropical Biological Research Unit- Collage of Science\

Baghdad University, and four were standard isolates. All the plates of isolates were transferred directly to the laboratory. All isolates were subcultured on potato dextrose agar and incubated at 30°C, then screened for lovastatin production as indicated by the procedure described by Upendra, *et at*. [8].

Lovastatin estimation

Lovastatin was estimated utilizing the method described by Mielcarek *et al.* [9] as follows: To one ml of trifluroacetic acid (1%), one ml of the supernatant was added and incubated for 10 min. A volume of 0.5 ml from the mixture was diluted 10 times with methanol and its absorbance was read at 238 nm utilizing UV-Visible Spectrophotometer. Lovastatin concentration in the sample was acquired by plotting the O.D values on a standard curve.

Screening the fungal isolates for lovastatin production

Via the single spore isolation technique, the spores from seventy three fungal isolates were collected utilizing 2 % Tween-20 solution, then diluted to 7×10^6 spores/ml. Spores counting was achieved by utilizing a hemocytometer [10]. A volume of 3.5 ml of spores suspension (7×10^6 spores/ml) was added to 50 ml of the production media (pH 6.0) composed of 10 gm Glucose, 5 gm Corn steep liquor, 40 gm Tomato paste, 10 gm Oat meal, and 10 ml of trace element solution which is composed, per liter, of 1gm MnSO4.4H2O, 1gm FeSO4.7H2O, 25 mg CuCl2.2H2O, 356 mg H3BO, 100 mg CaCl2.2H2O, 200 mg ZnSO4.7H2O, 19 mg (NH4)6Mo7O24.2H2O, and 1 liter distilled water. The culture was incubated in a rotary shaker-incubator at 28°C for 10 days at 140 rpm. Screening experiments were performed on the isolates for lovastatin production on this media. After 10 days of fermentation process, the broth pH was adjusted to 3.0 with 10 % of 1 N HCl. An equal volume of ethyl acetate was added to the broth for lovastatin extraction. This experiment was performed under shaking conditions (140 rpm) at 30°C for 2 hrs. The biomass of fungi was separated via filtration utilizing Whatman No. 1 filter paper. The organic phase was recovered by centrifugation at 5,000 rpm for 10 min [8]. Depending on the previous screening results, three local isolates were selected and again cultivated on the same production media (pH 6.0).

Bioassay of lovastatin detection utilizing *Candida albicans*

The approach for the detection of lovastatin produced was conducted as recommended earlier [11]. Wells were aseptically made utilizing a cork borer on the surface of the potato dextrose agar and a 24 hours old culture of *C. albicans* was swabbed onto the surface of the media. Lovastatin extracted from the fungal isolate by ethyl acetate was stacked into the well, while ethyl acetate was utilized as a negative control and standard lovastatin as a positive control. All the inoculated plates in triplicates were incubated at 28 ºC for 24−48 hours and observed for the inhibition zone.

Fermentation system

Two types of fermentation systems were employed for lovastatin production from the local isolate of *Aspergillus terreus* A50, namely the submerged and solid state fermentation systems. In submerged fermentation, the medium contained the same compounds mentioned above in **the** screening experiment, while the medium of solid state fermentation was composed of 10 gm Wheat bran and a moisture of 60% (v:w) of distilled water [12]. Flasks (250 ml) were used to contain all the components, while the pH was adjusted to 6.0. The medium was sterilized via autoclaving for 15 min at 121˚C and then utilized for lovastatin production from the local isolate of *A. terreus* A50. The flasks containing the production media were inoculated with 3.5 ml of the isolates (7×10^6 spores/ml). Part of the flasks used for the SmF approach were incubated at 28 ºC in the rotary shaking incubator (140 rpm), while the others were incubated without rotary shaking for 10 days. In the SSF approach, the production media were incubated at 28 ºC for 10 days. Lovastatin concentration was estimated according to a method described previously [8].

Extraction of lovastatin produced by SSF

After the end of the SSF, the concentration of lovastatin was measured; The culture was extracted in 250 ml Erlenmeyer's flask by utilizing ethyl acetate (pH 3.0). The mixtures were incubated in a rotating shaker with 140 rpm at 28 ºC for 2 h. Next, the extract was filtered with a Whatman filter paper (No. 1) for the separation of the biomass from the filtrate. Then the supernatant was stored in glass bottles at 4 ºC until utilized for additional examination [13]. Lovastatin concentration (µg/gm) was estimated according to the following equation [14]:

Lovastatin concentration (µg/gm substrate) = Lovastatin concentration (µg/ml) × Total volume of extraction (ml)/Amount of substrate (gm)

Optimum conditions for lovastatin production

Carbon source

Ten carbon sources were tested to determine the optimum carbon source for lovastatin production from the local isolate of *A. terreus* A50. These sources were Oat bran, Soybean powder, Sorghum grain, Wheat bran, Orange peel, Barley, Potato peel, Sugarcane Bagasse, Rice straw, and Sawdust. Ten gm of each source was placed in 250 ml Erlenmeyer flasks with a moisture of 60 % (v:w) of distilled water. The pH was adjusted to 6.0, then the mixtures were inoculated with 3.5 ml of the isolates (7×10^6 spores/ml) and incubated at 28 ºC for 10 days [15]. Lovastatin concentration was then estimated.

Effects of carbon mixture

So as to determine the better blend of carbon sources that provides the highest lovastatin production, various (C:C) mixtures were prepared. Ten gm of the medium was added in 250 ml Erlenmeyer flasks, each contained 5:5 gm of different C:C types. These types (mixtures) were Wheat bran: Oat bran, Wheat bran: Barley, Barley: Oat bran, and Barley: Oat bran: Wheat bran. Then, each flask was provided with humidity of 60 % of distilled water and the pH was adjusted to 6.0. The mixtures were then inoculated with 3.5 ml (7×10^6 spores/ml) of the local isolate of *A. terreus* A50 and incubated at 28 ºC for 10 days. Lovastatin concentration was then estimated.

Incubation temperature

The medium composed of 10 gm of Wheat bran: Oat bran (5:5 gm) and distilled water (6 ml) at pH 6.0 was inoculated with 3.5 ml (7×10^6 spores/ml) of the local isolate of *A. terreus* A50 and incubated for 10 days at various temperature (25, 30, 35, 40 , 45 , 50 ^{O}C) to determine the better incubation temperature for lovastatin production [14]. Lovastatin yield was then estimated.

Moisturizing solution

Seven different moisturizing solutions were tested to determine the most efficient solution for the production of lovastatin from the local isolate of *A. terreus* A50. These solutions, as described earlier [11] with some modification, were Sodium Acetate Buffer (0.2 M), tap water, corn steep liquor, distilled water which was employed as a control treatment, cellulose (1%), trace element stock solution (as mentioned in the screening experiment), and oil.

Six ml of each solution was added separately to 10 gm of Wheat bran: Oat bran (5:5 gm) (pH 6.0), then inoculated with 3.5 ml of 7×10^6 spores/ml of the local isolate of *A. terreus* A50 and incubated for 10 days at 28 ºC. Lovastatin concentration was then estimated.

Statistical method (Design of the experiments)

Central composite design (CCD) pattern dependent on three components and five scales was utilized to consider, for highest lovastatin generation by *A. terreus* A50, the impact and associations among: (A) moisture solution in the extent of 0.2:1 and 1.8:1 (v:w); (B) pH in the extent of 4 and 9; (C) Incubation time in the extent of 1 and 10 days,. The experiments were performed by utilizing the Design-Expert program (Stat-Ease Inc., Minneapolis, USA, ver. 7.0.0). Trials were conducted in triplicates (Table 2-6). A sum of 20 runs were utilized in CCD to evaluate arch and association impacts of the selected factors. Lastly, the significance of the model was tested by F-test (determined p- value) and integrity of fit by numerous connection R just as assurance R2 coefficients. The concentration of lovastatin (µg/g) was the deliberate exploratory reaction.

Analysis of variance (ANOVA) was utilized to evaluate the statistical parameters of the cultures. A probability estimation of P value <0.05 was utilized to determine statistically significance differences [11].

Results and discussions

Screening of fungal isolates for lovastatin production

In this study, seventy three local fungal isolates were acquired and exposed to the quantitative and qualitative techniques of screening. A straightforward strategy for screening was followed to decide lovastatin producing fungi on the used production media (pH 6.0). The efficiencies of fungal isolates were compared by determining the ratio of lovastatin production. The results of the first screening for lovastatin production showed that three local isolates produced the highest amounts of lovastatin compared with the other fungal isolates. These local isolates were *A. flavus* A13, *A. fumigatus* A61, and *A. terreus* A50, with concentrations of 5.85, 6.58, and 12.58 µg/ml, respectively. Depending on the second screening outcomes, the results in Figure-1 demonstrated that the local isolate *A. terreus* A50

was the most efficient isolate for lovastatin production, with 12.66 µg/ml concentration, compared with *A. fumigatus* A61 and *A. flavus* A13 isolates, which produced 6.32 and 5.74 µg/ml, respectively, after repeating the cultivation of the three isolates on the same production media.

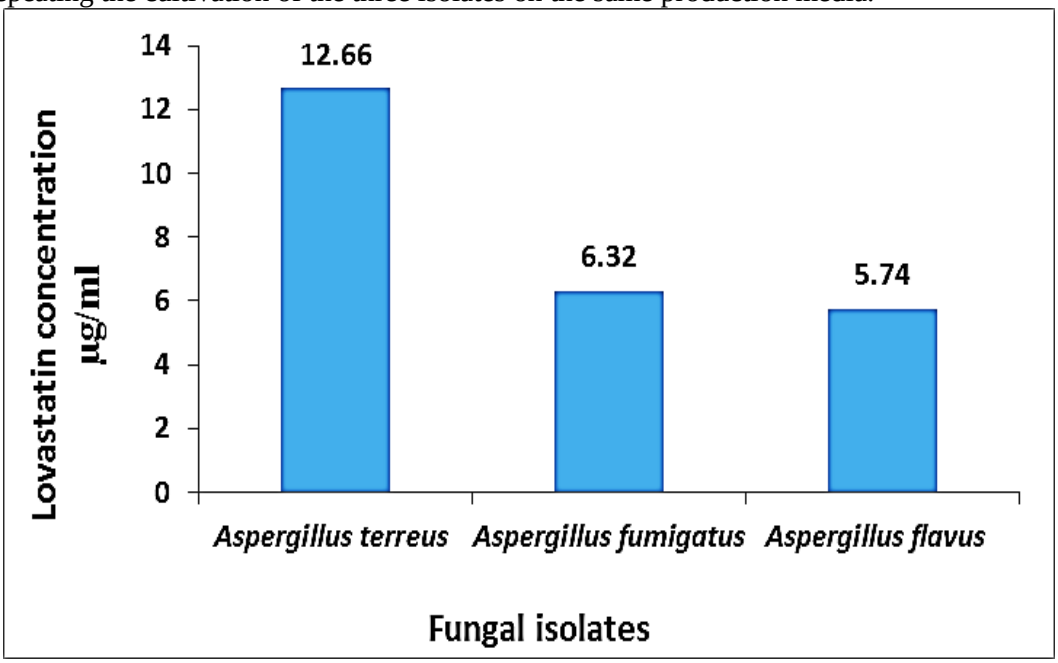

Figure 1-Screening of lovastatin production isolates.

Jaivel and Marimuthu [16] demonstrated that the results of the screening of various local isolates for lovastatin production through submerged fermentation revealed that the maximum lovastatin (138.4 mg/l) was produced by *A.terreus* JPM3, followed by *A.terreus* JPM1 (116.8 mg/l), *A.terreus* MTCC 479 (105.7 mg/l) and *A.terreus* JPM2 (97.6 mg/l), whereas other fungal isolates recorded lower yields of lovastatin. While Moussa *et al*. [17] utilized 19 isolates of *Aspergillus* spp. acquired from various localities for lovastatin production, with the outcomes showing that *A. terreus* (S57) was the most efficient isolate with a highest yield of 6.03 mg/l.

Bioassay of lovastatin detection utilizing *C.albicans*

The detection method of lovastatin production was conducted as proposed previously [11], depending on utilizing lovastatin produced as an antimicrobial agent against *C. albicans*. The obtained results revealed that lovastatin generally showed antimicrobial activity against the yeast isolate (Figure-2).

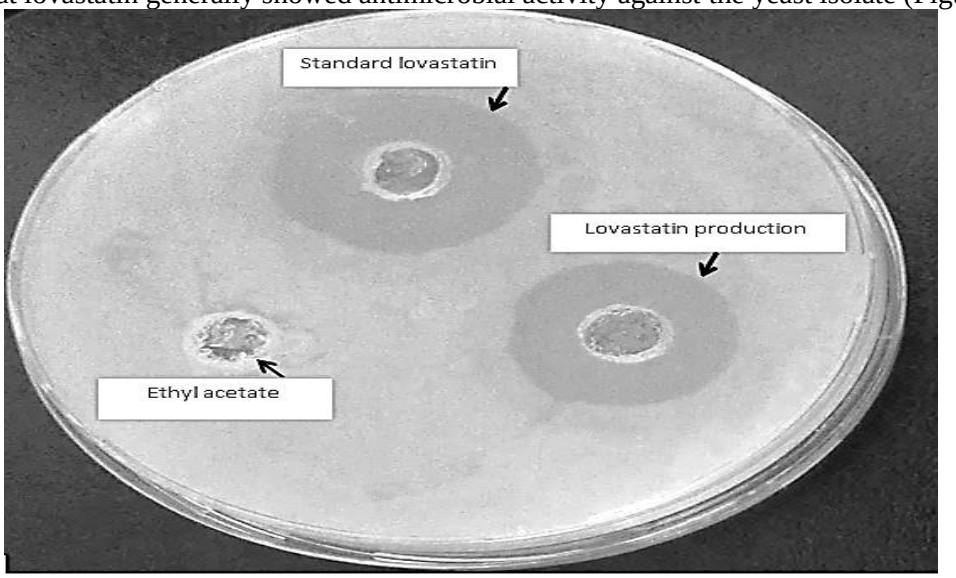

Figure 2-Bioassay of the detection of lovastatin produced from local isolate *A. terreus* A50 using *Candida albicans.*

Dhar *et al.* [3] applied the detection and screening of the produced lovastatin using the bioassay method. The detection and screening of the potentially produced lovastatin using molds were carried out by measuring the inhibition zone against *Neurospora crassa* and *C.albicans*, which was examined by employing the well method. Also, Pandey *et al*. [18] determined the produced lovastatin by the disc diffusion and agar well diffusion methods during a bioassay experiment with *N.crassa*.

Types of fermentation systems

Two types of fermentation systems were employed for the production of lovastatin from the local isolate of *A.terreus* A50, which are the submerged and solid state fermentation. The results of this experiment showed that the production of lovastatin from *A.terreus* A50 by the SSF demonstrated a higher yield of lovastatin as compare that by the SmF system, both with and without agitation. The concentration of lovastatin produced by the SSF was 17.27 µg/ml or 69.08 µg/gm, while SmF showed a lower concentration of lovastatin produced (Figure-3).

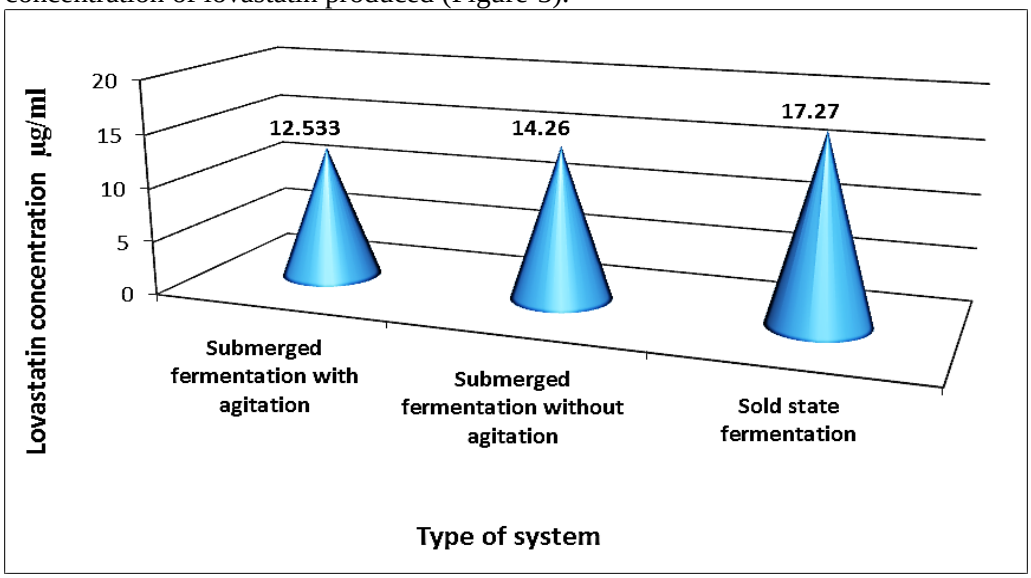

Figure 3-Production of lovastatin from the local isolate of *A.terreus* A50 by different fermentation systems with a moisture of 60% and pH 6.0, incubated at 28 °C for 10 days.

The findings agree with the results of Jaivel and Marimuthu [16], who found that SSF was more efficient than SmF in the production of lovastatin by *A.terreus* JPM 3. Praveen *et al*. [19] demonstrated a comparative work on lovastatin production in two strains of *A. terreus*, which established the advantage of SSF, with a 30 fold yield increase (9.56 mg/g and 9.7 mg/g) as compared to SmF (0.276 and 0.236 mg/ml). Supporting evidence is also provided by previously published reports that various types of solid substrates including wheat bran, orange peel, barley, sugarcane bagasse, rice straw… etc., can enhance the yield of lovastatin in *A.terreus* [20].

Optimum conditions for lovastatin production

Impact of carbon source

Impact of carbon source on the production of lovastatin was tested in the presence of various carbon sources. A higher yield of lovastatin (18 µg/ml or 72 µg/gm) was obtained in the culture containing wheat bran as compared to that produced with the other carbon sources (Figure-4).

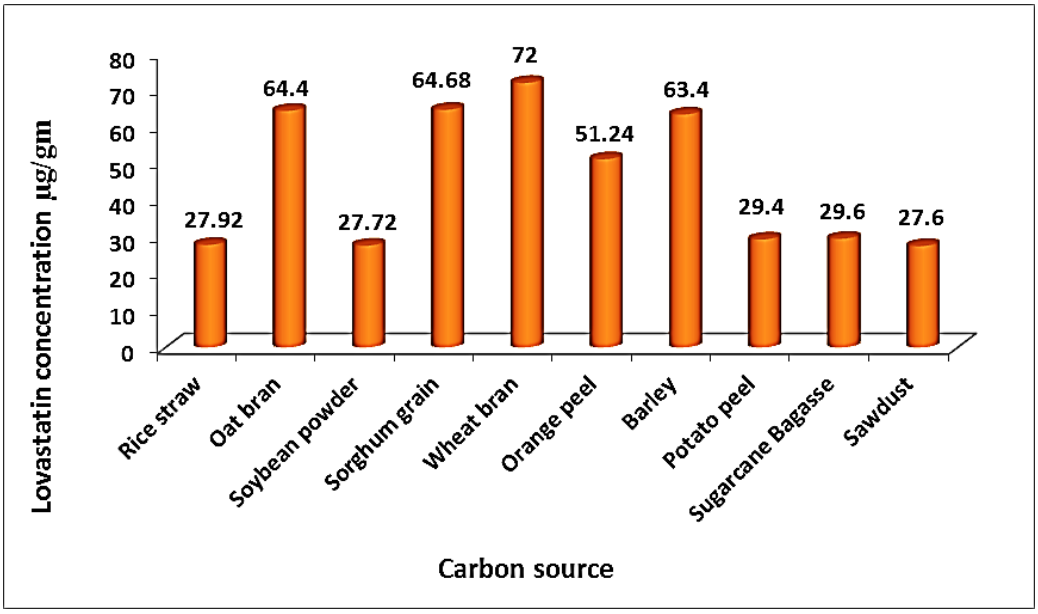

Figure 4-Effect of carbon source on lovastatin production by the local isolate *A. terreus* A50.

Carbon source is a factor that has a significant role in lovastatin production. Jaivel and Marimuthu [16] found that lovastatin produced by *A. terreus* JPM3 utilizing SSF was the highest when utilizing wheat bran as a substrate (982.3 µg/gm), followed by rice bran, sorghum grains and paddy straw. Szakacs *et al*. [21] reported that the production of lovastatin from *A. terreus* by SSF is superior to that by submerged fermentation. They utilized solid substrates such as wheat bran and sweet sorghum pulp for lovastatin production, and the highest production was noticed in sweet sorghum pulp. In general, wheat bran is a good source of protein, crude fiber, minerals, fat, pentose, vitamin (E), cellulose, and certain phytochemicals. It was described as an appropriate substrate for the production of lovastatin commercially [20].

Effects of carbon sources mixture

In order to investigate the most efficient carbon sources mixture that gives the maximum production of lovastatin from the local isolate of *A.terreus* A50, various mixtures of carbon sources were studied. The outcomes in Figure-5 show that the mixture of wheat bran and oat bran (1:1, w: w) was the superior mixture for lovastatin production by the SSF method, with 80.76 µg/gm concentration, while the other mixtures (wheat bran: barley, barley: oat bran, and barley: oat bran: wheat bran) produced lower yields of lovastatin.

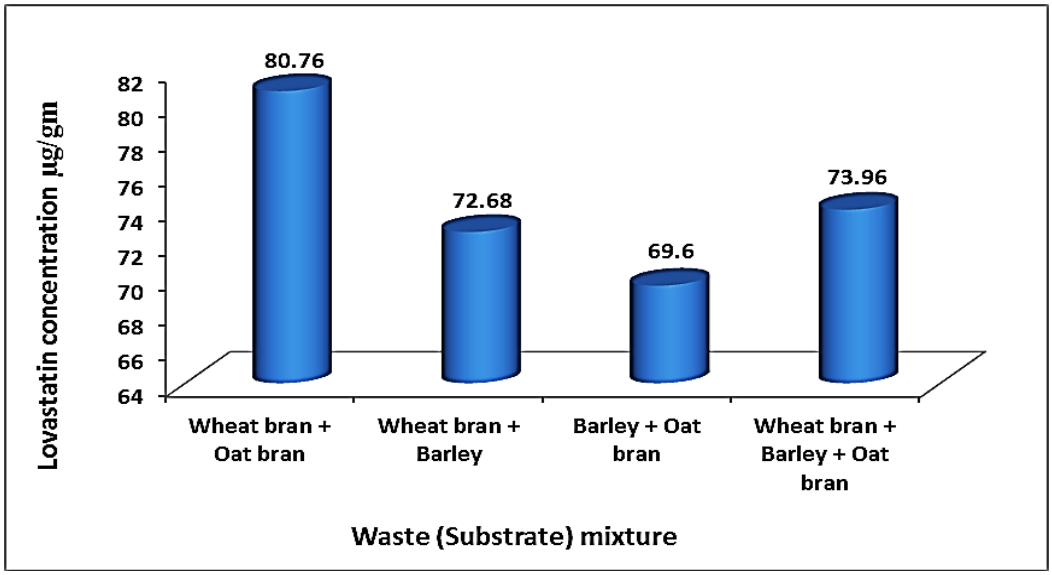

Figure 5- Impact of the carbon sources mixture on the production of lovastatin from the local isolate of *A. terreus* A50.

Wheat bran is a good source of many nutrients and protein, with relatively low calories. Wheat bran also has decent amounts of mineral salts such as iron, magnesium, zinc, and copper. There may be some disadvantages of wheat bran, including the requirement of some other minerals. Oat bran and rolled oats contain equivalent amounts of saturated fat, sodium, carbohydrates, phosphorus, sugars, and minerals such as calcium and iron. Additionally, oat bran contains significantly greater quantities of minerals such as potassium, phosphorus, folate and selenium, as well as the fatty acids omega-3 and omega-6 [22]. Therefore, mixing wheat bran with oat bran could provide essential nutrients and minerals for the production of lovastatin from the local isolate *A.terreus* A50.

Incubation temperature

The results in Figure-6 demonstrate the capacity of the local isolate of *A.terreus* A50 to grow and produce lovastatin at a wide scale of temperatures (25, 30, 35, 40, 45 and 50 ^{o}C). Lovastatin production was found to be maximum at 30 °C, with 80.72 µg/gm, while the yields at 25, 35, 40, 45 and 50 ^{o}C were 69.7, 67, 31.75, and 13.6 µg/gm, respectively. The maximum production of lovastatin at 30°C might be due to the fact that this temperature is the most suitable for the sporulation, growth, and proliferation of mycelia masses for the production of secondary metabolites. Temperature is one of the important parameters that determine the success of the SSF system. Temperature impacts the paces of biochemical responses either by prompting or preventing enzyme production. Temperature influences the microbial enzyme production by affecting the dissolvability of oxygen in the media, the vibration vitality of particles, and the speed of enzymatic responses in the cell, which is reflected negatively or positively on the production of lovastatin [23].

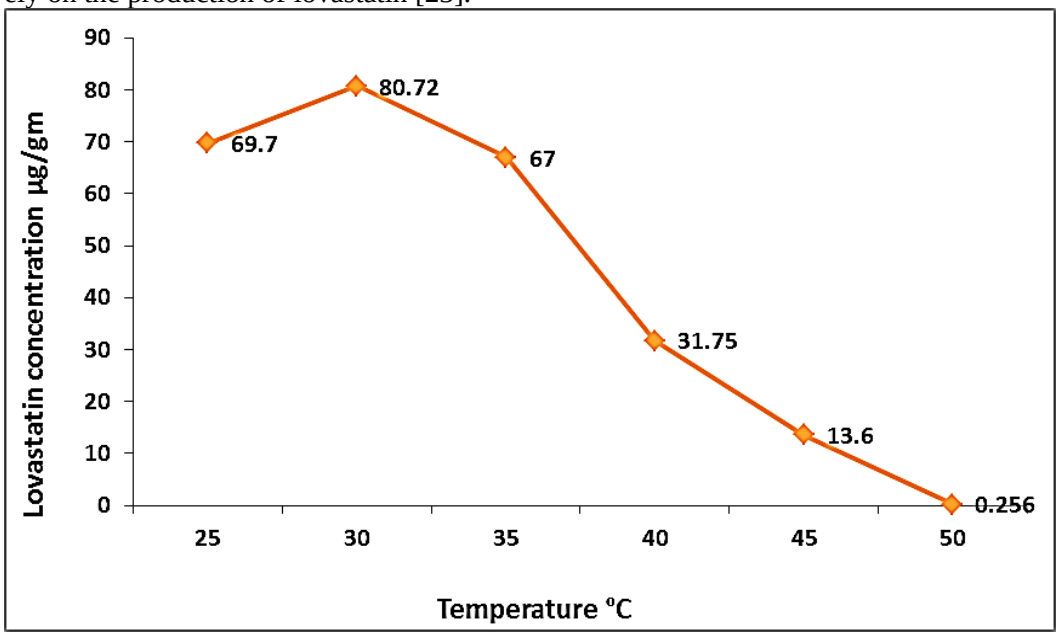

Figure 6-Impact of the incubation temperature on lovastatin production from the local isolate of *A.terreus* A50.

In a study by Ahmed *et al*. [24], the maximum lovastatin production (231.50 mg/l and 194.00 mg/l) was observed at 30°C in the fermentation broth and mycelial extract, respectively, of *A.terreus* using the submerged fermentation method. Mouafi *et al*. [25] reported that the most suitable incubation temperature for lovastatin production by an *A.fumigatus* isolate, using the SSF system and wheat bran as a substrate was 28 °C. Lovastatin yield of 3.353 mg/g was achieved with 70 % as initial moisture content, pH of 5.0, temperature of 28 °C, and incubation period of 12 days.

Moisturizing solution

Seven various moisturizing solutions were examined to determine the better solution for the production of lovastatin from the local isolate of *A. terreus* A50. These solutions were sodium acetate buffer, tap water, corn steep liquor, cellulose, trace element stock solution, oil, and distilled water which served as a control treatment. The results showed that the most efficient moisturizing solution was sodium acetate buffer (pH 6.0), with a highest yield of 89.2 µg/gm (Figure-7).

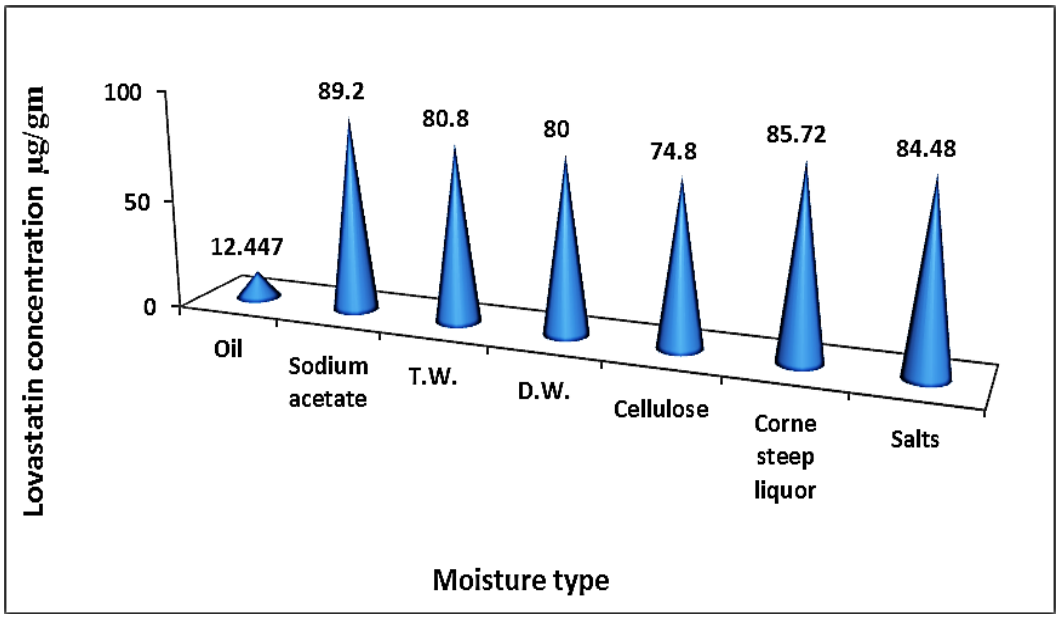

Figure 7-Impacts of moisturizing solutions on lovastatin production from the local isolate of *A.terreus* A50.

Kavith *et al*. [26] used distilled water for the production of lovastatin by SSF utilizing an interspecific protoplast fusion culture of wild strains of *A.terreus* and *A.flavus*. Kamath *et al*. [27] found that the maximum yield (3.50 mg/g) of lovastatin produced by *A. terreus* (KM017693) was achieved when 60 % of distilled water was utilized a as moisture content. Moisture level/water activity is one of the most critical factors in SSF, which often determines the success of the process because SSF is different from the submerged fermentation culturing in the characteristic that microbial growth and product formation occur at or near the surface of the solid substrate particles having low moisture contents [11].

Optimization utilizing Response Surface Methodology (RSM)

Lovastatin production requires optimization processes for the moisture content and well-controlled physical conditions such as pH and incubation time. The classical method of optimizing conditions involves changing one independent variable at a time while holding others at a fixed level. For many factors, this is highly time consuming and expensive. In contrast, the optimization process based on statistical methods helps to minimize the number of experiments. In addition, it assists to construct an approximation model that can be exercised to study the interaction between numbers of fermentation variables. In this study, the RSM was employed to build a model in order to evaluate the optimum effective factors for lovastatin production and study their interactions. It is an experimental strategy in which designed variables are varied together, instead of one factor at a time. The setting of RSM based on central composite design (CCD) was achieved utilizing a design expert software. The statistical optimization set included three factors, namely moisture content, pH and incubation time, to obtain the highest response (highest lovastatin concentration). The upper and lower bound of each factor is defined based on previous studies. The statistical optimization set was performed with one replication for each factor and five replications of the central point, generating 20 runs for the prediction of the response, as shown in Table-1 with the actual and predicted values of response.

Table 1-Experimental design and results of central composite design for lovastatin production with actual and predicted values of response

	Factor 1	Factor 2	Factor 3	Lovastatin µg/gm	
Run	A: Moisture solution ml	B: pH	C: Incubation time (day)	Actual	Predicted
1	1.0	7	10	49.6	55.12
2	1.0	4	6	81.96	84.64
3	0.5	8	8	72.12	74.1
4	1.0	7	6	98.4	98.24
5	1.0	9	6	103.56	97.09
6	1.0	7	1	0	-0.93
7	1.5	5	3	29.2	38.89
8	1.0	7	6	97.6	98.24
9	1.8	7	6	91.89	90.4
10	1.5	5	8	86.4	88.64
11	1.0	7	6	98.4	98.24
12	0.5	5	8	85.2	75.54
13	0.5	8	3	46.8	47.22
14	0.2	7	6	70.04	72.74
15	1.0	7	6	98.4	98.24
16	1.5	8	3	42.8	55.13
17	1.5	8	8	83.48	90.78
18	1.0	7	6	97.6	98.24
19	0.5	5	3	39.2	34.56
20	1.0	7	6	98.4	98.24

For more understanding of lovastatin production under the optimum conditions, the results of the experiments were analyzed by contour plots which described the response over the interactions among independent variables. The resulting graphics gave an excellent clarification for the effects of moisture content, pH, and incubation time on lovastatin production, as shown in Figures 8, 9, and 10. Figure-8 shows the impact of moisture content and pH on lovastatin production with an incubation time of 6 days. The contour plot demonstrated a maximum lovastatin concentration of 99.44 µg/gm that was achieved with the combination of 1.2 ml (v:v) moisture solution and pH of 7.0. Further increased moisture solution to about 1.2 ml (v:v) did not result in a significant enhancement in lovastatin production.

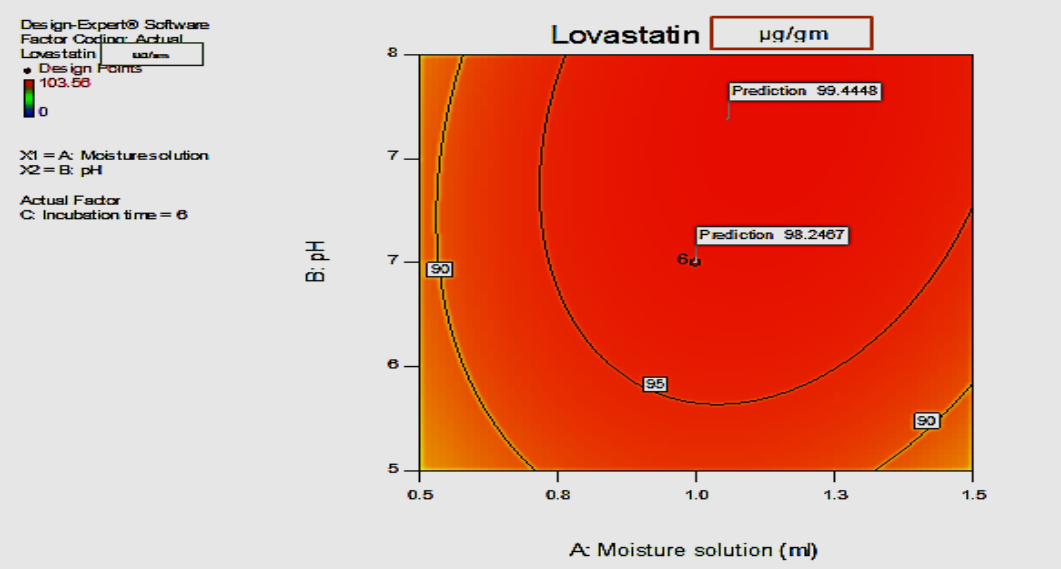

Figure 8-Impact of interaction factors of moisture solution and pH on lovastatin production from the local isolate of *A. terreus* A50 utilizing solid state fermentation.

Figure-9 explains the corresponding contour and response surface plot for lovastatin production based on the combination of moisture solution and incubation time. The moisture solution and incubation time were set at 1.2 ml (v:v) and 6 days, respectively. The maximum lovastatin production reached to 100.45 µg/gm at pH 7 whereas, the minimum concentration was 50 µg/gm.

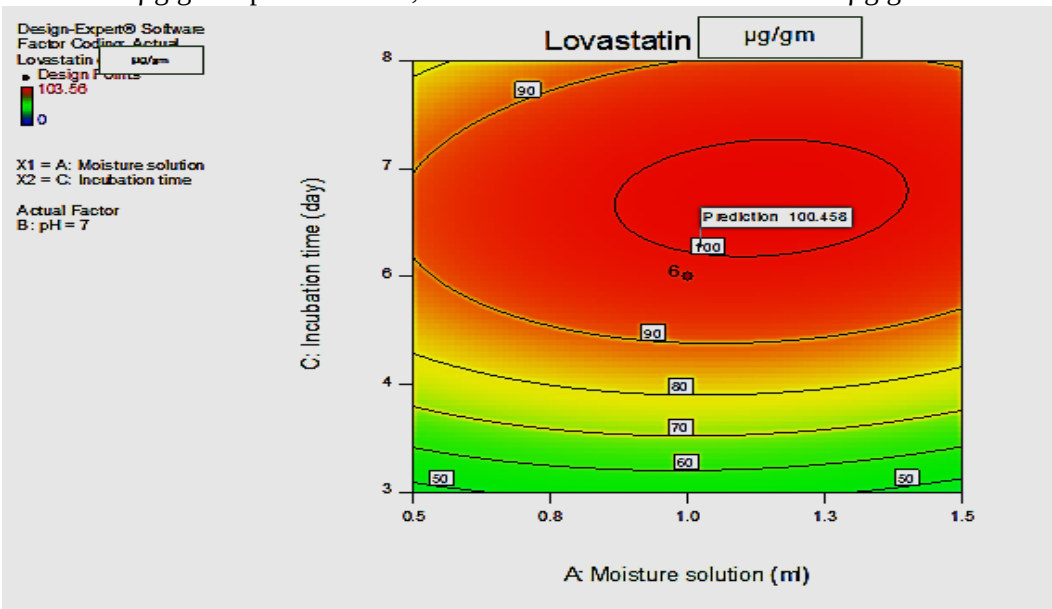

Figure 9-Impact of the interaction of the factors of moisture solution and incubation time on lovastatin production from the local isolate of *A. terreus* A50 utilizing solid state fermentation.

Finally, the results in Figure-10 shows the corresponding contour and response surface plot for lovastatin production with various pH values and different incubation times. The maximum lovastatin production of 100 µg/gm could be achieved at pH 7 and 6 days of incubation time with a moisture solution of 1.2 ml (v:v).

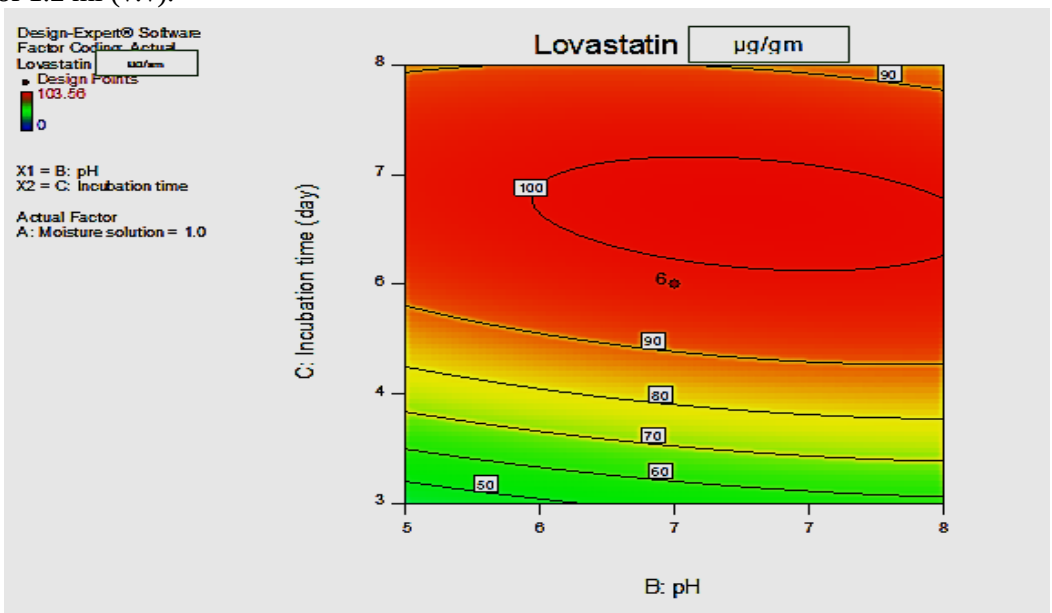

Figure 10-Effect of the interaction of the factors of pH and incubation time on lovastatin production from the local isolate of *A. terreus* A50 utilizing solid state fermentation.

Based on the results of the ANOVA test shown in Table-2, all of the term showed significant effects to the response. ANOVA is an efficient method to compare the means of different statistical populations and to test the significance of a model [28]. The experimental data were analyzed by fitting to a second order polynomial model, which was statistically validated by performing the analysis of variance equation to obtain a full actual model on lovastatin production.

Lovastatin (µg/gm) = 98.2467 + 2.46832 * A + 3.04072 * B + 18.4974 * C + 2.02 * AB + 3.32 * AC + -4.65 * BC + -6.88671 * A^2 + -2.64054* B^2 + -26.668 * C^2
where A is moisture solution in ml (v:v), B is pH, and C is the incubation time in days.

The F-test and ANOVA were used to test the statistical significance of the results of the equation, as given in Table-2. The 41.10 Model F value means that the model is significant, while the Prob > F model values, which is less than 0.05, also suggested that the terms of the model are important. Therefore, in this state, A, B, C are significant model terms. Values above 0.0500 suggested that the terms of the model are not significant, since p-value is utilized as a tool to test the significance of each factor. The model's goodness can be measured by determining the coefficient (R2) and the coefficient of correlation (R) [29]. In good accordance with the modified R-Squared of 0.95, the expected R-Squared is 0.8003. This result indicates that the selected model can describe 95% of the total lovastatin output variation data.

Table 2-Analysis of variance (ANOVA) for the quadratic modal of production of lovastatin obtained from the results of the experiments.

	Sum of		Mean	F	p-value
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**
Model	**15688.86**	**9**	**1743.21**	**41.10**	**< 0.0001**
A-Moisture solution	**83.21**	**1**	**83.21**	**1.96**	**0.0016**
B-pH	**126.27**	**1**	**126.27**	**2.98**	**0.0051**
C-Incubation time	**4672.77**	**1**	**4672.77**	**110.18**	**< 0.0001**
AB	**32.64**	**1**	**32.64**	**0.77**	**0.0009**
AC	**88.18**	**1**	**88.18**	**2.08**	**0.0099**
BC	**172.98**	**1**	**172.98**	**4.08**	**0.0010**
A^2	**683.48**	**1**	**683.48**	**16.12**	**0.0025**
B^2	**100.48**	**1**	**100.48**	**2.37**	**0.0048**
C^2	**10249.07**	**1**	**10249.07**	**241.66**	**< 0.0001**
Residual	**424.11**	**10**	**42.41**		
Lack of Fit	**423.26**	**5**	**42.65**		
Pure Error	**0.0**	**5**	**0**		
Cor Total	**16112.97**	**19**			

Regression analyzes can be utilized, in addition to correlation, to evaluate the better fitness of a line by employing the equation y= b0+b1x. The ideal line of the better fitness will have as small as possible sum of the squares of the distances between x and the fit line. The diagnosis of normal residuals shown in Figure-11 indicates that the residual behavior followed a normal distribution and was quadratic, which is the most important assumption for statistical modeling tests.

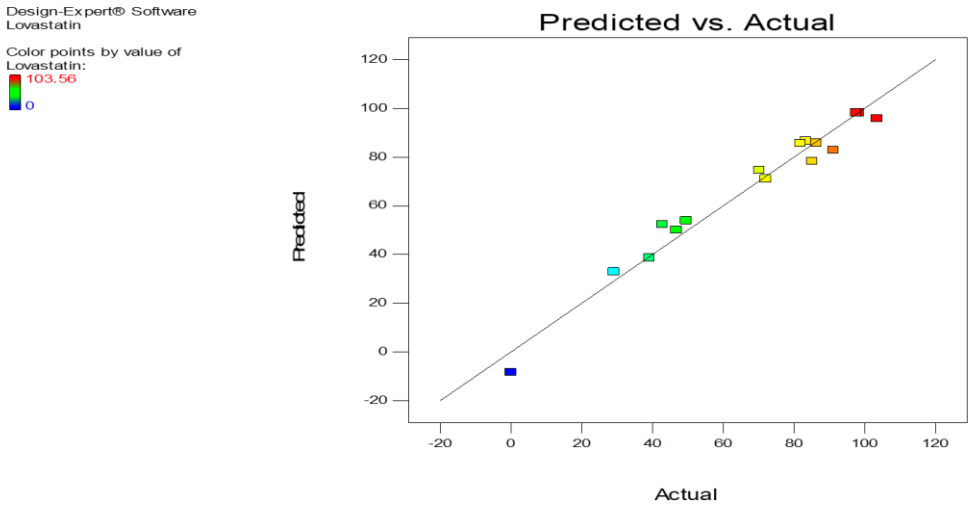

Figure 11-Normal probability plot of standardized residuals of quadratic model based on CCD for lovastatin production.

Figure-12 shows the predicted performance values versus actual experimental values for lovastatin production. It can be noted from this figure that the values measured utilizing the quadratic predictive model were in good agreement with the experimental values, with a reasonable correlation between those values. The developed model is therefore suitable for predicting the concentrations of lovastatin under suggested the composition.

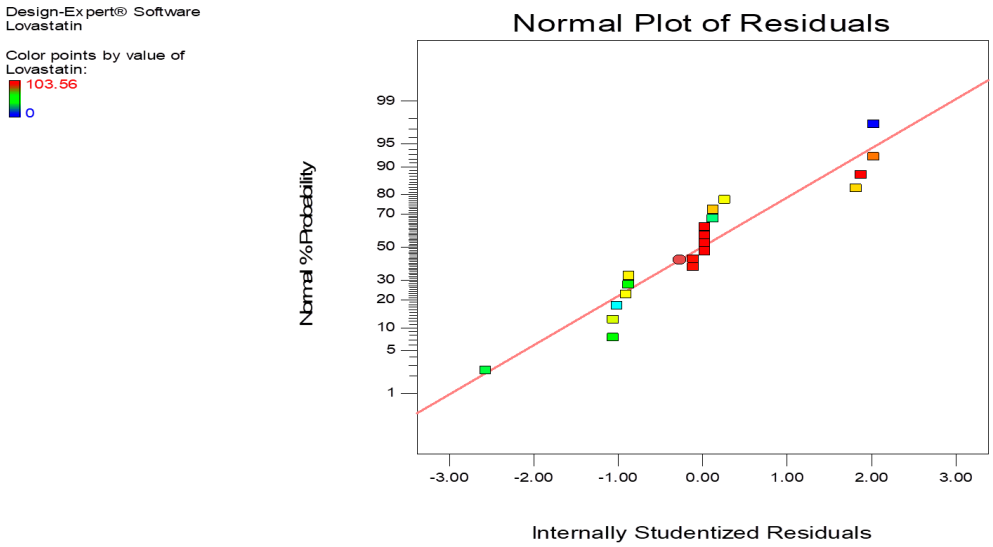

Figure 12-Actual versus predicted values for lovastatin production.

In the present study, the CCD was applied to determine the optimum process variables for lovastatin production from the local isolate of *A.terreus* A50. Depending on the regression model, the better plot could be created by employing the design expert 7 software to determine the optimum conditions for maximum response (lovastatin). As can be seen from the ramp charts presented in Figure-13, the suggested optimal of values pH, incubation time and moisture solution for the maximum lovastatin production (102.321 µg/gm) were 7.0, 6 days, and 1.2 ml (v:v), respectively.

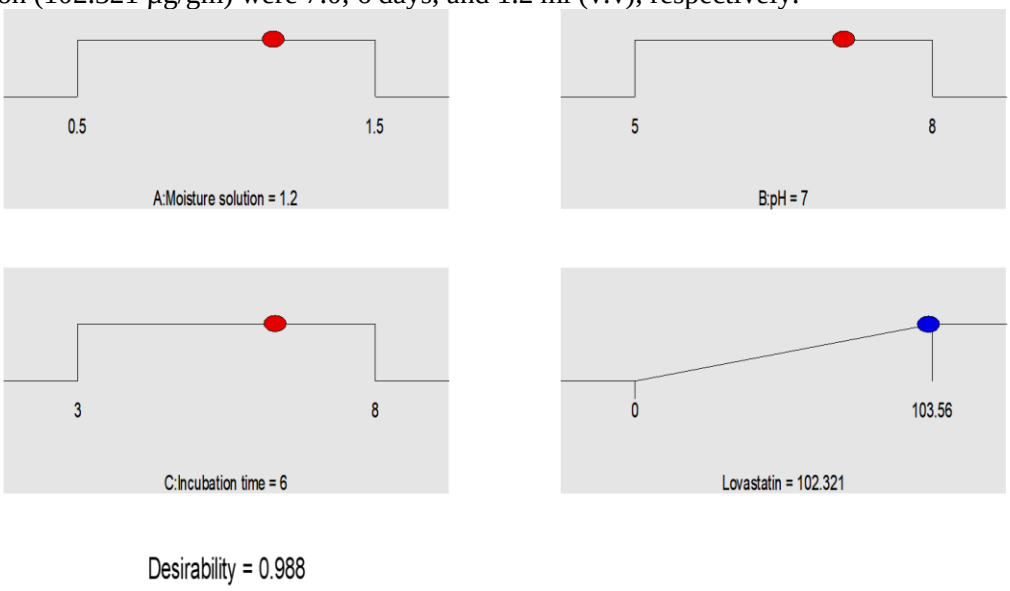

Figure 13-Ramp charts of the suggested optimal values of pH, moisture solution, and incubation time for maximum lovastatin production.

Lovastatin can be produced in much higher amounts under optimal physical and chemical conditions. Therefore, considerable differences in the environmental conditions can influence lovastatin

production. In fact, pH as well as moisture solution and incubation time play important roles in lovastatin production. Usually, the cultivation conditions affect lovastatin production directly and indirectly, through the stationary phase. Mouafi *et al*. [25] found that a lovastatin concentration of 3.353 mg/g was obtained in an optimum temperature of 28 ºC, initial moisture content of 70%, pH of 5.0, and incubation duration for 12 days, by utilizing the CCD. Pansuriya and Singhal [12] demonstrated the potential of SSF as an alternative to the submerged fermentation for the production of lovastatin by the isolate of *A. terreus* UV 1718. They showed that the highest yield (2914 ± 30 µg/gm) of lovastatin by employing SSF was achieved with a moisture content of 70 %, particle size of 0.525 mm, K2HPO4 content of 20 % (v/w), and trace ions concentration of 40 % (v/w).

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