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Research Article

Production Enhancement of Lovastatin Using Strategies of Screening and Response Surface Optimization in Solid State Fermentation

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Authors' Contributions
This work is a part of PhD research of TJ who conducted all the research. MA supervised the work. FH and HNB planned the research work and guide for research.

Keywords
Lovastatin, Optimization, *Pleurotus spodoecus*, Solid state fermentation

Abstract | High blood cholesterol, lipoproteins like low density lipoproteins, very low-density lipoprotein and decreased high density lipoprotein levels are the major risk factors involved in the development of cardio vascular diseases. Lovastatin is formed as secondary metabolic intermediate initiated by anion moiety (acetate) through polyketide chain reactions. The current work was put away to investigate the potency of *Pleurotus* strains for statin formation by cultivating them on lignocellulosic substrates (wheat straw, rice straw, banana stalk) in SSF for 8 days. Among *Pleurotus* species (*P. nebrodensis*, *P. sepidus*, *P. spodoecus*), *P. spodoecus* expressed best statin formation i.e., 9.1±1.4 mg/g. Four physical parameters including temperature, pH, inoculums size and fermentation time (each at five levels) were optimized using central composite design (CCD) design in 30 triplicate experimental runs. The maximum statin formation (19 mg/g) was achieved at 25°C, pH 5.5, inoculums size 3.5 mL and fermentation time 144 h on wheat straw as a growth substrate. The growth medium has an important effect on lovastatin production. Effect of carbon and nitrogen supplementation was investigated due to their restrictive ingredients role. Best yield (49.3±15 mg/g) was achieved by *P. spodoecus* using lactose (Carbon source) and $NaNO_3$ (Nitrogen source) under pre-optimize circumstances. Different C/N ratios were used to obtain the optimal ratio. Best statin production was attained at 25:1 C/N ratio that was 65.7 ± 5.5 mg/g by *P. spodoecus* using solid state fermentation.

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Introdution

Cardio vascular diseases (CVD) have close relation with hypercholesterolemia and controlled by lipoproteins (combination of lipids and proteins). Maintaining HDL (high density lipoprotein) level in a safe range is very crucial to avoid CVDs problem. High blood cholesterol, bad lipoprotein like low density lipoproteins, very low-density lipoprotein (LDL, VLDL) and decreased HDL level are the major risk factors involved in the development of coronary disease (Alam *et al.*, 2011). Natural products are helpful to cure the pathogenesis. They behave as leading metabolites in manufacturing of various potent medicines. Fungi play vital role for the formation of many pharmaceutical substances. They form many complex substances basically through the polyketide biosynthetic pathway. Statins are fungal derived metabolic compounds which become deep intention for researchers because they inhibit the production of cholesterol (Nidhiya *et al.*, 2012).

They were recognized by FDA (food and drug authority) as hypocholesterolemic agent in animals and human. The best control of lipid pathophysiology in CVD protection could not be attained without the use of statins (Taylor, 2012). Now a day, statins are the most regularly used hypolipidemic medicine. In submerged fermentation, the production of lovastatin is associated to the biomass

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assembly. It can also be formed by solid state fermentation (SSF). The substrates used in SSF required lesser pretreatment with higher production than submerged fermentation (Alarcón and Águila, 2006). In SSF the product yield is high and has better stability. Formerly, solid state fermentation was used basically for the production of industrial enzymes but recently, it is also being exploited for the formation of secondary metabolic intermediates (Dikshit and Tallapragada, 2015).

Many edible fungi of higher *Basidiomycetes* have capacity of forming hypocholesterolemic agents, specifically *Pleurotus* strains (Suryanarayan, 2003). Now a day, statins have recognized as an effective potential agent in curing different kinds of cancers because they suppressed tumor development inside the body through the restriction of biosynthesis of isoprenoid metabolites. Response surface methodology coupled with experimental designs was employed to investigate efficient statin production in a limited number of experiments (Wasser and Reshetnikov, 2002). In the present research work *Pleurotus spodoecus* was exploited for the lovastatin production in solid state fermentation using RSM methodology for optimization and screening of different nitrogen and carbon sources and ratios for production enhancement. According to our knowledge it is the first report on lovastatin production using *Pleurotus spodoecus* strain.

Materials and Methods

All the experimental and analytical work was done in Industrial Biotechnology laboratory (IBL), Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. Three strains of *Pleurotus* included *P. spodoecus, P. nebrodensis* and *P. sepidus* were used to investigate their statin production through solid state fermentation of three different lignocellulose substrates (wheat straw, rice straw, banana stalk). The physical factors (temperature, pH, inoculum size, fermentation time) were optimized by RSM using CCD and nutritional parameters (C. N sources, C/N) by two factors design experiments, to improve the statin formation by selected strains.

Microbial strains

Pure culture of *Pleurotus* strains were obtained from Industrial Biotechnology Lab., Department of Biochemistry, University of Agriculture Faisalabad, Pakistan. The fungal strains were grown on potato dextrose agar (PDA) slants having pH 4.5, and sub cultured monthly.

Substrate collection and seed culture preparation

Lignocellulosic substrates were dried out in oven at 50 °C and ground to form a 4 mm mesh size. The ground substrates were kept in caped storage jars to maintain moisture free environment. For seed preparation, *Pleurotus* strains were grown in modified Kirk basal medium with pH 4.5 included g/L, 0.22g Ammonium tartarate, 0.21g KH_2PO_4, 0.05g $MgSO_4.7H_2O$, 0.01g $CaCl_2$, 0.001g Thiamine, , 1g Chloramphenicol, 1 mL veratryl alcohol (100 mM), 10 mL trace element solution (The trace element solution contained g/L: $CuSO_4$, 0.08; H_2MnO_4, 0.05; $MnSO_4.4H_2O$, 0.07; $ZnSO_4$, $7H_2O$, 0.043; $Fe_2(SO_4)_3$, 0.05 and 1% (w/v) glucose. Flora *et al.* (2000).

Screening experiments with micro-organism and substrate selection

Pleurotus strains were grown on three growth substrates through SSF. Triplicates flasks consisted of 5g of particular substrate soaked with Kirk basal medium (50% moisture level) for statin production by *Pleurotus* strains (Tien and Kirk, 1988). Each flask was cleaned and uncontaminated by autoclaving and inoculated with 5 mL of freshly formed inoculum of respective fungus. The inoculated flasks were allowed to ferment for 8 days (30°C) in culture incubator. Following 8 days of fermentation time period, the fermented biomass was dried at 40°C for 24 h and then 25 mL methanol was mixed for extraction by keeping in an orbital shaking incubator at 180 rpm for 2 h. After that the cultures were filtered and filtrate was centrifuged at 1500 rpm for 15 min for statin extraction (Wei *et al.*, 2007). The supernatants were collected in caped test tubes and analyzed for statin production. Best selected fungal species and substrate on the basis of higher statin production in 8 days was selected for further studies.

Optimization of culture conditions by RSM under CCD

Central Composite Design (CCD) model of RSM was used to optimize the four parameters for fermentation process. Total of thirty triplicate experimental runs were carried out in 250 mL flasks for optimization. The data obtained from various runs employed were investigated and depicted by using design of expert (DOE) version 6.0.8. Temperature was maintained at 25, 26.25, 27.50, 28.75 and 30 °C, pH was adjusted at 4.00, 4.37, 4.75, 5.12 and 5.50, Inoculum size was kept at 3,5, 4, 4.5, 5 and 5.5 mL and fermentation time was conducted for 144, 168, 192, 216 and 240 hours in 30 experiments (Table 1).

Effect of carbon and nitrogen sources and ratios

For suitable nutritional parameters, both carbon and nitrogen sources and ratios were investigated to improve the production of lovastatin. Different carbon sources like lactose, glucose, fructose, glycerol and maltose were used with various inorganic nitrogen sources (ammonium nitrate (NH_4NO_3), sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and ammonium sulfate $(NH_4)_2SO4$) under preoptimized cultural conditions were used to sort out the best carbon and nitrogen source. The concentration of carbon and nitrogen sources were 1% and 0.2% respectively. For the evaluation of C/N ratio's effect on the statin pro-

duction, different C/N ratio were employed in the experiments.

Analysis of lovastatin

For UV spectrophotometric analysis, 1 mL of clear supernatant was mixed with 1mL of trifloro acetic acid and the incubation was given to mixture (10 minutes) to lactonize the hydroxyl acid form of statin (Wei *et al*., 2007). The resulting mixture was mixed with methanol and the absorbance was read at 238nm using UV-VIS Double Beam spectrophotometer (Dynamica). Standard curve of statin was constructed by plotting concentrations verses absorbances. Pure lovastatin lactone (99.9%) form (Sigma Aldrich) was dissolved with methanol to make pure mixture of varying concentrations (1.0mg-10.0mg/mL) in test tubes.

Results and Discussion

The current work was conducted to investigate the potency of various strains of *pleurotus* (*P. nebrodensis*, *P.sepidus* and *P. spodoecus*) through solid state fermentation using several different substrates (wheat straw, rice straw, banana stalk). Further growth conditions including pH, temperature, inoculum size and fermentation time used to optimize statin production. After optimizing physical parameters, growth medium also developed with supplementation of suitable carbon and nitrogen sources and their ratios as one carbon source with different N sources at a time and vice versa. The goal of this study was to quantify the process flow and determine conditions suited for scale up because *P. spodoecus* was secreened out as best lovastatin producer for the first time. The experiments were carried

Table 1: Central Composite Design for optimization of statin production by *P. spodoecus* on wheat straw in SSF.

Exp. Runs	A: temp (°C)	B: pH	C: In. size (mL)	D: Ferm. Time (h)	Response Statin prod.(mg/g)
1	27.50	4.75	4.5	192	9
2	30.00	5.50	3.5	240	13
3	30.00	4.00	5.5	240	6
4	27.50	4.75	4.5	192	8
5	26.25	4.75	4.5	192	10
6	30.00	4.00	5.5	144	10
7	25.00	5.50	3.5	240	17
8	30.00	5.50	3.5	144	16
9	30.00	5.50	5.5	144	12
10	28.75	4.75	4.5	192	7
11	27.50	4.75	5.0	192	7
12	25.00	4.00	5.5	240	9
13	30.00	4.00	3.5	144	14
14	27.50	4.75	4.5	192	8
15	25.00	5.50	5.5	240	10
16	27.50	4.37	4.5	192	9
17	25.00	4.00	5.5	144	13
18	25.00	5.50	3.5	144	19
19	27.50	5.12	4.5	192	9
20	27.50	4.75	4.5	216	6
21	30.00	5.50	5.5	240	7
22	30.00	4.00	3.5	240	10
23	27.50	4.75	4.0	192	9
24	25.00	4.00	3.5	144	18
25	27.50	4.75	4.5	192	8
26	27.50	4.75	4.5	192	7
27	27.50	4.75	4.5	168	7
28	27.50	4.75	4.5	192	9
29	25.00	5.50	5.5	144	14
30	25.00	4.00	3.5	240	14

out along different fronts to obtain a clear picture of the process conditions conducive for the production of high amounts of lovastatin by the fungus. *P. spodoecus*. It is unique strain for lovastatin production and no research work was observed on it previously for lovastatin production.

During two ways screening process maximum statin production 9.1±1.4 mg/g was observed with cultivation on wheat straw after 8 days of fermentation time (Figure 1). Other two *Pleurotus* strains (*P. nebrodensis, P. sepidus*) gave lower statin formation upon wheat straw i.e. 6.9±0.5 mg/g and 6.7±0.9 mg/g respectively. Diversity in statin formation with in various species on distinct materials is because of various enzymatic arrangements of every fungal species. Genetic differences and modifications within material architecture may be involved for this diversity.

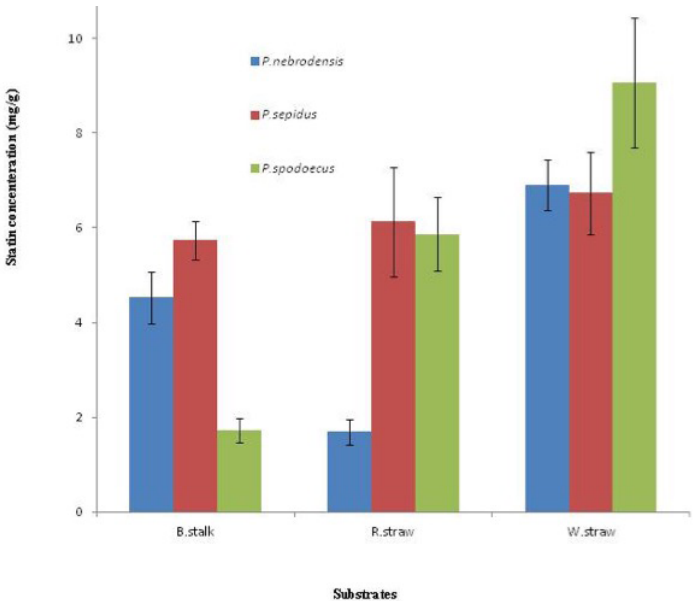

Figure 1: Effect of different substrates on statin production by different strains of *Pleurotus* in SSF.

Fungi has potential to produce statins (secondary metabolite) as reported previously on *P. ostreatus* (Ragunath *et al*., 2012) and on *Schizophyllum commune* (Lakshaman and Radha, 2012). But only little work has been carried out using *pleurotus* through SSF. SSF on natural solid substrates is being considered as the most common and the best option for production of microbial metabolites with use of cheap raw materials. Lovastatin production in SSF on natural solid substrates has been studied (Pushpa *et al*., 2016) with *Aspergillus flaviceps*, *Aspergillus terreus* (Tien and Kirk, 1988) and *Monascus ruber* (Alarcón and Águila, 2006). The observed lovastatin yields were 4-6 mg/g, 2-9 mg/g and 16-78 mg/g respectively. However, it may involve genes expression of lovE and lovF. The best formation (lovastatin) in SSF may influence with higher gene expression of loveE and lovF (two-fold) within specified culture situations (Valera *et al*., 2005). A study in which out of 36 fungi isolated from soil and observed the potential for lovastatin production through yeast growth bioassay method. *Cunninghamella blakesleeana* (C. *blakesleeana* strain) showed high potency for lovastatin formation producing 1.4 mg g–1 DWS prior optimization. TLC, HPTLC and HPLC used further confirmation (Balraj *et al*., 2018). *P.spodoecus* used in the study, is a new strain and having the potential for sufficiently high lovastatin production. The strain may be used at commercial level synthesis of lovastatin after scaling up studies.

Optimization for overproduction of statin by P. spdoecus

Four factors each at five levels in a Central Composite Design with 30 experimental runs in triplicate were performed in the study. Response (statin formation) from *P. spodoecus* was observed for the experiments. Maximum statin formation from *P. spodoecus* was achieved at 25°C, pH 5.5, inoculums size 3.5 mL and fermentation time 144 h (19 mg/g) on wheat straw as a substrate through RSM (Table 1).

ANOVA is used for the statistical analysis of the obtained data (Table 2). Coefficient of determination R^2 was frequently utilized to investigate the suitability of the predicted model and observed records. The value of R^2 lies between 0 and 1. The closer the R^2 value to 1, the better the fitness of the model to observed data (Barrios-González, 2008). R^2 mainly represent the variation percent of the data predicted by the used model (Pansuriy and Singhal, 2010; Chen *et al*., 2009).

Predicted R^2 value 0.9393 of the model (for statin production using *P. spodoecus*) is in accordance with adjusted R^2 i.e. 0.9671 value. Adjusted R^2 to actual R^2 value in statin formation by *P. spodoecus* showed that the linear, square and interaction terms could be described 98.30% (Table 3). The Model F-value of 61.90 revealed that the model is significant. The complete multiple regression model for statin production process by *P. spodoecus* is represented in the following

$$Y1 = (7.91) + (-1.67A) + (0.85B) + (-2.48C) + (-1.85D) + (3.18A^2) + (5.18B^2) + (1.18C^2) + (-4.82D^2) + (0.13AB) + (0.25AC) + (-0.13AD) + (-0.25BC) + (0.12BD) + (-0.25CD)$$

It was seen that the factors including temperature, pH, inoculum size and fermentation time had positive influence on statin formation by *P. spodoecus* in SSF. Effects of temperature, pH, Inoculum size, fermentation time, quadratic effects of pH fermentation time were found significant. Standard devation value of 0.66 showed that lack of Fit F-value was not significant in comparision to pure error and this showed the fitness of model (Table 2). It was already observed that pH significantly influenced the lovastatin production (Pratheeba *et al*., 2013). It is due to the fact that pH powerfully effects the transportation of many ingredients through the physiological membrane (Bizukojc *et al*., 2012). Inoculum sizes used for lovastatin

Table 2: Analysis of Variance (ANOVA) for statin formation (*P. spodoecus*).

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	Significance
Model	380.9066	14	27.20761	61.89719	< 0.0001	Significant
A	45.83333	1	45.83333	104.2706	< 0.0001	Significant
B	11.87879	1	11.87879	27.02418	0.0001	Significant
C	101.8788	1	101.8788	231.7737	< 0.0001	Significant
D	56.37879	1	56.37879	128.2615	< 0.0001	Significant
A2	1.679705	1	1.679705	3.821321	0.0695	not significant
B2	4.460208	1	4.460208	10.14695	0.0061	Significant
C2	0.230543	1	0.230543	0.524483	0.4801	not significant
D2	3.871093	1	3.871093	8.806717	0.0096	Significant
AB	0.25	1	0.25	0.568749	0.4624	not significant
AC	1	1	1	2.274995	0.1522	not significant
AD	0.25	1	0.25	0.568749	0.4624	not significant
BC	1	1	1	2.274995	0.1522	not significant
BD	0.25	1	0.25	0.568749	0.4624	not significant
CD	1	1	1	2.274995	0.1522	not significant
Residual	6.593421	15	0.439561			
Lack of Fit	3.760087	10	0.376009	0.663545	0.7287	not significant
Pure Error	2.833333	5	0.566667			
Cor Total	387.5	29				

Table 3: Statin production under various Carbon and Nitrogen sources by *P spodoecus* under pre optimized condition.

Carbon source	Nitrogen source				
	NH_4NO_3	$NaNO_3$	NH_4Cl	$NH_4H_2PO_4$	NH_4SO_4
Lactose	32.3±9.6	49.3±15.1	25.1±9.2	31.7±9.0	22.3±14.5
Glucose	30.3±6.3	20.5±13.4	25.1±12.5	26.1±16.7	33.9±9.5
Fructose	35.1±12.0	39.1±9.2	29.9±15.5	23.5±16.1	23.9±13.2
Glycerol	27.1±9.2	25.1±9.2	23.1±10.4	25.7±13.2	17.1±12.0
Maltose	28.2±10.5	21.1±12.5	15.1±12.5	21.3±9.4	27.1±15.3

production vary in different studies, however, much larger or too lower inoculums size reduces the lovastatin formation (Barrios-González *et al*., 2008; Foukia *et al*., 2016). Substrate, 5gm: pH, 5.5, Temperature, 25°C, Inoculum size, 3.5 ml, Incubation time, 144 h gave optimal statin production (Valera *et al*., 2005). Various studies revealed best lov gene expression (ten times) on SSF as compared to SmF, representing functional structures corresponding to SSF for greater lovastatin yield. No interaction effect for the model is significant as represented in Table 2. The predicted values of the model are very close to the observed values as indicated in Figure 2. The fact also represents the goodness of model fitness for the observed data.

A study reported in 2018 also showed the maximum lovastatin formation (13.40mg gdfs−1 at a temperature of 25.64°C, a fermentation time of 14.49 days, glucose concentration of 1.32% and peptone concentration of 0.20%, with 92.85% validity. In our study, highest production also obtained at same temperature but at different fermentation time as in this case 144 h. This is may be due to different strain used at different substrate along with other different parameters used for this study (Suraiya *et al*., 2018).

Aspergillus terreus FFCBP-1053 also reported the good architecture of lovastatin after screening among different *Aspergillus* strains and in optimized conditions to enhance the synthesis under CCD. Best formation Statistical analysis of the second phase data revealed that *Aspergillus* terreus FFCBP-1053 in medium having 70% moisture content on incubating at 35°C with pH 4.5 during SSF showed significant (P<0.05) response in the form of lovastatin production while independent on inoculum size (P>0.05) (Munir *et al*., 2018). The optimum temperature used in the study was different from the present study, however optimum pH is to the present study. The difference is may be due to the fungus of different sp.

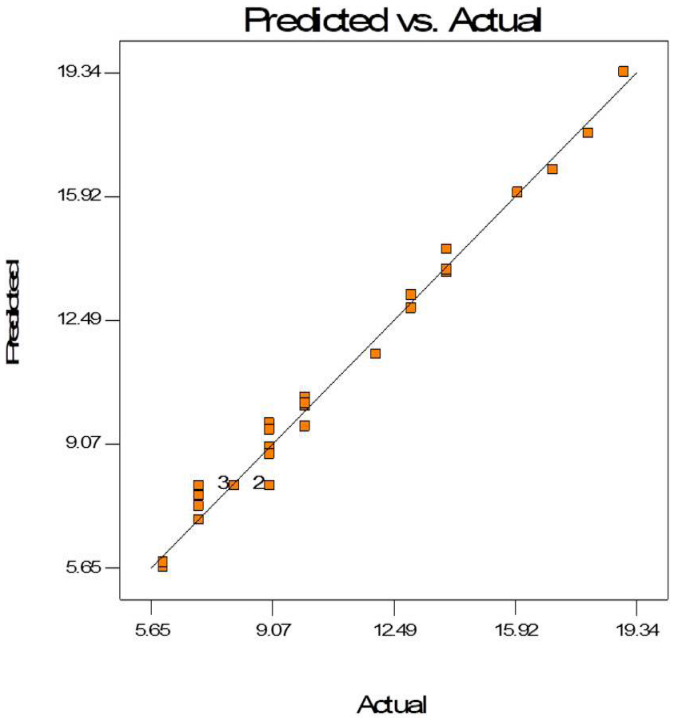

Figure 2: Correlation of observed data with predicted values by the model.

Effect of carbon and nitrogen sources

The growth medium has an important effect on lovastatin production. So that's why choice and arrangement for optimization of a proper medium is significant for maintaining lovastatin production. Among best growth conditions, carbon and nitrogen supplements usually take prominent part in fermentation production due to directly involvement of biomass and the metabolic intermediates with nutrients. Effect of carbon and nitrogen supplements was investigated, as nitrogen play as a restrictive ingredient. Best yield was achieved in *P. spodoecus* at lactose (Carbon source) and $NaNO_3$ (Nitrogen source) was 49.3±15.1under pre-optimize circumstances (Table 3). Generally, lovastatin synthesis in typically mixed-growth related materials is during early and late state of biomass formation. D galactose, D-arabinose and D-glucose responsible for least production while D-xylose and D-fructose for best production which means nature of carbohydrates also effect on lovastatin production (Rahim *et al.*, 2017).

Lovastatin biosynthesis also depends on the carbon and nitrogen sources, (Jahromi *et al.*, 2012). Carbon and nitrogen ingredients along with C/N ratio take part important role for providing initial sources and cofactors which straightly participate in the biomass formation of structural units and metabolic intermediates (Jahromi *et al.*, 2012; Hajjaj *et al.*, 2001). Lactose was observed as best source previously (Subhan *et al.*, 2016) for lovastatin biosynthesis in various fungal strains. Other research works reported sodium nitrate as enhancer for statin biosynthesis (Alarcón and Águila, 2006). Additional experiment by a scientist showed lactose as a good source for lovastatin biosynthesis at various carbon/nitrogen ratios in *A. terreus* ATCC 20542 (Chanakya *et al.*, 2012).

Effect of carbon and nitrogen ratio

Proper C/N ratio was also studied for the fermentation process of lovastatin. Best formation was achieved at C/N ratio of 25:1, which was 65.7 ± 5.5 in *P. spodoecus* (Table 4). Lovastatin production was enhanced in restricted inorganic nitrogen environment. The results are similar to the study of another research group (Casas *et al.*, 2004) in which inorganic nitrogen did not significantly influence lovastatin production. Similar results have been reported in studies performed with *Aspergillus terreus* (Jahromi *et al.*, 2012; Alarcón and Águila, 2006).

Table 4: Impact of Carbon and Nitrogen ratios for statin production by *P. spodoecus*.

Sr. No.	C/N ratio	Lovastatin production (mg/g)
1	5:1	37.5±15.7
2	10:1	49.3±15.4
3	15:1	53.5±6.6
4	20:1	57.7±12.7
5	25:1	65.7 ± 5.5

Conclusion

Fungus *P.spodoecus* produced 9.1±1.4 mg/g statin during two ways screening process that was further optimized by RSM under CCD using Design Expert 6.0.8 version and production enhanced to 19mg/g on wheat straw at 25°C, pH 5.5, inoculums size 3.5 mL and fermentation time of 144 h. The impact of carbon and nitrogen sources was also investigated. In case of *P. spodoecus* the mixing of lactose (Carbon source) and $NaNO_3$ (Nitrogen source) gave statin yield of 49.3±15 mg/g under pre-optimize conditions. C/N ratio was also optimized and maximum statin formation was achieved at C/N ratio of 25:1 that was 65.7 ± 5.5 mg/g in case of *P. spodoecus*.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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