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Production of lovastatin from rice straw using *Aspergillus terreus* in solid state fermentation

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Responce surface methodology (RSM) was used to optimize production of lovastatin from rice straw in solid state fermentation by *Aspergillus terreus* ATCC 74135. A four-factor-five-level central composite design (CCD) was used to examine the combining effects of temprature, moisture, inoculum and pH on lovastatin production. Results of the CCD study showed that only temperature and moisture contents significantly affected (P<0.01) lovastatin production. The maximum lovastatin production from experimentation and predicted by the CCD were 351.54 and 357.54 mg/kgDM, respectively, under the optimal conditions of 51.19% moisture, 28.29°C incubation temperature, inoculum size of 10.26% and pH of 6.31. Results of the present study showed that lovastatin can be produced from rice straw in solid state fermentation and the lovastatin enriched rice straw has the potential to be used as feed ingredient for reduction of ruminal methanogenesis.

Key words: Lovastatin, solid state fermentation, *Aspergillus terreus*, rice straw, responce surface methodology.

INTRODUCTION

Lovastatin is a potent drug for lowering the blood cholesterol by inhibiting 3-hydro-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is a key enzyme in the cholesterol biosynthesis pathway (Alberts et al., 1980). It is the first statin approved by the United States Food and Drug Administration (USFDA) in 1987 as a hypercholesterolemic drug (Tobert, 2003). In addition, lovastatin is a potential inhibitor of methanogenic archara because cell membrane production in archaea shares similar pathway with cholesterol biosynthesis (Miller and Wolin, 2001). Methane (CH_4) is the main metabolic product of methanogenic archaea and is a greenhouse gas with livestock activity contributes about 49% of the 165 Tg CH_4 annually production from agricultural activity (Johnson and Johnson, 1995).

Rice straw is one of the most abundant agricultural byproducts, with nearly 90% of the world production in Asia (Karimi et al., 2006). The traditional method for disposing rice straw after grain harvest is by burning (Summers and Jenkins, 2001) resulting in increasing concern in environmental pollution. Although rice straw is used as roughage source for ruminant livestock production, its use is limited by the high lignocelluloses and low nitrogen contents. In addition, there is a need to balance the feeding of agro-biomass such as rice straw to ruminants and the concomitant production of enteric

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CH_4. Although lovastatin is an inhibitor for methanogenesis (Miller and Wolin, 2001), pure lovastatin is too expensive to be used for CH_4 mitigation in ruminant livestock under farm conditions.

Different substrates including wheat bran, corn, glucose, fructose and rice have been used for lovastatin production in solid state fermentation (SSF) or submerged culture (Jaivel and Marimuthu, 2010; Pansuriya and Singhal, 2010; Wei et al., 2007). These substrate materials are expensive as they are human food and feed for livestock production. While the ability of *Aspergillus terreus* for production of lovastatin and cellulolytic enzymes has been extensively reported (Gao et al., 2008a, b; Jahromi et al., 2011; Jaivel and Marimuthu, 2010), the use of biomass such as rice straw for the production of lovastatin is not well documented. Thus the primary objectives of this study was to investigate the efficacy of using *A. terreus* for production of lovastatin using rice straw as substrates and to elucidate the fermentation conditions to optimize its production.

MATERIALS AND METHODS

Substrate, microorganism and preparation of spore suspension

Rice straw, collected from rice fields in the state of Selangor, Malaysia, was ground, sieved through mesh size 6 to obtain particles size of about 3.5 mm and dried in oven at 60°C for 48 h for later use in the study. *A. terreus* ATCC 74135 used in this study was obtained from the American Type Culture Collection (ATCC). It was maintained on potato dextrose agar (PDA) slants at 25°C for 7 days, stored at 4°C and sub-cultured every two weeks. Spore suspension (10^7 spores/ml) was prepared in the sterilized 0.1% Tween-80 solution.

Response surface methodology (RSM) was used for optimization of the fermentation factors which include temperature, moisture, inoculums size and pH for lovastatin production. Solid state fermentation was carried out in 500 ml Erlenmeyer flasks containing 20 g of rice straw with different concentrations of inocula and incubated for 8 days. The moisture content was adjusted using distilled-water, and taking into account the water content of the inoculums. Since adjustment of pH of solid sample is difficult, it was done by adjusting the pH of water (using 1N HCl or 1N NaOH) before it was added to the solid samples. A four-factor-five-level central composite design (CCD) with six replicates at the centre points (Table 1) was employed. A set of 30 runs with five levels for each variables designed by the Design-Expert® 8.0.6 software (Stat-Ease Inc., Minneapolis, MN, USA) was used for this study (Table 2). All the flasks were incubated for 8 days. All experimental designs and statistical data were analyzed using Design-Expert® 8.0.6 software. In addition, growth of *A. terreus* on the solid culture of rice straw was studied using Environmental Scanning Electron Microscope (Phillips XL30, Germany).

Extraction and determination of lovastatin

At the end of fermentation, lovastatin from the solid culture was extracted using methanol. After filtration with membrane filter (0.2 µm), the concentration of lovastatin in the filtrate was assayed using high performance liquid chromatography (HPLC) (Waters, USA, 2690) attached with an ODS column (Agilent, 250 × 4.6 mm i.d., 5 µm). The mobile phase consisted of acetonitrile and water (70:30 by volume) containing 0.5% acetic acid. The flow rate was 1 ml min^{-1}. The photo diode array detection range was set from 210 to 400 nm and lovastatin was detected at 237 nm. The sample injection volume was 20 µl, and the run time was 12 min. Since two forms of lovastatin (lactone and β-hydroxyl forms) are expected to present in the fermented culture, they were determined using HPLC. Commercial lovastatin (mevinolin K, 98%, HPLC grade, from sigma, M2147) used in this study is in the lactone form and β-hydroxyl lovastatin was produced from the lactone form using the method of Friedrich et al. (1995). Briefly, to prepare β-hydroxyl acid, the lactone lovastatin (Sigma) was suspended in 0.1 M NaOH and heated at 50°C for 1 h in a shaking incubator. Subsequently, the mixture was adjusted to pH 7.7 with 1 M HCl, filtered through 0.2 µm filters and used as standard for HPLC. The retention times of the hydroxyl and loctone lovastatins were 6.668 and 10.898 min, respectively. Different concentrations, ranging from 0.05 to 50 µg/ml of lovastatin were used to develop the standard curve.

RESULTS AND DISCUSSION

The prolific growth of *A. terreus* on the surface of rice straw (Figure 1) indicates the ability of this fungus to produce cellulolytic enzymes and hydrolyze the macromolecule lignocelluloses as energy source for their growth.

Lovastatin determination

Lovastatin was quantified as its β-hydroxyl acid and lactone forms by HPLC (Figure 2). The β-hydroxyl acid lovastatin is unstable (Jaivel and Marimuthu, 2010), thus its standard solution was prepared freshly from the lactone form according to Friedrich et al. (1995). Although the β-hydroxyl form of lovastatin was reported to be unstable (Jaivel and Marimuthu, 2010), results of this study show that the β-hydroxyl lovastatin is the dominant lovastatin in the fermented rice straw (> 74%). It has been reported that the conditions needed for conversion of the lactone form into the β-hydroxyl form are high pH (for example, by addition of NaOH), heating to 50°C and naturalization by acid (Friedrich et al., 1995). Since none of the above conditions were applied in this study, we believe that the β-hydroxyl lovastatin, the more active form of lovastatin for inhibition of HMG-CoA reductase (Alberts, 1988), present in the extract of the fermented rice straw in this study is a direct product of SSF and not from conversion of the lactone form.

Response surface methodology (RSM)

RSM was used to optimize the effects of moisture content, temperature, inoculums size and pH for lovastatin production using rice straw as substrate in this study. The

Table 1. Values of coded and actual factors for the CCD matrix.

Parameter	Levels				
	-2	-1	0	1	2
Temperature (°C)	22	27	32	37	42
Moisture content (%)	40	50	60	70	80
Inoculum (%)	5	7.5	10	12.5	15
pH	4	5	6	7	8

Table 2. Central composite design (CCD) matrix of independent variables with their corresponding responses by experiments and predicted values.

Run	A	B	C	D	Lovastatin production (mg/kgDM)	
	Moisture (%)	Temperature(˚c)	Inoculums (%)	pH	Experimental	Predicted
1	60	32	10	4	223.92	225.42
2 (cp)*	60	32	10	6	337.03	314.24
3	50	27	7.5	7	323.20	329.54
4	70	37	7.5	5	186.92	192.87
5	60	32	10	8	217.68	208.75
6	40	32	10	6	305.67	320.53
7	50	27	12.5	5	322.44	334.15
8	70	37	12.5	5	208.32	204.46
9	60	32	5	6	257.43	282.20
10	70	27	12.5	5	162.33	182.47
11	80	32	10	6	179.21	156.92
12 (cp)	60	32	10	6	305.98	314.24
13 (cp)	60	32	10	6	324.33	314.24
14 (cp)	60	32	10	6	271.99	314.24
15	70	37	7.5	7	186.46	177.23
16	50	37	12.5	7	200.78	204.69
17	60	22	10	6	281.24	254.69
18	50	27	12.5	7	334.10	333.11
19	50	27	7.5	5	356.13	346.92
20	60	32	15	6	329.57	297.37
21 (cp)	60	32	10	6	316.03	314.24
22	60	42	10	6	101.68	115.75
23	50	37	7.5	5	237.32	215.77
24	50	37	7.5	7	204.35	189.16
25	70	37	12.5	7	190.99	205.17
26 (cp)	60	32	10	6	325.04	314.24
27	50	37	12.5	5	212.25	214.96
28	70	27	7.5	7	174.17	176.42
29	70	27	7.5	5	184.27	182.83
30	70	27	12.5	7	168.37	192.41

*, Centre points.

experimental and predicted results of the RSM study in the different runs are presented in Table 2. Statistical analysis (Table 3) showed that only incubation temperature and moisture content and their interaction have significant effects on lovastatin production ($P<0.01$). The effects of incubation temperature and moisture content together with inoculums size and pH on lovastatin production are shown in Figures 3a and 3b, respectively.

Table 3. Analysis of variance table of response surface quadratic model.

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	1.302E+005	14	9298.34	16.89	< 0.0001
A-moisture	33026.86	1	33026.86	60.01	< 0.0001
B-temprature	30653.73	1	30653.73	55.70	< 0.0001
C-inoculum	396.11	1	396.11	0.72	0.4096
D-pH	457.73	1	457.73	0.83	0.3762
AB	19932.12	1	19932.12	36.22	< 0.0001
AC	153.99	1	153.99	0.28	0.6046
AD	120.33	1	120.33	0.22	0.6468
BC	142.94	1	142.94	0.26	0.6177
BD	85.13	1	85.13	0.15	0.6996
CD	267.11	1	267.11	0.49	0.4967
A^2	9825.51	1	9825.51	17.85	0.0007
B^2	28414.15	1	28414.15	51.63	< 0.0001
C^2	1030.49	1	1030.49	1.87	0.1914
D^2	16261.88	1	16261.88	29.55	< 0.0001
Residual	8255.51	15	550.37		
Lack of Fit	5665.52	10	566.55	1.09	0.4906
Pure Error	2589.99	5	518.00		
Cor Total	1.384E+005	29			
Coefficient of variation (CV%)		9.47	Adj R-squared		0.8847
R-Squared		0.9404	Pred R-squared		0.7426
Std. Dev.		23.46			

The high R^2 (0.9404) value (Table 3) indicates a high correlation between the experimental and predicted values of lovastatin production. The value of determination (R^2 = 0.7426) suggests that the response model accounted for 74.26% variations of the total. The value of adjusted determination coefficient (R^2 Adj = 0.8847) indicates the significance of this model. A mathematical model showing the significant importance is described as follow:

$$\text{Lovastatin (mg/kgDM)} = -704.96241-(6.90849\times A) + (41.25366\times B)\ (3.81883\times C) + (269.27829\times D) + (0.70591\times A\times B) + (0.12409\times A\times C) + (0.27424\times A\times D) + (0.23911\times B\times C)\ -(0.46133\times B\times D) + (1.63433\times C\times D) - (0.18881\times A^2) - (1.39298\times B^2) - (0.97834\times C^2) - (24.29021\times D^2)$$

Where, *A*, *B*, C and D indicate temperature, moisture content, inoculums size and pH, respectively. Point prediction of software was used to determine optimum conditions of the factors for maximum lovastatin production including 51.19% moisture, incubation temperature of 28.29°C, inoculum size of 10.26% and pH of 6.3. The maximum lovastatin production values from experimentation and predicted by the CCD were 351.54 and 357.54 mg/kgDM, respectively, under the optimal conditions.

Wei et al. (2007) reported maximum production of 2900 mg lovastatin/kg DM rice grain using *A. terreus* ATCC 20542 and with addition of peptone and glucose. Other studies reported production values from 982.3 to 3723 mg lovastatin/kg DM wheat bran using *A. terreus* (Jaivel and Marimuthu, 2010; Pansuriya and Singhal, 2010). Although the maximum production of lovastatin in this study (351.54 mg/kgDM) is lower than those reported in the literature, we believe the yield is substantial because rice straw is a poor quality biomass compared to the more expensive substrates used by the other researchers.

Approximately, one billion ton of rice straw is produced annually with 90% of this biomass in Asia. Majority of this biomass is burned away causing great environmental concerns. Conversion of low quality agro-biomass, which is a potential pollutant to the environment, into high value biomaterials was the primary aim of this study. Therefore, this study provides a new insight for production of lovastatin and/or other similar biomaterials of high value from agro-biomass, which otherwise may be sources of pollutant to the environment. In addition, SSF also breaks down the lignocelluloses and improves the nutritive value of the biomass as animal feed (Jalc et al., 1998). We have evidence of improvement in the nutritive quality of the treated rice straw as animal feed, including the potential use for mitigation of ruminal methane emission,

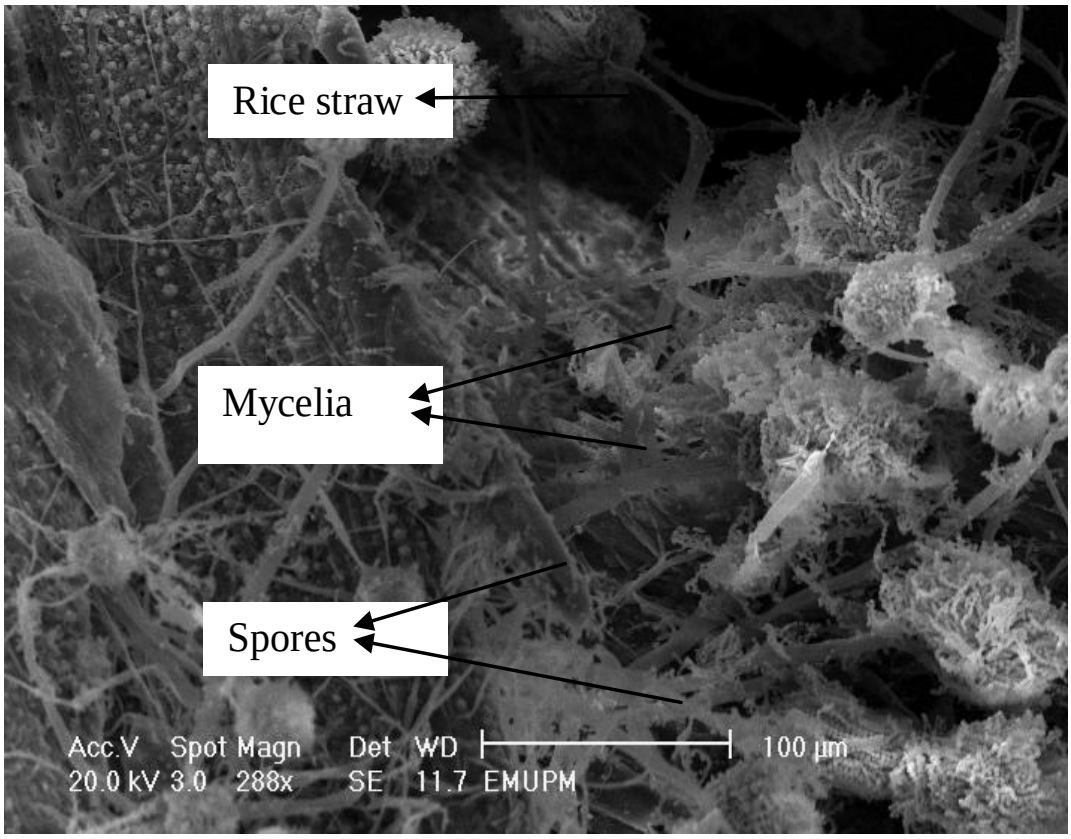

Figure 1. Prolific formation of mycelia and spores by *A. terreus* ATCC 74135 on surface of rice straw.

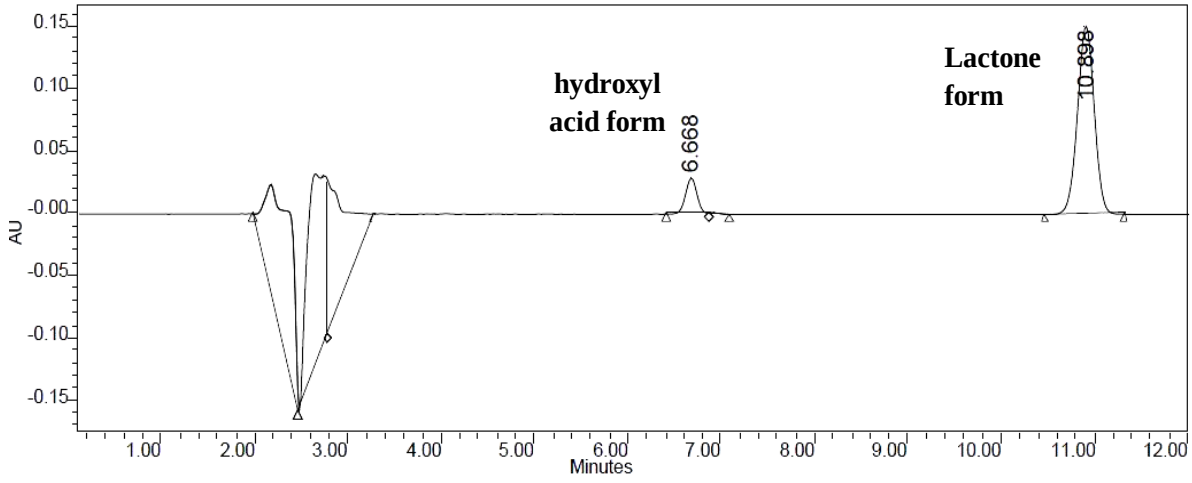

Figure 2. HPLC chromatogram of lovastatin in hydroxyl acid and lactone forms. HPLC conditions: column, Agilent C-18 (5mm i.d.×250 mm, 5 µm); mobile phase, acetonitrile/H2O/acetic acid = 70/30/0.5 (v/v/v); flow rate, 1 mL/min; detection, 237 nm. (Jahromi et al., 2013).

which often have been implied as a source of global warming (Jahromi, et al., 2013). In addition, the hydroxyl form of lovastatin, which is the more active inhibitor of HMG-CoA reductase, was dominant in the fermented rice straw and could serve as a better alternative to the currently available lovastatin in the market, which is in the

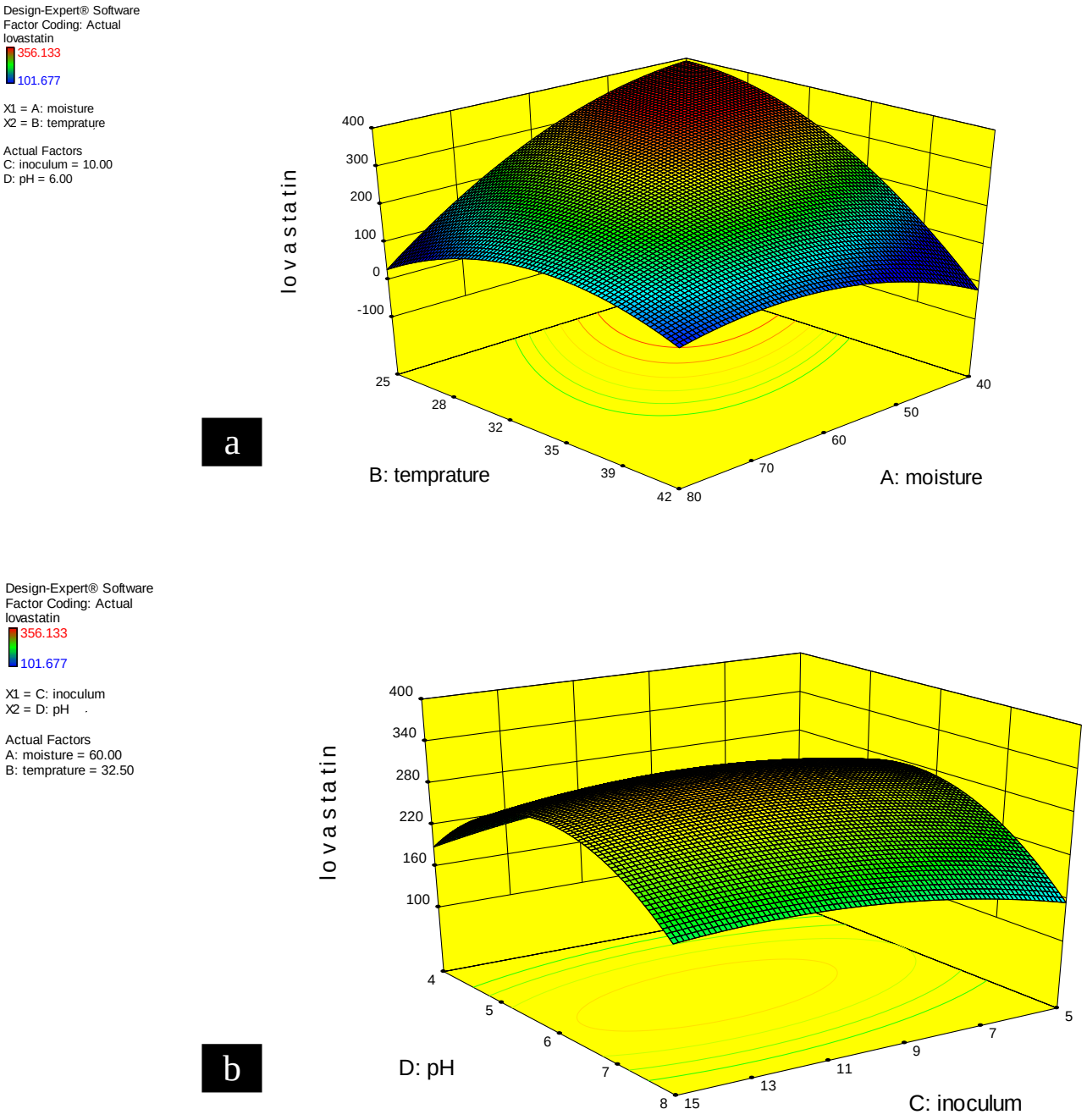

Figure 3. Response surface plots showing relative effects of different process parameters on lovastatin production (mg/kgDM) during solid state fermentation. a, Effect of moisture (%) and temperature (˚C); b, effect of pH and inoculums size (%) on lovastatin production.

form of lactone.

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ABBREVIATIONS

HMG-CoA, 3-Hydro-3-methylglutaryl-coenzyme A reductase; **SSF,** solid state fermentation; **PDA,** potato dextrose

agar; **RSM,** Response surface methodology; **HPLC,** high performance liquid chromatography.

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