Applied Biological Research 19 (2): 122-131; (2017)
DOI: 10.5958/0974-4517.2017.00018.0

STATISTICAL OPTIMIZATION OF CULTURAL CONDITIONS FOR MANGANESE PEROXIDASE PRODUCTION BY *Coriolus versicolor* MTCC 138 FROM DIGESTED CATTLE DUNG SLURRY

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(Received 14 November, 2016; accepted 20 April, 2017)

ABSTRACT

The present study reports the production of manganese peroxidase enzyme using digested cattle dung slurry as a substrate by *Coriolus versicolor* MTCC 138. Digested cattle dung slurry was analyzed for proximate (total solids, volatile solids and pH), chemical (cellulose, hemicellulose, lignin and silica) and biochemical (total proteins and manganese peroxidase) composition. Manganese peroxidase production by *C. versicolor* MTCC 138, was optimized by a conventional 'one variable at a time approach' and then by Placket-Burman design. Optimized fermentation conditions for the maximum manganese peroxidase (78.0 U mL^{-1}) enzyme production were 25% slurry concentration, 5 days of incubation, temperature 25°C and inoculum size of 10^6 spores mL^{-1}. This enzyme titre obtained by statistical optimization was 1.86-fold than that achieved by 'one variable at a time approach'. It revealed that the effect of different cultural conditions (slurry concentration, spore concentration and incubation period) have statistically significant effect on enzyme production.

Key words: *Coriolus versicolor* MTCC 138, digested cattle dung slurry, manganese peroxidase, Placket-Burman model

INTRODUCTION

Utilizing the organic wastes such as residues from forestry, agriculture, alimentary industries, etc. for the production of value-added products by solid state fermentation is at on rise during past few years (Kalogeris *et al.*, 2003). Utilization of such wastes minimizes environmental problems caused otherwise by their disposal. Manganese peroxidase (MnP) [EC 1.11.1.13] with a molecular weight of 45 to 47 kDa is an extracellular glycoprotein containing heme as a prosthetic group. It is an important enzyme in lignin degradation catalyzing the oxidation of Mn^{2+} to Mn^{3+} in an H_2O_2-dependent reaction. MnP acts as an active redox mediator attacking phenolic lignin structures, resulting in the production of unstable free radicals that disintegrate spontaneously (Zhao *et al.*, 2015).

Lignin, after cellulose and hemicellulose, is the most abundant aromatic polymer on earth comprising of three different phenyl propane units namely coniferyl, ρ-coumaryl and sinapyl alcohol. Complicate structure of lignin makes it more difficult to degrade as compared to cellulose or hemicellulose. Lignin degraders and their ligninolytic enzymes have become fascinating as they are expected to provide environment-friendly technologies for biofuel industry. MnP is one of the ligninolytic enzymes. Environmental factors and availability of nutrients have a major impact on MnP production (Elisashvili and Kachlishvili, 2009; Hiscox *et al.*,2010). Different groups of fungi

reportedly produce ligninolytic enzymes (Gianfreda and Rao, 2004). Among them, white-rot fungi including *Bjerkandera* sp. BOS55, *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* reportedly are capable of producing MnP (Hofrichter, 2002; Martinez *et al*., 2005).

Biogas slurry, an anaerobically digested organic material released as liquid by-product from the biogas plants, has a C:N ratio of is 20 and mainly contains 7.0-8.0% dry matter, 68.5-70.6% organic matter, 1.40-1.84% nitrogen, 1.10-1.72% P_2O_5, 0.84-1.34% K_2O, 35-38.4% organic carbon, 103-116 mg kg^{-1} Zn, 51-68 mg kg^{-1} Cu , 231-295 mg kg^{-1} Mn and (Anonymous, 2009). Since biogas slurry contains cellulose, hemicellulose, lignin, organic matter and many potential microbes, so it can be exploited for the production of value-added products like cellulolytic-, proteolytic- and lignolytic-enzymes; besides it can be used for biopulping bioremediation, bio-bleaching and bioremediation, etc. (Young and Akhtar, 1998).

Statistical experimental design "Response Surface Methodology (RSM)" is an efficient and lucrative approach to optimize the growth conditions for enhanced enzyme production. (Arantes *et al*., 2011; Levin *et al*., 2005; Macedo *et al*., 2008). Although the design has widely been applied for optimizating various other processes (Reddy *et al*., 2008), the use of this method of optimization of cultural factors for enhancing MnP production by *Coriolus versicolor* MTCC 138 from digested cattle dung slurry has not yet been reported. The present study reports the screening of cultural conditions for their significant main effects using Plackett–Burman design to optimize cultural conditions for optimum MnP activity by *C. versicolor* MTCC 138.

MATERIALS AND METHODS

Procurement of necessary materials

Digested cattle dung slurry was obtained from a biogas plant in biogas field laboratory of School of Renewable Energy Engineering, PAU, Ludhiana (India). The chemicals used were of analytical grade procured from Hi-Media, SRL, Sigma and SD Fine Chemicals. The standard culture of *Coriolus veriscolor* MTCC 138 was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India) and was maintained on malt extract Blakeslee`s agar at 28±2°C by monthly transfers. The culture was stored in a refrigerator at 4°C after sub-culturing.

Proximate and chemical analysis

Standard methods of AOAC (2000) were followed for proximate (total solids and volatile solids) and chemical (cellulose, hemicellulose, silica and lignin) analysis of digested cattle dung slurry.

Manganese peroxidase activity of digested cattle dung slurry

Fifty mililitre digested cattle dung slurry was taken from biogas plant in flasks in triplicate and analyzed for proximate and chemical composition. Manganese peroxidase (MnP) activity was calculated for both autoclaved and unautoclaved digested cattle dung slurry. Other sets of 15 flasks, each dispensed with 50 mL digested cattle dung slurry separately, were also autoclaved at 121°C for 20 min. Another set was prepared without autoclaving. Both sets were inoculated with 5 mm culture bit of *C. versicolor* MTCC 138 and incubated at 28±2°C for enzyme production. After incubation for 2, 4, 6 and 8 days, the samples were taken out from the incubator and centrifuged at 10,000 rpm for 15 min at 4°C to get clear supernatant. The supernatant was used as crude enzyme extract and analyzed for MnP activity as per the method of Paszczynski *et al.* (1988) and for protein content as per Lowry *et al*. (1951). Enzyme activities and protein were determined spectro-photometrically using UV-VIS spectrophotometer (Hitachi-2800 model).

Manganese peroxidase (MnP) (EC.1.11.1.13) Assay

Manganese peroxidase activity was determined by the method of Paszczynski *et al.* (1988). The enzyme filtrate (0.2 mL) was put in a cuvette along with 3 mL 0.05 M guaiacol solution, 0.2 mL 2

mM $MnSO_4$ and 0.2 mL 0.4 M H_2O_2 was thoroughly mixed and change in light absorbance recorded after every 10 sec upto 180 sec at 465 nm against a blank without H_2O_2 using UV-VIS spectrophotometer Hitachi-2800 model. An increase in optical density (OD) by 0.001 in 60 sec was taken as 1 unit.

Optimization of MnP production in submerged fermentation by 'one variable at a time approach'

The manganese peroxidase production by *C. versicolor* MTCC 138 from digested cattle dung slurry was first optimized by 'one variable at a time approach' to find the most important factors that affect the production of manganese peroxidase. The mold was grown in digested cattle dung slurry, as above, with different incubation temperatures (25, 30, 40 and 50ºC) to evaluate the effect of temperature on enzyme production. Similarly, to study the effect of different inoculums levels or spore concentrations, slurry concentrations and incubation periods on enzyme production, the mold was grown with different levels of inoculums (10^6, 10^7, 10^8, 10^9 spores mL^{-1}), slurry concentrations (25, 50, 75%) and incubation periods (2, 4, 6, 8, 10, 12 and 14 days) respectively. *C. versicolor* MTCC 138 was inoculated in flasks containing 10 g straw with 40 mL of digested cattle dung slurry. The experiment was performed in triplicate.

Experimental design using Placket-Burman model

Response surface methodology (RSM), using a Placket-Burman model, was used for the optimization of manganese peroxidase (MnP) production. Four process variables selected by 'one variable at a time' approach, were used in designing the experiment i.e. slurry concentration (25, 50 and 75%), incubation period (5, 10 and 15 days), spore concentration (10^6, 10^7 and 10^8 spores mL^{-1}) and temperature (25°C, 30°C and 35°C). MnP activity was analyzed and protein content was determined using these sets of conditions as shown in Table 3. Evaluation of the model and the determination of the operating conditions were done by examining the F test, P value, Coefficient of determination (R^2), three-dimensional (3D) response surface plots. The three-dimensional plots according to the fitted model were drawn using software Statgraphics Centurian XVI.I.

RESULTS AND DISCUSSION

Proximate and chemical analysis

The proximate and chemical composition of digested cattle dung slurry revealed that the digested slurry had 6.2% total solid concentration and 65.67% volatile solid content and was alkaline in nature (pH 8.06) (Table 1). Cellulose and hemicellulose were less (13.9 and 6.93%, respectively) in digested cattle dung slurry which may be due to the consumption of some part of organic matter like cellulose and hemicelluloses by natural consortia of microorganisms in biogas plant. The content of lignin and silica in digested slurry was 16.5 and 10.5%, respectively. Anonymous (2009) reported that the average total solid concentration and volatile solids content in digested slurry were 12.38 and 66.5%, respectively. The

Table 1: Proximate chemical composition (% dry weight) and manganese peroxidase activity (U mL^{-1}) of digested cattle dung slurry

Parameters	Digested cattle dung slurry
pH	8.06±0.10
Total solids (TS)	6.20±0.12
Total volatile solids (TVS)	65.67±2.16
Neutral detergent fibre (NDF)	50.20±0.94
Acidic detergent fibre (ADF)	43.27±0.31
Hemicellulose	6.93±0.42
Cellulose	13.90±0.23
Lignin	16.50±0.31
Silica	10.50±0.26
MnP activity of unautoclaved sample	14.30±1.42
MnP activity of autoclaved sample	10.80±1.32

*The data represents the mean of three determinations each; ±values indicate standard error

Table 2: Manganese peroxidase enzyme profile of digested cattle dung slurry inoculated with *C. versicolor* MTCC 138

Incubation period (days)	Manganese peroxidase (U mL^{-1})	
	Unautoclaved digested cattle dung slurry	Autoclaved digested cattle dung slurry
Control	14.3±0.76 (9.72)	8.4± 1.38 (5.25)
2	25.0± 0.66 (18.99)	18.8±0.92 (11.32)
4	48.6±1.21 (35.33)	34.5±0.61 (18.18)
6	54.0±0.97 (39.7)	31.4±1.2 (22.69)
8	32.6± 0.80 (22.02)	28.5±0.85 43 (19.07)
$CD_{0.05}$	1.64	1.88

#**Cultural conditions**: Incubation temperature: 28±2°C; pH :6.0; Inoculum concentration:10^6 spores mL^{-1} of *C. versicolor*. The data represents the mean of three determinations each; ±values indicate standard error; C.D: Critical difference at 5% level. Values in parenthesis indicate specific activity i.e. U mg^{-1} of protein.

manganese peroxidase enzyme activity was determined in both autoclaved and unautoclaved digested cattle dung slurry (50% concentration) and MnP activity was found higher in unautoclaved sample (14.3 U mL^{-1}) of digested cattle dung slurry than in autoclaved sample (10.8 U mL^{-1}). The autoclaved samples showed more reduction in enzyme activity than the unautocalved one. This indicates that the slurry can be used without autoclaving, thus saving energy. The maximum manganese peroxidase (54 U mL^{-1}) was found on 6th day of incubation; thereafter, a reduction in enzyme activity was observed (Table 2). The findings are in agreement with Dritsa and Rigas (2006). Iqbal *et al*. (2011) found maximum production of manganese peroxidase, lignin peroxidase and laccase after 5 and 6 days of incubation in media containing rice straw and wheat straw as substrate respectively.

Factors affecting enzyme activity: Single factor optimization

C. versicolor MTCC 138 grew well in digested cattle dung slurry and secreted MnP (42.0 U mL^{-1}). The MnP production started off in early stage of incubation and reached a peak on 8th day and this was close to that of 6th and 10th days, respectively (Fig. 1a). The highest MnP titre was found at 25°C

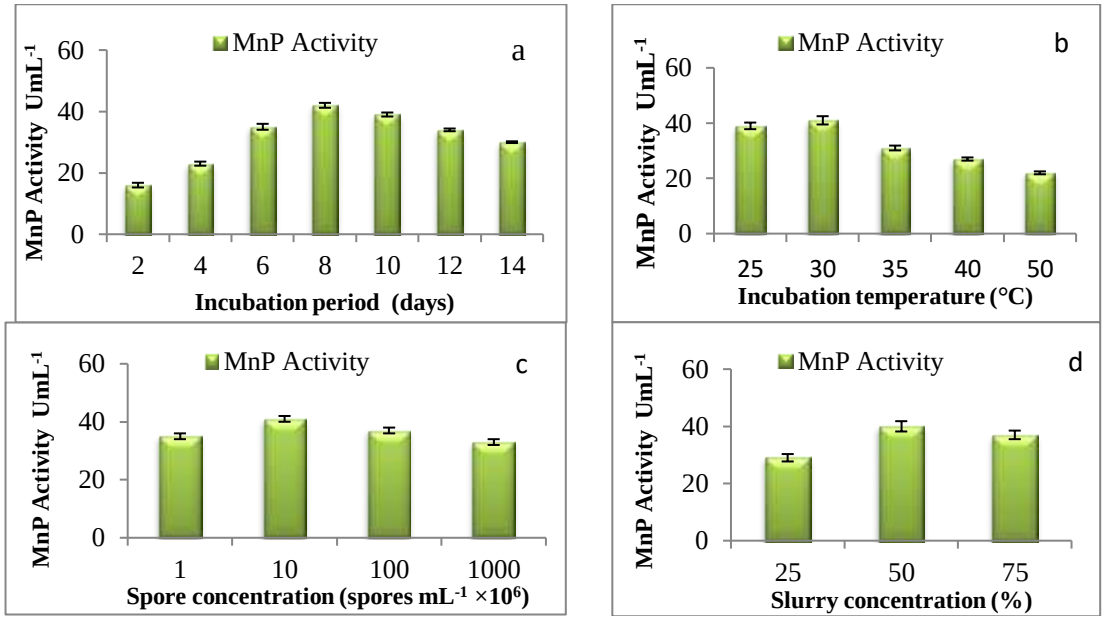

Fig. 1: Effect of various growth parameters on MnP production a) Incubation period; b) Incubation temperature; c) Spore concentration; and d) Slurry concentration

(Fig. 1b), which was close to that of 30°C. MnP production by *C. versicolor* MTCC 138 was highest at 25°C which was in optimum temperature range (25-30°C) for its growth. The optimization of inoculum size revealed that 10^7 spores mL^{-1} yielded maximum (41 U mL^{-1}) MnP (Fig. 1c). At lower and higher spore concentrations, a decrease in enzyme production was noticed because low inoculum load is insufficient for enzyme production, while higher inoculum size affects yield due to nutrient competition. This is in line with Dar and Phutela (2015). Maximum enzyme activity (40 U mL^{-1}) was noted for 50% slurry concentration (Fig. 1d). This could probably be due to moisture content, which being below or above, determines the optimal level leading to enzyme inhibition and greater enzyme diffusion away from the substrate, respectively.

Process variable optimization using Placket-Burman design

For optimization, various cultural parameters were assessed using Placket-Burman statistical design. It being a fraction of two-level factorial design allows the investigation of n-1 variables in at least n experiments. This design takes into account equality of frequency of each process variable at each level and that in each test the number of high and low variables are kept equal. This leads to the cancellation of effects of other variables while evaluating the effect of a particular variable. The main effect was determined as the difference between average of measurements made at high-level setting (+1) and at low-level setting (-1) of each factor. The independent variables included digested slurry concentration (25, 50 and 75%), incubation period (5, 10 and 15 days), spore concentration (10^6, 10^7 and 10^8 spores mL^{-1}) and temperature (25, 30 and 35°C). Twelve runs, as suggested by Placket-Burman model, were performed. The results are shown in Table 3. The 3D plots (Fig. 3a-f) depict the effect of various factors on one another.

The multiple regression analysis was performed to fit the high order polynomial to MnP experimental results (Table 4). The standard error of each effect is shown which measures their sampling error. The probability (*p*) of model equation was 0.0004, implying that the model is significant ($P \leq 0.05$). In this case, all the 4 factors have P-values ≤ 0.05, indicating that they were significantly different from zero at 95.0% confidence level. The R-squared statistic or coefficient of determination (R^2) indicated that the model as fitted explained 92.8% variability in MnP. This gives very good correlation between input factors and their responses indicating that the model is correlated well with the measured data and was statistically significant. The adjusted R-squared statistic, the most suitable for comparison of models with different numbers of independent variables, was 88.70%. The standard deviation of estimate was 7.05. Experimental results were very close to predicted ones for MnP production (Fig. 2).

Table 3: Manganese peroxidase profile of digested cattle dung slurry inoculated with the spores of *C. versicolor* MTCC 138 as per Placket Burman model parameters of optimized conditions

Runs	Process variables				MnP activity (U mL^{-1})
	Spore count (spores mL^{-1})	Incubation period (days)	Slurry concentration (%)	Temperature (°C)	
1	10^6	5	25	35	72.0±0.04
2	10^8	15	25	25	63.0±0.56
3	10^8	5	75	25	51.0±0.99
4	10^6	15	75	35	23.0±2.36
5	10^8	5	75	35	58.0±1.44
6	10^6	5	25	25	78.0±2.54
7	10^6	15	75	35	23.0±0.87
8	10^6	15	25	25	57.0±1.57
9	10^6	5	75	25	28.9±0.54
10	10^8	5	25	35	74.0±1.42
11	10^8	15	25	35	75.8±1.37
12	10^8	15	75	25	34.0±0.32

#MnP: Manganese peroxidase; Data represents the mean of 3 determinations; ±values indicate standard error.

Table 4: The regression analysis of the fitted model for manganese peroxidase

Source	Sum of squares	d.f.	Mean square	F-ratio	P value	Prob>F
Model	4485.94	4	1121.48	22.59	0.0004	Significant
A: Slurry concentration	3396.97	1	3396.97	68.43	<0.0001	
B: Spore concentration	455.10	1	455.10	9.17	0.0192	
C: Incubation period	617.77	1	617.77	12.45	0.0096	
D: Temperature	16.10	1	16.10	0.32	0.5868	
Residual	347.47	7	49.64			
Cor. Total	4833.41	11				
Standard deviation (SD)	7.05					
R-squared	0.9281					
Mean	53.14					
Adj R-squared	0.887					
Predicted R-square	0.7887					
Adequate precisior	13.263					

The regression equation of the fitted model for manganese peroxidase is as follows:
MnP = +51.0833 - 0.673* slurry concentartion + 6.1583* spore conc. - 1.435* incubation period + 0.2316* temperature

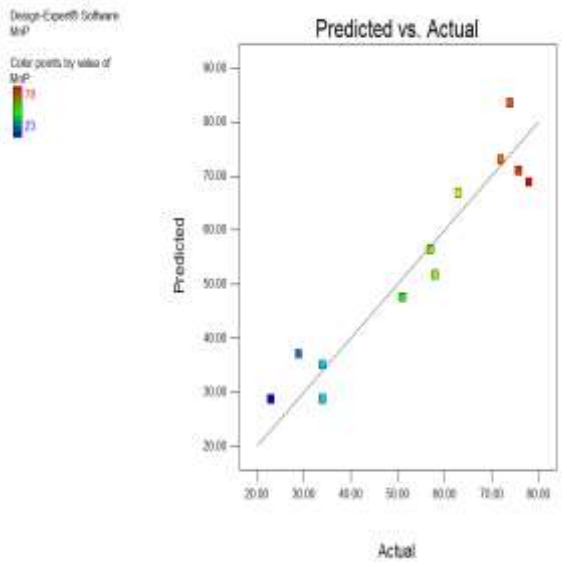

Fig.2: Plot showing predicted and observed value of MnP

Parameters like slurry concentration (25-75%) were studied for optimization of ligninolytic enzyme (MnP) production from *C. versicolor* MTCC 138 inoculated-digested slurry. The 25% slurry concentration was observed found optimum for MnP production which is in line with Kundu *et al*. (1983) who conducted an experiment at 30°C from 1 to 10 days and recorded maximum enzyme activities after 5th day of incubation on banana stalk. Similarly, Regina *et al*. (2008), Shaheen *et al*. (2008) and Bhatti and Nawaz (2009) observed that increasing moisture level from 50-66.6% increases fungal growth and ligninases production. However, further enhancement caused a significant downfall in enzyme activity. Higher and lower moisture levels were inhibitory, lowering the activity of ligninases in secondary growth, because of poor nutrient accessibility and limited aeration. Optimum moisture and water holding capacity determine the end product and water requirement of fungus (Kim *et al*., 1985). Incubation period has profound effect on enzyme activity. In present study, 5 days incubation period was found optimum for MnP activity. The findings are in close proximity with Castillo *et al*. (1997) who reported that LiP and MnP activities in straw extracts due to *Phaneochaete chrysosporium* BKM-F-176 during fermentation were high after 6 days of incubation. Different white rot fungi reportedly produce maximum ligninolytic activity after different time periods due to genetic variation among the strain as well as in the nature and composition of substrate used (Patel *et al*., 2009). To optimize temperature for maximum MnP production, samples were incubated at 25, 30 and 35°C. When cultivated at a temperature >25°C, the ligninolytic enzyme activities were substantially decreased and for MnP production optimized temperature was 25°C, followed by 30°C. Tekere *et al*. (2001) found that the optimum temperature for cultures of *P. chrysosporium* and *C. versicolor* for ligninase synthesis in SSF varied between 25-37°C. A significant influence of incubation temperature on

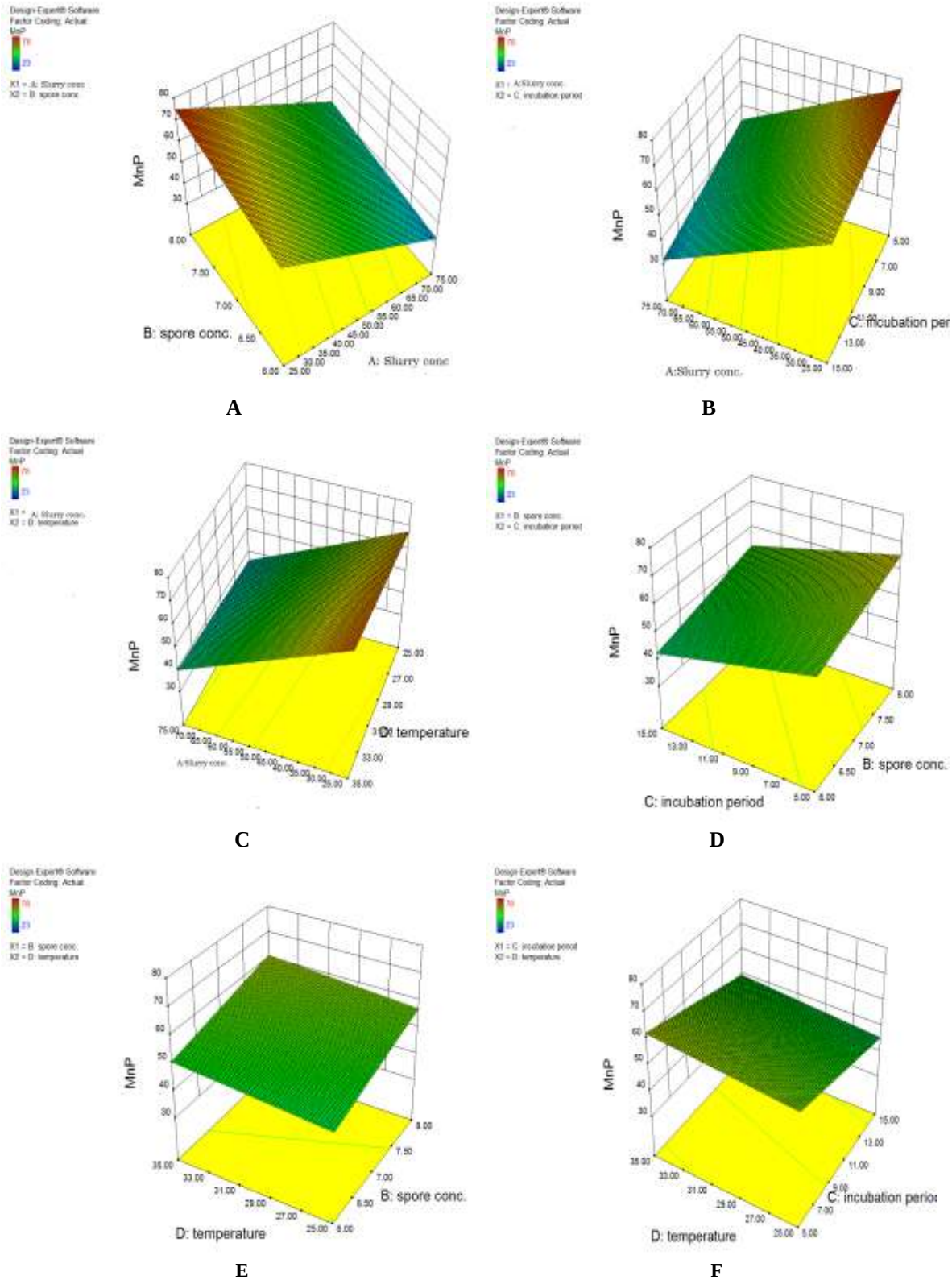

Fig. 3: Effect of various variable on Mn production a) Slurry concentration and spore concentration; b) Slurry concentration and incubation period; c) Slurry concentration and temperature; d) Spore concentration and incubation period e) spore concentration and temperature f) incubation period and temperature

ligninolytic enzymes of *Pleurotus* sp., *Dichomitus squalens* and other white rot fungi has been reported (Arora and Gill, 2000; Tripathi *et al*., 2008). The temperature ranging from 25 -37°C has been found optimum for ligninase production by different white rot fungi (Zadraril *et al.,* 1996; Arora and Gill, 2000; Tripathi *et al*., 2008). The inoculum size appeared to be a crucial parameter for enzyme production. Highest MnP activity was noted at spore concentration of 10^6 spores mL^{-1}. It appears that low inoculum level perhaps results in insufficient biomass generation causing reduced product formation, whereas higher inoculum may be a route to excessive biomass and drain out the substrate which is essential for product formation (Sabu *et al*., 2006; Vivekanand *et al*., 2011). Our results are also in harmony with Dar and Phutela (2015) who observed low enzyme activity at higher and lower spore concentrations of *Thermoascus auranticus* MTCC 375.

Model validation

The model was validated by repeating the experiments under the optimized conditions (conditions obtained through Placket-Burman design), which resulted in the MnP production of 74.20 U mL^{-1} (Table 5), which is close to that of the value obtained using the Placket-Burman design, showing strong agreement between them, thus validating the model.

Table 5: Model validation experiments with the experimental responses of manganese peroxidase (MnP) enzyme obtained by RSM (Placket–Burman design)

Spore count (spores mL^{-1})	Incubation period (days)	Slurry concentration (%)	Temperature (°C)	MnP activity (Um L^{-1})	MnP activity (RSM) (Um L^{-1})
10^6	5	25	25	74.2±2.5	78±2.54

Conclusion: In present study, inoculum load of 10^6 spores mL^{-1}, 5 days incubation period, 25% slurry concentration and 25°C temperature were found optimum conditions for MnP production (78 U mL^{-1}) from digested cattle dung slurry. Digested cattle dung slurry contains organic nutrients like cellulose, hemicellulose, lignin, organic matter and potential microorganisms. So, it can be further used for large scale production of MnP besides other value-added products like cellulolytic, proteolytic and lignolytic enzymes.

Acknowledgement: The authors thankfully acknowledge the financial support provided by AICRP-BCT (All India Coordinated Research Project, Bioconversion Technology), Indian Council of Agricultural Research, New Delhi (India) for pursuing this project.

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