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Optimization of lovastatin production by *Fusarium nectrioides* (MH173849) using response surface methodology and fuzzy logic system

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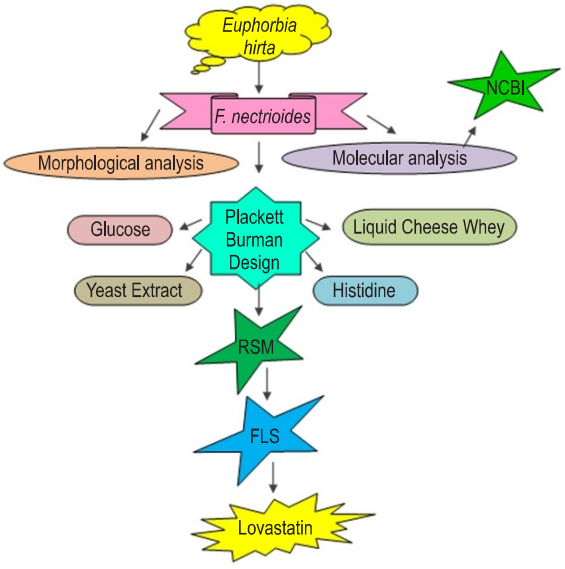
Abstract

Aim : To enhance the productivity of lovastatin from *Fusarium nectrioides* isolate with liquid cheese whey as a major carbon source and to optimize the media components using Response Surface Methodology (RSM) and Fuzzy Logic System (FLS).

Methodology : *Euphorbia hirta* was collected, surface sterilized and incubated on potato dextrose agar medium amended with ampicillin and streptomycin sulphate. *F. nectrioides* was isolated from *E.hirta* and identified using morphological and molecular methods. Primarily, media components were screened by Plackett Burman design (PBD). Further, the effect of significant nutrients was predicted using RSM and FLS and compared with experimental yield.



Results : Molecular identification by gene sequencing confirmed the isolate to be *F. nectrioides*, given an accession number (MH173849) the sequence was submitted in the gene bank. PBD revealed that peptonized milk (which is an enzymic digest of high grade skimmed milk powder), corn steep liquor, liquid cheese whey and histidine were significant variables. The optimum levels of these significant variables in different combinations were studied by RSM in which the predicted yield of lovastatin was 1.2 gl^{-1}. Further, it was analyzed by FLS with 14 set of fuzzy rules and the maximum production obtained was 1.8 g100 ml^{-1} which was closer to the experimental yield of 1.75 g100 ml^{-1}. Therefore, compared to RSM, FLS was more suitable technique to determine the optimum levels of significant nutrients for enhanced lovastatin production.

Interpretation : This study suggests that *F. nectrioides* (MH173849) can be used as a potent producer of lovastatin and the production highly influenced by glucose, corn steep liquor, liquid cheese whey and histidine.

Key words: Cheese Liquid whey, *Euphorbia hirta*, *Fusarium nectrioides*, Fuzzy Logic system, Lovastatin

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Introduction

Lovastatin is a potent anti-cholesterol compound and competitive inhibitor of 3 hydroxyl 3 methylglutaryl co-enzyme A reductase (HMG-CoA) which has a huge demand in the pharmaceutical and health sectors (Tandon *et al.,* 2005; Alberts *et al.,* 1980). It is also used to prevent stroke, reduce the development of peripheral vascular disease and treat bone fractures (Pahan, 2006). Due to its pleiotropic effects (Davies *et al.,* 2016), it has received increasing recognition and clinical applicability across a broad range of cardiovascular and non-cardiovascular conditions, including Alzheimer's, cancer, dementia, Parkinson's, multiple sclerosis and rheumatoid arthritis (Davignon and Leiter, 2005). Owing to its significant importance in medicinal field, the cost effective production of lovastatin in large scale is highly solicited. Therefore, industries are seeking assistance of strong scientific technology and statistical tools for enhancing the production of Lovastatin (Seenivasan *et al.,* 2008).

Lovastatin is a secondary metabolite produced during the exponential phase of selective endophytic fungi under optimal conditions. Isolating a potential endophytic fungi and designing a fermentation medium is crucial for the enhanced production of lovastatin (Hutchinson *et al.,* 2000; Goswami *et al.,* 2012). Lovastatin is produced from multiple genera and types of filamentous fungi, including *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopolariopsis, Doratomyces, Phoma, Phythium, Gymnoascus, Hypomyces* and *Pleurotus* (Casas López *et al.,* 2003). Endophytic fungi from medicinal plants have gained greater attention due to their rich diversity (Pinruan *et al.,* 2010) towards the production of secondary metabolites. The concentration of biomass and production of lovastatin mainly depends on selection of carbon sources (Dhar and Nigam, 2015; Gonciarz *et al.,* 2016; Boruta *et al.,* 2017).

Liquid cheese whey is a yellowish liquid, remaining after milk coagulation during cheese production. It is a valuable byproduct of cheese industry and has huge commercial applications due its high lactose content and milk nutrients (Lievore *et al.,* 2015). Although several efforts have been employed for the utilization of cheese-whey, almost half of its global production is left untreated and is discarded as an effluent (Wang *et al.,* 2017). Hence, in this study, liquid cheese whey has been used as a carbon source in addition to glucose for the growth of fungal species, *Fusarium nectrioides* and significant nutrients have been optimized using RSM and FLS.

Materials and Methods

Collection of weed plants and surface sterilization of leaves: Healthy and matured leaves of a pantropical weed, *Euphorbia hirta*, were collected from in and around Sathyamangalam region, Erode district, Tamil Nadu. Samples were collected in a sterile zip-lock bag and processed within 24 hrs at the Endophytic Fungal Research Laboratory, Bannari Amman Institute of Technology. Authentication of plant specimen was done at Botanical Survey of India, Tamil Nadu Agriculture University, Coimbatore. Fresh and healthy leaves of weed plants were washed thoroughly with sterile water to remove soil and debris from the leaf surface. The surface sterile leaves were cut into 50 small segments of 0.5 cm x 0.5 cm using sterile scalpel and placed equidistantly on the freshly prepared Potato Dextrose Agar plates (PDA) amended with ampicillin (50 $\mu g ml^{-1}$) and streptomycin sulphate (250 $\mu g\ ml^{-1}$). The segments inoculated in PDA plates were incubated in fungal rack at 25°C±1°C provided with 12 hr of light followed by 12 hr of dark cycles till the colony appeared (Dobranic *et al.,* 1995).

Isolation and identification of endophytic fungi : Individual colonies were isolated and subcultured subsequently to obtain pure culture. Endophytic fungi, *F. nectrioides* was isolated and confirmed through morphological and molecular analysis (White *et al.,* 1990) with forward primer (ITS-1F) TCCGTAGGTGA ACCTGCGG and reverse primer (ITS-4R) TCCTCCGCTT ATTGATATGC. The gene sequence of the same was submitted in gene bank to obtain accession number. Stock culture of *F. nectrioides* was maintained on PDA incubated at 28°C for 7 days and stored under refrigeration at 5- 10°C.

Growth media and culture conditions

Seed medium: Spore suspension of *F. nectrioides* (MH173849) was inoculated in the seed media containing Glucose, Liquid cheese whey, Yeast extract, Magnesium sulphate at pH 6. The flask was incubated at 28°C for 40 hrs in a shaking incubator at 180 rpm (Samiee *et al.,* 2003; Su *et al.,* 2003).

Production medium: Grown seed culture ($5x10^7$ spore ml^{-1}) was transferred into the production medium comprising Glucose, Yeast extract, Potassium Di hydrogen Phosphate, Peptonized milk, Magnesium sulphate, Histidine, Liquid cheese whey, at pH 6 in triplicates. All the flasks were incubated in shaker incubator at 28°C and 180 rpm for 15 days (Karthika *et al.,* 2013; Hajjaj *et al.,* 2001; Casas lopez *et al.,* 2003).

Extraction of lovastatin: After 12 days of fermentation, the culture broth was separated by filtration using sterilized filter cloth. The culture filtrate was adjusted from pH 6 to pH 2 and kept in a rotatory shaker with equal volume of ethyl acetate at 100 rpm for 2 hrs in room temperature. After the extraction process, the broth was separated from ethyl acetate using separating funnel and concentrated to 20 ml using rotatory evaporator. The presence of Lovastatin in the fermentation broth was confirmed by UV spectrometry at 238 nm. The extract obtained from the culture was analyzed using HPLC with C18 column as a stationary phase and acetonitrile and water (65:35 v/v) as mobile phase. An isocratic condition was maintained in the mobile phase.

The extract was filtered through 0.45 µm Millex-LH filter (Millipor corp., Bedford, MA 01730) and a clear extract (20 µl) was analyzed using high pressure liquid chromatography (Friedrich *et al.,* 1995; Kysilka and Kren, 1993). The flow rate was maintained as 0.8 ml min^{-1} throughout the run and detection was carried out at

238 nm. This was further compared with the retention time (5.124 min) of standard lovastatin.

Media optimization for batch fermentation of lovastatin: One factor at a time technique has short comings in locating the region of optimum response in the media optimization. To overcome this issue, Plackett Burman Experimental Design (PBD) is used to screen significant media nutrients (Plackett and Burman, 1946). This technique analyzes the impact of nine assigned factors such as glucose, yeast extract, peptonized milk, soya flour, potassium dihydrogen phosphate, magnesium sulphate, histidine, liquid cheese whey, corn steep liquor and two dummy variables such as

Table 1: High and low levels of factors used in Plackett–Burman Design

Code	Factors	Low level (-1)	High level (+1)
A	Glucose	15	100
B	Yeast extract	25	30
C	Peptonized milk	15	28
D	Soya flour	3	5.8
E	Potassium Di hydrogen Phosphate	0.3	2
F	Magnesium sulphate	0.05	0.8
G	Ferrous sulfate heptahydrate (DV1)	0.04	0.2
H	Histidine	4	7
I	Calcium chloride (DV2)	0.04	0.2
J	Liquid cheese whey	10	25
K	Corn steep liquor	3	5

Table 2 : Twelve runs-Plackett-Burman design matrix for nine variables with coded values

	Media Components (g l^{-1})										
Run	A	B	C	D	E	F	G	H	I	J	K
1.	100	30	28	5.8	2	0.05	0.04	4	0.04	25	3
2.	15	30	28	3	2	0.8	0.2	4	0.04	10	5
3.	100	25	28	5.8	0.3	0.8	0.2	7	0.04	10	3
4.	15	30	15	5.8	2	0.05	0.2	7	0.2	10	3
5.	15	25	28	3	2	0.8	0.04	7	0.2	25	3
6.	15	25	28	5.8	0.3	0.05	0.2	4	0.2	25	5
7.	100	25	15	3	2	0.05	0.2	7	0.04	25	5
8.	100	30	28	3	0.3	0.05	0.04	7	0.2	10	3
9.	100	30	15	3	0.3	0.8	0.2	4	0.2	25	3
10.	15	30	15	5.8	0.3	0.8	0.04	7	0.04	25	5
11.	100	25	15	5.8	2	0.8	0.04	4	0.2	10	5
12.	15	25	15	3	0.3	0.05	0.04	4	0.04	10	5

Table 3 : Fourteen set of FUZZY rule and their crispy outputs

1.	If(GLU is L) and (YE is HH) and (HIS HHH) and (LCW is LLLL) then (Productivity is LESS)
2.	If(GLU is L) and (YE is LL) and (HIS HHH) and (LCW is HHHH) then (Productivity is LESS)
3.	If(GLU is L) and (YE is LL) and (HIS LLL) and (LCW is HHHH) then (Productivity is LESS)
4.	If(GLU is L) and (YE is HH) and (HIS HHH) and (LCW is HHHH) then (Productivity is MEDIUM)
5.	If(GLU is L) and (YE is LL) and (HIS LLL) and (LCW is LLLL) then (Productivity is LESS)
6.	If(GLU is L) and (YE is HH) and (HIS LLL) and (LCW is LLLL) then (Productivity is LESS)
7.	If(GLU is H) and (YE is LL) and (HIS LLL) and (LCW is LLLL) then (Productivity is MEDIUM)
8.	If(GLU is H) and (YE is HH) and (HIS HHH) and (LCW is LLLL) then (Productivity is MEDIUM)
9.	If(GLU is H) and (YE is LL) and (HIS HHH) and (LCW is LLLL) then (Productivity is MEDIUM)
10.	If(GLU is H) and (YE is LL) and (HIS LLL) and (LCW is HHHH) then (Productivity is HIGH)
11.	If(GLU is M) and (YE is LL) and (HIS LLL) and (LCW is HHHH) then (Productivity is HIGH)
12.	If(GLU is H) and (YE is HH) and (HIS MMM) and (LCW is LLLL) then (Productivity is MEDIUM)
13.	If(GLU is H) and (YE is MM) and (HIS MMM) and (LCW is HHHH) then (Productivity is MEDIUM)
14.	If(GLU is M) and (YE is LL) and (HIS MMM) and (LCW is MMMM) then (Productivity is MEDIUM)

Table 4 : Twelve runs -Plackett-Burman design matrix for nine variables with coded values along with the yield

Run	Media components (gl^{-1})									Yield (g 100 m l^{-1})
	A	B	C	D	E	F	H	J	K	
1	100	30	28	5.8	2	0.05	4	25	3	1.7
2	15	30	28	3	2	0.8	4	10	5	0.8
3	100	25	28	5.8	0.3	0.8	7	10	3	0.9
4	15	30	15	5.8	2	0.05	7	10	3	0.2
5	15	25	28	3	2	0.8	7	25	3	0.7
6	15	25	28	5.8	0.3	0.05	4	25	5	0.7
7	100	25	15	3	2	0.05	7	25	5	1.1
8	100	30	28	3	0.3	0.05	7	10	3	0.8
9	100	30	15	3	0.3	0.8	4	25	3	1.4
10	15	30	15	5.8	0.3	0.8	7	25	5	0.7
11	100	25	15	5.8	2	0.8	4	10	5	0.8
12	15	25	15	3	0.3	0.05	4	10	5	0.2

Table 5 : ANOVA for Central Composite Design

Source	SS	df	MS	F Value	P - value Prob> F	
Model	0.872372	14	0.062312	15.24563	< 0.0001	Significant
A	0.136504	1	0.136504	33.39778	< 0.0001	
B	0.078204	1	0.078204	19.13382	0.0005	
H	0.009204	1	0.009204	2.251937	0.1542	
J	0.024704	1	0.024704	6.044244	0.1266	
AB	0.008556	1	0.008556	2.093414	0.0216	
AH	0.006806	1	0.006806	1.665251	0.2164	
AJ	0.163423	1	0.033306	8.148872	0.0311	
BA	0.567831	1	0.567831	0.013762	0.9082	
BH	0.112446	1	0.009506	2.325846	0.0142	
BJ	0.021756	1	0.021756	5.322992	0.1957	
A^2	0.226824	1	0.226824	55.49591	< 0.0001	
B^2	0.233103	1	0.233103	57.03205	< 0.0001	
H^2	0.168753	1	0.168753	41.28786	< 0.0001	
J^2	0.142931	1	0.142931	34.97027	< 0.0001	
Residual	0.061308	15	0.004087			
Lack of fit	0.050158	10	0.005016	2.249253	0.1920	Non-significant
Pure error	0.01115	5	0.00223			
Cor Total	0.93368	29				

Where, SS = Sum of Square; df = degrees of freedom; MS = Mean Square

ferrous sulfate heptahydrate and calcium chloride at a high level (coded + 1) and low level (coded -1) (Table 1) for lovastatin production. Significant parameters were determined using Design Expert Version 8.0 software with 12 experimental designs (Table 2) (Box and Hunter 1957; Sayyad *et al.,* 2007).

RSM AND Fuzzy inference system: The significant variables screened by PBD were optimized using RSM employing Central Composite Design (CCD). Their possible interaction and optimum operational conditions were analyzed by 25 runs of experiments. Significance of each component was determined by F-test and P-value. Probability level, $P < 0.05$ was considered to be statistically significant. The significant factors were also analyzed using FLS, where Fuzzy rule viewer constructed the system with 14 set of rules (Gupta *et al.,* 2009) (Table 3) for determining optimum concentration of input variables (A, B, H and J) and output variables (Lovastatin production) (Honda and Kobayashi, 2000). The mamdani fuzzy inference system adjusts the membership functions to control the relation between input and output variables using MATLAB Version 7.3 (Fig. 1). It involves five subsequent steps from fuzzification to defuzzification where the crisp values of input variables (gl^{-1}) such

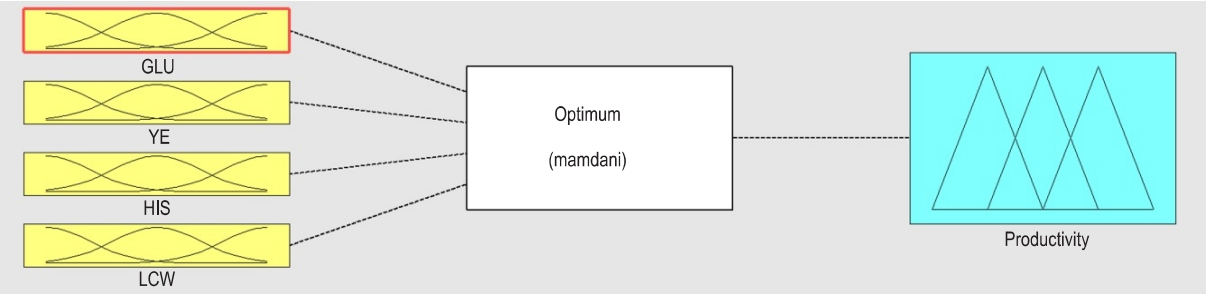


Fig. 1: Fuzzy inference system in MATLAB Software version 7.3 (Where GLU - Glucose, YE - Yeast Extract, HIS - Histidine and LCW - Liquid Cheese Whey).

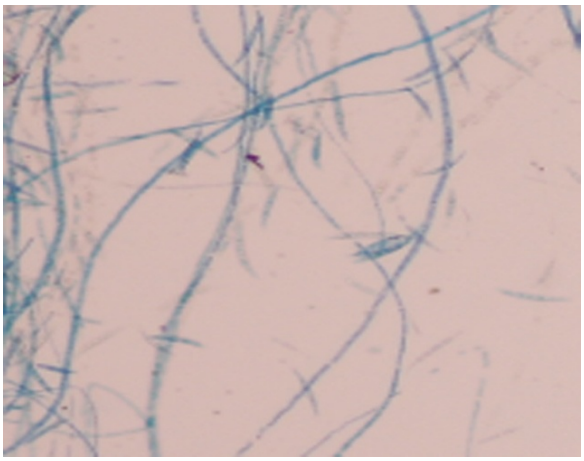
as glucose (0-100), yeast extract (0-30), histidine (0-7), LCW (0-25) were fuzzified into degree of membership with respect to fuzzy sets and finally extract a precise quantity out of the range of fuzzy set to output variable (Monton *et al.,* 2013). Accuracy in the prediction of lovastatin yield via RSM and Fuzzy logic system was compared.

Results and Discussion

F. nectrioides was isolated and its sequence was submitted in National Center for Biotechnology Information (NCBI) with accession number MH173849 (Fig. 2). The presence of lovastatin in the fermentation broth was confirmed using HPLC with silica gel (60-120 mesh size) as a stationary phase in the column (300mm x18mm). Retention time of fermentation broth was 5.047 min, which was almost close to the retention time of standard lovastatin (5.124 min) (Fig 3a, b). Increase in lovastatin production was then analyzed further using RSM and FLS. Experiments conducted through PBD screened the media components, A to I and indicated that glucose (A), yeast extract (B), histidine (H) and liquid cheese whey (J) were most significant variables. The positive and negative effects of the media components are represented graphically in Pareto chart (Fig. 4) and their experimental yield is listed in Table 4.

The effect of these four significant variables A, B, H and J on the lovastatin production was studied by RSM. To optimize the actual concentrations, CCD preceded further with 25 runs of experiments. All the experiments were done in 250ml Erlenmeyer flask containing 30 ml of media. The results were further analyzed statistically and interpreted with analysis of variance (ANOVA) in Table 5. Arulmathi and Elangovan (2016) reported that F- test with

Endophytic fungi propagules emerging from *E. hirta*



F. nectrioides

Fig. 2 : Propagation and microscopic images of *F. nectrioides* (MH173849) from *E. hirta.*

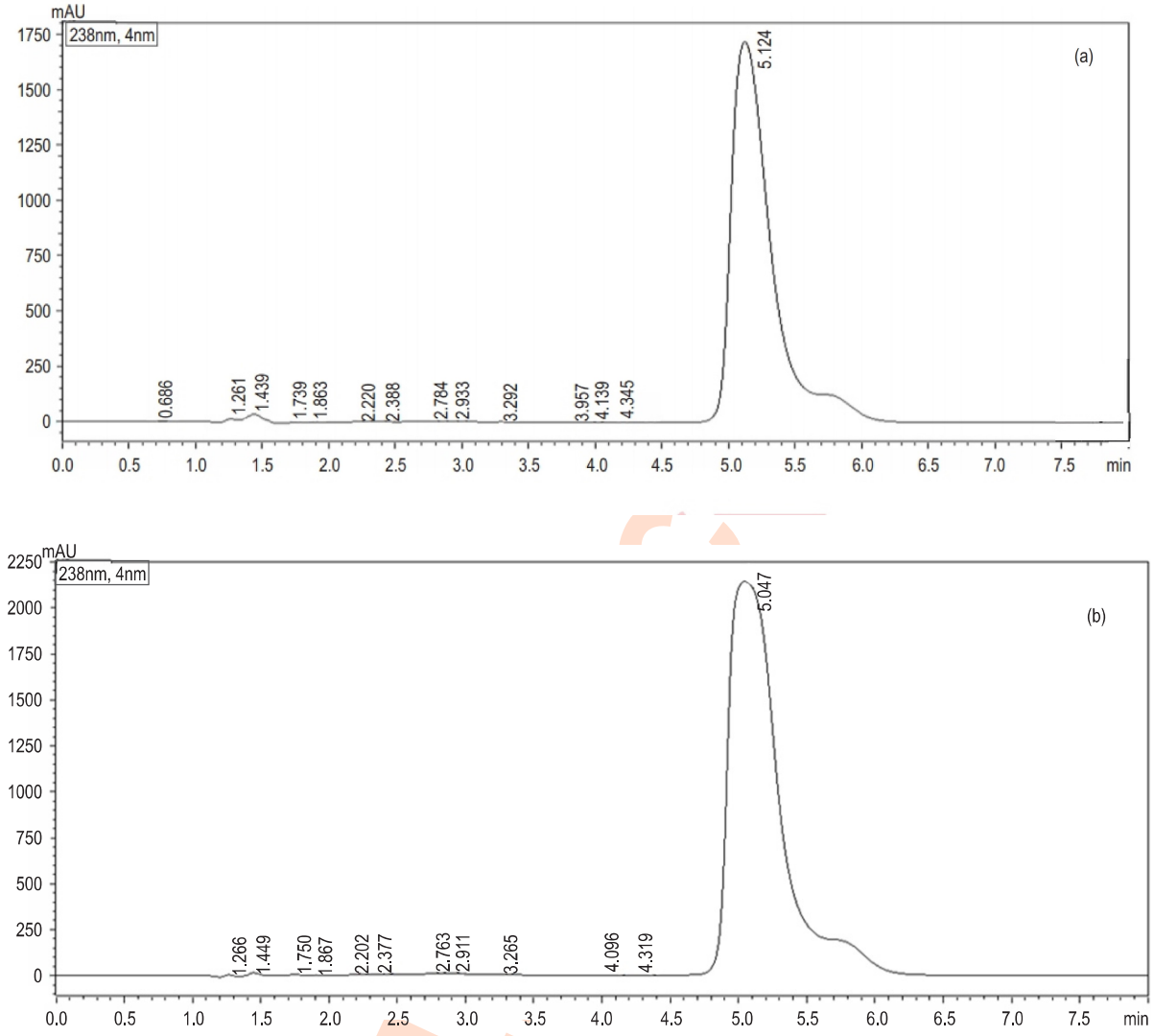


Fig. 3 : HPLC chromatogram showing peaks of (a) standard lovastatin and (b) lovastatin produced by *F. nectrioides.*

a very low probability revealed high significance for the regression model. The model F-value of 15.24 implies that the model was significant (Table 5) and there was only 0.01% chance that a "Model F-Value" could occur due to noise. Values of "Prob > F" less than 0.0500 indicate that the model terms were significant. Luthra *et al.* (2015) also reported similar results. In this case glucose, yeast extract, histidine and liquid cheese whey were significant model terms where values greater than 0.1000 indicate that the model terms were not significant as reported by Pansuriya and Singhal (2010). The "Lack of fit F-value" of 2.24 implies that the curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space was not significant relative to noise. The R-square value and "AdjR-Square" value of this model was 0.9905 and 0.9479, which were higher than the reported values of Mouafi *et al.* (2016). Adequate precision measures the signal to noise ratio. This model can be used to navigate the design space. The optimum level of variables and interaction effects were found by 3D contour plots (Fig. 5) as mentioned by Luthra *et al.* (2015).

Graphical representation of response surface and contour plots determine the interaction effects of four significant factors on the response. Each of the actual response was compared with the predicted value. The predicted and the

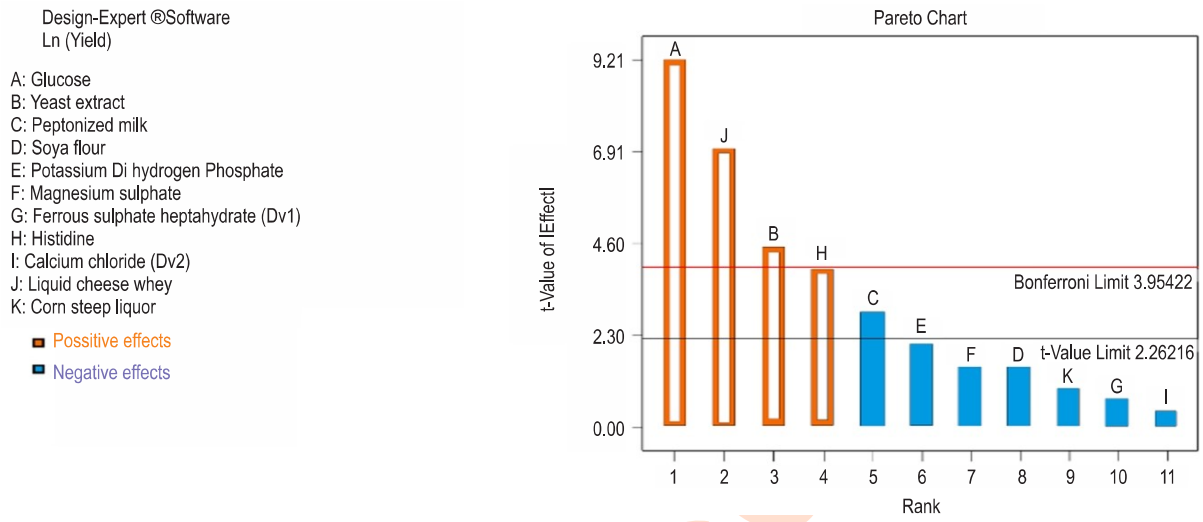


Fig. 4 : Pareto chart for positive and negative effects of variables in PBD.

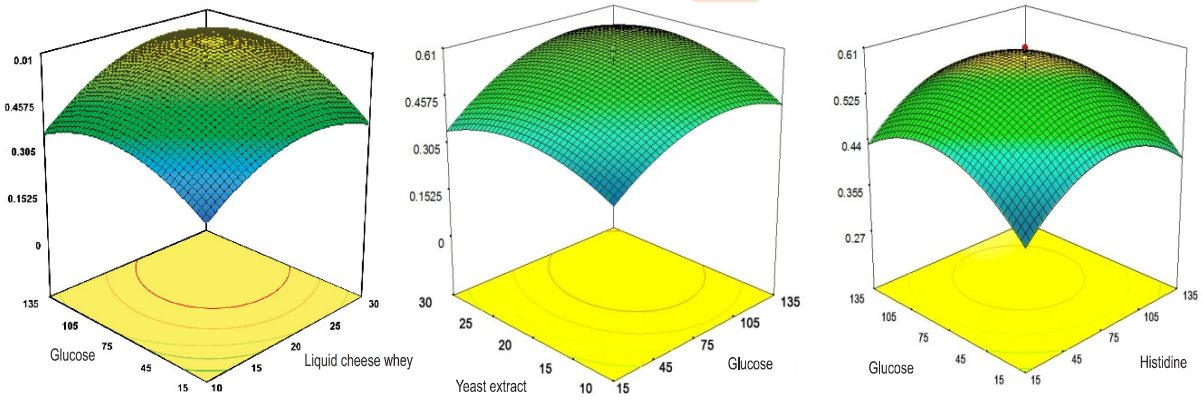


Fig. 5 : 3D surface and contour plots for optimum lovastatin production.

observed results of Lovastatin yield were 1.2 g 100 ml^{-1} and 1.75 g 100 ml^{-1}, whereas Karthika *et al.* (2013) reported the predicted and observed results of lovastatin yield such 0.034 g 100 ml^{-1} and 0.036 g100 ml^{-1}, respectively. Lovastatin obtained from *F. nectrioides* (MH173849) isolate had a good model fit due to high value of R^2. Thus, optimization of significant media components increased the lovastatin production by *F. nectrioides* (MH173849).

The polynomial equation derived from multiple regression analysis is as follows:

Lovastatin Yield (Y) = 0.575+0.075417* Glucose+ 0.057083* Yeastextract+ 0.019583* Histidine+ 0.032083* Liquid cheese whey+ 0.023125* Glucose* Yeast extract+ 0.045625* Glucose* Histidine+ 0.020625* Glucose* Liquid cheese whey+ 0.024375* Yeast extract* Histidine+0.036875* Yeast extract* Liquid cheese whey+ 0.001875* Histidine* Liquid cheese whey- 0.09094 Glucose* Glucose - 0.09219 Yeast extract* Yeast extract- 0.07844 Histidine* Histidine -0.07219 Liquid cheese whey* Liquid cheese whey. Ozlem *et al.* (2015) reported that Fuzzy rule viewer with different set of rules provided crispy output for the numerical range of input variables as that of our output (Fig. 6). Surface plot for the effect of significant factors on lovastatin production was constructed (Fig. 7, b) and is represented in Table 6. MATLAB ingrained fuzzy rule based system predicted the best combination and interaction of input variables and their predicted yield was compared with the theoretical yield. The surface plot of FLS between liquid cheese whey and glucose produced maximum lovastatin of 1.8 g100 ml^{-1} compared to RSM (1.2 g100 ml^{-1}). Thus, the predicted value of lovastatin productivity by means of liquid cheese whey and glucose was found maximum and closer to the theoretical value

Fig. 6 : Fuzzy rule viewer for lovastatin production.

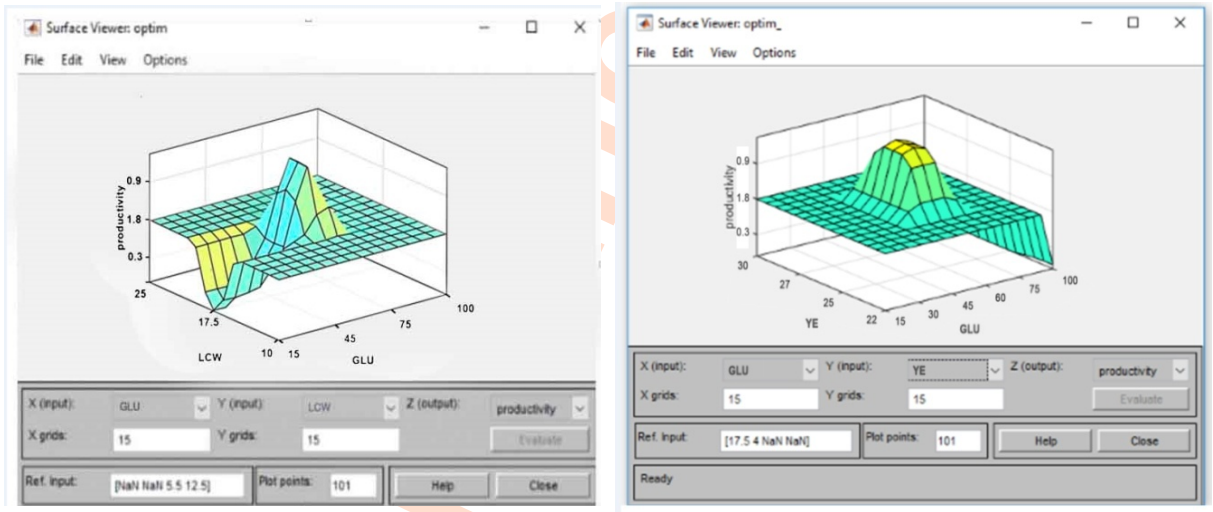


Fig. 7 : Surface plot demonstration on lovastatin yield due to the effect of (a) Liquid Cheese Whey and Glucose; (b) Yeast extract and Glucose.

(1.75 g 100 ml^{-1}). Lovastatin produced from *F. nectriodes* was higher than those reported from *Aspergillus terreus* and *Rhizopus oryzae* (Rajkumar *et al.,* 2018). Hence, from this investigation it was concluded that a novel strategy was adopted to enhance lovastatin production by new isolate, *F. nectriodes* utilizing industrial waste such as liquid cheese whey, as a cheap carbon source, with different combination of glucose, histidine and yeast extract. This is the first report to use liquid cheese whey and *F. nectriodes* for enhanced lovastatin production. RSM and Fuzzy models were utilized and, thus, the more accurate prediction was achieved by FUZZY models compared to RSM models.

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