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Optimization of Growth Parameters for Elevated Production of Mycoprotein - *Fusarium venenatum* using Response Surface Methodology

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***Fusarium venenatum* – Mycoprotein are rich in protein, sugars and essential fatty acid and supplemented as dietary food for human and animal consumption in the various parts of the world. Biomass production is highly prejudiced by supplying nutritional factors in the media. Response surface methodology (RSM) is an important tool to optimize the various essential factors for the production of biomass and yield the desired concentration of metabolite that satisfy the requirement in bioprocess industries. In the present study, Response surface methodology via Central composite Design (CCD) was used to optimize the medium components to improve biomass yield. The medium components involved are liquefied jaggery, date extract, KH_2PO_4 and K_2HPO_4. Thus maximum biomass was observed in 7^{th} trial, the medium components were liquefied jaggery 2.5 ml, date extract 8ml, KH_2PO_4 500mg, K_2HPO_4 100mg and 5% of inoculum with 72 hrs.**

Key words: *Fusarium venenatum* , Mycoprotein, Response surface methodology (RSM), biomass.

A growing range of food constituents used in the manufacture of meat substitute products and single cell proteins that reflecyts technological, novel developments and consumer demand for high quality alternative food commodities. The key technological developments to date are the nutritional value, health benefits and potential supply to public health of such foods, and relevant safety issues. Future developments in the sector will lead to improved eating quality and further increases in variety and choice. (Michele J. Sadler, 2004) *Fusarium venenatum* is a micro-fungus belongs to Nectriaceae family used commercially production of mycoprotein in the trade name of 'Quorn' in United Kingdom. Studies on economically important fungi at the genomic scale are an innovative approch to unravel the mystery of mycotoxin biosynthesis and also to help better understand the biology, evolution, biochemical function and genetic regulation of the genes in fungal systems. It will provide vital clues for identifying antifungal gene(s), eliminating mycotoxin contamination of pre-harvest crops, improving high efficiency production of industrial enzymes and accelerating drug development in the near future. (Yu *et al*, 2004). *Fusarium venenatum* was chosen at the year of 1960 and after intensive testing the Mycoprotein for 12 years it was approved for sale as consumable by the Ministry of Agriculture (Wiebe 2002). The product is now available in Six European Countries only and the filaments of the fungi were used as mycoprotein which is rich in protein content (44%) and less cholesterol. Consumption of mycoprotein significantly reduces total and Low Density Lipoprotein (LDL) cholesterol levels and raises High Density Lipoprotein (HDL) levels in the serum (Turnbull *et al*., 1992). It is also less energy dense

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than equivalent meat products and does not have animal fats and cholesterol. It is used as flavoring, and nutritional agent. Milk, Meat, and other food stuffs, animal feeds and anti-tumor agent.

Production of adequate quantities of a good quality inoculum is an essential component of the mycoprotein production by the following methods (1) Relatively small quantities of the inoculum for laboratory experimentation and field-testing during the development of mycoprotein and (2) Development of a basic production system for large-scale production by following the labor intensive and economically viable methods for relatively small size markets. Many protein rich sources are available in the natural resources like animals and plants. But which sources are needed to cultivate in long period. So the alternative sources like microbial proteins are selected to the living beings. The microbial proteins are derived from various sources like bacteria, fungi and algae. Animal and plant protein sources would not be insufficient to human and animals requirements. Among the sources fungal protein has rich nutrients like protein, carbohydrates and fiber sources.

Response surface methodology (RSM) is an innovative tool, involving Central composite design giving number of input (independent) factors and their corresponding relationship between one or more measured dependent responses. RSM is widely used for multivariable optimization studies in several biotechnological processes such as optimization of media, process conditions, catalyzed reaction conditions, oxidation, production, fermentation, etc., (Rajasimman and Subathra, 2009). The objectives of this work are to find out the optimum production medium by Response Surface Methodology for the production of Mycoprotein by *Fusarium venenatum*

MATERIALS AND METHODS

Fungal strain

Fusarium venenatum was purchased from Fungal Biodiversity Centre; Netherland as lyophilized form and the fungi was activated in oats meal medium. An activated fungus was maintained on the oats meal agar slant as monosporic culture at 4°C.

Inoculum preparation

Fungal spore was collected from 15 days old culture by scrapping off with a sterilized glass rod from SDA plate. A homogenous spore suspension was prepared in sterile distilled water by adding a few drops of the wetting agent Tween80 (0.01%). The spore concentration of the suspension was determined using an improved Neubauer haemocytometer (Germany). One hundred milliliter (100 ml) of the media with the above mentioned nutrient composition was prepared in 250 ml conical flask. One milliliter (1ml) of the spore suspension (10^6 spores/ml) was inoculated into the flask. *Fusarium venenatum* inoculum was prepared in Vogel's minerals medium which consisted of 10 g glucose, 2.6 g $Na_3C_6H_5O_7\cdot2H_2O$, 2.52 g KNO_3, 2.88 g $(NH_4)H_2PO_4$, 1.6 g KH_2PO_4,0.2 g $MgSO_4\cdot7H_2O$, 0.1 g, $CaCl_2\cdot2H_2O$, 2.5 mL of biotin solution and 5 mL of trace elements per liter. The trace elements solution consisted of 0.1 g Citric acid, 0.1 g $ZnSO_4\cdot7H_2O$, 0.02 g $FeSO_4\cdot(NH_4)_2SO_4\cdot6H_2O$, 5 mg $CuSO_4\cdot5H_2O$, 1 mg $MnSO_4\cdot H_2O$,1 mg H_3BO_3, 1 mg $Na_2MoO_4\cdot2H_2O$ per 100 mL. The pH of the medium was adjusted to 5.8.

Preparation of media

50g and 100g of jaggery and dates were dissolved separately in 1000ml of distilled water, filtered and the collected filtrate was mixed with trial sources of K_2HPO_4, KH_2PO_4, $MgSO_4$ (Table 2), sterilized by cold sterilization. After sterilization, 5% of inoculum was added and incubated at 72 hours under shaking condition

Experimental Design and Procedure

Response surface methodology is used in this study. The experimental variables at different levels used for the production of Mycoprotein by *Fusarium venenatum* using CCD is given in Table 1. A total of 50 runs are used to optimize the medium. The average from two replicated values of each run is taken as dependent variables or response or yield. The experimental design is carried out using Design Expert 7.0.0 (Stat Ease, USA). Central composite design (CCD) is used to identify the optimum operating condition in order to obtain maximum Mycoprotein production (R1) as response. The collection of experiments delivers an effective means for optimization through these process variables. The design allow to estimate of all main and interaction results. Similarly, the purpose of the center points is to estimate the pure error and curvature. A second-degree quadratic polynomial can be used to represent the function in the range of interest (Rajasimman and Subathra, 2009).

Biomass (R1) = + 0.32 - 0.029A - 9.847E - 003B + 0.046C - 0.032D - 3.407E - 003E + 1.875E - 003AB - 0.026AC +0.018 AD - 0.013AE + 0.031BC - 0.040BD -0.041BE -0.047CD + 0.051CE + 0.017DE.

Where A, B, C, D, E are the variable and the constant in numerical.

Biomass estimation

Biomass was estimated from the broth by wet weight method. Prior to biomass estimation, the broth kept at 64 -65°C for 20-30 min to remove RNA content (Wiebe, 2002). After the heat treatment, media was filtered through Whatman No.1 filter paper, the filtrate was discarded. Biomass transferred to pre dried Whatman No.1 filter paper, kept in sterile Petri plate, dried using an oven at 60°C to a constant weight.

RESULTS AND DISCUSSION

O'Donnell.K *et al.*, 1998 analyzed cellular and genetic relationship, structural, and mycotoxin data were used to investigate the relationships and identity of the Quorn mycoprotein fungus within *Fusarium* and to examine commercial Quorn food products for trichothecene mycotoxins which are powerful inhibitors of protein synthesis. No trichothecene mycotoxins were detected in commercial Quorn products marketed for human consumption in England. It was evident that there was no trichothecene present in *F. venenatum*. Brownsell *et al.,* 2001, Williams *et al.,* 2001 used plastein to remove RNA because the concentration above 8% is not acceptable in consumable products but in this extraction procedure, heating 64^{o}C - 65^{o}C for 20 to 30 minutes will remove RNA.

Murray Moo-Young *et al.*, 1993 reported that from *N.sitophila* at 37^{o}C, 6.5 pH, agitation of 250 rpm and 38hour there was maximum mycoprotetin production. Banerjee *et al.*, 1995 reported that nature of substrate – corn stover's particle size and the addition of NaOH influence the positive production of mycoprotein from *N.sitophila*. The optimum condition was obtained by maximum production of mycoprotein, from the design which resulted from the trails and given in Table-1. Multiple regressions were used to analyze the data and thus polynomial equation was derived from regression analysis. Regression analysis of the experimental data showed that date extract, jaggery and KH_2PO_4 had positive linear effect on mycoprotein production ($P< 0.05$). Among the five variables date extract has highest impact on biomass production as given by highest linear coefficient, followed by jaggery water source and K_2HPO_4. During this process the lower value were estimated for Date extract which was considered significant hence other constituents were elimination in the medium. All rest terms had significant effects on biomass production by low P values (<0.05). In this study the P value is <0.0001 (Table3.11) and the model is significant. The R^2 value is 97.5%. . Similarly (Hosseini *et al.,* 2009 and 2011) reported that date extract has highly significant component for mycoprotein production. (Prakash *et al.*, 2013) reported that growth was observed in Vogels minerals medium followed by Sabouraud Maltose yeast extract broth. When continuous aeration is provided more production of Mycoprotein of food-grade carbohydrate substrates by *F.venenatum* was reported (Miller and Dwyer, 2001)

CONCLUSION

A detailed study was on the optimization of media requirement for extensive production of *Fusarium venenatum* – Mycoprotein in this paper. Mycoprotein are rich in nutritive components and

Table 1. Experimental variables at different levels used for the production of Mycoprotein by *Fusarium venenatum* using RSM-CCD

Variables	Code	Factors Level		
		-1	0	1
Liquid Jagerry (ml/L)	A	10	20	30
Date extract (ml/L)	B	40	60	80
K_2HPO_4 (ml/L)	C	500	750	1000
KH_2PO_4 (ml/L)	D	500	1000	1500
$MgSO_4$ (ml/L)	E	100	200	300

Table 2. Experimental conditions and observed response values of 50 central composite design

Std	A:Jagerry mg/L Factor 1	B:Date extract mg/L Factor 2	C:K_2HPO_4 mg/L Factor 3	D:KH_2PO_4 mg/L Factor 4	E:$MgSO_4$ mg/L Factor 5	Biomass g/L Response 1
1	10	40	500	500	100	2.9
2	30	40	500	500	100	2.8
3	10	80	500	500	100	3.9
4	30	80	500	500	100	3.6
5	10	40	1000	500	100	4
6	30	40	1000	500	100	2.1
7	10	80	1000	500	100	5.7
8	30	80	1000	500	100	4.7
9	10	40	500	1500	100	3.4
10	30	40	500	1500	100	4
11	10	80	500	1500	100	2.6
12	30	80	500	1500	100	3.1
13	10	40	1000	1500	100	2.3
14	30	40	1000	1500	100	1.8
15	10	80	1000	1500	100	2.5
16	30	80	1000	1500	100	2.5
17	10	40	500	500	300	2.6
18	30	40	500	500	300	1.9
19	10	80	500	500	300	1.7
20	30	80	500	500	300	1.2
21	10	40	1000	500	300	5.4
22	30	40	1000	500	300	3.8
23	10	80	1000	500	300	5.3
24	30	80	1000	500	300	4.1
25	10	40	500	1500	300	3.8
26	30	40	500	1500	300	3.7
27	10	80	500	1500	300	1.4
28	30	80	500	1500	300	1.4
29	10	40	1000	1500	300	4.5
30	30	40	1000	1500	300	3.7
31	10	80	1000	1500	300	3.8
32	30	80	1000	1500	300	2.2
33	10	60	750	1000	200	3.8
34	30	60	750	1000	200	2.5
35	20	40	750	1000	200	3.5
36	20	80	750	1000	200	2.8
37	20	60	500	1000	200	2.1
38	20	60	1000	1000	200	4.2
39	20	60	750	500	200	4.2
40	20	60	750	1500	200	2.3
41	20	60	750	1000	100	3.3
42	20	60	750	1000	100	3.1
43	20	60	750	1000	200	3.7
44	20	60	750	1000	200	3.7
45	20	60	750	1000	200	3.2
46	20	60	750	1000	200	3.2
47	20	60	750	1000	200	3.2
48	20	60	750	1000	200	3.2
49	20	60	750	1000	200	3.2
50	20	60	750	1000	200	3.2

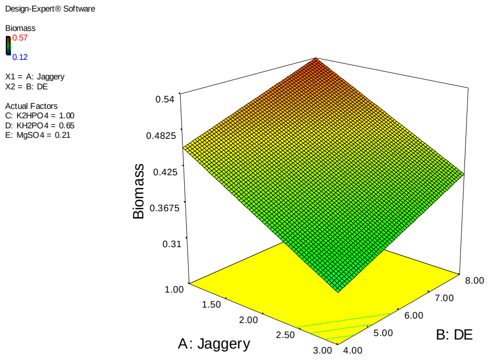
Table 3. Analysis of Variance (ANOVA) for Response Surface Quadratic model

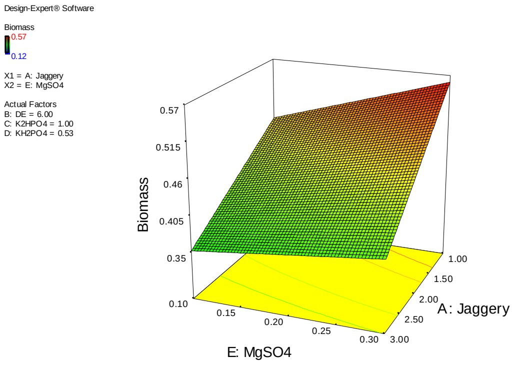
Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F	
Model	0.51	15	0.034	89.10	< 0.0001	significant
A-Jagerry	0.04	1	0.037	96.88	< 0.0001	
B-Date extract	0.00	1	0.004	10.94	0.0022	
C- K_2HPO_4	0.09	1	0.090	235.65	< 0.0001	
D- KH_2PO_4	0.04	1	0.045	116.51	< 0.0001	
E- $MgSO_4$	0.00	1	0.001	1.31	0.2605	
AB	0.00	1	0.000	0.29	0.5918	
AC	0.02	1	0.022	57.44	< 0.0001	
AD	0.01	1	0.011	27.38	< 0.0001	
AE	0.01	1	0.006	14.36	0.0006	
BC	0.03	1	0.030	78.18	< 0.0001	
BD	0.05	1	0.051	133.37	< 0.0001	
BE	0.05	1	0.054	141.84	< 0.0001	
CD	0.07	1	0.070	183.16	< 0.0001	
CE	0.08	1	0.082	213.63	< 0.0001	
DE	0.01	1	0.010	25.53	< 0.0001	
Residual	0.01	34	0.000			
Lack of Fit	0.01	27	0.000	0.64	0.8084	Not significant
Pure Error	0.00	7	0.001			
Cor Total	0.53	49				

The "Lack of Fit F-value" of 0.64 implies the Lack of Fit is not significant relative to the pure error. There is a 80.84% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good - we want the model to fit.

Std. Dev.	0.019593	R-Squared	0.97519097
Mean	0.3224	Adj R-Squared	0.96424581
C.V. %	6.077284	Pred R-Squared	0.943045182
PRESS	0.029965	Adeq Precision	0.454558069

The "Pred R-Squared" of 0.9430 is in reasonable agreement with the "Adj R-Squared" of 0.9642.





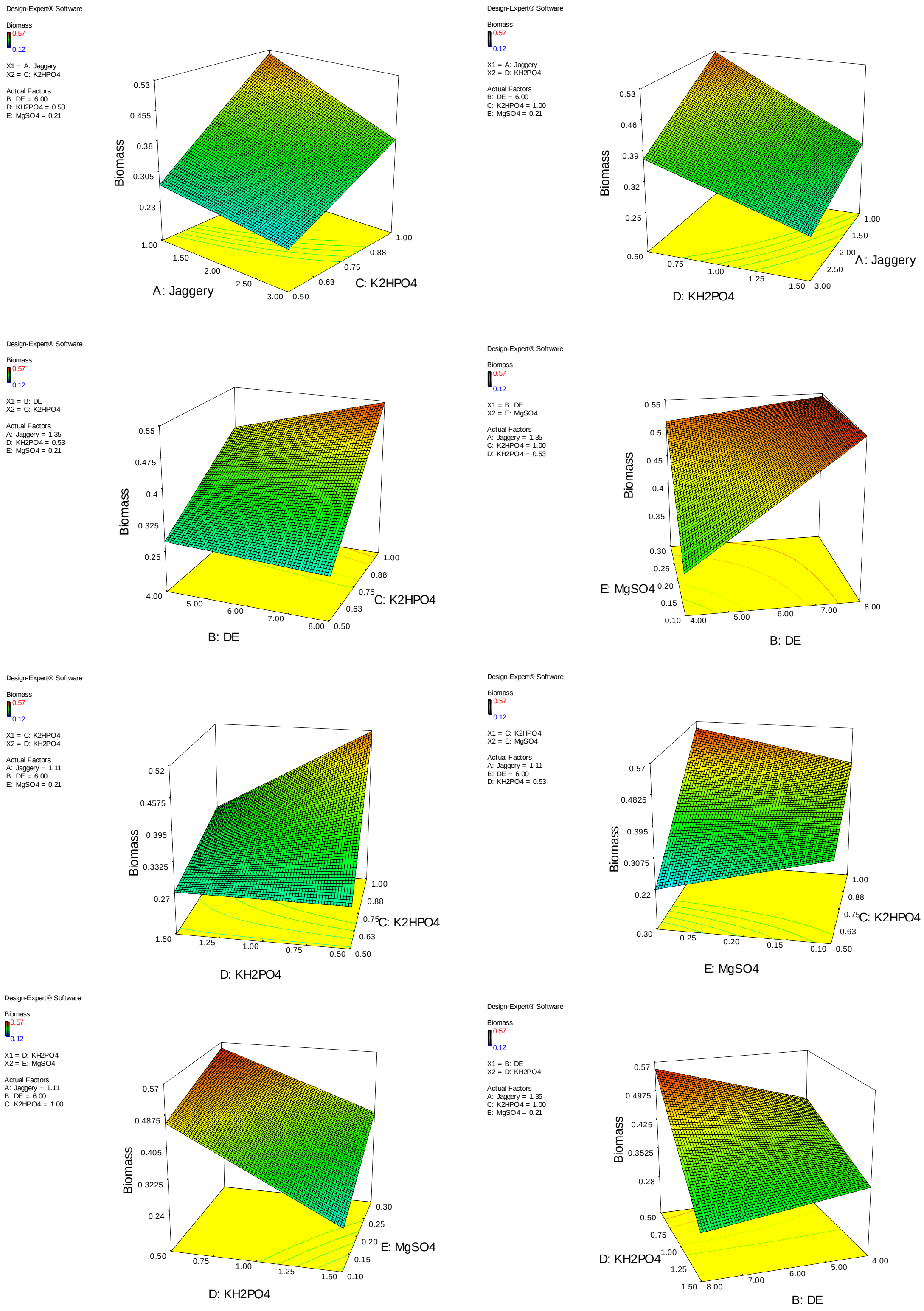


Fig. 1. 3D plot of the combined effect

act as supplementary dietary food for human and animal consumption. Biomass production was enhanced by design of experiment done in Response surface methodology (RSM) - Central composite Design (CCD) to improve biomass yield. The medium components involved are liquefied jaggery, date extract, KH_2PO_4 and K_2HPO_4. Thus maximum biomass was observed in 7th trial, the medium components were liquefied jaggery 2.5 ml, date extract 8ml, KH_2PO_4 500mg, K_2HPO_4 100mg and 5% of inoculum with 72 hrs. from this study the effect of date extract and jaggery were analyzed and its influence on other components was also studied

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