Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Suppl 1, 2014

Research Article

STATISTICAL DESIGNING OF ENRICHED PECTIN EXTRACT MEDIUM FOR THE ENHANCED PRODUCTION OF PECTINASE BY ASPERGILLUS NIGER

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Received: 18 Dec 2013, Revised and Accepted: 10 Feb 2014

ABSTRACT

Objective: The present research focused on the production and process optimization of pectinase using orange peel as substrate by solid state fermentation.

Methods: Orange peel, a major by product from fruit processing industry has been used to formulate a prescribed liquid medium named as Enriched Pectin Extract Medium (EPEM) for enhanced production of exo-pectinase and endo-pectinases. EPEM was designed by adopting two step procedures. Firstly four factors peptone, KH_2PO_4, $MgSO_4$ and $CuSO_4$ out of eleven variables greatly influencing the pectinase production were identified by Plackett-Burman design followed by response surface modeling to formulate an EPEM for enhanced pectinase production.

Results: Exo-pectinase of 575.58 IU/ml and endo-pectinase of 52.8 IU/ml were obtained from the models at optimum concentration of medium components. The results from the models were validated optimal concentrations of variables resulting in enzyme activities of exo-pectinase (573 IU/ml) and endo-pectinase (52 IU/ml) thus corroborating the predicted models.

Conclusion: The cost of raw materials is a vital factor that determines the overall economy of any fermentation process. Use of food industrial wastes as substrates has become an attractive alternative renewable source for enzyme production, consequently reducing the raw materials cost involved in the fermentation process.

Keywords: Pectinase, Orange peel, Plackett- Burman design, Response surface method, Central composite design.

INTRODUCTION

Orange peels account for 40-45% of the total bulk fruit mass. They constitute a major part of by-products along with waste pulp from fruit processing industries. Orange peels if left untreated may cause potential environmental concerns, particularly water pollution[1]. These orange peels are rich sources of pectin (about 30%) [2], which can be extracted using aqueous solvents. Conventionally, Soxhlet extraction apparatus has been used to extract the soluble portion of the citrus-peel using aqueous solvents [3]. This aqueous extract which is rich in natural pectin can act as an effective medium in the production of pectinases through submerged fermentation [4]. The residual pectin-extracted orange peel pellets, which are rich sources of cellulosic compounds, can be used as solid substrates for hydrolytic enzyme production such as cellulose and xylanase. If strategically used nothing goes as waste from orange peel. Recently industries widely extracting the enzymes from natural sources such as agricultural wastes, plant litter, manure of farmyard, citrus peels and cow dung[5].

Pectinases are group of enzymes which catalyze the breakdown of pectin-containing substrates. Pectinases consist of polygalacturonase (EC 3.2.1.15), pectin esterase (EC 3.1.1.11), pectin lyase (EC 4.2.2.10) and pectate lyase (EC 4.2.2.2), categorized based on their mode of action [6, 7, 8]. They are extensively used in biotechnological applications, namely, in food industry (i.e. fruit juice extraction, coffee and tea fermentation, oil extraction, textiles, improvement of chromaticity and stability of red wines) and paper and pulp industries and waste-water treatment [9].

Various agro-industrial wastes such as wheat bran [10], sugarcane bagasse [11], coffee pulp [12], lemon peel[13], orange peel[4], apple pomace[14] and deseeded Sunflower heads [15]have been explored and used as solid substrates for microbial production of pectinases using solid state fermentation. Though solid state fermentation has been proved to be cost-effective in the production of enzymes, it has its own limitations such as process scalability, reproducibility and poor control over process parameters like pH and temperature, which makes it less attractive over submerged fermentation for industrial production of enzymes. The potent producer of pectinase were produced from *Penicillium citrinum* isolated from soil, spoiled fruits, vegetables and rotten tomatoes[16].Pectinase production from orange peel extract and dry orange peel pellets as substrates was compared in Submerged and solid-state fermentation respectively using *Aspergillus niger*[4]. The objective of the current study is to screen different substrates to formulate an enriched media which can augment the nutritious capacity of existing Pectin Extract Medium (PEM) for enhanced production of endo-pectinases and exo- pectinases using the fungus *Aspergillus niger*.

The study evaluates the most suitable dilution of the peel extract for optimal pectinase production, followed by statistical screening of the most influencing media components by Plackett- Burman Design (PBD) and optimization of Enriched Pectin Extract Medium (EPEM) by Central Composite Design (CCD).

MATERIALS AND METHODS

Microorganism and culture maintenance

Fungal culture *Aspergillus niger*(MTCC 3323) was obtained from Microbial Type Culture Collection, Chandigarh, India. It was periodically sub cultured on Potato-Dextrose Agar (PDA)medium and maintained at 4 °C. Fungus was grown on PDA plates for 5-6 days to obtain sufficient quantity of matured spores. These spores were scrapped out, suspended in 0.1% w/v Tween-80 and then transferred to sterile test tubes. These suspended spores were used as seed culture spores for inoculum preparation.

Preparation of citrus peel

Orange peels collected from local fruit-juice shops were sorted and picked manually for their fine texture and rigidity. The peels were minced to pieces and dried in hot air oven at 55°C until constant weight was attained. The dried peels were diminuted using ball mill and clarified in a sieve shaker. The peel fractions from the mesh size 12 were used for pectin extraction using Soxhlet extraction apparatus. Extraction was carried out as described by [2]. Briefly, about 100 gram of dried orange peel pellets was placed in the thimble and 1.6 L of deionized water was taken in round-bottom extract collector of the Soxhlet extraction apparatus. The apparatus was placed over the heating mantle, and extraction process was carried out until the extract returning to the flask changed colorless.

Preparation of seed inoculums

One ml of spore suspension was inoculated in 100 ml of 50 % v/v sterilized orange peel extract containing 0.5% glucose and incubated in an orbital shaker maintained at30°C for 12 h.

Pectin Extract Medium (PEM) dilution and formulation of Enriched Pectin Extract Medium (EPEM)

The most suitable dilution of the PEM for achieving maximum pectinase activity was evaluated by diluting PEM with de-ionized water ranging 50 to 90 % by volume. The dilution yielding maximum pectinase activity was chosen for the statistical screening experiments. Enriched nutrient medium was formulated according to the Plackett-Burman design table for which the low (-1) and high (+1) levels of the medium variables are tabulated in Table 1.

Pectinase assay

The culture sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for pectinase assay. Exo-polygalacturonase/ Exo-pectinase activity was determined by incubating the culture supernatant, for 1 h at 50°C, with 0.5% (w/v) polygalacturonic acid in 50 mM citrate buffer pH 4.8. The enzyme activity was determined from a calibration curve by using galacturonic acid as standard. Reducing sugars were determined by the dinitrosalicylic acid method[24]. One enzyme unit is defined as the amount of enzyme required for formation of 1 µmol of reducing sugars per minute at 50°C. Endo-Polygalacturonase/ Endo-pectinase activity was assayed by determining the percentage decrease in apparent viscosity of a mixture of 12 ml of enzyme and 12 ml of 3% pectin solution in 0.2 M citrate-phosphate buffer, pH 6.5 at 40 °C, using a Fenske-Ostwald viscosimeter as described by Fellows & Worgan. The results were subtracted from experimental controls when denatured enzyme was used, according to the following formula:

$$\mathbf{Apparentviscosityreduction}(\%) = \frac{(V_C - V_R)}{(V_C - V_S)} * 100 \quad (1)$$

Where: V_C = flow time of control, V_R= flow time of sample; V_S = flow time of water.

One unit of endo-pectinase activity was defined as the amount of enzyme which reduced the initial viscosity of pectin solution by 50% in 10 min.

Statistical screening of important nutrient components by Plackett-Burman method

To screen the most vital variables in a medium, Plackett-Burman design was used. This technique allows the assessment of "N-1" variables by "N" experiments. "N" should be in multiples of 4, e.g. 8, 12, 16, 20, 24 etc. The design presumes that there are no interactions between different medium constituents, X_i, in the range of variables under consideration and a linear approach is taken sufficient for screening.

$$Y = \beta_0 + \sum \beta_i X_i \ (i = 1, \ldots K) \quad (2)$$

Where *Y* is the estimated target function or response and β_i are the regression coefficients.

In designing the matrix each horizontal row represents a trial and each vertical column represents the +1 (High) and -1 (Low) values of one variable in all the trials. The statistical software package design expert® Ver. 7.0.0 was used to generate Plackett-Burman design matrix and in the evaluation of statistical parameters. The media thus prepared were sterilized and inoculated with 2% (v/v) of 12 hrs seed culture suspension. They were maintained in the shaker at 180 rpm and 30°C. After 34 hrs of fermentation the culture broth was estimated for the Pectinase activity as described later. All the experiments were performed in triplicates and the response (pectinase activity IU/ml) was the mean value of triplicates.

The effect of an independent variable on the response is the difference between the average response for the N/2 experiments at the high (+1) level and the average value of N/2 experiments at the low level (-1).

$$\mathbf{Effect} = \sum \frac{r(+1)}{4} - \sum \frac{r(-1)}{4} \quad (3)$$

Where r (+1) = responses when variable is at (+1) levels,

r (-1) = responses when variable is at (-1) levels.

The standard error of the concentration effect is assessed as the square root of variance of an effect and the significance level (p-value) of each concentration effect is calculated from Student's t-test

$$t(X_i) = \frac{E(X_i)}{S.E} \quad (4)$$

Where *t* (X_i) is the effect of the variable *X*

Optimization of media constituents of EPEM using Central Composite Design (CCD)

The screened components which were found to be influencing the production of endo-pectinases and exo-pectinases were optimized by a central component design. A 2^4 full factorial Central Composite Design (CCD) for four independent media variables each at five levels with eight star points and six replicates at the centre points was taken to fit a 2nd order polynomial model which showed that 30 experiments were required for this method[14,15].

For four factors the model takes the following form

$$Y_i = b_0 + b_1x_{1i} + b_2x_{2i} + b_3x_{3i} + b_4x_{4i} + b_{11}x_{1i}^2 + b_{22}x_{2i}^2 + b_{33}dx_{3i}^2 + b_{44}x_{4i}^2 + b_{12}x_{1i}x_{2i} + b_{13}x_{1i}x_{3i} + b_{14}x_{1i}x_{4i} + b_{23}x_{2i}x_{3i} + b_{24}x_{2i}x_{4i} + b_{34}x_{3i}x_{4i} + r_i \quad (5)$$

Where Y_i is the response. The coefficient b_0 is the intercept. The term r_i allows for discrepancies between the predicted model and the measured value.

The test variables were coded according to the equation

$$x_i = \frac{X_i - X_{i*}}{\Delta X_i} \quad (6)$$

Where x_i is the coded value of the *i*th independent variable, X_i is the uncoded value of the *i*th independent variable, X_{i*} is the uncoded value of the *i*th independent variable at the centre point and ΔX_i is the step change value.

Regression and graphical analyses of the data were acquired using "Design Expert" software, Ver. 7.0.0 (Stat-Ease, Inc., Minneapolis, USA). Exo-pectinases and endo-pectinases activities were taken as the responses for design of experiments. The optimal concentrations of the critical medium contents were obtained by the response contour plots.

Results and discussion

Optimal PEM dilution

Different concentrations of diluted pectin extract medium (50 -90 % v/v) were studied for their pectinase production. The dilution, 80% v/v PEM in water showed maximum endo-pectinases, exo- pectinase activities and the results are shown in Table 1.

Table 1: Optimum PEM dilution

Pem dilution (%v/v)	Exo-pectinase activity (iu/ml)	Endo-pectinase activity (iu/ml)
50	78	5.2
60	87	5.7
70	87.6	6.6
80	94.5	7.8
90	90.4	7.5
100 (Without dilution)	56	5.1

It was observed that increase in dilution from 50 to 80% increased the exo-pectinase activity; however the dilution caused no significant changes in endo-pectinase activity. Maximum pectinase activity (data not shown here) was attained at 34 hrs of

fermentation. Surprisingly the undiluted PEM showed lesser pectinase activities when compared to diluted PEM. Thus the optimal dilution of 80% v/v was chosen for the medium optimization studies.

Screening of the most important medium components influencing the Pectinase production

In the screening of important factors to formulate an (EPEM), eleven media components (Glucose- $C_6H_{12}O_6$, di-potassium hydrogen phosphate-K_2HPO_4, potassium di-hydrogen orthophosphate-KH_2PO_4, Sodium Chloride-NaCl, Manganese Sulphate- $MnSO_4$, Magnesium Sulphate- $MgSO_4.7H_2O$, Calcium Chloride- $CaCl_2$, Ferrous Sulphate-$FeSO_4$, Copper Sulphate- $CuSO_4.5H_2O$& Zinc Sulphate- $ZnSO_4$) were chosen. Experiments were performed for different combinations of high (+1) and low (-1) levels of media components as given in Table 2. Responses (Pectinase activity) for all the 12 experiments are also tabulated.

Table 2: Concentration levels of media components in Plackett-Burman design

Component code	Component	High level (+1) G/l	Low level (-1) G/l
x_1	Glucose	1	10
x_2	Peptone	0.5	3
x_3	K_2HPO_4	0.1	0.5
x_4	KH_2PO_4	0.1	1
x_5	NaCl	$1x10^{-3}$	0.01
x_6	$MnSO_4$	$1x10^{-4}$	$25x10^{-4}$
x_7	$MgSO_4.7H_2O$	0.2	0.8
x_8	$CaCl_2$	$5x10^{-3}$	0.01
x_9	$FeSO_4$	$1x10^{-3}$	$1x10^{-2}$
x_{10}	$CuSO_4.5H_2O$	$0.5x10^{-4}$	$30x10^{-4}$
x_{11}	$ZnSO_4$	$2.5x10^{-4}$	$20x10^{-4}$

Among the media components peptone, $MgSO_4$ and $CuSO_4$ were found to be greatly influencing the exo-pectinase production ($p<0.05$). Peptone shows positive effect of 1236 on exo-pectinase production, whose contribution accounts for 53.65%. This shows that high concentration of peptone in the PEM will increase the exo-pectinase synthesis. At the same time peptone shows an F-value of 29.86. This shows that nitrogen source is an important limiting nutrient in formulation of EPEM. Organic nitrogen sources like yeast extract, peptone, soy bean meal were reported to be the ideal nitrogen sources for pectinases production [14,4]. It is surprising to notice that contribution of glucose to exo-pectinase is as low as 1.26%. Although the effect of glucose is positive, its p-value is 0.3048. This is probably due to the repressive effect of glucose as reported earlier [19].

In contrast to the studies reported [20,21]where pectin in conjunction with carbon sources like glucose and sucrose enhanced pectinase production, in our study the presence of carbon source glucose showed no or unnoticeable influence over pectinase production in PEM, which is also evident from its high p-value. The next most influencing substrate is $MgSO_4$ which is the major source of Mg^{2+} ions, whose contribution to total exo-pectinase activity is 12.3%, with F- and p- values of 6.85 and 0.0397 respectively. However, it shows a negative effect of -592 on exo-pectinase production which implies $MgSO_4$ at low concentrations in the EPEM will enhance the exo-pectinase synthesis. The other medium components like NaCl and KH_2PO_4 show less effect on exo-pectinase production. Their presence at low concentrations can reasonably improve the pectinase production. The remaining factors such as $CaCl_2$, $ZnSO_4$, Glucose, $FeSO_4$,$MnSO_4$and K_2HPO_4 showed less/no significant enhancement in exo-pectinase production as their p-values exceeded well above 0.1 and at the same time the total contribution of them to the exo-pectinase production is just 10%.

For endo-pectinase, peptone shows a contribution of 44.9% of total, and respective F- and p- values are 781.5 and 0.0001. Thus the presence of peptone in the formulation of EPEM is inevitable. Other components like $CuSO_4$ and $MgSO_4$ which are responsible for enhanced exo- pectinase production also show significant contribution to the endo-pectinase production. The last factor which is considered for EPEM optimization is KH_2PO_4, whose contribution is 8.1% for endo-pectinase while it is 6.96% for exo-pectinase. Thus medium constituents (Peptone, $MgSO_4$, KH_2PO_4 and $CuSO_4$) were chosen based on the statistical results obtained from PBD and at the same time which influence preferentially the exo-pectinase synthesisTable 3 & 4.

Table 3: Plackett-Burman design matrix for 11 variables and experiment wise responses

Components												R_1	R_2
Runs	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9	x_{10}	x_{11}	Exo-pectinase activity (U/ml)	Endo-pectinase activity (U/ml)
1	+	+	-	+	+	+	-	-	-	+	-	420.0	63
2	-	+	+	-	+	+	+	-	-	-	+	483.4	60.4
3	+	-	+	+	-	+	+	+	-	-	-	375.6	22
4	-	+	-	+	+	-	+	+	+	-	-	351.2	43.3
5	-	-	+	-	+	+	-	+	+	+	-	340	21.1
6	-	-	-	+	-	+	+	-	+	+	+	256	52
7	+	-	-	-	+	-	+	+	-	+	+	281	31.2
8	+	+	-	-	-	+	-	+	+	-	+	564	34
9	+	+	+	-	-	-	+	-	+	+	-	429.8	58.6
10	-	+	+	+	-	-	-	+	-	+	+	477	56
11	+	-	+	+	+	-	-	-	+	-	+	341.2	44.4
12	-	-	-	-	-	-	-	-	-	-	-	390	24.3

+ High level (+1), - Low level (-1)

Table 4: Statistical parameters for Plackett-Burman design

Component	Effect		Sum square		% contribution		P-Value		F- value	
	Ex-P	En-P	Ex-P	En-P	Ex-P	En-P	Ex-P	En-P	Ex-P	En-P
Glucose	190	-0.65	108300	1.26	1.26	0.047	>0.1	>0.1	-	-
Peptone	1236	20.05	$4.58x10^6$	1206	53.65	44.9	0.0016	0.0001	29.8	781.57
K_2HPO_4	308	2.45	284592	18	3.33	0.67	>0.1	0.0420	-	11.67
KH_2PO_4	-445	8.517	594965	217.6	6.96	8.10	0.0365	0.0013	3.88	141.02
NaCl	-459	2.75	632961	22.68	7.41	0.844	0.0886	0.0313	4.12	14.7
$MnSO_4$	281.3	-0.83	237445	2.34	2.77	0.087	>0.1	>0.1	-	-
$MgSO_4$	-592	4.117	$1.05x10^6$	50.84	12.30	1.89	0.0397	0.0105	6.85	32.95
$CaCl_2$	114	-15.8	38988	753.6	0.45	28.05	>0.1	0.0002	-	488.4
$FeSO_4.7H_2O$	-241	-0.58	174725	1.02	2.04	0.038	>0.1	>0.1	-	-
$CuSO_4.5H_2O$	-502	8.91	758021	238.5	8.87	8.88	0.0680	0.0011	2.94	154.12
$ZnSO_4$	160	7.61	76800	174	0.899	6.47	>0.1	0.0018	-	112.79

Ex-P → Exo-pectinase, En-P → Endo-pectinase, - → removed factors

Table 5: Experimental range and levels of variables used in the Central Composite Design

Variables (g/l)	Levels				
	-2	-1	0	1	2
Peptone (x_1)	1	2	3	4	5
KH_2PO_4 (x_2)	0.1	0.2	0.3	0.4	0.5
$MgSO_4.7H_2O$ (x_3)	0.05	0.2	0.35	0.5	0.65
$CuSO_4.5H_2O$ (x_4)	$0.25x10^{-4}$	$0.5x10^{-4}$	$0.75x10^{-4}$	$1x10^{-4}$	$1.25x10^{-4}$

A central composite design matrix with 30 experiments at different levels viz., -2, -1, 0, +1 & +2 and their corresponding endo-pectinase & exo-pectinase activities as responses are shown in Table 6.

Table 6: Central Composite Design matrix for four test components along with their responses

Runs	Components				Response			
	x_1	x_2	x_3	x_4	Y1 Exo-pectinase activity (IU/ml)		Y2 Endo-pectinase activity (IU/ml)	
					Actual	predicted	Actual	Predicted
1	-1	-1	-1	-1	470	453	54.2	52.7
2	1	-1	-1	-1	572	592	53	54.0
3	-1	1	-1	-1	420	429	34	35.3
4	1	1	-1	-1	557	543	40	39.5
5	-1	-1	1	-1	430	430	42.9	42.9
6	1	-1	1	-1	547	526	43	41.2
7	-1	1	1	-1	400	398	32	29.4
8	1	1	1	-1	450	453	30	30.5
9	-1	-1	-1	1	363	375	28	27.2
10	1	-1	-1	1	546	531	35	36.4
11	-1	1	-1	1	394	398	32	32.6
12	1	1	-1	1	498	513	45	44.7
13	-1	-1	1	1	420	417	43	32.3
14	1	-1	1	1	547	534	40	38.4
15	-1	1	1	1	420	416	43	41.7
16	1	1	1	1	496	492	50.3	50.6
17	-2	0	0	0	350	337	36	37.8
18	2	0	0	0	536	548	48.4	48
19	0	-2	0	0	532	547	39	40.1
20	0	2	0	0	512	497	34.7	34.9
21	0	0	-2	0	521	501	40	38.6
22	0	0	2	0	433	453	32	34.8
23	0	0	0	-2	465	462	42	42.9
24	0	0	0	2	415	418	37	37.4
25	0	0	0	0	543	549	46	52
26	0	0	0	0	546	549	51	52
27	0	0	0	0	547	549	53	52
28	0	0	0	0	557	549	52.1	52
29	0	0	0	0	551	549	55.3	52
30	0	0	0	0	550	549	55	52

Optimization of EPEM by Central composite design

A Central composite design was used to study the interaction between the most influencing media components such as peptone, $MgSO_4$, $CuSO_4$ and KH_2PO_4 and to design an (EPEM) to produce optimal levels of endo-pectinase & exo-pectinases.

The statistical treatment combinations in the range of -2 to +2 for the experiments are shown in Table 5.

x_1 Peptone; x_2 KH_2PO_4, X_3 $MgSO_4.7H_2O$ & x_4 $CuSO_4.5H_2O$

Equations for exo-pectinase and endo-pectinase productions were developed based on regression analysis of experimental data

$$Y_1 = +549 + 52.83x_1 - 12.5x_2 - 11.92x_3 - 10.92x_4 - 10.12x_1x_2 - 9.75x_1x_3 + 5.25x_1x_4 - 6.00x_2x_3 + 7.75x_2x_4 + 17.13x_3x_4 - 26.48x_1^2 - 6.73x_2^2 - 17.98x_3^2 - 27.23x_4^2 \quad (7)$$

&

$$Y_2 = +52.07 + 2.58x_1 - 1.31x_2 - 0.96x_3 - 1.37x_4 + 0.71x_1x_2 - 0.77x_1x_3 + 1.96x_1x_4 + 0.98x_2x_3 + 5.71x_2x_4 + 3.73x_3x_4 - 2.29x_1^2 - 3.62x_2^2 - 3.84x_3^2 - 2.96x_4^2 \quad (8)$$

Where Y_1 and Y_2 are the responses (activity IU/ml) of exo-pectinases and endo-pectinases respectively, x_1 peptone, x_2 KH_2PO_4, x_3 $MgSO_4.7H_2O$ & x_4 $CuSO_4.5H_2O$. The regression equations indicate that the value of R^2 for exo-pectinases & endo-pectinase are 0.964 and 0.952 respectively (a value of R^2>0.75 indicates good fit of the model). The basic purpose of statistical analysis is to identify the experiment factors, which generate signals that are large in comparison to the noise [18].The adequate precision measures the signal to noise ratio and the ratio greater than 4 is desirable. In our model signal-noise ratio is 20.622 for exo-pectinase and 14.98 for endo-pectinase. This validates the model and it can be used to navigate the design space. The F-value for models exo-pectinase and endo-pectinase are 29.26 and 21.39, which imply that the models are significant. Further p-values of the models are less than 0.0001 0.0057, which is well below p-value of 0.05. From the above generated statistical data it can be concluded that the models are good enough to predict the responses of the system.

The results of Analysis of Variance (ANOVA) for Response Surface quadratic model are summarized in Table 7.

Table 7: ANOVA for quadratic model

Sources of variations		SS	df	Mean square	F-Value	p-value Prob>F
Ex-p	Model	$1.21x10^5$	14	8937.18	29.26	<0.0001
	Error	4581.33	15	23.6		
	Corrected Total	$1.297x10^5$	29			
Other statistics: C.V = 3.6%; R^2 = 0.965; Adj. R^2 = 0.9316; Pred R^2 = 0.86 & R=0.98						
En-p	Model	1929.7	14	137.84	21.39	<0.0001
	Error	96.66	15	15.45		
	Corrected Total	2026.37	29			
Other statistics: C.V = 6.06%; R^2 = 0.95; Adj.R^2 = 0.91; Pred.R^2 = 0.85; R = 0.975						

SS- Sum of squares; df- degrees of freedom; Ex-p –Exo-pectinase; En-p –Endo-pectinase

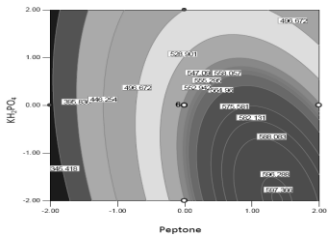

Fig.1: A

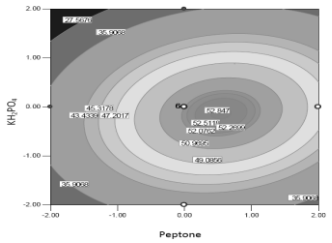

Fig.1: B

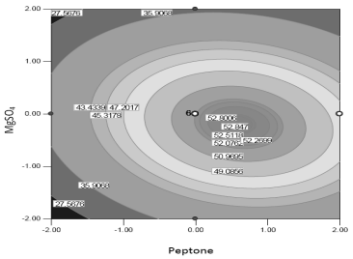

Fig.2: A

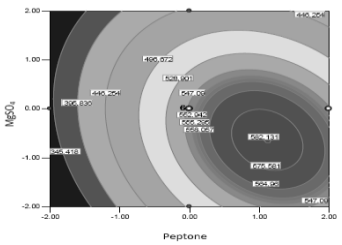

Fig.2: B

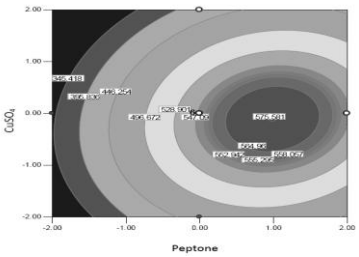

Fig.3: A

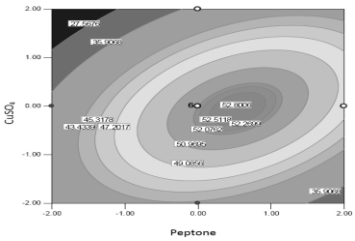

Fig.3: B

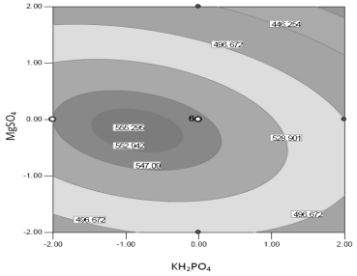

Fig.4: A

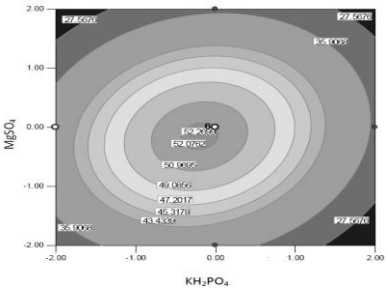

Fig.4: B

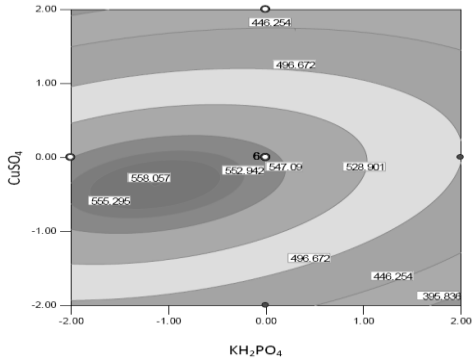

Fig.5: A

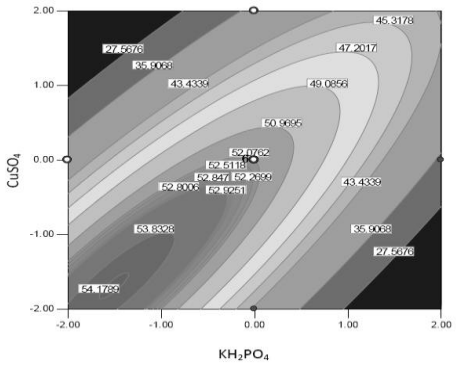

Fig.5: B

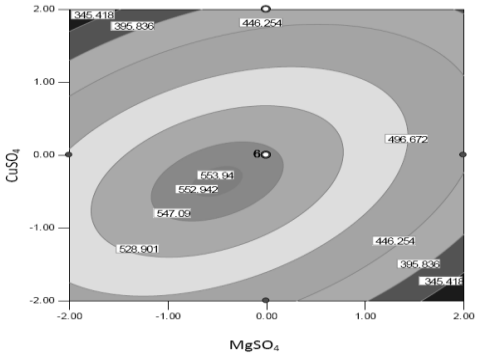

Fig.6: A

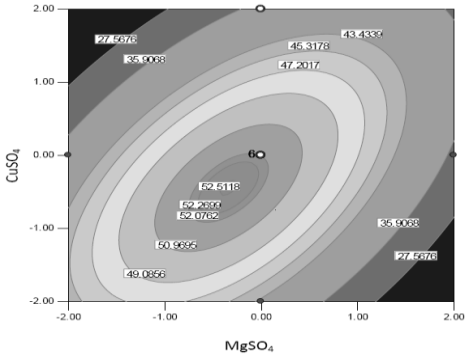

Fig.6: B

The optimal concentrations of the EPEM yielding maximum pectinase activities can be obtained from the contour plots. The 2-D contour plots showing the interaction between different variables are shown in Fig 1 – 6**.**

Fig. 1. Response surface contour plots showing the effect of interactions between Peptone and KH_2PO_4 on; a) Exo-pectinase activity, b) Endo-pectinase activity. Other parameters are maintained at their zero level.

Fig. 2. Response surface contour plots showing the effect of interactions between Peptone and $MgSO_4$ on a) Exo-pectinase activity, b) Endo-pectinase activity. Other parameters are maintained at their zero level.

Fig. 3. Response surface contour plots showing the effect of interactions between Peptone and $CuSO_4$ on a) Exo-pectinase activity, b) Endo-pectinase activity. Other parameters are maintained at their zero level

Fig. 4. Response surface contour plots showing the effect of interactions between $KH_2PO_4$and $MgSO_4$ on a) Exo-pectinase activity, b) Endo-pectinase activity. Other parameters are maintained at their zero level.

Fig. 5. Response surface contour plots showing the effect of interactions between $KH_2PO_4$and $CuSO_4$ on a) Exo-pectinase activity, b) Endo-pectinase activity. Other parameters are maintained at their zero level.

Fig. 6. Response surface contour plots showing the effect of interactions between $MgSO_4$and $CuSO_4$ on a) Exo-pectinase activity, b) Endo-pectinase activity. Other parameters are maintained at their zero level.

The maximum possible yield (exo-pectinase, endo-pectinase activity) is traced in the contour surface confined in the smallest ellipse [16, 18]. Table 8

Table 8: Optimal responses from 2D- contour plots

Pectinase	Optimal concentration (g/l)				Activity (IU/ml)
	Peptone	KH_2PO_4	$MgSO_4$	$CuSO_4$	
Exo-	4.38	0.104	0.35*	0.75x10-4*	597.366
Endo-	3.55	0.29	0.35*	0.75x10-4*	52.847
Exo-	4.10	0.3*le	0.259	0.75x10-4*	582.131
Endo-	3.6	0.3*	0.321	0.75x10-4*	52.847
Exo-	4.01	0.3*	0.35*	0.73x10-4	575.58
Endo-	3.54	0.3*	0.35*	0.74x10-4	52.8
Exo-	3*	0.2	0.321	0.75x10-4*	555.295
Endo-	3*	0.28	0.325	0.75x10-4*	52.2
Exo-	3*	0.19	0.35*	0.66x10-4	558.08
Endo-	3*	0.15	0.35*	0.33x10-4	54.178
Exo-	3*	0.3*	0.275	0.66x10-4	553.94
Endo-	3*	0.3*	0.296	0.64x10-4	52.518

(*) Concentration of components at zero levels

shows the maximum predicted yield obtained from confined surface of the smallest ellipse in the 2-D-contour plots for two interacting variables while other variables maintained at "0" levels. It can be observed that the exo-pectinase activity ranged between 553.94 IU/ml to 597.366 IU/ml

and endo-pectinase activity ranged between 52.2 IU/ml to 54.178 IU/ml for the assigned design levels of variables.

Since our objective is to design an EPEM supplemented with components giving maximum yield of both endo-pectinases and exo-pectinases, it is desirable to find the optimal concentrations of interacting components. A closer scrutiny of the data reveals that concentrations of peptone x_1, KH_2PO_4 x_2, $MgSO_4$ x_3& $CuSO_4$ x_4 giving optimal activities of exo-pectinase & endo- pectinase (575.58 IU/ml and 52.8 IU/ml) are found to be 4 g/l, 0.3 g/l, 0.35 g/l & $0.75x10^{-4}$ g/l respectively.

A test experiment carried with EPEM containing predicted optimal concentrations of the four components resulted in exo-pectinases and endo-pectinase activities of 573 IU/ml and 52 IU/ml.

CONCLUSIONS

The study aims at enhancing the pectinase (exo-pectinase & endo-pectinase) production in the (PEM) obtained from orange peel using *Aspergillus niger*. EPEM was formulated by screening components greatly influencing the pectinase production using Plackett-Burman design followed by medium optimization using Response surface modeling based on CCD. Four significant variables affecting pectinase production in PEM were identified as peptone, KH_2PO_4, $MgSO_4$& $CuSO_4$ and their corresponding optimal concentrations in EPEM are 4 g/l, 0.3 g/l, 0.35 g/l & $0.75x10^{-4}$ g/l respectively thereby resulting in pectinase activities of 575.58 IU/ml of exo-pectinase and 52.8 IU/ml of endo-pectinase.

Thus (EPEM) can act as an effective alternative to the pre-existing commercial media for industrial production of pectinases through submerged fermentation. It justifies the extract from orange peel acts as an effective inducer of pectinase synthesis even in the absence of simple sugars such as glucose, fructose and sucrose. Also the residual pectin extracted solid mass can be used as substrate in the production of other important enzymes like cellulase, xylanase etc.

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