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A Combinatorial Statistical Approach For Hyperproduction Of Pectinase From Aspergillus Niger C-5 Via Solid State Fermentation Of Wheat Bran And Its Kinetic Characterization

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***Abstract*—In the present study, a two step combinatorial statistical approach was employed to optimize various nutritional and fermentation conditions for the hyper production of pectinase from *Aspergillus niger* C-5 on wheat bran based medium. Appreciable enzyme yield was obtained on simple wheat bran based basal medium without any exogenous supplementation of nutrients. For further augmenting the enzyme yield, Plackett-Burman design was first employed to screen important process parameters affecting the productivity. Of 23 variables screened, four significant variables including Tryptone, SDS, NH_4Cl, NaCl were selected to further study their interactive effects and to determine their optimal concentrations in central composite design of response surface methodology. This combinatorial approach led to 3.17 fold increase in the enzyme productivity revealing the yield of 304.14 IU/g. The characterization of the partially purified enzyme preparation revealed it to be active over a broader temperature and pH range and was also found to be quite thermostable and pH stable retaining much of its residual activity after more than 216 h of incubation at higher temperatures as well as wider pH ranges.**

Keywords—Solid State Fermentation; Pectinase; Plackett-Burman Design; Aspergillus niger; Response Surface Methodology; Wheat bran

I. INTRODUCTION

Pectin is a complex high molecular weight, heterogeneous and acidic structural polysaccharide found mainly in the primary cell wall of cereals, skin of fruits and vegetables. It ranks third as a cell wall constituent after cellulose and hemicelluloses. The main component of pectin backbone is galacturonic acid linked by α1-4 linkages with neutral sugars [1]. Pectinases break down this complex polysaccharide and splits polygalacturonic acid into monogalacturonic acid by opening the glycosidic linkages. Through this process, it softens the cell wall and increase the yield of juice extract from the fruits [2]. The two major sources of the enzyme production are plants and microorganisms, but from both technical and economic point of view, microbial sources are considered more favourable [3]. Even though, occurrence of pectinolytic enzymes have been reported in a large number of bacteria and fungi, most commercial preparations of pectic enzymes are obtained from fungal sources because the pH optima of enzymes produced by fungal strains are in a range naturally found in materials to be processed [4]. Pectinases have also been reported to be produced in combination with cellulases, hemicellulases and amylases in fungi including *Aspergillus* sp., *Botrytis cinerea, Fusarium moniliforme, Rhizoctonia solani, Rhizopus stolonifer, Trichoderma* sp., *Neurospora crassa, Penicillium* and *Fusarium* [5,6]. Pectinases find wide application in the field of textile industries, food industries, tea industries, paper and pulp industries, vegetable oil extractions, saccharification of agricultural residues and in alcoholic beverages [7,8]. Now a days, pectinase is considered to be one of the most important enzymes in food processing industries and is mainly used for the extraction and clarification of fruit juices and wines [9,10]. It shares about 25% of global sale in the food enzymes. Because of the widespread applications of this enzyme, it is necessary to use inexpensive and readily available raw material for its production. Utilization of cheaper substrates such as fruit processing waste, various lignocellulosic materials, brans and straws etc and use of simple technologies like solid state fermentation (SSF) can help increase enzyme productivity at a low cost [11,12]. SSF offers additional advantages of low capital investment, simple, higher reactor volume, production of concentrated products and ease of product recovery [13]. Several reports exist where SSF has been employed for the production of industrially important enzymes such as cellulases, polygalacturonase, xylanase, pectinase, mannanase and amylases [14-22].

As these hydrolytic enzymes are to be used in bulk for various industrial applications, the availability of low cost pectinases is a necessity. The enzymes obtained from microorganisms are generally extracellular and their yield as well as cost is highly affected by the components of the medium such as carbon and nitrogen ratio, inorganic nutrients, as well as fermentation conditions like temperature, pH, aeration and agitation. Therefore, development of an economically viable production medium requires selection of process parameters and their optimization strategies. Optimization of media components by changing one factor at a time is not only tedious and cumbersome but also becomes non feasible, in cases where large numbers of variables are to be screened. The combinatorial statistical approaches of Plackett-Burman (PB) and response surface methodologies (RSM) can be employed to screen large number of factors and then design a simple low cost medium for enhancing the product yields. PB based statistical designs are first employed for screening various cultural and environmental factors in order to understand their significance on the product formation and then few better factors that show a significant role are selected for subsequent optimization studies by RSM. This provides models and graphs showing the effects of independent variables on enzyme yield and also give the predictive responses of each combination, the interactive effects of variables and the optimum levels of each independent variable in the growth medium.

The aim of the present study was to develop a low cost, readily available medium and environment friendly production of pectinase from *Aspergillus niger* C-5 by statistically optimizing the media constituents of wheat bran based solid media and employing simpler technology of solid state fermentation. The enzyme preparation was also kinetically characterized for its properties so as to be used in wide range of biotechnological applications.

II. Materials and Methods

A. Microorganism

The pectinolytic strain of *Aspergillus niger* C-5 used in the present study was isolated from the rotten food samples and soil of Chandigarh city [16]. It was grown and maintained on potato dextrose agar plates at 28^{o}C for 4 days to allow the development of spores and then stored at 4^{o}C until use.

B. Enzyme production by solid state fermentation

The production of pectinase enzyme was carried out under solid state cultivation conditions in 250 ml Erlenmeyer flasks containing 5g wheat bran moistened with 5 ml of distilled water. The flasks were autoclaved and inoculated in triplicate with 2.5 ml of fungal spore suspension (2.8×10^{7} spore/ml) and incubated at 30^{o}C in stationary state for 4 days which was found to be the optimum time period for maximum fungal growth [16]. The enzyme was extracted by adding 200 ml of distilled water to each flask and churning the contents in a blender. After churning, the contents were filtered through metallic sieve and the solid residue was thoroughly pressed to extract the remaining liquid. The suspension from each flask was then centrifuged at 10,000 rpm for 10 min at 4^{o}C, and the supernatant analysed for enzyme activity.

C. Assay of pectinolytic activity

Pectinase activity was estimated by using pectin as the substrate [23] and determining the μmoles of galactouronic acid liberated/min, using dinitrosalicylic acid reagent [24]. The enzyme activity was expressed in terms of International units (IU). One IU of pectinase activity was defined as equivalent to the enzyme that releases one μmole of galactouronic acid from pectin, in one min under standard assay conditions (50°C and pH 4.0).

D. Statistical optimization of medium components for pectinase production by PB design

Enzyme production is highly influenced by many factors including media constituents, various cultural and environmental parameters. For screening the effect of these parameters on enzyme productivities, 23 different process variables designated as X_1, X_2, X_3 ……X_{23}, (urea, NH_4SO_4, KH_2PO_4, peptone, yeast extract, meat extract, soyabean meal, tryptone, $CaCl_2$, $MgSO_4$, $COCl_2$, $ZnSO_4$, $FeSO_4$, water, Tween 80, $MnCl_2$, malt extract, incubation time, SDS, $MnSO_4$, NH_4Cl, $NaNO_3$, NaCl) were chosen and examined, in one block as shown in Table I, at low (-1) and high (+1) levels depicted in Table II using first order Plackett-Burman factorial design:

$$Y = \beta o + \Sigma \beta i Xi \qquad \ldots\ldots\ldots\ldots (1)$$

Where, Y is the response, βo is the model intercept, βi is the linear coefficient, and Xi is the level of the independent variable.

E. Standardization of important screened parameters for further optimization of pectinase production by Aspergillus niger C-5 using RSM

In order to determine the optimal concentrations of most significant factors screened during PB design, four independent variables including Tryptone (X_8), SDS (X_{19}), NH_4Cl (X_{21}), NaCl (X_{23}) affecting pectinase productivity were further chosen to investigate the first- and higher-order main effects of each factor and interactions amongst them for further optimization through RSM. All other factors including X_1, X_2, X_4, X_5, X_6, X_7, X_9, X_{10}, X_{11}, X_{12}, X_{13}, X_{14}, X_{16}, X_{17}, X_{18}, X_{20}, at the levels of 0.60 mg, 0.65 mg, 81.42 mg, 10.28 mg, 99.97 mg, 96.26 mg, 1.06 mg, 0.05 mg, 0.002 mg, 0.006 mg, 0.025 mg, 8.85 ml, 0.006 mg, 74.05

mg, 3 days, 0.58 mg were kept constant. The flasks were inoculated with 2.5 ml of fungal spore suspension having 2.8×10^7 spores/ml, incubated at 30ºC, pH 4.0 in stationary state for 72 h. A 2^4 factorial central composite experimental design resulting in 30 experimental runs as shown in Table 3 was generated by (Design Expert, Version 9.0, Stat-Ease Inc., Minneapolis, MN). The relation between coded and actual values is described according to equation:

$$xi = (Xi - X0i) / \Delta Xi \quad (2)$$
$$i = 1,2,3\ldots j$$

Where xi = coded (dimensionless) value of the variable Xi,
Xi = actual value of the i^{th} variable
X0 = the value of Xi at the center point,
ΔX = the step change value.

The behavior of the system was explained by the following second order polynomial equation:

$$Y= bo+\sum bixi+\sum\sum bij\ xi\ xj + \sum bii\ x^2i+e \quad (3)$$

Where Y= measured response; bo, bi, bij, bii are constant and regression coefficients of model; xi and xj are levels (codes values) of independent variables; e is random error.

F. Statistical analysis of data

The software package, Design-Expert version 9, Stat-Ease (Inc, Minneapolis, MN) which provides highly efficient design of experiments was employed. Multiple linear regression analysis was carried out to estimate t-values, p-values and F- values to evaluate the significance of experimental design. Contour plots were also obtained to illustrate the relationship between the variables. Accuracy and general ability of the model was evaluated by coefficient of determination (R^2). The statistical significance of model coefficient was evaluated by ANOVA.

G. Concentration, partial purification and kinetic characterization of enzyme

The pectinase obtained from solid state cultures of *Aspergillus niger* C-5 was first concentrated and partially purified by ammonium sulphate precipitation and subsequently characterized for its temperature and pH activity profiles, the effect of temperature and pH on enzyme stability and the role of various metal ions and chelating agent on enzyme activity was also studied. The enzyme preparation was first centrifuged at 10, 000 rpm for 10 min. The cell free supernatant was then subjected to ammonium sulphate precipitation step wise from 0- 80 % [25]. The pellets obtained were individually dissolved in a minimum amount of acetate buffer (pH 4.0) and dialysed against the same buffer for 24 h at 4°C to remove traces of ammonium sulphate and other low molecular weight impurities. The protein content and enzyme activity was observed in crude as well as fractions saturated with 40, 60 and 80% ammonium sulphate by the method of [26] and [27] respectively. Optimum temperature for enzyme was determined by assaying its activity at different temperatures (30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC) at pH 4.0 in (0.1M) acetate buffer. The pH activity profile was studied by assaying the pectinase activity at 50°C using substrate solutions and enzyme dilutions made in different buffers using three buffer systems (0.1M) acetate buffer, pH 3.0-5.0; phosphate buffer, pH 6.0-7.0 and Tris-HCl buffer, pH 8.0-10.0.

The thermostability profile of partially purified enzyme was studied by preparing the enzyme dilutions in 0.1M acetate buffer, pH 4.0 and separately incubating at 50ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC with and without the addition of calcium chloride at a final concentration of 10 mM in the reaction mixture. The samples were withdrawn at an interval of 4 h upto 216 h and the residual activity was determined by the standard assay under normal conditions and expressed in terms of % of control. The pH stability profile was also studied by preparing the enzyme dilutions, separately in buffers ranging from pH 4.0 to pH 10.0 at room temperature. 0.1% sodium azide was added to each tube containing enzyme to prevent bacterial contamination. The samples were withdrawn immediately after incubation and after 2, 4, 6, 24 h and the residual activities was determined by the standard assay under normal conditions and expressed in terms of % of control.

The effect of metal salts and EDTA on enzyme activity was studied by supplementing various metal salts including $CaCl_2$, $CoCl_2$, $HgSO_4$, KCl, $MgSO_4$, $MnCl_2$, $MnSO_4$, NaCl, $ZnSO_4$ and EDTA), separately, in the reaction mixtures at a final concentration of 5 mM. The relative enzyme activity was determined under optimal pH and temperature conditions and expressed in terms of % of control. The effect of various substrate concentrations (0.05 to 0.5 %) on the enzyme activity was studied under the above mentioned standard assay conditions of pH (4.0) and temperature (50ºC). The Michealis-Menten constant (K_m) and maximum velocity (V_{max}) of the enzyme were obtained using the reciprocal plot (Line-weaver–Burk plot).

III. RESULT AND DISCUSSION

A broad range of agricultural residues have been successfully employed by various microorganisms for the production of large number of hydrolytic enzymes [14,28,17,8,5]. Several crop residues, in the form of flours, brans, straws, hulls, residues of the fruit processing industries, waste of the oil processing mills have been successfully used in solid state fermentation by many workers [29,30,16,17]. Wheat bran has been the prime among many substrates employed in solid state fermentation processes, owing to its particularly rich nutritional composition: wheat bran is particularly rich in dietary fiber and essential fatty acids and contains significant quantities of carbo-

hydrates, proteins, vitamins and minerals [15,28,31, 16]. In the present study also, wheat bran was used as a substrate for fungal growth and production of pectinase under solid state fermentation conditions. When used as a solid state fermentation substrate, wheat bran was able to remain loose in moist conditions and supported good fungal growth and sporulation. The organism colonized well on this substrate and produced higher titres of pectinase revealing the yield of 101±3.03 IU per gram dry substrate after 96 h incubation period at 28°C on simple wheat bran based solid medium without the addition of any exogenous nutrients.

A. Statistical optimization of media components for pectinase production by Plackett-Burman design

PB is a set of small and efficient experimental design which has been recently employed by many research groups aiming to enhance various enzyme yields utilizing different agro-industrial substrates [32,33]. The usefulness of the design lies in the fact that in determining the effect of one variable, the net effect of changing other variables cancel out, so that the effect of each variable on the system can be independently determined. Based upon our preliminary studies and literature survey, a set of 23 independent variables, designated as X_1, X_2, X_3 …… X_{23}, were chosen and examined in the present study with their respective responses shown in Tables I and II. The main effects of the examined variables on pectinase production was calculated as the difference between the average measurements made at higher level (+1) and lower level (-1) of that factor. In the model, it was observed that supplementation of peptone, soyabean meal, manganese chloride, incubation time, ammonium chloride exerted enhanced positive effect while potassium dihydrogen orthophosphate, Tween 80 and sodium nitrate exerted enhanced negative effect.

TABLE I. RANDOMIZED PLACKETT-BURMAN EXPERIMENTAL DESIGN FOR EVALUATING THE FACTORS INFLUENCING PECTINASE PRODUCTION

Run	Urea (X_1)	NH_4SO_4 (X_2)	KH_2PO_4 (X_3)	Peptone (X_4)	Yeast Extract (X_5)	Meat Extract (X_6)	Soyabean meal (X_7)	Tryptone (X_8)	$CaCl_2$ (X_9)	$MgSO_4$ (X_{10})	$CoCl_2$ (X_{11})	$ZnSO_4$ (X_{12})	$FeSO_4$ (X_{13})	Water (X_{14})	Tween 80 (X_{15})	$MnCl_2$ (X_{16})	Malt Extract (X_{17})	Incubation Time (X_{18})	SDS (X_{19})	$MnSO_4$ (X_{20})	NH_4Cl (X_{21})	$NaNO_3$ (X_{22})	NaCl (X_{23})	Pectinase (IU/gds)
1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	227
2	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	252
3	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	104
4	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	+1	-1	+1	+1	+1	+1	+1	104
5	-1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	104
6	-1	-1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1	+1	+1	+1	+1	+1	+1	-1	222
7	-1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	+1	-1	-1	-1	+1	+1	+1	104
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	+1	-1	-1	-1	-1	-1	-1	104
9	-1	-1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	217
10	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	104
11	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	+1	-1	208
12	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	84
13	+1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	77
14	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	+1	+1	-1	+1	+1	-1	-1	252
15	+1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	218
16	+1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	+1	-1	+1	+1	-1	-1	+1	104
17	-1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	74
18	+1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	192
19	-1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	+1	+1	-1	-1	-1	-1	+1	134
20	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1	59
21	+1	-1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	+1	-1	+1	-1	+1	-1	-1	104
22	+1	+1	+1	+1	+1	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	163
23	-1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	+1	+1	+1	-1	+1	-1	120
24	+1	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1	+1	-1	+1	-1	+1	-1	-1	+1	+1	+1	+1	84

TABLE II. LEVELS OF INDEPENDENT VARIABLES USED FOR MEDIUM OPTIMIZATION IN PLACKETT BURMAN DESIGN

Variables	Levels	
	Low (-1)	High (+1)
X_1:Urea	0	1.5 mg
X_2:NH_4SO_4	0	7.0 mg
X_3:KH_2PO_4	0	100 mg
X_4:Peptone	0	100 mg
X_5:Yeast extract	0	100 mg
X_6:Meat extract	0	100 mg
X_7:Soyabean meal	0	100 mg
X_8:Tryptone	0	100 mg
X_9:$CaCl_2$	0	1.5 mg
X_{10}:$MgSO_4$	0	1.5 mg
X_{11}:$CoCl_2$	0	0.01 mg
X_{12}:$ZnSO_4$	0	0.01 mg
X_{13}:$FeSO_4$	0	0.03 mg
X_{14}:Water	5	12 ml
X_{15}:Tween 80	0	0.01 mg
X_{16}:$MnCl_2$	0	0.5 mg
X_{17}:Malt extract	0	100 mg
X_{18}:Incubation time	72 h	144 h
X_{19}:SDS	0	0.6 mg
X_{20}:$MnSO_4$	0	0.5 mg
X_{21}:NH_4Cl	0	1.5 mg
X_{22}:$NaNO_3$	0	5.0 mg
X_{23}:NaCl	0	1.5 mg

B. Standardization of important screened parameters for further optimization of pectinase production using RSM designs

Response surface methodology (RSM) is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results [34, 35]. A second order model like central composite design (CCD) is widely used in RSM because it can take on wide variety of functional forms and this flexibility allows it to predict the true response surface more closely. In the present study, to determine the optimum response regions for the maximum production of pectinase, a 2^4 factorial central composite experimental design was generated by (Design Expert, Version 9.0, Stat-Ease Inc., Minneapolis, MN) to evaluate the nature of the response surface in the experimental region and to identify optimal values for the chosen significant variables including Tryptone (X_8), SDS (X_{19}), NH_4Cl (X_{21}), NaCl (X_{23}). The experimental designs resulting in 30 experimental runs with the observed responses for enzyme(s) production is presented in Table III. All other factors which showed positive behavior during screening by Plackett-Burman model including X_1, X_2, X_4, X_5, X_6, X_7, X_9, X_{10}, X_{11}, X_{12}, X_{13}, X_{14}, X_{16}, X_{17}, X_{18}, X_{20}, at the levels of 0.60 mg, 0.65 mg, 81.42 mg, 10.28 mg, 99.97 mg, 96.26 mg, 1.06 mg, 0.05 mg, 0.002 mg, 0.006 mg, 0.025 mg, 8.85 ml, 0.006 mg, 74.05 mg, 3 days, 0.58 mg were kept constant. The flasks were inoculated with 2.5 ml of fungal spore suspension having 2.8×10^7 spores/ml, incubated at 30°C, pH 4.0 in stationary state for 72 h. The regression equation was developed using RSM, allowing for the analysis of interacting factors by identifying which significant factors contribute to the regression model and determining the optimal values of the most significant independent variables [36].

To decide about the adequacy of each of the model for enzyme production, different tests were carried out [37]. The statistical significance of the ratio of mean square variation due to regression and mean square residual error was tested using the analysis of variance (ANOVA). The goodness of fit for each of the model was tested by analyzing their F value, p-value. Accuracy and general ability of polynomial model was evaluated by coefficient of determination (R^2). P-values greater than 0.1000 indicate the model terms are not significant.

A determination coefficient R^2 value close to 1 indicates that the model describes and represents the experimental data well. The value of Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The graphical representation of the regression equation for each of the response surface model has been presented in terms of isoresponse contour plots. From the contour plots, it is easy and convenient to understand the interactions between two variables and also to locate the optimum levels. Each curve represents an infinite number of combinations of two test variables with the other variables maintained at constant level.

Various models including linear, 2F1, quadratic and cubic were tried and tested in the present study to enhance pectinase production. Each model was analysed on the basis of their F value, P value, determination coefficient (R^2), standard deviation and PRESS values. Of all the models analyzed, the quadratic regression model was found to be significant with a lower p value.

The ANOVA test for the response surface quadratic model is shown in Table IV. The resulting model F-value of 74.05 implies that the model is significant, with only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X_8, X_{19}, X_{21}, X_{23}, X_8^2, X_{19}^2, X_{21}^2, X_{23}^2, X_8X_{19}, X_8X_{21}, X_8X_{23}, $X_{19}X_{23}$ are significant model terms. The goodness of the fit of the model was checked by the 'determination coefficient' R^2 which was calculated to be 0.9812, indicating that 98.12 % of variables fit the response. The "Pred R-Squared" of 0.9449 is in reasonable agreement with the "Adj R-Squared" of 0.9680. The value of "Adeq Precision is 26.638 which indicates an adequate signal. Hence, this model can be used to navigate the design space.

The ANOVA analysis for pectinase model indicates a linear relationship between the main effects of the tryptone, SDS, ammonium chloride, sodium chloride, the interaction between tryptone and SDS, tryptone and ammonium chloride, tryptone and sodium chloride, SDS and sodium chloride, the quadratic relationship with tryptone, SDS, ammonium chloride, sodium chloride. However, some regression coefficients i.e. CD and BC were found to be unnecessary having p values >0.05 suggesting their insignificance and hence were removed by backward elimination step. Thus, by neglecting the insignificant terms, the final model equation for pectinase in terms of coded factors may be written as:

Pectinase = $+302.50 + 3.64 \times X_8 + 1.44 \times X_{19} + 2.42 \times X_{21} + 1.50 \times X_{23} + 2.44 \times X_8X_{19} - 2.39 \times X_8X_{21} + 2.28 \times X_8X_{23} - 3.24 \times X_{19}X_{23} - 3.51 \times X_8^2 - 3.19 \times X_{19}^2 - 5.04 \times X_{21}^2 - 1.37 \times X_{23}^2$

Where X_8, X_{19}, X_{21} and X_{23} are Tryptone, SDS, NH_4Cl, NaCl respectively.

TABLE III. CENTRAL COMPOSITE DESIGN MATRIX OF RESPONSE SURFACE METHODOLOGY WITH EXPERIMENTAL VALUES OBTAINED FOR PECTINASE PRODUCTION BY *Aspergillus niger* C-5

Run	Tryptone (X_8) (-2) = 100 mg (-1) = 200 mg (0) = 300 mg (+1) = 400 mg (+2) = 500 mg	SDS (X_{19}) (-2) = 0.40 mg (-1) = 0.60 mg (0) = 0.80 mg (+1) = 1.00 mg (+2) = 1.20 mg	NH_4Cl (X_{21}) (-2) = 1.00 mg (-1) = 1.50 mg (0) = 2.00 mg (+1) = 2.50 mg (+2) = 3.00 mg	NaCl (X_{23}) (-2) = 1.00 mg (-1) = 1.50 mg (0) = 2.00 mg (+1) = 2.50 mg (+2) = 3.00 mg	Pectinase (IU/gds)
1	-1	+1	+1	+1	285
2	0	0	0	0	301
3	0	0	-2	0	277
4	+1	+1	-1	+1	299
5	-1	+1	-1	-1	284
6	0	0	0	+2	299
7	-1	+1	-1	+1	276
8	+1	-1	+1	+1	298
9	0	0	0	0	305
10	-2	0	0	0	282
11	-1	-1	+1	+1	294
12	0	0	0	0	303
13	-1	+1	+1	-1	292
14	0	0	0	0	304
15	-1	-1	-1	-1	278
16	-1	-1	+1	-1	289
17	0	0	0	0	302
18	0	-2	0	0	287
19	0	0	+2	0	288
20	+1	-1	+1	-1	281
21	-1	-1	-1	+1	285
22	+2	0	0	0	295
23	+1	+1	-1	-1	296
24	+1	-1	-1	+1	295
25	+1	+1	+1	+1	297
26	+1	-1	-1	-1	283
27	0	0	0	-2	296
28	+1	+1	+1	-1	296
29	0	0	0	0	300
30	0	+2	0	0	293

Std. Dev. 1.52; R-Squared 0.9812; Mean 292.02; Adj R-Squared 0.9680; C.V. % 0.52; Pred R-squared 0.9449 ; PRESS 114.64; Adeq Precision 26.638

Coefficient estimates in the reduced quadratic model indicated that all the factors X_8, X_{19}, X_{21} and X_{23} had a positive effect on pectinase yield. The interactions between the factors X_8X_{19}, X_8X_{23} had positive effects whereas X_8X_{21} and $X_{19}X_{23}$ had a negative effect on enzyme yields. The larger t- test values of factor X_8 indicates a higher positive effect of the variable on the response.

The graphical representation of the regression equation is presented in the form of contour graphs indicating the interactions between two factors for the optimization of conditions for Pectinase production. The contour graph obtained as a function of tryptone concentration versus SDS concentration indicated that pectinase production increased with the increase of both tryptone and SDS but at higher concentration of both, the enzyme productivity decreased. The maximum production of pectinase corresponding to 304.14 IU/gds was obtained in the wheat bran based optimized medium where the concentrations of supplemented tryptone and SDS were 377.98 mg and 0.88 mg respectively (Fig. 1) while NH_4Cl and NaCl were held at 0, 0 coded levels equivalent to 2 and 2 mg respectively. The contour graph obtained as a function of tryptone concentration versus NH_4Cl concentration showed that the enzyme productivity continue to increase with the increase in the concentration of both the parameters. However, the interactive effect of both the parameters showed a downfall in the enzyme yield after a certain point. The maximum productivity of 303.45 IU/gds occurred at a point where the concentration of tryptone was 345.69 mg and that of NH_4Cl was 2.0 mg with SDS and NaCl held at 0, 0 coded levels equivalent to 0.80 and 2 mg respectively (Fig. 1). Fig. 1 shows the effect of Tryptone and NaCl on pectinase production. Increase in the concentration of both Tryptone and NaCl promoted the pectinase productivity up to a certain level. Thereafter further increase in the concentration of both produced a slight enhancement in the yields.

Maximum productivity corresponding to 305.11 IU/gds was obtained at 418 mg and 2.74 mg of tryptone and NaCl respectively while SDS and NH_4Cl, were held at 0,0 coded levels equivalent to 0.80 and 2 mg respectively. Fig. 1 shows the effect of SDS and NaCl on pectinase production. Increase in the concentration of both SDS and NaCl led to the gradual increase in the productivity, but at higher levels the mutualistic effect of both parameters led to a decline in the yields after a certain value. The maximum productivity corresponding to 302.08 IU/gds was obtained at 0.63 mg and 2.97 mg concentration of SDS and NaCl respectively with Tryptone and NH_4Cl held at 0,0 coded levels equivalent to 300 and 2 mg respectively.

To evaluate the accuracy of statistical model of response surface methodology design for pectinase production, attempts were made to maximize the yields using significant variables. Numerical optimization for pectinase production attempted with Design Expert using X_8 (Tryptone 381.10 mg), X_{19} (SDS 0.81 mg), X_{21} (NH_4Cl, 2.0 mg), X_{23} (NaCl, 2.2 mg), inoculated with 2.5 ml of fungal spore suspension having 2.8×10^7 spores/ml, incubated at 30°C in stationary state for 72 h in 5g wheat bran based medium containing urea, ammonium sulphate, peptone, yeast extract, meat extract, soyabean meal, calcium chloride, magnesium sulphate, cobalt chloride, zinc sulphate, iron sulphate, water, manganese chloride, malt extract, incubation time, manganese sulphate at the levels of .60 mg, .65 mg, 81.42 mg, 10.28 mg, 99.97 mg, 96.26 mg, 1.06 mg, .05 mg, .002 mg, .006 mg, .025 mg, 8.85 ml, .006 mg, 74.05 mg, 3 days, .58 mg predicted the yield of 305 IU/gds. To validate the optimum concentrations, an experiment with the above specified conditions was performed and the result was 300 IU/gds which is quite close to the predicted value, hence validating that the model chosen for optimization was correct.

TABLE IV. STATISTICAL ANALYSIS OF CENTRAL COMPOSITE DESIGN SHOWING SUM OF SQUARES, F-VALUE, p-VALUE, COEFFICIENT ESTIMATE, STANDARD ERROR, t-TEST VALUE, CONFIDENCE LEVEL FOR EACH VARIABLE AFFECTING PECTINASE ACTIVITY AFTER BACKWARD ELIMINATION REGRESSION ANALYSIS

Source	Sum of squares	F-value	P -value	Coefficient estimate	Standard error	t- test	Confidene level	
Model	2041.35	74.05	< 0.0001	302.50	0.62	487.90	99.99	Significant
X_8-Tryptone	318.21	138.52	< 0.0001	3.64	0.31	11.74	99.99	
X_{19}-SDS	49.57	21.58	0.0002	1.44	0.31	4.64	99.98	
X_{21}-NH_4Cl	140.60	61.20	< 0.0001	2.42	0.31	7.80	99.99	
X_{23}-NaCl	53.73	23.39	0.0002	1.50	0.31	4.83	99.98	
X_8X_{19}	95.01	41.36	< 0.0001	2.44	0.38	6.42	99.99	
X_8X_{21}	91.25	39.72	< 0.0001	-2.39	0.38	-6.28	99.99	
X_8X_{23}	83.22	36.23	< 0.0001	2.28	0.38	6.00	99.99	
$X_{19}X_{23}$	167.77	73.03	< 0.0001	-3.24	0.38	-8.52	99.99	
X_8^2	338.30	147.26	< 0.0001	-3.51	0.29	-12.10	99.99	
X_{19}^2	278.59	121.27	< 0.0001	-3.19	0.29	-11.00	99.99	
X_{21}^2	695.89	302.92	< 0.0001	-5.04	0.29	-17.37	99.99	
X_{23}^2	51.44	22.39	0.0002	-1.37	0.29	-4.72	99.98	

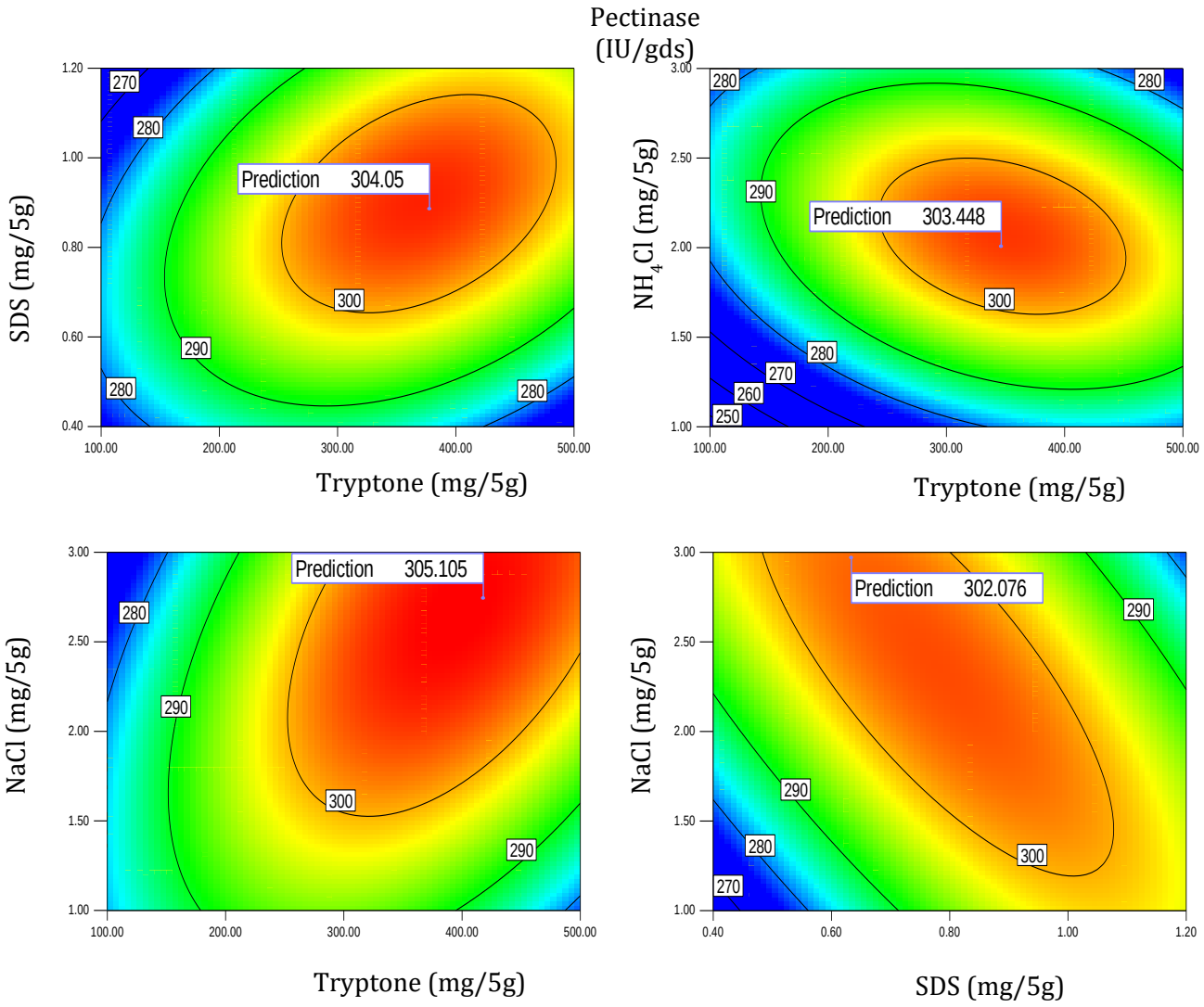

Fig. 1. *Contour graphs representing pectinase yield (IU/gds) from solid state culture of Aspergillus niger C-5 on wheat bran as affected by Tryptone and SDS, Tryptone and NH_4Cl, Tryptone and NaCl, SDS and NaCl concentration in quadratic model of RSM.*

The pectinase yield of 101±3.03 IU/g obtained in the present study on simple wheat bran based medium is quite appreciable as compared to the already published reports. A comparison of the yields of fungal pectinase studied in the present work with already published reports is depicted in Table V. Application of combinatorial approach of statistical tools in the present study proved to be quite useful in augmenting the productivity with enzyme yields reaching 304.14 IU/g on wheat bran based medium leading to 3.17 fold increase in the productivity as compared to under un-optimized conditions. This approach of enhancing the enzyme yield by developing a low cost culture medium will certainly help reduce the cost of overall production of pectinases that are also an important class of enzymes from the industrial point of view.

TABLE V. COMPARISON OF PECTINASE PRODUCTIVITY FROM *A. niger* C-5 WITH THE YIELDS OF SOLID STATE CULTURES OF OTHER FUNGAL STRAINS

Microorganism	Pectinase (IU/gds)	Ref.
Aspergillus niger IM-6	85.54	[2]
Rhodotorulla sp.	82.95	[38]
Rhodotorulla sp.	16.12	[38]
Mucor mucorales	46.05	[38]
A. niger LFP-1	8.90	[39]
A. oryzae	139.56	[40]
Penicillium citrinum	40.95	[41]
A. niger URM 4645	31.35	[42]
Trichoderma reesei	499.90	[43]
A. niger	2925.00	[44]
A. terreus NCFT 4269	6500.00	[6]
A. niger C-5	304.14	Present study

C. Partial purification of pectinase produced by Aspergillus niger C-5

In-house produced crude enzyme preparations in addition to desired enzyme, may contain several undesirable metabolites of the microorganism. Purification is the series of processes aspired to isolate single type of protein from a complex mixture having a desired property. In the present study also, the enzyme preparation was concentrated and partially purified by means of ammonium sulphate precipitation to characterize the kinetic properties of enzyme. The total activity of enzyme was found to be 4.65 IU/ml; with a specific activity of 0.20 IU/mg of protein. The concentration and partial purification of the in-house produced enzyme preparation led to an increase in the specific activity corresponding to 3.85 from initial value of 0.20 IU/mg. Highest specific activity was observed in 80% fraction which might be due to the maximum removal of proteinaceous impurities from the crude sample. The protein content of the sample decreased from 23 mg/ml in the crude extract to 5.12 mg/ml in the 80% saturated ammonium sulphate fraction. The enzyme was purified 19.05 folds with a lower recovery of 42.43. Several reports exist where ammonium sulphate precipitation has been employed to partially purify and concentrate various industrial enzymes [1,6,44]. An increase in the specific activities and purification folds have also been observed for partially purified enzyme preparations. In a study by [45] partial purification was carried out for pectin methyl esterase (PME). They found that as the concentration of ammonium sulphate was increased from 0 to 80%, the PME activity increased from 8.25 to 21.50 IU/g. The protein content in the sample decreased from 62 mg/ml in crude extract to 21 mg/ml in 80% ammonium sulphate fraction. Similar results have been reported earlier by [46] where they reported a 2.4 fold purification of PME from apple fruit juice using ammonium sulphate fractionation.

D. Kinetic characterization of partially purified enzyme from Aspergillus niger C-5

The temperature versus activity profile revealed that the enzyme preparation was active over a wider range of temperature from 30°C to 100°C. At a temperature of 30 and 40°C, the enzyme showed a relative activity of 60%. On further increasing the temperature to 50°C, it was found that the activity increased and at this temperature, maximum activity of enzymes was observed. Further increase in assay temperature to 60°C led to a slight decrease in the activity relative to the maximum activity which was observed at 50°C which was taken as control (Fig. 2). When the temperature was raised to 80 and 90˚C, relative activity corresponding to 38% and 17% were obtained respectively compared to maximum activity. Even at 100˚C, the enzyme preparation was still active exhibiting a relative activity of 15%.

It is generally desirable that industrial enzymes should be active at high temperatures to economize the process; therefore, there has been a continued research for the isolation of more thermophilic and thermostable enzymes [47]. The enzymes in the present study prove to be thermophillic in nature revealing appreciable activity even at higher temperatures of 80, 90 and 100°C. This feature makes the enzyme preparation a potential candidate to be used in a wide range of biotechnological processes where higher temperatures are desirable to achieve optimal results. The optimum temperature of the purified polygalacturonase from *R. pusillus* was recorded as 55°C [48] and that from *Aspergillus niger* grown on citrus peel was found to be 50°C [44]. De Andrade [49] reported the optimum temperature for polygalacturonase enzyme between 60–70°C. Optimum temperature for pectinase by *Rhodotorulla* spp was reported to be at 35°C while that of *Mucor mucorales* was 45°C [38]

The data on the effect of pH on the activity of pectinase is depicted in Fig. 2. From the results, it was deduced that pH activity profile of partially purified pectinase showed a broader range of pH for its activity. The relative activity of pectinase was found to be maximum at pH 4.0 (100%) and at pH 5.0 (97%) while the activity decreased with the further increase in pH value. The relative activity decreased to 90, 80, 50, 40 and 30% compared to the maximum relative activity when pH of the enzyme preparation was increased from 6.0 to 10.0 respectively. This showed that the enzyme preparation from *Aspergillus niger* C-5 works well over a wider range of pH optimas ranging from 3.0 – 10.0 without much loss of activity. This feature of being active at a broader pH ranges makes the enzyme preparation a potential candidate to be exploited for use over wider range of biotechnological processes. Our results are in accordance with the results of Meena et al.,[50] who reported pH of 4.5 as optimum for maximum pectinase activity. In support of above obtained results many workers reported optimum pH for polygalacturonase from various *Aspergillus* sp. to be in the range of 5.0-5.5 [48] while polygalacturonase from *Sporotrichum thermophile* [51] has been reported to be active at pH of 7.0.

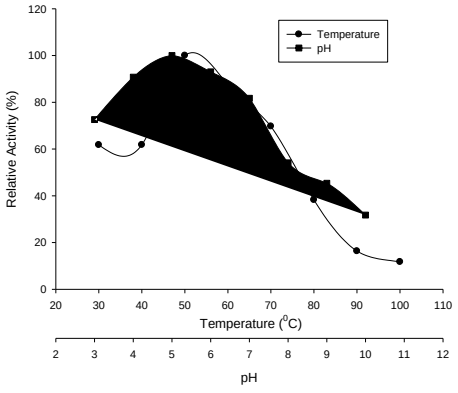

Fig. 2. *Temperature and pH activity profiles of pectinase produced by Aspergillus niger C-5.*

E. Thermostability profiles

The enzyme stability is crucial factor in the application of enzymes in any industrial processes. Enhanced stability and maintenance of a desired level of activity over a long period are two important points to be considered for the selection and design of enzymes [52]. The thermostability profile of enzyme preparation at 50°C is depicted in Fig. 3. At this temperature which was also the optimum temperature of the enzyme preparation, it was found that the enzyme retained residual activity of 6 % even after 216 h of incubation period. The enzyme showed a half life ($T_{1/2}$) of more than 72 h and retained 25 % of the residual activity even after 120 h of incubation. At 60°C, the half life was found to be 72 h and was reduced to 75 h at 70°C. At 80°C, the enzyme preparation lost its stability completely after 8 h of incubation with half life of 4 h. On further incubation, it was found that the enzyme preparation became inactive and rapidly lost its complete activity after 12 h of incubation. At 90°C, the enzyme preparation was found to be active for only upto 2 h of incubation. With further incubation at this temperature, denaturation of enzyme took place which completely deactivated the enzyme leading to a loss of activity. At still higher temperature of 100°C, the enzyme became inactive only after half hour of incubation (Fig. 3). The presence of thermostabilizer did not have any significant effect on the stability enzyme, hence eliminating the need of a stabilizing agent in maintaining the thermostability of enzymes. Hence, enzyme showed a wider thermostability profile which makes it a suitable candidate for use in agro-industrial conversion processes.

Thermostability of enzyme as recorded in the present study is much high than most of the already published reports. The optimum temperature for the stability of the polygalacturonase from *R. pusillus* was recorded as 55℃ and the enzyme was stable at 50℃ [48]. After two hours, the PG activity was only 8.2 and 9.30% at 60 and 70°C, respectively, and then the enzyme became suddenly inactive. From 80°C onwards the enzyme activity was lost during the first hour itself which is similar to the results obtained in the present study. Thakur et al. [53] reported stability of polygalactouranase from *Mucor circinelloides* for up to 4 h at 42℃ and 2 h at 60°C. Polygalacturonase from *M. flavus* has been reported to be stable up to 40℃ for 4 h [54]. De andrade et al., [49] reported the stability of polygalacturonase between 60–70℃ and the enzyme retained about 82 and 63% of its activity at 60 and 70℃, respectively, after 2 h of incubation. Our results strongly suggest that the enzyme preparation is thermophillic as well as thermostable in nature, possessing a wider temperature range for maximum activity of the enzyme, maintaining more than 15% of the residual activity even after 216 h of incubation at the temperature of 50 and 60°C, that is considered optimum for carrying out enzymatic hydrolysis of ligncellulosic substrates. Hence, the in-house produced enzyme preparation from *Aspergillus niger* C-5 can be considered ideal for use in many biotechnological applications.

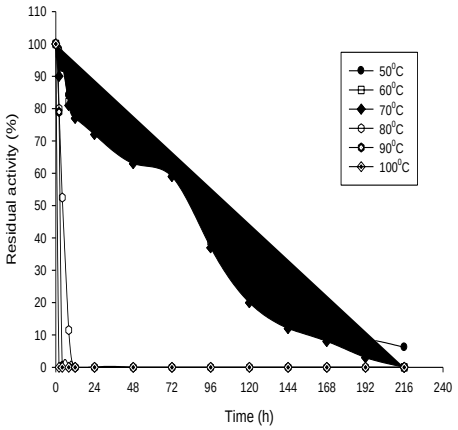

Fig. 3. *Thermostability profile of enzyme preparation from Aspergillus niger C-5 at temperature of 50, 60, 70, 80, 90 and 100°C.*

F. pH stability profiles

The pH stability profile of enzyme also showed a broader range of pH. The higher half life ($t_{1/2}$) of 146 h was observed at pH 3.0 (Fig. 4). At pH 4.0, which was also the optimum pH range, the enzyme was stable for more than 216 h revealing the residual activities of 33.45% after 216 h of incubation. As the pH was raised to 5.0, residual activity showed a slight decline, but there was not much effect on the stability as evident from the half lives of 120 h. With further increase in the pH to 6.0, the residual enzyme activities began to decline but the enzyme preparation was quite stable even at this pH exhibiting residual activities of 23.90 %. At pH 7.0, the half lives of 72 h was observed. On further increasing the pH to 8.0 and 9.0, the enzyme started losing its stability with prolonged incubation. Lower half life values corresponding to 50, 30 h was obtained at pH 8.0 and 9.0 respectively. Furthermore, investigating the enzyme stability profiles at a higher pH of 10.0, showed that at this pH, the enzyme suddenly lost its activity and became inactive over this pH range retaining a minimal residual activities. The results obtained in the present study confirm the broader pH stability profiles of the enzyme, making it a potential candidate to be employed in wide range of industrial processes.

G. Effect of various metal salts and EDTA on enzyme activity

The effect of various metal ions and chelating agents on enzyme preparation from *Aspergillus niger* C-5 did not had much influence on pectinase activity. The results depicted in Fig. 5 shows that pectinase do not require any supplementation of metal ions to promote its activity.

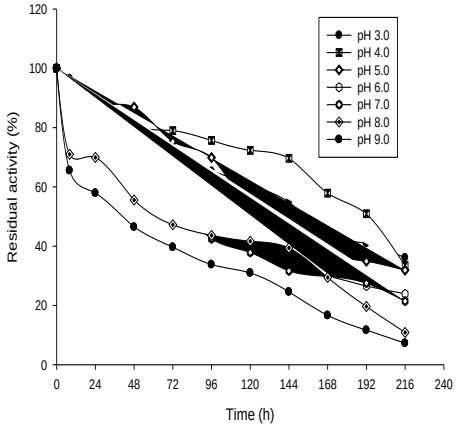

Fig. 4. *pH stability profile of enzyme preparation from Aspergillus niger C-5 at various pH ranges from 3.0-9.0.*

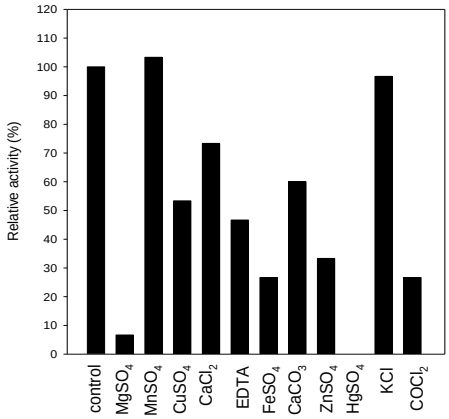

Fig. 5. *Effect of various metal ions and chelating agents on pectinase obtained from solid state cultures of Aspergillus niger C-5.*

The results obtained in the present study are in accordance to the findings of various published reports where various metal ions have been tested to act as activators and inhibitors of the activity of various enzymes. In a study, pectinase showed a decrease in enzyme activity in presence of Mn^{+2}, Co^{+2}, and Mg^{+2}, Fe^{+3}, Zn^{+2}. Thus, the enzyme did not require any metal ions to express its activity. Mercuric chloride was the strongest inhibitor of enzyme at 1mM concentration followed by ascorbic acid [53]. A considerable decrease (>80% inhibition) in activity was observed in the presence of Co+, Cu^{2+}, and Mn^{2+}. These ions are already known in the literature to be inhibitors for several microbial enzymes [55-57].

H. Determination of Michalies-Menten (K_m) constant and V_{max}

The Michalies-Menten constant (K_m) is defined as the affinity of an enzyme for a particular substrate, a low K_m value, represents a high affinity and a high K_m implies a low affinity of the enzyme for its substrate. This concept of affinity of an enzyme for its substrate is useful in applied enzymology in determining the most useful substrate concentration ranges for faster conversion rates. In the present study, enzyme activity was measured under standard assay conditions as described earlier and enzyme activity (IU/ml) against concentration of substrate (mg) was plotted, which yielded a hyperbolic curve as shown in Fig. 6. The Line Weaver – Burk plot was used to determine the V_{max} and K_m of the enzyme. V_{max} of pectinase, was found to be 18.22 IU/ml/min with a K_m value 1.36 mg/ml. The K_m values of the enzyme show a wider range, but for most of the industrially used enzymes, the values lie in the range of 10^{-1}- 10^{-5} M when acting on biotechnologically important substrates [58]. The K_m and V_{max} values of PGases of *Rhizomucor pusillus* were estimated as 0.22 mg/ml and 4.34 IU/ml, respectively, by plotting the Lineweaver Burk plot [48]. Saad et al., [59] reported a K_m of 1.88 mg/ml and V_{max} of 0.045 mole/ml/min for pectinase from *Mucor rouxii*. Michaelis Menten value for pectinase by *Rhodotorulla* spp was 3.0 mg/ml while the maximum velocity (V_{max}) was 0.043364 IU/mg/min [38].

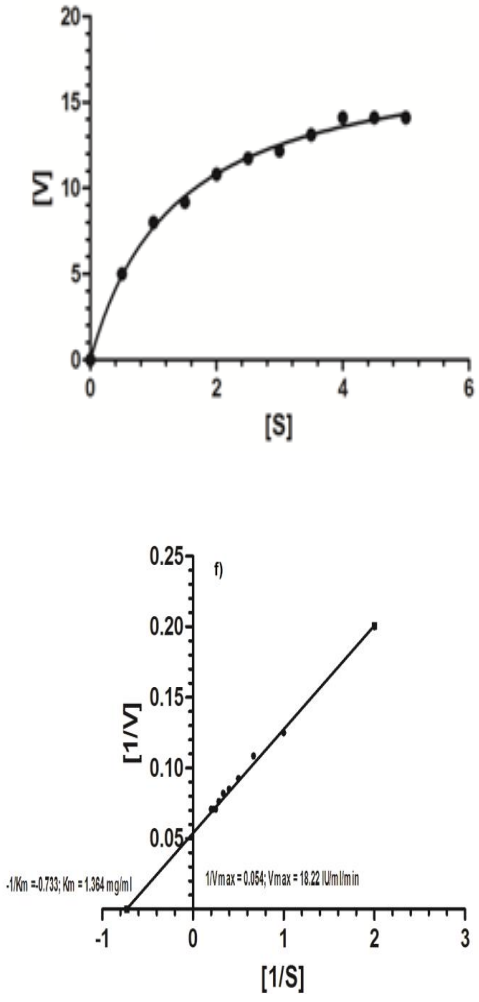

Fig. 6. Substrate concentration versus rate of reaction and Lineweaver Burk plot representing Km and Vmax values for Pectinase produced from *Aspergillus niger* C-5.

CONCLUSION

Successful attempts have been made in the present study to lower down the production cost of pectinase, an industrially important enzyme, by optimizing the nutritional and cultural parameters affecting the solid state fermentation of wheat bran employing a combinatorial statistical approach of Plackett-Burmann and response surface methodology designs. This led to appreciable fine tuning of productivity revealing a higher yield of 304 IU/g with 3.17 fold increase as compared to the yield of 101 IU/g observed under unoptimized conditions. Further, the enzyme preparation was active over broader temperature (50-90°C) and pH ranges (3.0-9.0), thus making this preparation a potential candidate for a wide range of industrial applications.

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