Optimization of reaction parameters for the degumming of buel (Grewia Optiva) bast fibres using alkaline pectinase with the RSM

Thakur Pitambri[1], Singh Abhishek Kumar[2] and Mukherjee Gunjan[1*]
1. University Institute of Biotechnology, Chandigarh University, Mohali, Punjab, 140413, INDIA
2. Department of Biotechnology Engineering, University Institutes of Engineering, Chandigarh University, Mohali, Punjab, 140413, INDIA
*gunjanmukherjee@gmail.com

Abstract

In the present work, response surface methodology (RSM) and central composite design (CCD) were used to optimize the reaction parameters for the synthesis of galacturonic acid from beul (Grewia Optiva) bast fibres catalyst with Bacillus tropics alkaline pectinase by degumming process. Central composite design was used in response surface methodology, for preparing a second order model for the response variable. Using a 2^4-factorial experimental design, the effects of pH between 7 to 11, temperature between 27 to 47 ^oC, incubation time of 1 to 2.4 hr and pectinase concentration 0.5 to 2.5g/l were studied.

The highest reaction product especially galacturonic acid was 487mmole/g at 42^oC, reaction pH 8, pectinase concentration 2g/L and incubation time 1.25hr. Present findings have potential implications for replacing the chemical degumming process by green degumming process in textile industry.

Keywords: Degumming, *Bacillus tropicus*, Alkaline pectinase, Galacturonic acid, Response surface methodology.

Introduction

Beul fibers are excellent natural fibers, but beul fibers contain approx 35% to 40% beul gum which contain pectin and hemicelluloses. In the regular degumming process, gum is removed with hot alkaline solution containing 12-20% NaOH solution[3]. This process is causing serious environmental problems along with high energy consumption. According to Said et al[17] degumming is the process of the removal of non-cellulosic massive gluey material of the plant fibers for its important utilization in textile making. Thus, enzyme has been proposed to solve this problem by reducing consumption of chemicals and energy[15]. Recently many textile chemists explored the probability of substitution of the traditional alkaline degumming process with environment friendly enzyme process.

Many enzymes such as pectinases[1,3,11,15,17], cellulases[8,20] and cutinases[5] were screened for their application in retting and degumming processes. Pectinases proved to be most effective due to degradation of pectic substances which are attached to cellulose and hydrophobic waxy materials as cement[20]. Breakdown of pectins with alkaline pectinase will make fibers more hydrophilic without worsening fiber. Pectic substances are a mixture of polysaccharides containing α-D-galacturonic acid units[10].

Breakdown of pectin is done by various enzymes due to their different reaction mechanism and specificities. Pectin lyase (EC 4.2.2.10) and pectate lyase cleave α-1, 4-glycosidic bond polygalacturonase (EC 3.2.15) decompose same linkage in pectic acid and pectin esterases (EC 3.1.11.1) degrade methyl ester bonds in pectin. In general, all pectinases are divided into two major categories: acidic pectinases[4, 11, 20] and alkaline pectinases[6,7]. At present most used industrial pectinases is alkaline pectinase giving activity at most 3000 Alkaline Pectinase standard unit (APSU) known as Bioprep 3000 L from Novozymes. But due to its high cost and inferior quality of fabrics, its application is restricted in China[1,21].

At present we have flourishingly optimized the alkaline pectinase production from *Bacillus tropicus* with better degrading capacity to break beul fibers pectins. For the optimization process, "change-one-factor-at-a-time" is the conventional method in which one independent variable is varied while all others are fixed at a specific level. But due to existence of factor interactions, it is not a definite method and may bulge to uncertain and less authentic results[14].

Response surface methodology (RSM) is a widely used statistical and mathematical technique for used for the purpose of designing experiments and optimize various processes parameters. For dealing with multifactor experiments RSM, CCD was implemented to measure a second-degree polynomial model to optimize the response variables of the interest (reaction time and reaction temperature etc) and finally linear regression was used to find the results. RSM helps to decrease the number of experimental trials required to evaluate multiple parameters and their interactions. RSM helps to predict optimal value from the estimated surface shape if it is simple hill. The estimated surface is complicated or away from the experiment region, the surface shape can be evaluated to confirm direction to which new experiments should function[13].

In recent past, response surface methodology has been applied strongly in diverse biotechnological facet[14], but no work is reported on the optimization of beul fiber degumming and retting processing. Enzymatic degumming

process has certain advantages over conventional chemical degumming process such as environment friendly operation, limited fiber damage and easy quality control[23]. The aim of this work was to optimize the process parameters for synthesis of galacturonic acid from beul (*Grewia Optiva*) bast fibres in the presence of *Bacillus tropics* alkaline pectinase by using RSM and CCD.

Material and Methods

Materials: *Bacillus tropicus*, Bihul (*Grewia optiva*) fibres obtained from Naggar, Distt Kullu, Himachal Pradesh, India. Glucose, yeast extract, magnesium sulfate heptahydrate, corn steep liquor, calcium carbonate, tris hydrochloride and phosphate phosphate were purchased from Glaxo India Ltd., Mumbai, India. Ammonium sulphate, Sephadex G-75, Sodium dodecyl sulphate, Coomassie brilliant blue r-250 and Cellulose ion exchange chromatograph were bought from Sigma Chemical Louis, USA. Gauze-filter was obtained from Whatman International Ltd., UK. D-galactose purchased from Loba Chemie, Mumbai, India. Sodium hydroxide was purchased from Merck India Ltd., Mumbai, India.

Alkaline pectinase production: Alkaline pectinase produced by the isolate *Bacillus tropicus* was used for the present study. 500 ml Erlenmeyer flask with 50 ml of medium containing yeast extract 1 %(w/v) and pectin 0.25 %(w/v) was used for fermentation and 250 ml of the flask containing 25 ml of medium was used to prepare inoculums and both were incubated at 200 rpm for 24 hrs and 12 hrs respectively at 37° C. After 24 hrs to remove miscellaneous proteins, the contents of the flasks were gauze-filtered after checking pectinase maximum activity. Ammonium sulphate upto 60% saturation was added to remove proteins with mild agitation. After centrifugation at 10000×g for 10 minutes to remove supernatant and precipitates were stored in 20 mM phosphate buffer (pH 8). Then dialysis was performed up to 24 hrs with same buffer.

For further purification, cellulose ion exchange chromatograph was utilized for ion exchange chromatography followed by Sephadex G-75 gel filtration chromatography. Further SDS-PAGE (Sodium dodecyl sulphate - polyacrylamide gel electrophoresis) was performed by using 12.0% gel containing standard proteins ranging from 15-98 KDa molecular weights and stained with coomassie brilliant blue dye. SDS-PAGE showed the single band corresponding to molecular weight of 44 KDa. Enzyme was stored at 4°C for further use.

Alkaline Pectinase Assay: 3, 5-Dinitrosalicylic acid (DNS) method given by Miller[12] with slight modifications was employed for the determination of purified alkaline pectinase activity by the measurement of the amount of galacturonic acid production. The reaction mixture was incubated at 37°C for 10 minutes. In this experiment, standard graph was prepared by using mono-D-galacturonic acid and 1.0% citrus pectin was used as substrate. Under the standard assay conditions, one unit of enzyme activity was defined as 1µmol/ml of product formed from the pectins on beul fibers per minutes by using alkaline pectinase.

Degumming of Beul (*Grewia optiva*) bast fibres

Bacterial Treatment: By inoculating autoclaved 1.0 g of decorticated beul fibers with 2% (w/v) bacterial culture, 2 ×102 CPU, 1 ml in the final mixture was obtained. For optimum degumming, different moisture contents (80, 85, 90 and 95%) were adjusted in the beul fibres. Samples were withdrawn periodically up to 2.40 hrs to assess amount of galacturonic acid released. Final pH and dry weight of the fibers were assessed and galacturonic acid content was estimated by DNS method. The treated Beul fibers were air dried and all the experiments were repeated.

Optimization of enzyme dose and time for degumming of Beul fibres: Baract-Pereira method was applied for enzymatic and chemical treatment of bihul fibers[2]. The pectinase level optimization for fiber treatment was implemented by treating 1 gm of bihul fibers with 10 ml of 0.01 M Tris-HCl buffer pH 9.0 with different levels of 100-500 U/ml. The reaction time, temperature, pH and concentration of alkaline pectinase were varied according to the experimental design (Table 2). 0.1 ml sample fractions were taken periodically from the reaction mixture which was on the continuous stirring mode at 500 rpm. For qualitative and quantitative analysis, supernatant was collected after centrifugation at 3000 g for 5 minutes and enzyme was separated.

Chemical Treatment: Sharma's[16] method was applied for the chemical treatment of the Bihul fibers by incubating 2gm of decorticated beul with 10 ml NaOH (2% w/v) solution under static conditions for 96 h in 90° C set water bath. For assessing, degumming samples were periodically withdrawn.

Experimental Design: The experimental design techniques were used for optimizing the process parameters for maximum synthesis of galacturonic acid studied with RSM and CCD. This system is optimizing the effective variables with a minimum number of experiments[19]. The four independent variables are studied at five levels (−2, −1, 0, +1, +2) (Table 1). A five- level and four-factor central composite rotatable design (CCRD) requiring a total of 30 runs was used to determine the experimental data. Reaction pH (X_1), temperature (X_2,°C), incubation time (X_3, hr) and pectinase concentration (X_4, g/l) are selected as independent variables and yield of galacturonic acid (mmole/g) as dependent variables (Table 2).

Statistical analysis: The effects of process parameters on response were analyzed by the RSM procedure to fit the second-order polynomial equation[19]. Statistica (StatSoft, Inc., USA) software was used to evaluate the experimental data. The basis form of the model equation is:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (1)$$

where Y represents the predicted response; β_o is the mean effect, β_i, β_{ii}, β_{ij} are the regression coefficients for linear, quadratic and cross-product coefficients and X_i and X_j represent the coded independent variables.

Analytical assessment of degumming of fibers: The assessment of the degumming was done by considering the amount of galacturonic acid released due to hydrolysis of pectic substances and finding the reduced weight of the fibers. For achieving these samples were withdrawn up to 24 hrs from bacterial and chemical treatment process.

Table 1
Experimental design of four process variables in terms of coded values

Variables				
Coded levels	**pH (X_1)**	**Temperature (ºC) (X_2)**	**Incubation Time (Hrs) (X_3)**	**Enzyme Concentration (g/l) (X_4)**
-2	7	27	1	0.5
-1	8	32	1.25	1
0	9	37	1.50	1.5
+1	10	42	2.15	2
+2	11	47	2.40	2.5

Table 2
Experimental design matrix and the experimental and predicted responses of the CCD design.

Run	pH (X_1)	Temperature (ºC) (X_2)	Incubation Time (hr) (X_3)	Enzyme Concentration (g/l) (X_4)	Yield (mmole/g)	
					Experimental	Predicted
1	8	32	1.25	2.0	460	461
2	8	32	2.15	1.0	311	310
3	8	42	1.25	1.0	352	353
4	8	42	2.15	2.0	458	459
5	10	32	1.25	1.0	300	296
6	10	32	2.15	2.0	412	411
7	10	42	1.25	2.0	416	414
8	10	42	2.15	1.0	303	302
9	9	37	1.5	1.5	450	448
10	9	37	1.5	1.5	445	448
11	8	32	1.25	1.0	350	354
12	8	32	2.15	2.0	423	421
13	8	42	1.25	2.0	485	487
14	8	42	2.15	1.0	319	320
15	10	32	1.25	2.0	402	399
16	10	32	2.15	1.0	302	303
17	10	42	1.25	1.0	285	283
18	10	42	2.15	2.0	440	437
19	9	37	1.5	1.5	449	448
20	9	37	1.5	1.5	447	448
21	7	37	1.5	1.5	430	427
22	11	37	1.5	1.5	320	325
23	9	27	1.5	1.5	412	412
24	9	47	1.5	1.5	433	432
25	9	37	0.6	1.5	391	391
26	9	37	2.4	1.5	413	412
27	9	37	1.5	0.5	184	183
28	9	37	1.5	2.5	422	423
29	9	37	1.5	1.5	448	448
30	9	37	1.5	1.5	447	448

Results and Discussion

Statistical analysis and model fitting: Response surface optimization is more appropriated model compared to the traditional single parameter optimization in that it saves reaction time, space and raw material. The levels of variables (pH, enzyme concentration, incubation time and temperature) and the effects on synthesis of galacturonic acid were determined by RSM. RSM using CCD consisting of 30 experiments was using different mixtures of variables. The results obtained from the CCD for synthesis of galacturonic acid are shown in table 1. From the statistical experimental design (Table 1), ANOVA and eq. (1), the second-degree polynomial response indicating the correlation between synthesis of galacturonic acid and reaction variables are shown in eq. (2):

$$Y = 447.97 - 19.71 X_1 + 6.42 X_2 - 5.09 X_3 + 60.60 X_4 - 18.14 X_1^2 - 6.26 X_2^2 - 11.49 X_3^2 - 36.14 X_4^2 - 2.87 X_1X_2 + 12.60 X_1X_3 - 0.87 X_1X_4 + 2.85 X_2X_3 + 6.87 X_2X_4 + 1.24 X_3X_4 \quad (2)$$

where *Y* is the galacturonic acid yield and X_1, X_2, X_3, X_4 and X_5 are the reaction pH, incubation time and temperature and enzyme concentration respectively. A quadratic regression model (Eq. 2) describing the influence of the reaction variables on synthesis of galacturonic acid was predicted. The reaction variables for galacturonic acid synthesis were impute to the independent variables of reaction temperature, pH, incubation time and enzyme concentration.

The statistically significant of the second order polynomial equation was calculated by the F-test analysis of variance. The analysis of variance (ANOVA) showed (Table 3) the significance of square term and first-order interaction terms of variables for synthesis of galacturonic acid. The experimental and expected results match practically well with high determination coefficient (R^2) as 0.98 for galacturonic acid yield. Thus, the model is acceptable for the estimation of such a reaction parameter[9].

The independent variables were more significant for larger *F* ratio and smaller P values[18]. The effects and interactions of variables were discussed from the Pareto chart shown in fig. 1. The bar length in the pareto chart is proportional to the absolute value of regression coefficient. A variable can be significant if its related bar crosses this vertical line.

From the Pareto chart (Fig. 1), linear coefficients term of reaction pH (X_1), temperature (X_2), incubation time (X_3) and enzyme concentration (X_4), quadratic coefficients term of reaction pH (X_1^2), temperature (X_2^2), incubation time (X_3^2) and enzyme concentration (X_4^2) and the first order interaction terms like X_1X_2, X_1X_3, X_2X_3 X_2X_4, X_3X_4 were significant ($p < 0.05$) factors for synthesis of galacturonic acid. The first order interaction terms like X_1X_4 were the statistically insignificant terms ($p > 0.05$) for galacturonic acid synthesis.

The effect and interaction of process parameters on the synthesis of galacturonic acid are shown in figures 2 and 3. figure 2 and 3 (a) show the three-dimensional surface and contour plots of effect of reaction pH and incubation time on galacturonic acid synthesis at fixed temperature (37°C), enzyme concentration (1.5 g/l). It can be seen from the plot that the yield of galacturonic acid increases with increase of reaction pH from 7 to 8, but yield decreased by further increasing of reaction pH. The effect of incubation time on the synthesis of galacturonic acid is significant. The yield increases with the incubation time increase from 1 to 1.25 hr, but the yield decreased by further increasing of incubation time. Wang et al[22] reported maximum bioscouring effect on cotton knitted fabrics with an alkaline pectinase at 1.25 hr reaction time.

Table 3
ANOVA for regression representing yield of galacturonic acid

Source of variance	Sum of squares	Degrees of reedom	Mean squares	F-ratio	P-value
X_1	9049.1	1	9049.08	3050.25	-
X_1^2	8976.6	1	8976.59	3025.82	-
X_2	960.4	1	960.39	323.73	0.000010
X_2^2	1071.4	1	1071.44	361.16	0.000007
X_3	629.4	1	629.44	212.17	0.000028
X_3^2	4088.4	1	4088.36	1378.10	-
X_4	85499.7	1	85499.65	28820.11	-
X_4^2	35623.5	1	35623.51	12007.93	-
X_1X_2	132.3	1	132.25	44.58	0.001139
X_1X_3	2711.5	1	2711.52	914.00	0.000001
X_1X_4	12.2	1	12.25	4.13	0.097856
X_2X_3	138.9	1	138.88	46.81	0.001018
X_2X_4	756.2	1	756.25	254.92	0.000018
X_3X_4	26.5	1	26.50	8.93	0.030487
Lack of fit	90.1	10	9.01	3.04	0.115955
Pure error	14.8	5	2.97		
Total	145364.7	29			

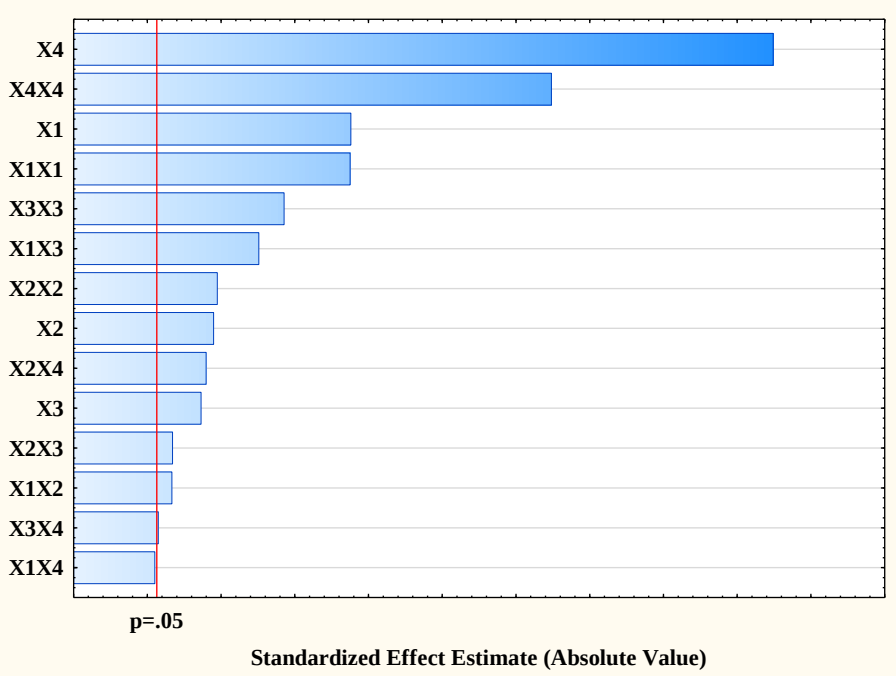

Fig. 1: Pareto chart of standardized effect estimate of reaction conversion.

(a) **(b)**

(c) **(d)**

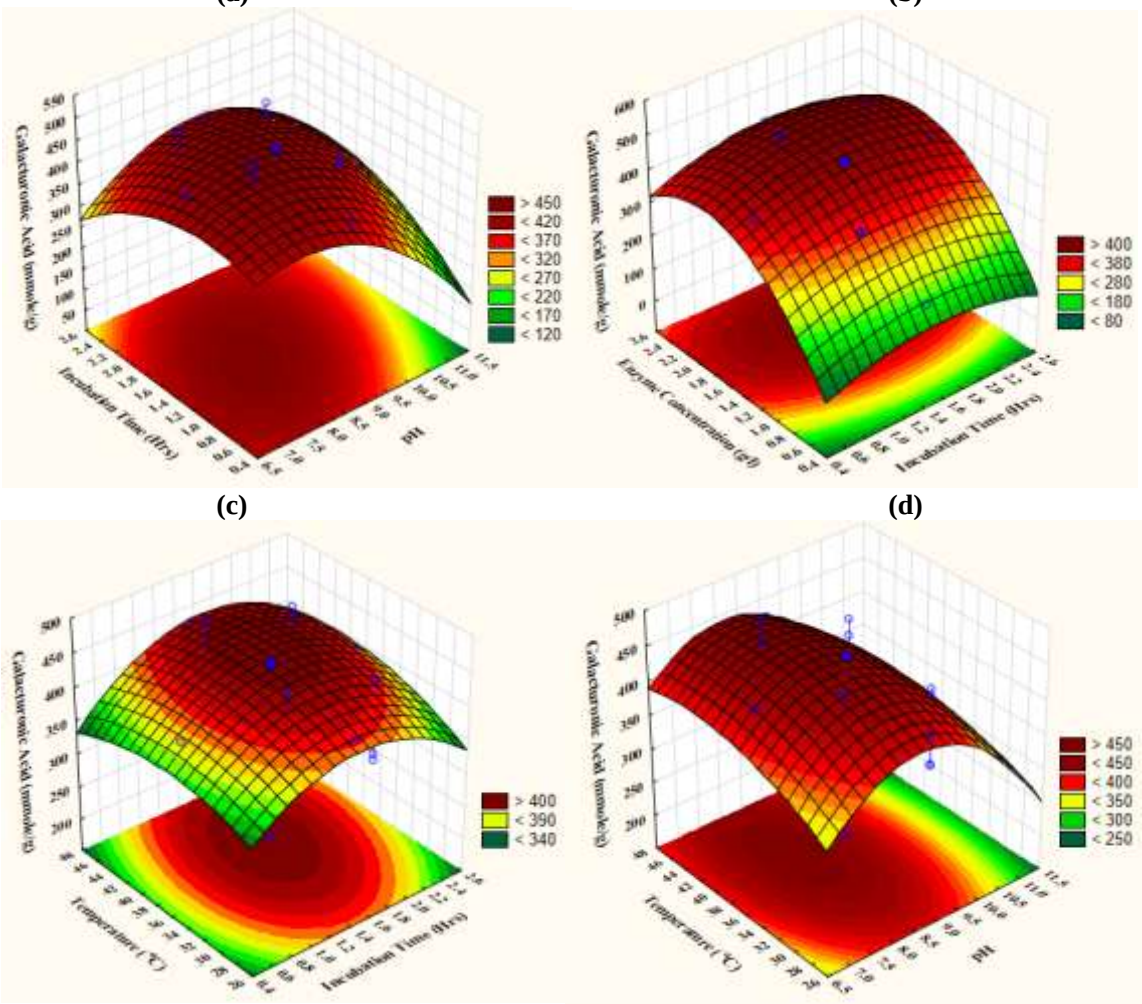

Fig. 2: Response surface plot (3-D) showing the relation between the yield of galacturonic acid and: (a) At varying reaction pH and incubation time (b) at varying incubation time and enzyme (pectinase) concentration (c) at varying incubation time and temperature and (d) at varying pH and reaction temperature.

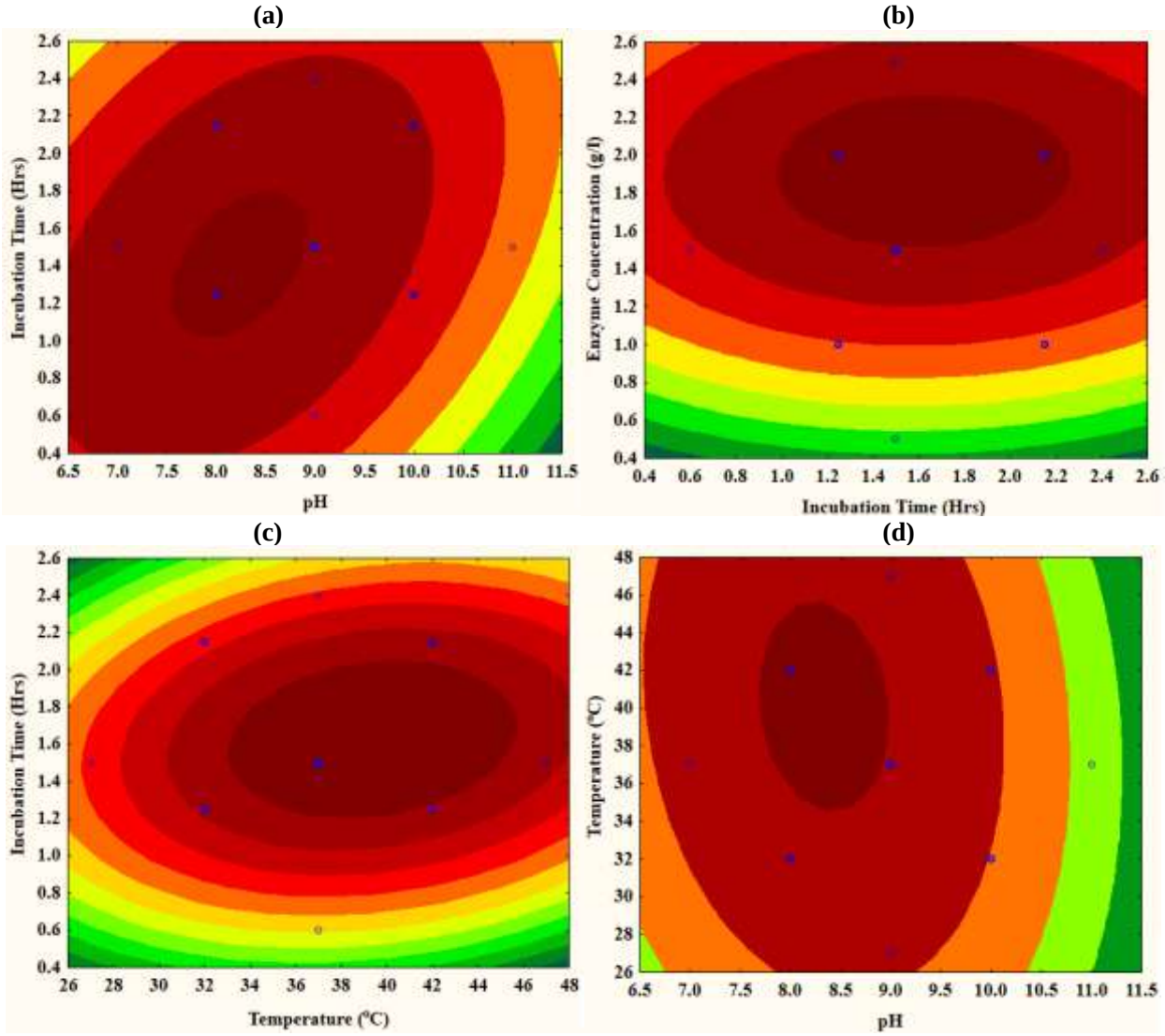

Fig. 3: Contour plot showing the effect of (a) the reaction pH and incubation time (b) incubation time and enzyme (pectinase) concentration (c) incubation time and temperature and (d) pH and reaction temperature on the galacturonic acid synthesis.

Figures 2 and 3 (b) show the three-dimensional surface and contour plots at different incubation time and enzyme concentration at fixed reaction pH (9), temperature (37ºC). The yield of galacturonic acid increased with the increase of enzyme concentration.

Figures 2 and 3 (c) show the three-dimensional surface and contour plots at varying incubation time and reaction temperature at fixed reaction pH (9) and enzyme concentration (1.5g/l). The effect and interaction between incubation time and reaction temperature were significant on galacturonic acid synthesis. As can be seen, the galacturonic acid yield increases with increasing the reaction temperature from 27 to 42 ºC and decreased rapidly above 42 ºC.

Figures 2 and 3 (d) show the three-dimensional surfaces and contour plots of effect of reaction pH and reaction temperature on the galacturonic acid synthesis. It can be seen that enhancing the reaction pH (7 to 8) and reaction temperature (27 to 42ºC) increased the yield of galacturonic acid, but a further increase in reaction pH and temperature lead to decrease of galacturonic acid content.

Validation of Regression model: Four random statistical parameters are selected to calculate the galacturonic acid yield by regression as in eq. 2. The CCD was used to find the exact values of the experimental conditions for the synthesis of galacturonic acid. From all experimental data, model predictions indicate good agreement with the experimental data. The optimal experimental and predicted galacturonic acid of 487mmole/g were obtained under the optimum conditions of pectinase concentration of 2g/L, reaction pH of 8 and incubation time of 1.25hr at 42ºC. These results show that the regression model was efficient to experimental and predicted conditions.

Conclusion

Bacillus tropicus alkaline pectinase was used as a catalyst in synthesis of galacturonic acid from beul (Grewia Optiva) bast fibres. RSM was used to optimize the reaction

parameters for the synthesis of galacturonic acid and second order response model was calculated. Reaction pH, incubation time, temperature and enzyme concentration were the factors affecting on the synthesis of galacturonic acid. The highest yield of galacturonic acid was 485mmole/g. The optimum reaction conditions for galacturonic acid synthesis were obtained as incubation time of 1.25hr, reaction pH of 8, alkaline pectinase concentration of 2g/l and temperature of 42ºC.

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