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Application of response surface methodology to the modeling of cellulase purification by solvent extraction

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ABSTRACT

Central composite design (CCD) concerning the purification of cellulase from the *Bacillus* sp. *JS*14 in a solvent extraction was established with Response surface methodology (RSM). Solvent concentration, pH, temperature and retention time were selected as process variables to evaluate the purification impact factor in solvent precipitation, including the purification fold and % recovery. An experimental space with 13 purification fold and 23 recovery percentage recovery is achieved through the optimized condition based on the model. The molecular weight of the purified enzyme was estimated to be 32.5 KDa. Optimum activity of purified enzyme was at pH and temperature 6.5˚C and 40˚C respectively. Enzyme showed maximum activity with carboxymethyl cellulose as substrate with compare to rice husk, wheat straw and sucrose. The purified cellulase activity was inhibited by Na^+, Cl^-, Mg^{2+} Tween 80 and EDTA.

Keywords: Cellulase; Purification; Solvent Extraction; Response Surface Methodology

1. INTRODUCTION

Cellulosic material is the most abundant renewable carbon source in the world [1,2]. Cellulose is possible substitutes for diminishing fossil energy resources and becoming increasingly important. With the shortage of petroleum fuels and air pollution due to the incomplete combustion of fossil fuel, there has been increasing worldwide interest in the production of bioethanol from lignocellulosic biomass. To utilize these materials and to avoid waste pollution, one of the most important approaches is to find appropriate cellulase enzyme to hydrolyze the lignocellulosic biomass to produce glucose, which is used for the production of ethanol, organic acids etc. Cellulase (E.C 3.2.1.4) refers to a class of enzymes that catalyze the hydrolysis of 1,4 β-D glycosidic linkages in cellulose are mainly produced by fungi, bacteria and protozoans [3] and have broad range of applications especially in animal feed, textile, waste water treatment, brewing and wine-making [4-7]. An important obstruction in the exploitation of cellulase is expensive purification effecting the overall cost of hydrolysis [8-11].

The problems in the purification of enzyme are an impenetrability in the development of economically feasible bioprocess. Impact of combinatorial interactions of different parameters for the solvent extraction of desired compound is abundant. Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of bioprocesses [12-14]. To illuminate the relationship among the recovery percentage and solvent concentration a statistical model is established by response surface methodology and validated with experimental data in this research paper. The present communication illustrate efficiency of statistical techniques for designing solvents extraction system for the purification of cellulase enzyme from the fermented broth with respect to the costly chromatography reliant purification matrixes. The results revealed the development of a practical criterion for solvent extraction of enzyme

2. MATERIALS AND METHODS

2.1. Microorganism and Enzyme Production

The *Bacillus* sp. *JS*14, isolated from soil in laboratory, was cultured in a medium containing (g/l) KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; CMC, 10.0; Yeast extract, 1.0; Peptone, 1.0; Na_2HPO_4, 2.5; $(NH_4)_2SO_4$, 1.0; pH 7.0. The culture was kept at agitation speed of 150 rpm and 37˚C. The supernatant was harvested after 36 h cultivation by centrifugation at 10000 g for 20 min and stored at 4˚C for further use.

2.2. Experimental Design and Statistical Analysis for Solvent Extraction of Cellulase Enzyme

Response surface methodology (RSM) involving a cen-

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tral composite design (CCD) and a second-order polynomial equation was employed to identify the relationship between four significant variables that influence cellulase extraction significantly. The central values (zero level) chosen for experimental design were; acetone (X_1), 50%; pH (X_2), 5.5; Temperatures, (X_3) 17˚C and incubation Time (X_4) 6.5 h in CCD I (**Table 1**). Different combination of variables was used according to the CCD design I (**Table 2**) for the determination of purification fold and % recovery.

Enzyme purification achieved by acetone was further extracted with ethanol solvent according to the CCD design II (**Tables 1** and **2**) .The Design expert 8.0.1 software, was used for regression and graphical analyses. The optimal concentrations of critical solvents were obtained by ridge analysis and contour plots. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA) for the determination of significant variables.

2.3. Validation of Model

The optimized conditions generated during response surface methodology implementation were validated by conducting extraction experiment on given optimal setting conditions. Study was carried out in triplicate to confirm the results.

2.4. Cellulase Assay and Protein Determination

The cellulase (CMCase) activity was assayed according to Stewart and Leatherwood (1976). Appropriately enzyme solution (0.5 ml) was added to 0.5 ml CMC (0.5% CMC dissolved in sodium acetate buffer 0.2 M, pH 5.0) and incubated at 60˚C for 30 minutes. The reaction was stopped by the addition of 3.0 ml of 3,5-dinitrosalicylic acid reagent [15] and A_{540} was measured in a Shimadzu UV-160A spectrophotometer. One international unit (IU) of enzyme activity was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1.0 µM glucose min^{-1} under assay conditions. The protein concentration of the crude and purified enzyme fractions was determined by the method of Lowry *et al.*, [16] using bovine serum albumin (BSA) as standard.

2.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% (w/v) acrylamide slab gel with 25 mM Tris for the determination of molecular weight, as described by Laemmli [17].

2.6. Optimum Temperature, pH and Thermal Stability of Purified Cellulase

Optimum temperature for activity enzyme was determined by carrying out cellulose hydrolysis at various temperatures *i.e.* 20˚C, 30˚C, 40˚C, 50˚C and 60˚C. In each case, the substrate was preincubated at the required temperature before the addition of enzyme. The optimum pH was determined by monitoring cellulase activity at pH values 4.0, 5.0, 6.0, 6.5, 7.0 and 8.0 using different buffers.

2.7. Determination of Kinetic Parameters

Enzyme was incubated with various concentrations of carboxy methyl cellulose (0.05%, 0.1%, 0.2%, 0.5% and 1.0%) at pH 6.5 and temperature 40˚C. Kinetic parameters K_m and V_{max} were calculated by linear regression from Line weaver Burk plot [18]. The substrate specificity of enzyme was tested for CMC, rice husk, wheat straw and sucrose. Chemical factors NaCl, $MgSO_4$, Tween 20 and EDTA at 5 mM final concentration was used to

Table 1. Different parameters for purification using Response Surface Methodology.

Factor	CCD Design (I) for Acetone Extraction				
	Name	Units	Low Actual (−1)	Value (0)	High Actual (+1)
X_1	Acetone	%	10.00	50	90.00
X_2	pH		4.00	5.5	7.00
X_3	Temp.	˚C	4.00	17	30.00
X_4	Time	Hrs	1.00	6.5	12.00
	CCD Design (II) for Ethanol Extraction				
X_1	Ethanol	%	10.00	50	90.00
X_2	pH		4.00	5.5	7.00
X_3	Temp.	˚C	4.00	17	30.00
X_4	Time	Hrs	1.00	6.5	12.00

Table 2. A central composite design (CCD) for extraction of cellulase using Response surface methodology by acetone and ethanol solvents.

CCD I for Acetone Extraction						CCD II for Ethanol Extraction					
Values of Factors*				Response I	Response II	Values of Factors**				Response I	Response II
X_1	X_2	X_3	X_4	Purification Fold	% Recovery	X_1	X_2	X_3	X_4	Purification Fold	% Recovery
10	7	4	1	0.04	7.9	130	5.50	17	6.50	0	0
10	7	4	12	0.03	5.34	50	5.50	17	6.50	0.93	13.4
50	5.50	17	6.50	2	29.36	10	7	30	12	1.68	16.66
10	7	30	1	0.05	7.93	30	5.50	17	6.50	1.45	14.2
10	4	4	1	1.01	7.94	50	2.50	17	6.50	1.77	13.4
10	7	30	12	0.03	48	50	5.50	17	4.50	1.24	13.4
50	5.50	17	17.50	0.47	14.2	10	4	4	1	6	15
90	4	30	12	1.31	18.6	**10**	**4**	**4**	**12**	**13**	**23**
10	4	4	12	0.61	2.07	50	5.50	17	17.50	2.1	4.8
90	7	30	1	0.128	48	90	4	30	12	3.85	1.4
30	5.50	17	6.50	0.81	10.23	10	7	4	1	1.58	9.7
50	8.50	17	6.50	0.42	14.2	10	7	4	12	2.57	8.57
50	2.50	17	6.50	0.85	14.2	90	7	4	12	4.435	1.4
50	5.50	43	6.50	0.85	14.2	90	7	30	1	0.88	0.89
90	7	30	12	0.056	23.3	50	8.50	17	6.50	1.18	4.8
90	4	4	12	1.21	18.6	10	4	30	1	1.42	6.9
90	**4**	**4**	**1**	**1.6**	**77**	90	7	30	12	4.26	0.96
130	5.50	17	6.50	0	0	50	5.50	43	6.50	1.027	4.8
50	5.50	9	6.50	1.07	14.2	10	7	30	1	3.8	6.9
10	4	30	1	0.37	2.07	90	4	4	1	2.7	0.84
10	4	30	12	0.426	2.07	90	4	30	1	3.8	0.84
50	5.50	17	4.50	0.65	14.2	10	4	30	12	3.38	17
90	7	4	12	0.069	23.3	90	4	4	12	2.6	0.96
90	4	30	1	1.37	71.4	90	7	4	1	2.7	0.84
90	7	4	1	0.67	48	50	5.50	9	6.50	1.1	4.8

*X_1 (Acetone), X_2 (pH), X_3 (temperature), X_4 (retention time); **X_1 (Ethanol), X_2 (pH), X_3 (temperature), X_4 (retention time).

verify the rate of enzyme activation and inhibition.

3. RESULTS AND DISCUSSION

3.1. Response Surface Experimental Design

Optimum levels of the key factors and their effect of interactions were determined by central composite design of RSM. Purification fold and % recovery of 1.7 and 77 respectively was achieved with optimum parameters *i.e.*; acetone (X_1) 90%, pH (X_2) 4, temperature (X_3) 4˚C and incubation period (X_4) 1 h. (**Table 2**). Regression equation obtained after the analysis of variance gave the

level of response as a function of three independent variables. A quardratic model was attempted to fit the data by least squares and all terms regard less of their significance were included in the following equation:

$$y = 284.09494 + 37.31286X_1 + 225.23766X_2 43.47243X_3 + 790.58161X_4 + 28.95995X_1X_2 + 0.043204X_1X_3 + 0.28792X_1X_4 + 64.21602X_2X_3 + 0.011021X_2X_4 + 1.14198X_3X_4 - 2.47136X_1^2 - 261.78389X_2^2 \quad (1)$$

where y is the measured response, and X_1, X_2, X_3 and X_4 are coded independent variables.

After the purification with the acetone another solvent ethanol was used for the extraction of cellulase by central composite design with Quadratic Design to determine the optimum levels of variables. Purification fold and % recovery of 13 and 23 respectively was achieved with the conditions having ethanol (X_1) 10%, pH (X_2) 4, temperature (X_3) 4˚C and retention time (X_4,) 12 h. Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second order model equation was checked by an F-test (ANOVA) and the data are shown in **Table 3**. The regression model for cellulase production was highly significant with a satisfactory value of determination. The Model F-value implied the model is significant. There is 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 indicated that the model terms were significant. The "Pred R-Squared" was as close to the "Adj R-Squared" as one might normally expect. "Adeq Precision" value measures the signal to noise ratio and greater than 4 is desirable as an adequate signal. These facts indicated that the model equation as expressed in **Eq.1** provided a suitable model to describe the response of the experiment pertaining to percentage recovery. It can be noticed from the degree of significance (**Table 4**) that the regression coefficients of linear and quadratic coefficients of X_1, X_2 and X_3 were significant at 1% level This model can be used to navigate the design space. The 3D contours response surface graph based on dependent variables are shown in **Figures 1-4**. The canonical analysis revealed a effects of variables for maximum purification fold and percentage recovery.

3.2. Model Application

The purification fold and percentage recovery of 13 and 23 respectively (16.7 IU·mg^{-1} protein) (**Table 5**) was achieved with the conditions having ethanol (X_1) 10%, pH (X_2) 4, temperature (X_3) 4˚C and retention time (X_4), 12 h with the CCD experimental results of cellulase purification. Established model is satisfactory and confirms

Table 3. ANOVA results for cellulase purification obtained from ethanol extraction.

CCD I1 Response I (Purification Fold)						CCD I1 Response II (% Recovery)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-Value Prob > F**	**Source**	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-Value Prob > F**
Model	7.07	14	0.51	4.12	0.0050 Significant	Model	9762.48	14	697.32	4.64	0.0027
X-Ethano	0.96	1	0.96	7.85	0.0134	X-Ethano	3256.97	1	3256.97	21.69	0.0003
X_2-pH	2.85	1	2.85	23.24	0.0002	X_2-pH	46.00	1	46.00	0.31	0.5881
X_3-Temp.	0.21	1	0.21	1.68	0.2151	X_3-Temp.	0.33	1	0.33	2.218E−003	0.9631
X_4-Time	0.22	1	0.22	1.76	0.2046	X_4-Time	1505.66	1	1505.66	10.03	0.0064
X_1X_2	0.32	1	0.32	2.65	0.1244	X_1X_2	693.00	1	693.00	4.61	0.0484
X_1X_3	9.882E−003	1	9.882E−003	0.081	0.7802	X_1X_3	274.68	1	274.68	1.83	0.1963
X_1X_3	0.025	1	0.025	0.20	0.6573	X_1X_3	2630.48	1	2630.48	17.52	0.0008
X_2X_3	0.024	1	0.024	0.19	0.6673	X_2X_3	201.19	1	201.19	1.34	0.2652
X_2X_4	2.004E−003	1	2.004E−003	0.016	0.8999	X_2X_4	1080.18	1	1080.18	7.19	0.0171
X_3X_4	0.15	1	0.15	1.25	0.2817	X_3X_4	200.74	1	200.74	1.34	0.2657
Residual	1.84	15	0.12			Residual	2252.47	15	150.16		
Lack of Fit	1.84	10	0.18			Lack of Fit	2252.47	10	225.25		
Pure Error	0.000	5	0.000			Pure Error	0.000	5	0.000		
Cor Total	8.91	29				Cor Total	12014.94	29			

Table 4. ANOVA results for cellulase purification obtained from acetone extraction.

CCD 1 Response I (Purification Fold)						CCD 1 Response II (% Recovery)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-Value Prob > F**	**Source**	**Sum of Squares**	**Df**	**Mean Squares**	**F Value**	**p-Value Prob > F**
Model	7.51	14	0.54	4.47	0.0034 significant	Model	9168.98	10	916.90	3.06	0.0172 Significant
X_1	0.10	1	0.10	0.84	0.3750	X_1	2678.04	1	2678.04	8.95	0.0075
X_2	2.05	1	2.05	4.47	0.0009	X_2	12.15	1	12.15	0.041	0.8425
X_3	0.066	1	0.066	0.55	0.4699	X_3	213.14	1	213.14	0.71	0.4093
X_4	0.059	1	0.059	0.49	0.4960	X_4	1041.73	1	1041.73	3.48	0.0776
X_1X_2	0.16	1	0.16	1.35	0.2629	X_1X_2	1010.94	1	1010.94	3.38	0.0818
X_1X_3	0.041	1	0.041	0.34	0.5683	X_1X_3	0.27	1	0.27	8.868E−004	0.9766
X_1X_3	2.326E−004	1	2.326E−004	1.935E−003	0.9655	X_1X_3	3061.99	1	3061.99	10.23	0.0047
X_2X_3	4.523E−003	1	4.523E−003	0.038	0.8488	X_2X_3	11.90	1	11.90	0.040	0.8441
X_2X_4	0.002	1	0.022	0.18	0.6740	X_2X_4	1126.93	1	1126.93	3.76	0.0673
X_3X_4	0.025	1	0.025	0.20	0.6576	X_3X_4	11.90	1	11.90	0.040	0.8441
Residual	1.80	15	0.12			Residual	5688.07	19	299.37		
Lack of Fit	1.80	10	0.18	2703.51	0.0001 Significant	Lack of Fit	5688.07	14	406.29		
Pure Error	3.333E−004	5	6.667E−005			Pure Error	0.000	5	0.000		
Cor Total	9.32	9				Cor Total	14857.05	29			

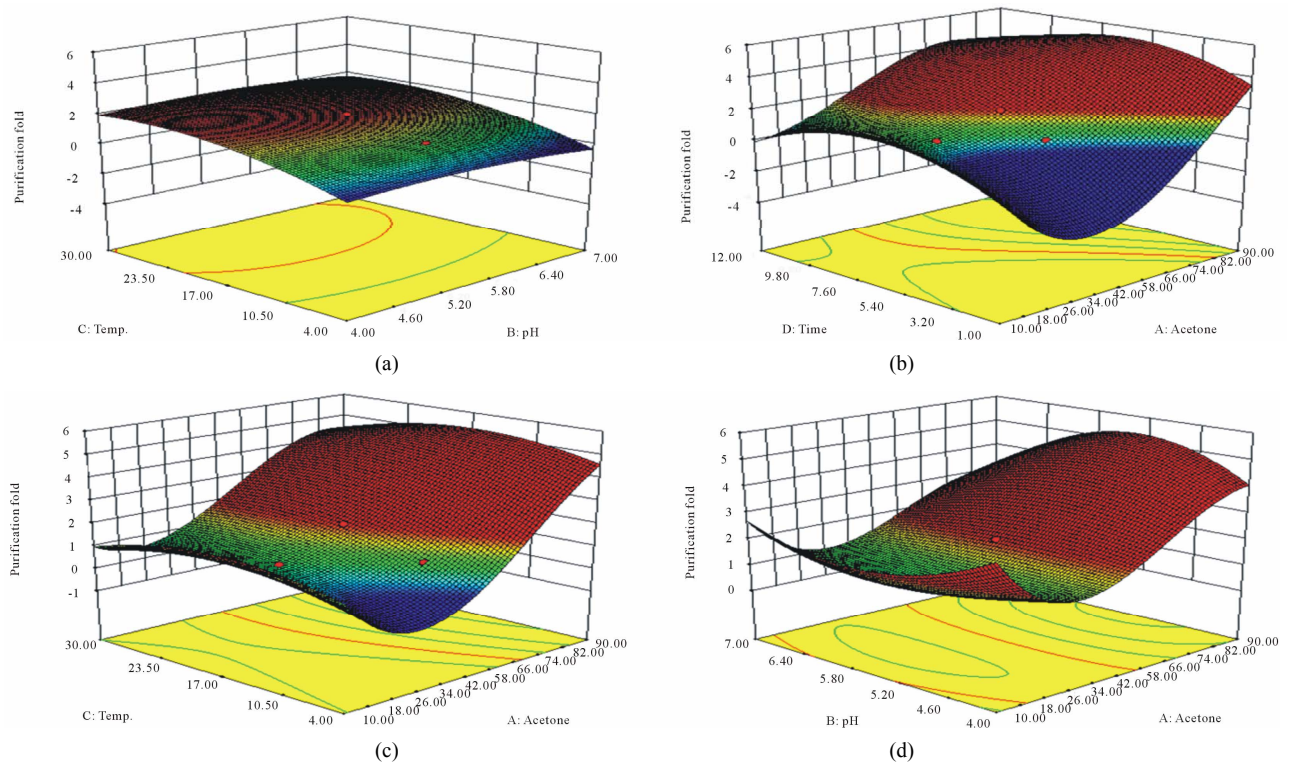

Figure 1. Surface plot for the effect of: (a) Temp and pH; (b) Time and acetone; (c) Temp and acetone; (d) pH and acetone conc. on purification fold by acetone extraction.

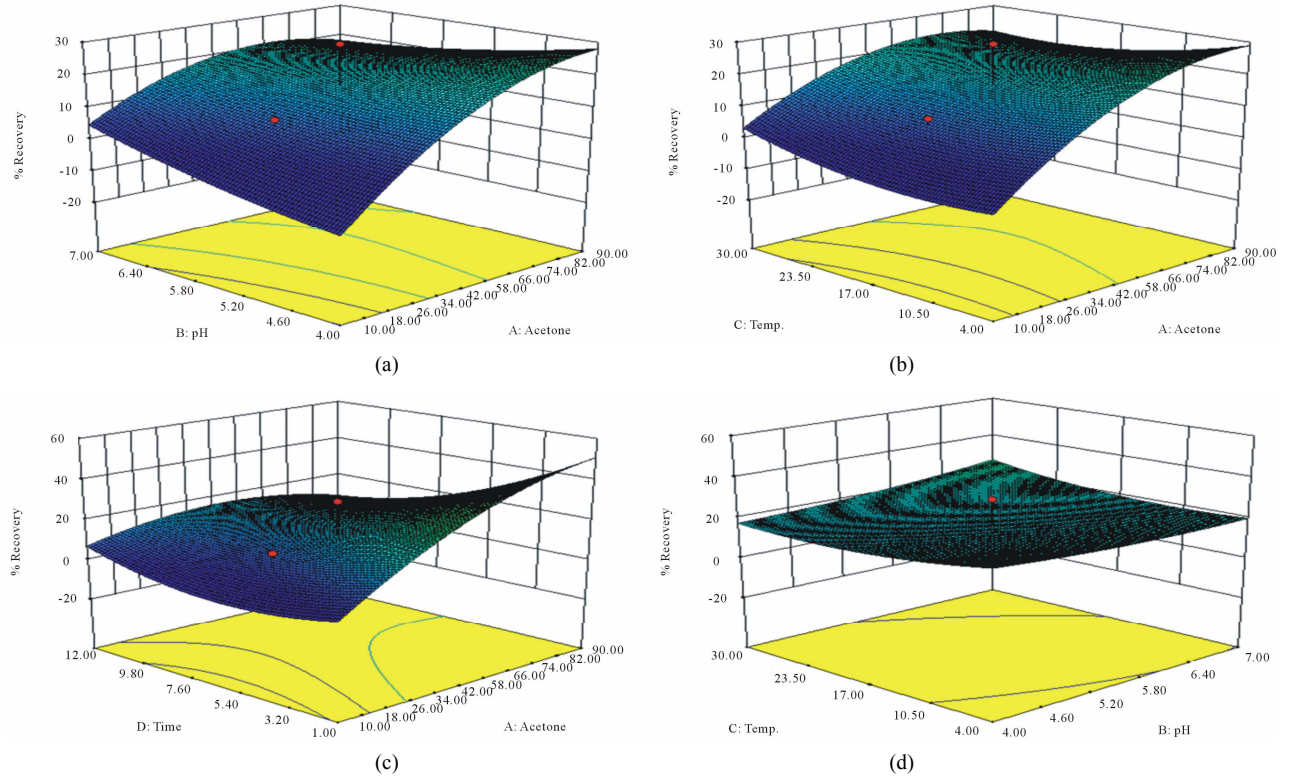

Figure 2. Surface plot for the effect of: (a) pH and acetone; (b) Temp and acetone; (c) Time and acetone; and (d) Temp and pH on % recovery by acetone extraction.

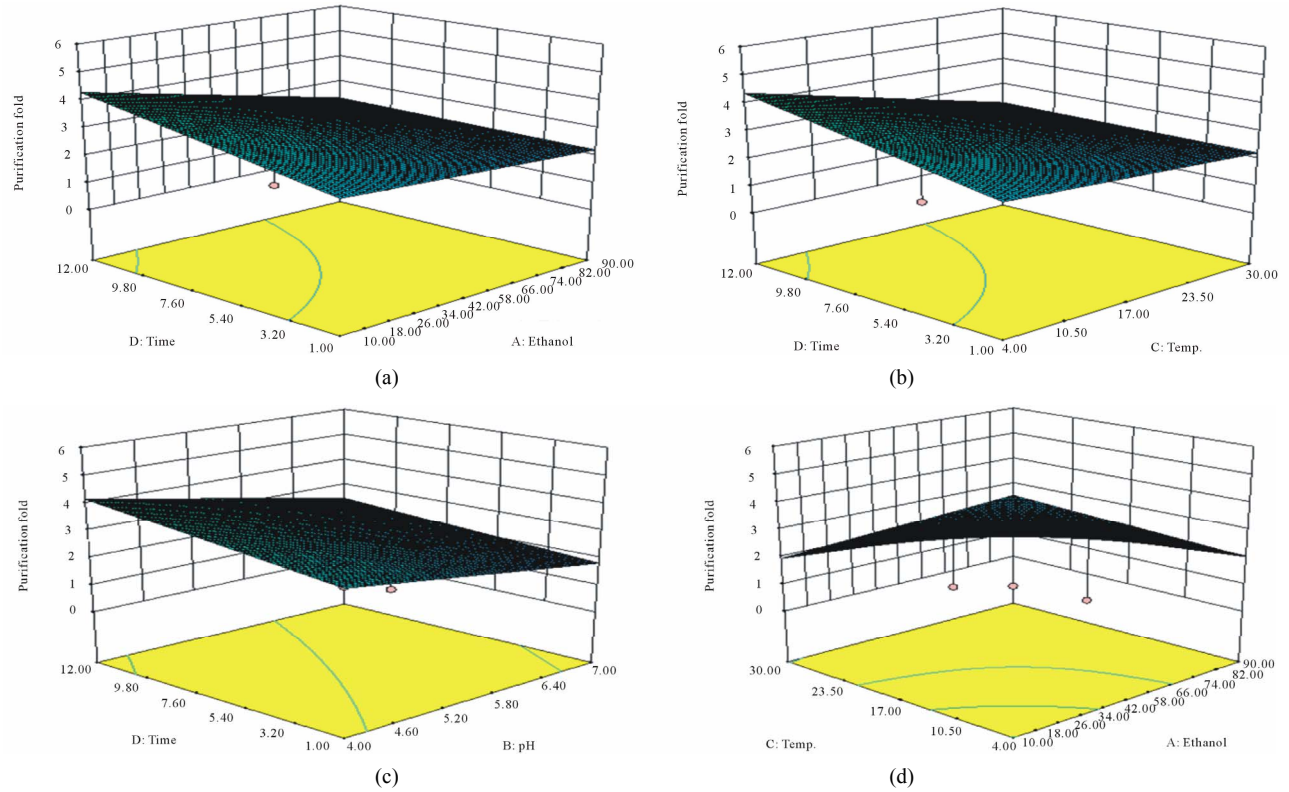

Figure 3. Surface plot for the effect of: (a) Time and ethanol con.; (b) Time and temp; (c) Time and pH; (d) Temp and ethanol conc. on purification fold by ethanol extraction.

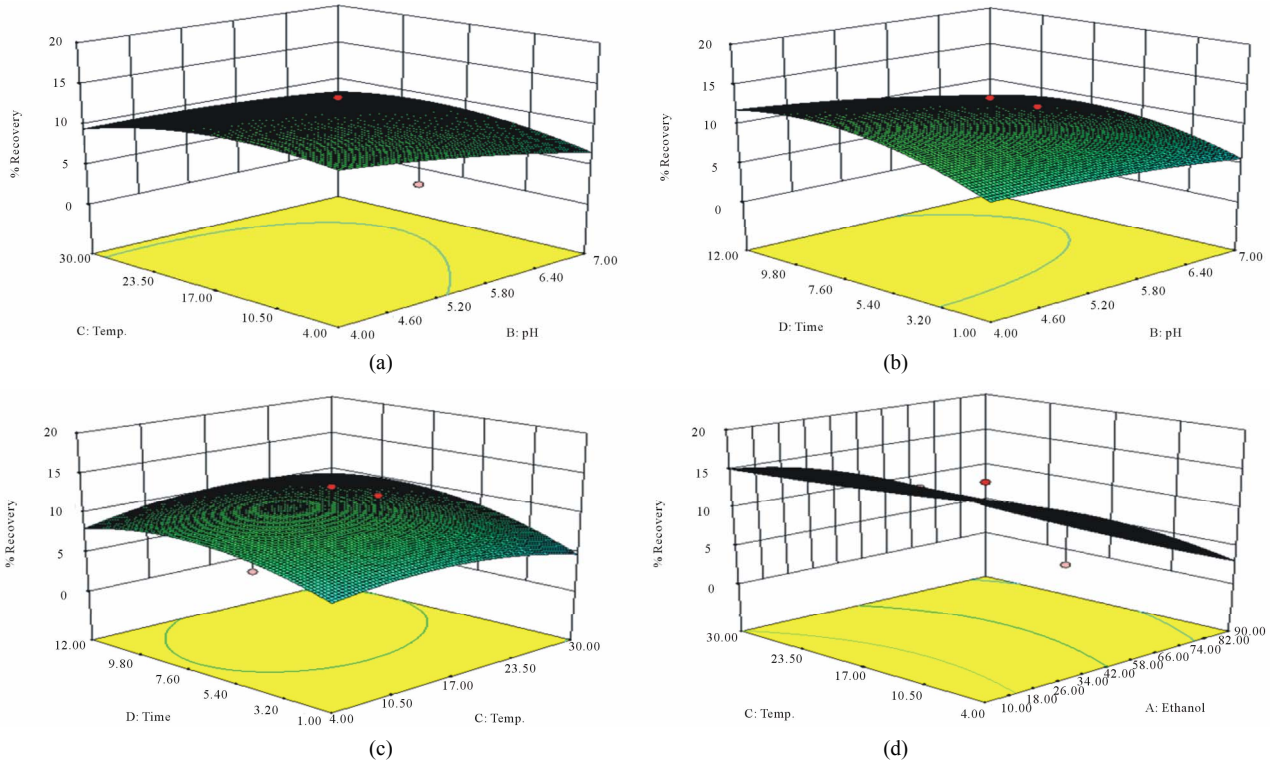

Figure 4. Surface plot for the effect of: (a) Temp and pH; (b) Time and pH; (c) Time and temp; (d) Temp and ethanol conc. on % recovery by ethanol extraction.

Table 5. Over all purification of cellulase from *Bacillus* sp. *JS*14.

Methods	Enzyme Activity (IU)	Protein (mg)	Specific Activity (IU/mg of protein)	Purification-Ion Fold	Recovery (%)
Culture Filtrate	1700	2200.0	0.77	--	100
Acetone Precipitation	1300	1040	1.25	1.7	77
Ethanol Precipitation	299.9	18.40	16.25	13	23

response surface methodology a promising tool in a design of complex process of purification in the selection of operating variables.

3.3. Cellulase Characterization after Purification

Results of Electrophoresis of the enzyme on SDS–PAGE showed that enzyme consist single polypeptide with a molecular weight of 32.5 KDa (**Figure 5**) smaller than cellulase from *C. thermophilum* (41 KDa), *C. thermophilum* (36 KDa), *M. thermophila* (100 KDa) and *H. grisea* (63 kDa) [19-23]. The optimum pH and temperature for activity of the purified enzyme from *Bacillus* sp. J14 for standard assay conditions were 6.5˚C and 40˚C respectively (**Figures 6(a)** and **(b)**). This pH value is comparable to the optimum pH value of 7.0 for purified enzyme from *Sinorhizobium fredii* [14] and higher with respect to the optimum value of 5 for cellulase from *Trichoderma viride* [24]. At 40˚C the cellulase was 98%

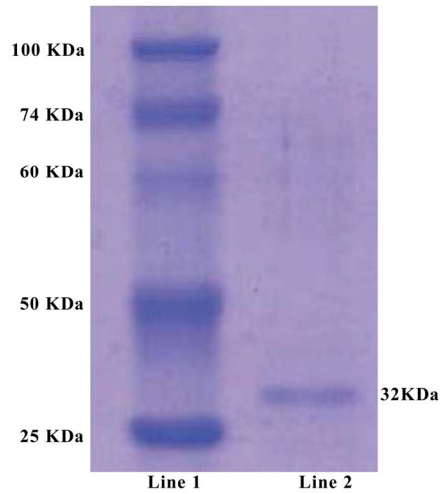

Figure 5. SDS-PAGE of purified cellulase from *Bacillus JS*14: Line 1 protein markers; Line 2: purified cellulase.

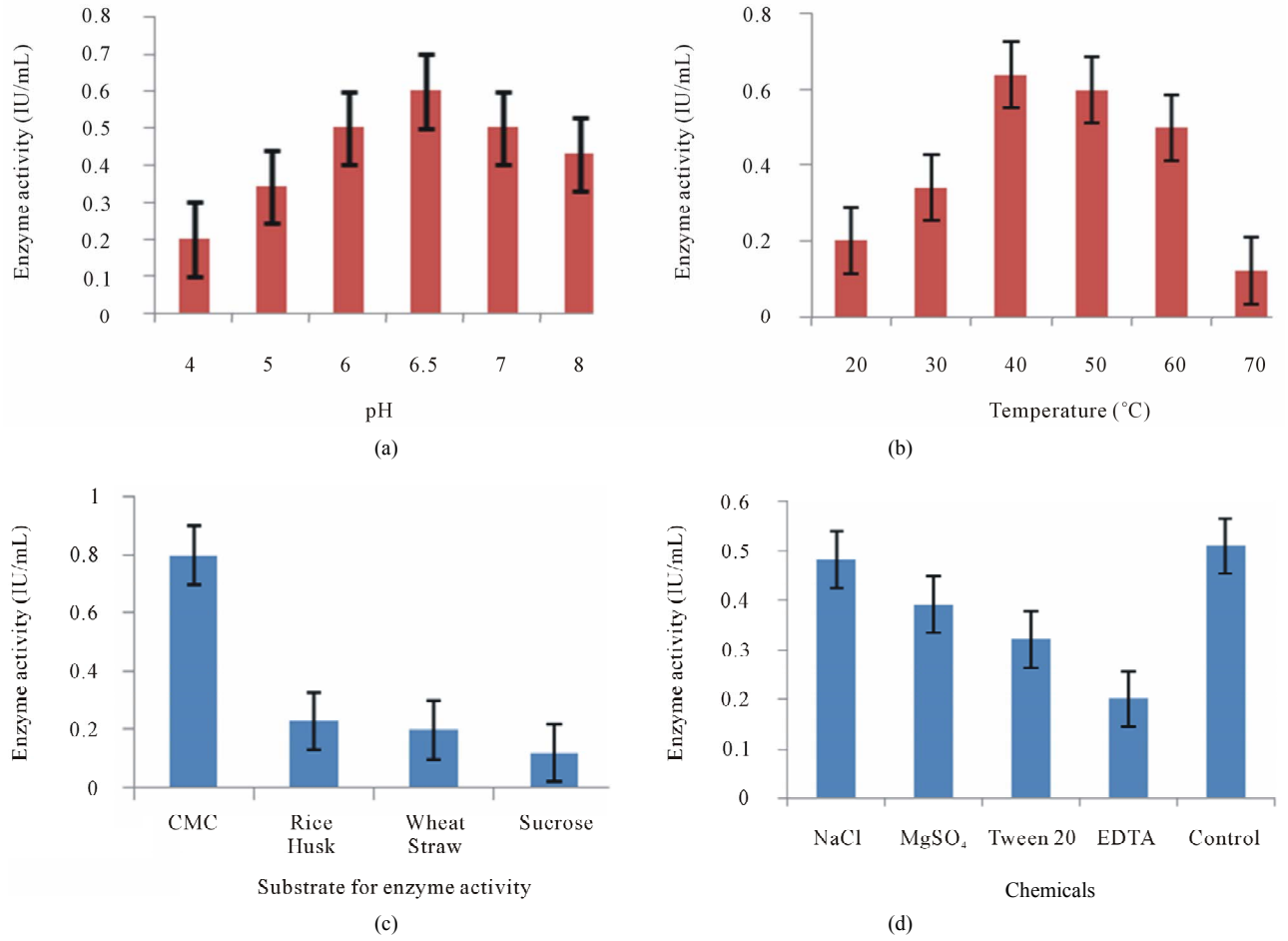

Figure 6. Effect of: (a) pH; (b) Temperature; (c) Substrates; and (d) Chemicals factors on the activity of purified enzyme.

stable and sharp decrease in activity was observed above 40˚C. Kinetic studies of the purified cellulase illustrated that V_{max} and K_m for purified enzyme was 66 IU/mg and 2.5 mg·mL^{-1} respectively. Whereas V_{max} and K_m for cellulase was found to be 84 U/mg and 3.6 mg·mL^{-1} respectively isolated from *Bacteroides succinogene* [25]. The K_m is lower than the enzyme from the fungus *M. thermophila* (3 mg·mL^{-1}) [23] and *T. aurantiacus* (3.9 mg·mL^{-1}) [26].

Purified enzyme illustrated highest activity with CMC as compare to rice husk, wheat straw and sucrose (**Figure 6(c)**), whereas cellulase enzyme produced from *Trichoderma koningii* reveal little ability to attack CMC as compare to cellotetraose and cellohexaose [27]. NaCl, $MgSO_4$, Tween 20 and EDTA are inhibitors of the enzyme and decrease the activity 5.32%, 3.55%, 5.32% and 21.66% respectively with respect to control (**Figure 6(d)**).

4. CONCLUSION

Purification fold and recovery percentage of 13 and 23 respectively was achieved with solvent extraction process chosen as optimum for cellulase purification by RSM. The molecular weight of the enzyme was estimated to be 32.5 KDa. The K_m of the purified enzyme for carboxy methyl cellulose, sodium salt (CMC), was 2.5 mg·mL^{-1} at pH and temperature 6.5˚C and 40˚C, respectively. Enzyme exhibited maximum substrate specificity for carboxymethyl cellulose with compare to rice husk, wheat straw and sucrose. The data obtained in this study will be used to shape the process for cellulase extraction for different aspects of higher purification yields.

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