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Utilization of peanut shells as substrate for cellulase production in submerged fermentation through Box-Behnken Design

Abstract: Increasing environmental pollution and global warming encourage bioengineers and biotechnologists to find renewable and environmental friendly sources of energy. Conversion of biomass especially agro-industrial biomass consisting of lignocellulosic material into different products and energy is the modern and promising scheme for this purpose due to their abundance in nature, lower cost and easy handling. These properties made them potential producers of biofuels in near future. In current research, *Bacillus paralichniformis* potential for cellulase production was analyzed using submerged fermentation, for which peanut shell waste was used as a substrate. Nutritional factors were optimized for best enzyme production using Box-Behnken Design (BBD) of response surface methodology (RSM). Results illustrated the maximum enzyme production of 12.838 IU for CMCase and 40.956 IU for FPase after 24 h of fermentation period. These results were obtained at 3 g/mL susbtrate concentration, 0.45 g/mL yeast extract and 0.01 g/mL $MgSO_4$ for CMCase and 3g/mL substrate, 0.45 g/mL yeast extract and 0.3 g/mL $MgSO_4$ for FPase. These results proposed the possibility of utilization of this strain for production of cellulase to endorse its use in industry.
Key words: *Bacillus* sp., cellulase, peanut shells, RSM, CCD, submerged fermentation.

Introduction

Increasing environmental pollution and global warming encourage bioengineers and biotechnologists to find renewable and environmental friendly sources of energy [1]. Conversion of biomass especially agro-industrial biomass consisting of lignocellulosic material into different products and energy is the modern and promising scheme for this purpose due to their abundance in nature, lower cost and easy handling. These properties made them potential producers of biofuels in near future [2]; if the product recovery and purification steps use limited and optimized water, solvents and energy [3; 4]. On the other hand conversion of lignin gasification (LG) biomass into simple units remains the major problem due to complex chemical nature of LG material. However, enzymatic hydrolysis, physical or biological degradation methods made it easy and economical for industrial uses [5]. These processes effect saccharification of cellulose enzymatically [6]. Cellulose is the most abundant and naturally renewable source of energy which can compete with many of the existing but expensive and nonrenewable resources of energy. It is a plant biopolymer with complex structure having a hydrolytic enzyme system for the cleavage of bonds and its conversion into simple sugar like D-glucose units [7].

Cellulases are the enzymes that catalyze cellulose into fermentable sugars which is now considered the feasible process to reduce the risk of environmental pollution, easy and useful dumping and degradation of plant and cellulosic waste. In industry these have great importance in paper and pulp, textile, detergents, food and feed additives, bio-ethanol production and processing of chemicals. The prerequisite for easy and economical use of cellulases is to search for best microbial strains which produce high enzyme titer and to optimize media that could be affordable for industry. There are many microorganisms studied and searched for the production of cellulases but lesser number of them are able to produce it in economically feasible

and industrially significant quantities of enzyme [8]. Microorganisms are genetically the most diverse group due to high metabolic flexibility and multiple enzyme based reactions which in turn catalyze complex structures like cellulose in D-glucose with the help of enzymes called cellulase. Microorganisms have high impact on biotechnological applications due to these significant features [7]. Bacteria are emerging as hotspots of versatility and variety genetically and functionally. They can degrade lignocellulosic materials involving complex system of lignocellulolytic enzymes [9].

The complex cellulase enzyme system consists of three enzymes which are endoglucanases, exoglucanases and cellobioases [10]. Endoglucanases are β-1,4-D glucan-4-glucano-hydrolase and carboxymethyl cellulase, while exoglucanases are β-1,4-D glucan-4-gluco-hydrolase and cellobiohydrolase and cellobioases termed as CBH and cellobioases are β-D glucoside glucohydrolase and β-1,4-D-glucosidase, all free enzymes present in 57 of glycosyl hydrolase families [11; 12]. Cellulolytic enzymes would be of great industrial use with the promising environmental and economical sustainability if their media could be improved with the proper optimized conditions for achieving best enzyme titer that eventually lessens its cost [13].

The optimization of cellulase production and selection of ideal substrate are major and important steps having significant enzyme titer at the end. Limited information was available for *Bacillus* species enzymology and its cellulytic activities. Bacterial cellulases usually reported to be extracellular and production can be optimized by adjusting nutritional parameters and physical properties like temperature and pH, etc. The major factor has always been carbon source but nitrogen phosphorus and metal ions sources are also of great importance [14].

Determination of cellulolytic potential of microorganism is carried out via fermentation process. There are two types used commonly one is solid state fermentation and second is submerged fermentation. Solid state fermentation is carried out in the absence of free liquids as microorganisms grow on firm surfaces holding up as substrates [15]. This type has been used mostly for filamentous fungi where solid substrate acts as natural surface for filamentous growth of fungi [16].

Submerged fermentation usually denoted as SMF is a type of fermentation which requires large amount of liquid substrates as compared to solid state fermentation. These may be water, molasses or broths. The products which mainly are secondary metabolites or enzymes are secreted in and collected form liquid media. Continuous replenishment of substrates or nutrient may be required according to the use of substrates in fermentation process. Microbes which need high moisture content for their growth such as bacteria usually best for this type of fermentation. The advantage of this technique is easy purification and product recovery [17; 18]. Submerged fermentation is preferred for bacterial cultures due to ease of purification, sterilization and process control. The culture conditions and media optimization are the major steps to be considered [19]. During early 1970's, cellulase production was started commercially via submerged fermentation. Mega scale use of cellulase as animal feed additive and for stonewashing denim was practiced in industry during 1980's [20].

Formulation of media is specific for the organism and its optimization is necessary for best production of required substance hence any general media composition cannot be used for optimum growth and cellulase production [21]. Response surface methodology (RSM) is the statistical analysis modeling used to optimize and evaluate many biotechnological processes and enzymatic hydrolysis [22]. RSM is affected by many parameters and variable factors affecting the results and then these are optimized for the best conditions using different designs of RSM with the interpolation of first or second polynomial equations in sequential testing procedure [23; 24]. This technique, RSM integrates mathematical and statistical approaches and analyzes defined independent parameters on response without having any previous information about the relation between response function and variable parameters [25-27]. RSM is being used now statistically, as an appropriate methodology for experiment designing, statistical model building, evaluation of factors affecting the optimum conditions for required response and in turn, decreasing the number of experiments for the required response [28]. In biotechnological processes, RSM evaluate the optimum conditions for the growth of microorganism and product formation [29; 30]. Here, RSM was used to determine optimal conditions for novel bacterial strain *Bacillus parlichniformus* and the factors that affect the response of cellulase production. Peanut shell waste has been taken as carbon source and cellulolytic potential of *Bacillus parlichniformis* was investigated in submerged fermentation with optimal medium conditions.

Materials and methods

Object. Samples of *Bacillus paralichniformis* isolated from soil were provided by the Laboratory of Microbial Biotechnology, Department of Biotechnology, University of Sargodha. The strain was maintained on nutrient agar slants and used for cellulase production in subsequent study.

Enzyme production. Submerged fermentation was performed for the production of enzyme having medium ingredients of peanut shell waste, yeast extract and $MgSO_4$. Concentration of these ingredients was optimized as per experimental design. The medium components were sterilized and inoculated with 1ml of 24 h old vegetative cell culture and placed in a shaking incubator for 24 h with a shaking speed of 120 rpm at 35°C. Culture broth was consequently centrifuged at 10,000 x g and 4°C for 10 min. Centrifuged pure extract without cellular material was used as source of crude enzyme extract for further processing.

Cellulase assay. Carboxymethyl cellulase (CMCase) and filter paper activity (FPase) was estimated as described in our earlier reports [31].

Saccharification of peanut shell. In 500 mL conical flask, 100 mL of crude cellulase enzymes with 4% substrate was incubated at 50 °C for various time intervals. After termination of enzymatic hydrolysis, centrifugation of material was performed at 10,000 rpm for 10 min. Saccharification (%) was calculated using the following formulae [32].

$$\text{Saccharification (\%)} = = \frac{\text{Reducing sugars released (mg/ ml)}}{\text{Substrate used (mg/ ml)}} \times 100$$

Experimental design. Box–Bhenken design (BBD) was used for optimization of medium components in this study. The independent and noteworthy variables used were peanut shell (substrate) concentration (X1), yeast concentration, (X2) and $MgSO_4$ (X3) and their levels are mentioned in Table 1. The relation between actual and coded values was described by the following equation;

$$xi = \frac{Xi - X0}{\Delta Xi},$$

where xi and Xi are the coded and actual values of the independent variable, Xo is the actual value of the independent variable at the center point and DXi is the change of Xi. The response is calculated from the following equation using STATISTICA software (99th ed.).

$$Y = \beta_O + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^{2+} \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Y is the response, X_1, X_2 and X_3 are the independent variables, β_O is the intercept, β_1, β_2 and β_3 are linear coefficient, β_1^1, β_2^2 and β^3_3 are square coefficients, β_{12}, β_{13} and β_{23} are interaction coefficients.

Results and discussion

Cellulases were produced by *Bacillus paralichniformis* in SMF. Media optimization for best enzyme titer was carried out using three independent variables such as substrate (peanut shell waste) concentration (X1), Yeast extract (X2) and $MgSO_4$ (X3) and their levels are mentioned in Table 1. The response was calculated by second degree polynomial regression equation (Eq. 3; 4) using Minitab software version 9.

With the optimized conditions of media, the best enzyme production obtained for CMCase was 12.838 IU with optimized conditions of substrate concentration of 3 (%), yeast extract 0.45 (%) and $MgSO_4$ 0.01(%)after 24 h of incubation. This value was in close proximity with the predicted value of 12.38850 IU as shown in table 2.Highest FPase production (40.956 IU) was observed with substrate concentration of 3 (%), 0.45% of yeast extract and $MgSO_4$ concentration of 0.3 (%) having predicted value of 31.5108 IU.

Table 1 – Coded and actual levels of the factors for three factors Box-Behenken design

Independent variables	Symbols	Coded and actual values		
		-1	0	+1
Peanut shell waste	X_1	0.5	1.75	3
Yeast extract	X_2	0.1	0.45	0.8
$MgSO_4$	X_3	0.01	0.155	0.3

$$\text{CMCase (IU)} = -3.55 + 7.13\ X_1 + 18.15\ X_2 + 12.96\ X_3 - 0.574\ X_1{*}X_1 - 19.62\ X_2{*}X_2 - 64.2\ X_3{*}X_3 - 3.366\ X_1{*}X_2 - 4.59\ X_1{*}X_3 + 20.59\ X_2{*}X_3 \quad \text{Equation (3)}$$

$$\text{FPase (IU)} = 10.7 + 4.5\ X_1 + 30.4\ X_2 - 40\ X_3 + 1.36\ X_1{*}X_1 - 40.4\ X_2{*}X_2 - 141\ X_3{*}X_3 - 9.8\ X_1{*}X_2 + 17.1 X_1{*}X_3 + 88\ X_2{*}X_3 \quad \text{Equation (4)}$$

Table 2 – Cellulase production by *B. paralicheniformis* using Box-Behenken design from peanut shells

Run #	X_1	X_2	X_3	CMCase activity (IU)			FPase activity (IU)		
				Observed	Predicted	Residual	Observed	Predicted	Residual
1	0.5	0.45	0.01	3.204	3.49975	-0.29575	7.238	16.68313	-9.4451
2	1.75	0.8	0.01	5.04	4.61963	0.420375	17.979	8.13738	9.84162
3	0.5	0.45	0.3	3.055	3.50450	-0.44950	7.238	6.48912	0.74888
4	3	0.8	0.155	6.557	7.42688	-0.86987	11.746	20.83875	-9.0927
5	0.5	0.8	0.155	3.024	3.14863	-0.12462	10.2	10.59650	-0.3965
6	1.75	0.1	0.3	4.085	4.50538	-0.42037	0.901	10.74263	-9.8416
7	3	0.45	0.3	9.358	9.06225	0.295750	40.956	31.51088	9.44513
8	3	0.1	0.155	12.043	11.91838	0.124625	36.45	36.05350	0.39650
9	1.75	0.45	0.155	9.358	9.36167	-0.00366	17.541	21.84300	-4.3020
10	1.75	0.45	0.155	9.019	9.36167	-0.34266	25.063	21.84300	3.22000
11	3	0.45	0.01	12.838	12.38850	0.449500	28.592	29.34088	-0.7488
12	1.75	0.1	0.01	7.682	8.25613	-0.57412	24.084	23.73163	0.35237
13	1.75	0.8	0.3	5.623	5.04888	0.574125	12.75	13.10238	-0.3523
14	1.75	0.45	0.155	9.708	9.36167	0.346333	22.925	21.84300	1.08200
15	0.5	0.1	0.155	2.62	1.75013	0.869875	17.709	8.61625	9.09275

All the data was statistically analyzed using analysis of variance for significance of the model (Table 3). The significance of model and response for coefficients is mainly dependent on F-value and P-values. The higher the F-value resulting in lower P-value described the high accuracy and significance of regression model [33]. Therefore, higher computed Fischer's F-value for CMCase was 26.07 and for FPase 1.14 with P-value 0.001 and 0.468 respectively. The model for CMCase was highly significant while FPase was found not significant. The fitness of model was further analyzed by the determination coefficient R^2 for CMCase and FPase. The R^2 value for CMCase and FPase were 97.91% and 67.17%, which revealed that 2.09% and 32.67% variation was not determined by model respectively. Higher value of R square of CMCase showed the accuracy of the model (Figure 1).

Interaction effect of parameters. The interaction effect of substrate concentration (X1), yeast extract (X2) and $MgSO_4$(X3) has been described in contour plots. Different pattern of colors in these plots depicted levels of enzyme production with one variable constant or zero level and two parameters with different levels (Figure 2). These plots indicated that each parameter significantly affect enzyme production.

The results were validated further by repeated experiments of optimized values of significant parameters as predicted in desirability diagrams (Figure 3).Results were in the close range with predicted values. This figure depicted that at optimized levels of peanut shell waste 1.75%, yeast extract 0.45% and $MgSO_4$ 0.155%, the maximum CMCase production was 13.678 IU which was confirmed by repeated experiments. The predicted optimized value for FPase was 1.75% peanut shell waste, 0.45% yeast extract and 0.155%$MgSO_4$ yielded 41.016 IU enzyme production which were almost similar after experiments.

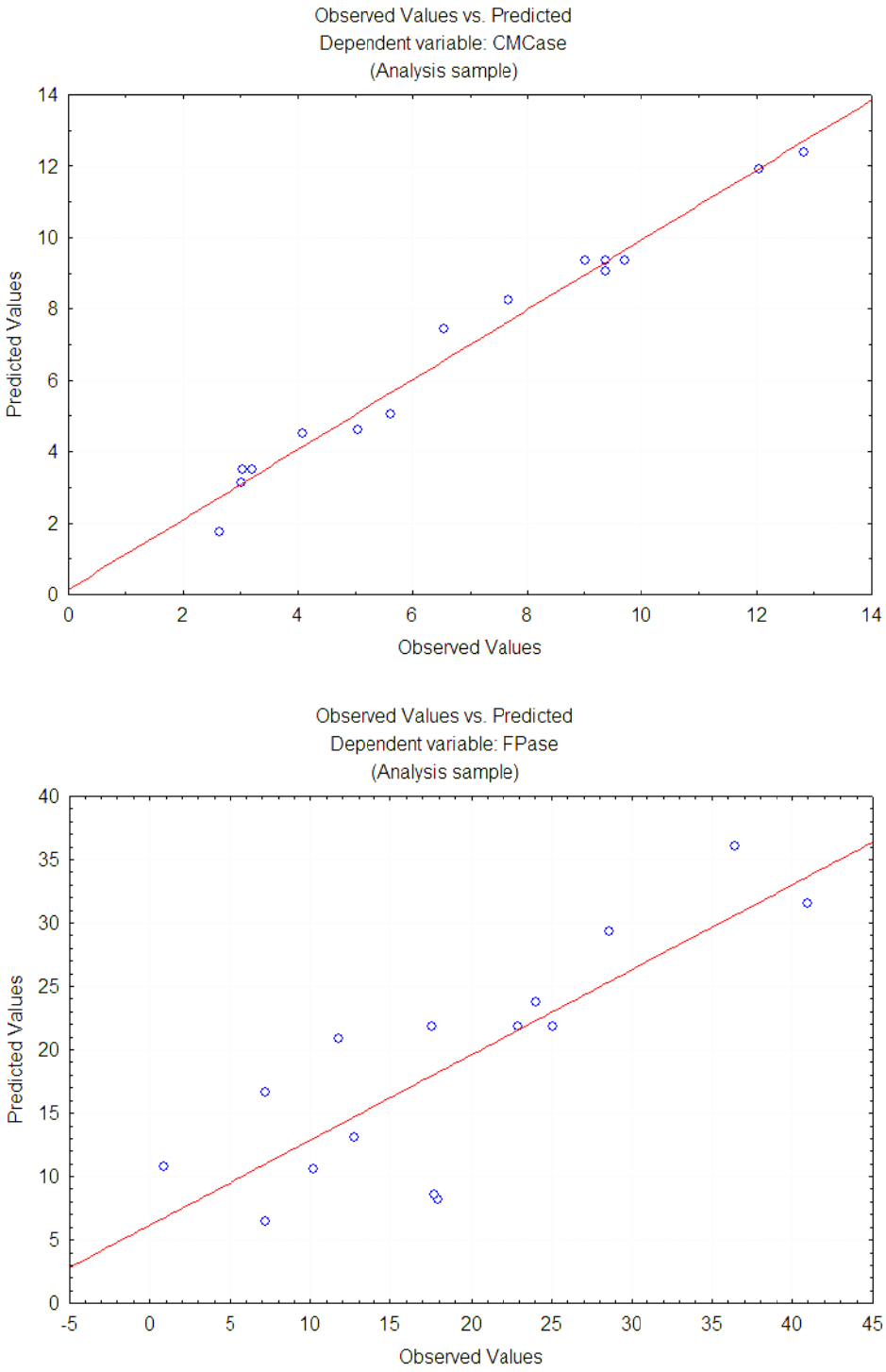

Figure 1 – Observed vs predicted values of independent variables for maximum enzyme production

Table 3 – Analysis of variance for cellulase production from *B.paralichniformis* in submerged fermentation

	Sources	DF	Adj SS	Adj MS	F value	P value
CMCase (IU)	Model Linear	9 3	158.320 114.650	17.591 38.217	26.07 56.64	0.001 0.000
	X_1	1	104.351	104.351	154.66	0.000
	X_2	1	4.783	4.783	7.09	0.045
	X_3 Square	1 3	5.516 27.855	5.516 9.285	8.18 13.76	0.035 0.008
	X_1^2	1	2.972	2.972	4.41	0.090
	X_2^2	1	21.329	21.329	31.61	0.002
	X_3^2 2 Way interaction	1 3	6.736 15.815	6.736 5.272	9.98 7.81	0.025 0.025
	X_1*X_2	1	8.673	8.673	12.85	0.016
	X_1*X_3	1	2.774	2.774	4.11	0.098
	X_2*X_3	1	4.368	4.368	6.47	0.052
	Error Lack of fit Pure error Total	5 3 2 14	3.374 3.136 0.237 161.693	0.675 1.045 0.119	8.81	0.104
FPase (IU)	**Sources**	**DF**	**Adj SS**	**Adj MS**	**F value**	**P value**
	Model Linear	9 3	1164.71 829.64	129.41 276.55	1.14 2.43	0.468 0.181
	X_1	1	709.87	709.87	6.24	0.055
	X_2	1	87.58	87.58	0.77	0.421
	X_3 Square	1 3	32.19 142.35	32.19 47.45	0.28 0.42	0.618 0.749
	X_1^2	1	16.76	16.76	0.15	0.717
	X_2^2	1	90.37	90.37	0.79	0.414
	X_3^2 2 way interaction	1 3	32.51 192.72	32.51 64.24	0.29 0.56	0.616 0.662
	X_1*X_2	1	73.92	73.92	0.65	0.457
	X_1*X_3	1	38.22	38.22	0.34	0.587
	X_2*X_3	1	80.59	80.59	0.71	0.439
	Error Lack of fit Pure error Total	5 3 2 14	569.22 539.18 30.05 1733.93	113.84 179.73 15.02	11.96	0.078

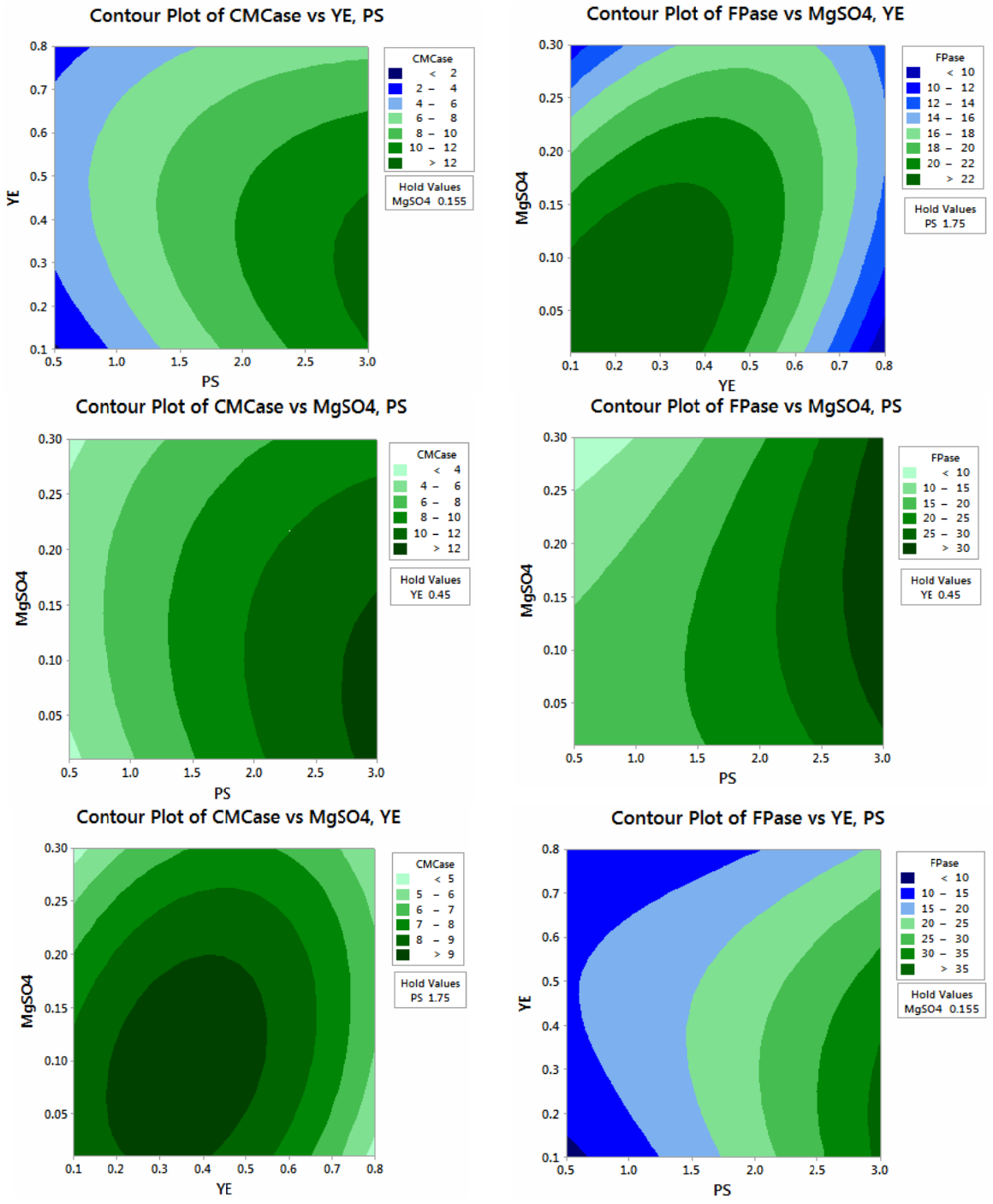

Figure 2 – Contour plots for CMCase (IU) and FPase (IU) production from peanut shells by *Bacillus paralicheniformis*in submerged fermentation

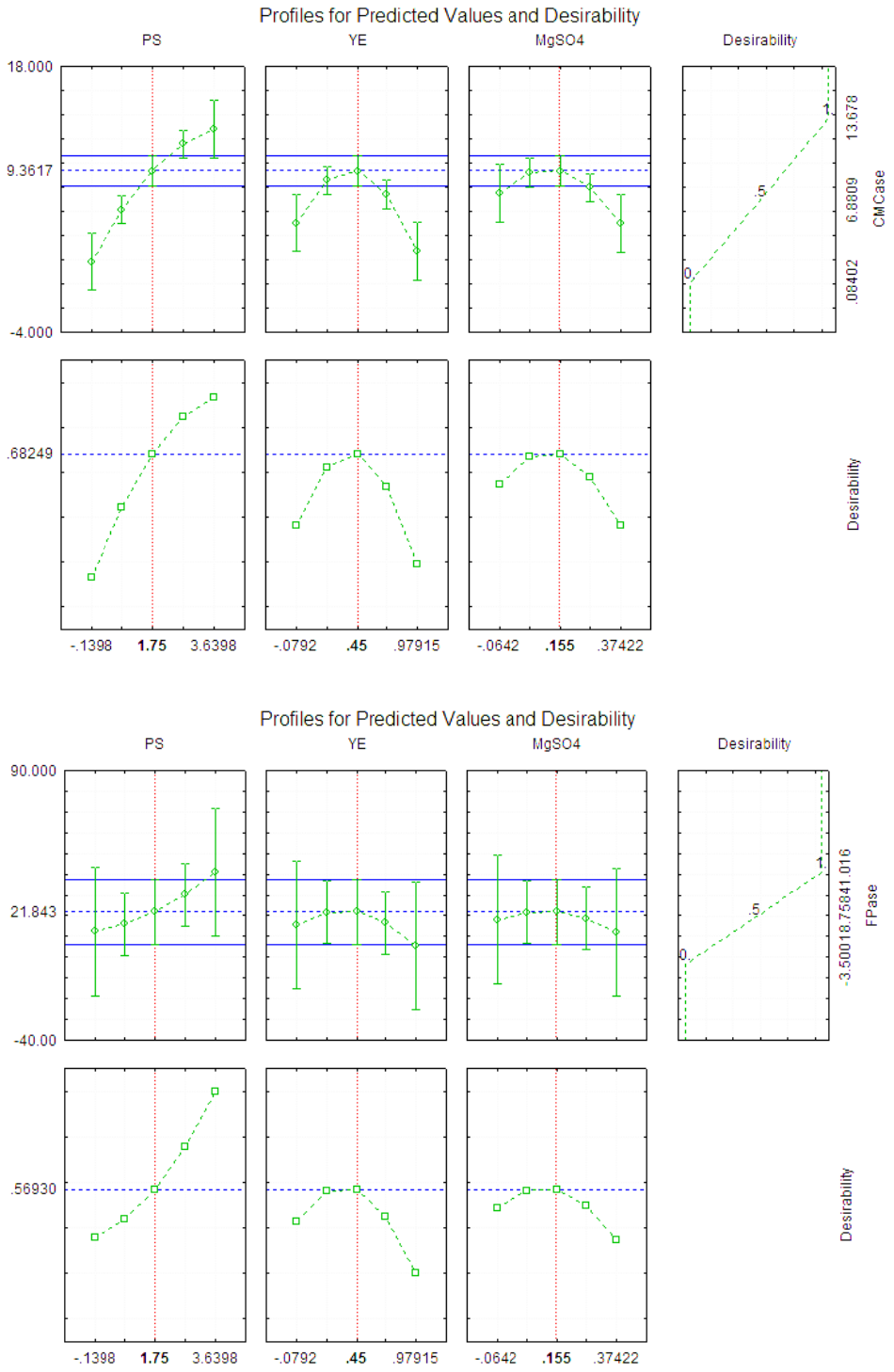

Figure 3 – Desirablity of CMCase and FPFase production

Saccharification. The crude enzyme which was produced from *Baccilus paralichniformis* in submerged fermentation was applied to hydrolyze peanut shell waste. The experiment was carried out for different time periods and results (Figure 4) depicted the maximum total sugar (38.0 ± 0.23 mg/mL) production was observed at 8 h. Further increased time of incubation declined sugar production.

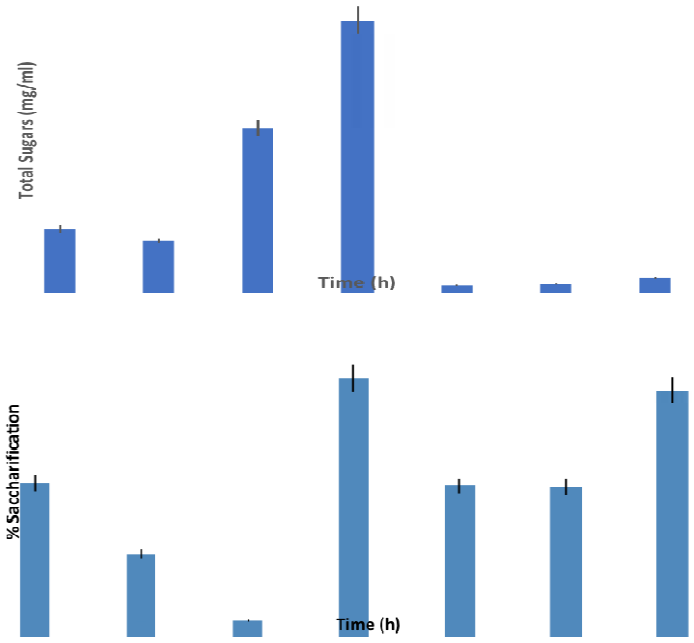

Figure 4 – Sugars released after enzymatic hydrolysis at various time period

As we know cellulase has major industrial role and now the increasing demands of renewable energy sources motivate biotechnologist to search for novel and better microbial strains and easy renewable sources of substrate, like agro-industrial wastes for the production of enzymes, using novel strain. *Bacillus subtilis* K18 had tremendous potential of cellulase production using a variety of substrates like saw dust [34], eucalyptus leaves [35], cotton stalk, peanut shells [36], potato peels [46], *Saccharum spontaneium* [37] and banana peduncle [31] through response surface methodology. Another study reported 0.037 IU/mL/min of CMCase from *Bacillus* sp. C1AC5507 using bagasse through RSM in submerged fermentation [38]. *Bacillus aquimaris* isolated from the gut of *Labeo rohita* has maximum endoglucanase producing potential of 437.3833 IU/mL/min in submerge fermentation [39].

Bacillus pumilis strain had potential of using sugarcane bagasse as carbon source for CMCase 13.6 IU/mL/min production in submerge fermentation [40]. *Aeromonas bestiarum* isolated from the gut of *Labeo rohita* gave the enduglucanase titer of 3.766 IU using sugarcane bagasse as substrate [41]. Medium components and type of substrate significantly affects cellulase production in submerge fermentation as described in previous study [42].

The ongoing study has been carried out with this perspective of easily available and renewable plus economical agro waste, i.e. peanut shell waste with a novel strain of *Bacillus paralichniformis*. The cellulase enzyme produced from this bacterium was further used for saccharification of peanut shells, which gave maximum sugar after 8 h of incubation period (Figure 4).

Another study reported maximum sugar production at 6 h for sugar beet pulp [43]. Saccharification of *Saccharum spontaneum* releasing maximum total sugars of 12.71 mg/mL after 20 h of incubation at 50°C [37]. Cellulase produced by the *Bacillus subtilis* K-18 has stimulated the 54.389% saccharification of pine needles [44]. Cellulase mediated saccharification by *Bacillus cereus* produced maximum total sugars of 31.42 mg/mL released after 6 h of incubation at 50 °C [45]. Wheat straw fermented with *Saccharomyces cerevisiae* under optimized condition of 2 % wheat straw, 0.5% enzyme concentration and 6 h of time period has been resulted in 40.15 % of saccharification [33]. Asghar et al. also reported 8 h of incubation time for maximum saccharification of wheat straw [46].

Conclusion

Results obtained within the current study prove that the novel strain *Baccilus paralichniformus* has

good industrial potential for cellulase production to perform cellulolytic functions, and it can also be used to convert lignocellulosic biomass into ethanol by saccharification.

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