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ENDOGLUCANASE PRODUCING POTENTIAL OF *BACILLUS* SPECIES ISOLATED FROM THE GUT OF *LABEO ROHITA*

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Key words: *Bacillus* sp. 16S rDNA, Cellulase, RSM, *Labeo rohita*.

ABSTRACT

In the current research, a microorganism, *Bacillus aquimaris*, had been isolated from the gut of *Labeo rohita* and classified by 16S ribosomal DNA genome sequencing. Sugarcane bagasse (substrate) was used for producing endoglucanase in submerged fermentation using this bacterium. Various factors affecting endoglucanase production were screened by Plackett-Burman design and optimization of significant parameters were carried out by the Box-Behnken design of response surface methodology. Results revealed that among nine parameters screened, concentrations of substrate, $MgSO_4$ and $(NH_4)_2SO_4$ were found significant. Maximum yield of endoglucanase (437.3833 IU) was achieved at 2.5% sugarcane bagasse, 0.01% $MgSO_4$ and 0.5% $(NH_4)_2SO_4$ after 24 h of the fermentation period. These results suggested the potential utilization of this strain for nutritional purpose to promote the growth of fishes for commercialization of aquaculture.

I. INTRODUCTION

Cellulose is a very important agricultural waste component (Bhat and Bhat, 1997). It is generally regarded as a never-ending raw material with many impressive and interesting structures and properties. Cellulose consists of β-1-4 glycosidic linked D-glucose units. In these units, glucose molecules are present as 6-membered rings that are accompanied with single oxygen atoms (Siro and Plackett, 2010). The cellulose linkage could easily be broken down with the help of enzymes. Cellulase is a class of enzymes that catalyzes the hydrolysis of cellulose. Simple metabolizable sugars can be produced from these fibrinolytic enzymes by disturbing and unsettling the cellulosic element (Murad and Azzaz, 2010).

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Cellulases belong to a group of hydrolases produced principally by bacteria, fungi, protozoans and termites (Watanabe et al., 1998; Lee et al., 2008). There are also many other types of organisms such as plants, mollusks and animals which produce cellulases (Watanabe and Tokuda, 2001). Cellulases have been accessible commercially not only for academic research but also widely used in detergents formulation, animal feed, wine production, juice clarification and paper industries (Singh, 1999; Singh et al., 2007; Sadhu et al., 2013). Enzyme yields, in general, depend on temperature, inoculum size, presence of inducers, pH, growth time and rate, aeration, medium additives and so on (Immanuel et al., 2006). Considering these facts, isolation and identification of potential cellulolytic bacteria from the gut of *Labeo rohita* and enhanced production of cellulose enzyme by optimization of process parameters by response surface methodology was the major purpose of this research.

II. MATERIALS AND METHODS

1. Microorganism

To study the cellulase-producing potential of bacteria, *Bacillus cereus* (KF625169), *B. pumilus* (KF625178) and *B. aquimaris* (KF551974) were isolated and identified from the intestine of freshwater fish, *Labeo rohita* caught from river Ravi, Pakistan. Briefly, 1 g of gut essence was extracted and settled in 9 ml autoclaved saline solution (0.9%) and reserved at 4°C in labeled glass vials. For the isolation of cellulase producing bacteria, 100 µl of given gut content dilution was spread on carboxymethyl cellulose agar plates (nutrient agar complemented with 1 % CMC) following incubation at 37°C for 24 h. The well separated colonies were processed for pure culturing and further study.

Genomic DNA was isolated and then16S ribosomal DNA was intensified with the help of universal primers, "27" forward 5'AGAGTTTGATCMTGGCTCAG3' and "1492" reverse 5'TACGGYTACCTTGTTACGACTT3' according to Shakir (2013). The PCR reaction mixture (50 µL) consisted of 10X PCR buffer (5 µl), DNA extract as template (5 µl), 2.0 U/µl Taq polymerase (2 µl), 25 mM $MgCl_2$ (5 µl), 1 mM dNTPs (5 µl), 10 pM forward primer (5 µl), 10 pM reverse primer (5 µl) and dH_2O (18 µl). A thermocycler was used for execution of PCR amplification. PCR conditions were as follow. Initial basic dena-

Table 1. Range of parameters used for Placket Burman design.

Sr. No.	Parameter	Label	Codes	
			+1	−1
1	Sugarcane bagasse Concentration (%)	X_1	2.5	0.5
2	pH	X_2	8.0	5.5
3	$MgSO_4$ (%)	X_3	0.2	0.01
4	Inoculum size (%)	X_4	5	1
5	Fermentation time (h)	X_5	72	24
6	Yeast Extract (%)	X_6	0.8	0.2
7	NaCl (%)	X_7	1.0	0.2
8	Peptone (%)	X_8	1.25	0.25
9	$(NH_4)_2SO_4$ (%)	X_9	1.0	0.2

turation for 5 minutes at 95°C which was then followed by 35 cycles of 94°C for 45 seconds of denaturation, 53°C for 45 sec. of annealing, 72°C for 1 minute of extension and 72°C of final extension for 7 min. Amplified outputs were monitored on 1 % agarose gel electrophoresis and then DNA was cleared and purified using GenJET™ kit. The purified amplicons were then sequenced commercially. The nucleotide sequences were then aligned using NCBI BLASTn and were deposited in GeneBank. The sequence retrieved was ordered using CLUSTAL W 1.81 (Thompson et al., 1994). A Neighbor-Joining method in MEGA 5.0 (Molecular Evolutionary Genetics Analysis, version 5.0) software was used for the construction of Phylogenetic tree (Tamura et al., 2011).

2. Enzyme Production

Fermentation medium executed from RSM (response surface methodology) was placed in Erlenmeyer flask of 250 ml capacity and then autoclaved at 121°C, for 15 minutes and 15 Psi. Following the sterilization, 1 ml of the 24 h old vegetative cell culture was taken from nutrient broth and inoculated under aseptic conditions to each of the respective fermentation flask. After the inoculation of medium, the fermentation flasks were incubated at 35°C with shaking speed of 120 rpm for 24 h in shaking incubator. After the termination of incubation period, muslin cloth was used for the filtration of fermented broth followed by centrifugation (Kokusan H-1500ER) for 10 minutes at 10,000 x g and 4°C for the replacement of cell mass and all the other unwanted particles. The clear cell-free essence retrieved after centrifugation was treated as a source of crude enzyme extract. Each of the experiment was carried out in triplicate independently.

3. Cellulase Assay

Carboxymethyl cellulase activity was measured and regulated as explained by Ghosh (1987). It was determined by incubating the reaction mixture consisting of 0.5 ml of aliquot of enzyme source and 0.5 ml of 1% Carboxymethylcellulose dissolved in 0.05M citrate buffer pH-5 at 50°C for 30 minutes. After the incubation, the reaction was stopped by adding 1.5 ml of dinitro salicylic acid (DNS) solution and test tube was placed in boiling water bath for 10 minutes. Absorbance was measured as OD_{540}, with the help of spectrophotometer (Spectrophotometer Cecil, CE 2042). One unit (U) of enzyme activity was described as the total extent of enzyme, which released 1 μmol of glucose under the standard assay conditions.

4. Experimental Design

Plackett-Burman experimental design was employed for screening and evaluation of the relative importance of medium's different constituents as 12 run experiment for producing the endoglucanase in submerged fermentation. Each variable was designated and used with a high (+) and a low (−) concentration (Table 1). The nutrient factors tested included concentration of sugarcane bagasse, $MgSO_4$, Yeast Extract, NaCl, Peptone, and $(NH_4)_2SO_4$. Moreover, the physical variables pH, fermentation period and inoculum size were also counted due to possible potential changes in the zones of these variables in the existence of numerous nutrients (Table 1).

For optimizing the process conditions for cellulase production, Box-Behnken design (BBD) was practiced. The independent parameters used for optimization were yeast extract (X_6), concentration of sugarcane bagasse (X_1) and $MgSO_4$ (X_3) and their levels have been mentioned in Table 3. This layout is most appropriate and satisfactory for quadratic response surface and set up for second order polynomial regression model. The relationship amid actual and coded values was defined by the equation given below,

$$x_i = \frac{X_i - X_\circ}{\Delta X_i} \tag{1}$$

where *xi* and *Xi* are the coded and actual values of the independent variable respectively, *Xo* is the actual charge of the independent variable at the central point and ΔXi is the transition of *Xi*. The response is estimated from the following equation with the help of the STATISTICA software (99th edition).

$$y = \beta_\circ + \sum_{i=1}^{k} + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_i \sum_j \beta_{1j} X_i X_j \tag{2}$$

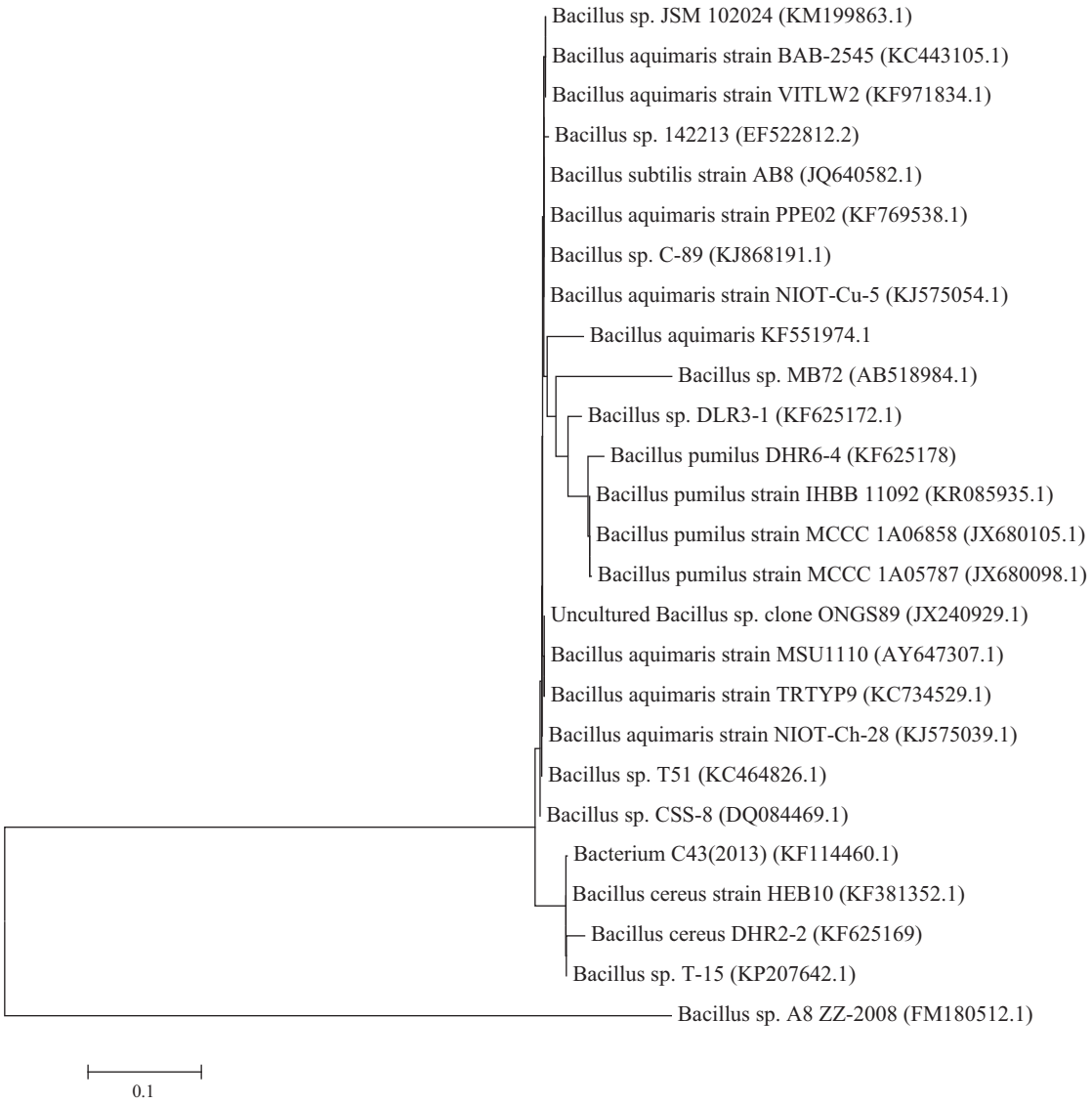

Fig. 1. Phylogenetic studies of a newly isolated *Bacillus sp.* from the gut of fish.

In the above equation Y is the response, k is the number of variables, β_0 is the intercept, X_i and X_j are independent absolute variables, βi is th *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient and β_{ij} is the interaction coefficient.

III. RESULTS AND DISCUSSION

In this investigation, *Bacillus* species isolated from the gut of *Labeo rohita* were identified and evaluated for cellulolytic potential. The bacterium with highest cellulolytic potential was classified as *Bacillus aquimaris* using the 16S ribosomal DNA sequencing and deposited in gene bank under the accession number of KF551974. The phylogenetic analysis of this strain revealed that it shared 97% homology with *Bacillus aquimaris* strain PPE02 (KF769538.1), *Bacillus aquimaris* strain NIOT-cu-5 (KJ575054.1) and others as shown in Fig. 1. Sreeja et al. (2013) isolated and identified two bacteria *Bacillus altitudinis* APS MSU and *Bacillus licheniformis* APS2 MSU from the gut of *Etroplus suratensis* which had the potential of cellulolytic activity. Ray et al. (2007) reported two bacteria *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 from the gut of common carp which exhibited cellulase activity. The gut of murrel and rohu contained bacterial community which had cellulolytic and proteolytic potentials (Kar and Ghosh, 2008).

The isolated *Bacillus* strains were assessed for cellulolytic production potential in submerged fermentation employing the sugarcane bagasse as substrate. Results (Fig. 2) showed that *Bacillus aquimaris* yielded highest endoglucanase production among all the tested strains. The fermentation period was also studied by sampling with regular interval of 24 h and best enzyme titer was achieved after 24 h of the fermentation period. The decrease in enzyme production as a result of increased fer-

Table 2. Plackett-Burman design (PBD) for the screening of parameters for endoglucanase production in submerged fermentation.

Run No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	Endoglucanase activity (IU)		Residual
										Observed	Predicted	
1	+1	+1	+1	+1	+1	+1	+1	+1	+1	207.000	260.922	-53.922
2	-1	+1	-1	+1	+1	+1	-1	-1	-1	64.0167	67.0514	-3.0347
3	-1	-1	+1	-1	+1	+1	+1	-1	-1	74.3667	71.3319	3.0347
4	+1	-1	-1	+1	-1	+1	+1	+1	-1	630.200	576.277	53.922
5	-1	+1	-1	-1	+1	-1	+1	+1	+1	49.2583	-4.6639	53.922
6	-1	-1	+1	-1	-1	+1	-1	+1	+1	181.700	184.734	-3.034
7	-1	-1	-1	+1	-1	-1	+1	-1	+1	50.4083	104.330	-53.922
8	+1	-1	-1	-1	+1	-1	-1	+1	-1	273.700	327.622	-53.922
9	+1	+1	-1	-1	-1	+1	-1	-1	+1	377.008	373.973	3.0347
10	+1	+1	+1	-1	-1	-1	+1	-1	-1	242.458	245.493	-3.0347
11	-1	+1	+1	+1	-1	-1	-1	+1	-1	55.0083	51.9736	3.0347
12	+1	-1	+1	+1	+1	-1	-1	-1	+1	175.183	121.261	53.922

Table 3. Box-Behnken Design for optimization of process parameters for endoglucanase production by *Bacillus aquimaris* in submerged fermentation.

Run No.	X_1	X_3	X_6	Endoglucanase activity (IU)		Residual
				Observed	Predicted	
1	1.5	0.11	0.5	286.7333	286.7333	0.0000
2	2.5	0.11	0.8	287.6917	337.4813	-49.789
3	2.5	0.2	0.5	343.85	342.9094	0.9406
4	2.5	0.11	0.2	403.2667	377.9337	25.333
5	2.5	0.01	0.5	437.3833	413.8674	23.5159
6	0.5	0.2	0.5	58.65	83.4531	-24.8031
7	1.5	0.01	0.2	91.80833	139.3240	-47.5156
8	0.5	0.11	0.8	100.4333	125.7663	-25.3330
9	1.5	0.2	0.8	230.575	179.1055	51.4695
10	0.5	0.11	0.2	21.85	-27.9396	49.7896
11	0.5	0.01	0.5	50.6	50.2534	0.3466
12	1.5	0.01	0.8	260.2833	236.6302	23.6531
13	1.5	0.2	0.2	131.4833	159.0903	-27.6070

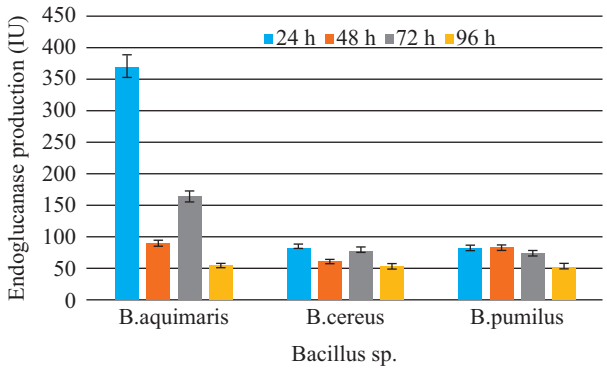

Fig. 2. Effect of fermentation time on endoglucanase production by respective *Bacillus* species.

mentation time might be due to the deficiency of nutrients or due to substrate limiting factor (Shahid et al., 2016). Various researchers reported that *Bacillus* sp. produce maximum cellulase after 24 h of fermentation period (Shabeb et al., 2010; Heck et al., 2002).

For screening, numerous medium ingredients have been checked with the help of the Plackett-Burman statistical design (PBD) that is a chunk of a two-level factorial design and allows the analysis of n-1 variables in somewhat n experiments (Plackett and Burman, 1946). Nine different parameters with their minimum (–1) and maximum (+1) values (Table 1) were taken in 12 runs for study and all the experiments for the Plackett-Burman design and executed in duplication and their average was taken as a response. Plackett-Burman design experiments showed extensive fluctuation in cellulase activity (Table 2). This fluctuation reflected the usefulness of optimization to earn maximum productivity. The average values were regarded as observed or experimental values. Substrate concentration, $MgSO_4$ and yeast extract were found significant parameters, whereas other six factors appeared to be insignificant for cellulase production.

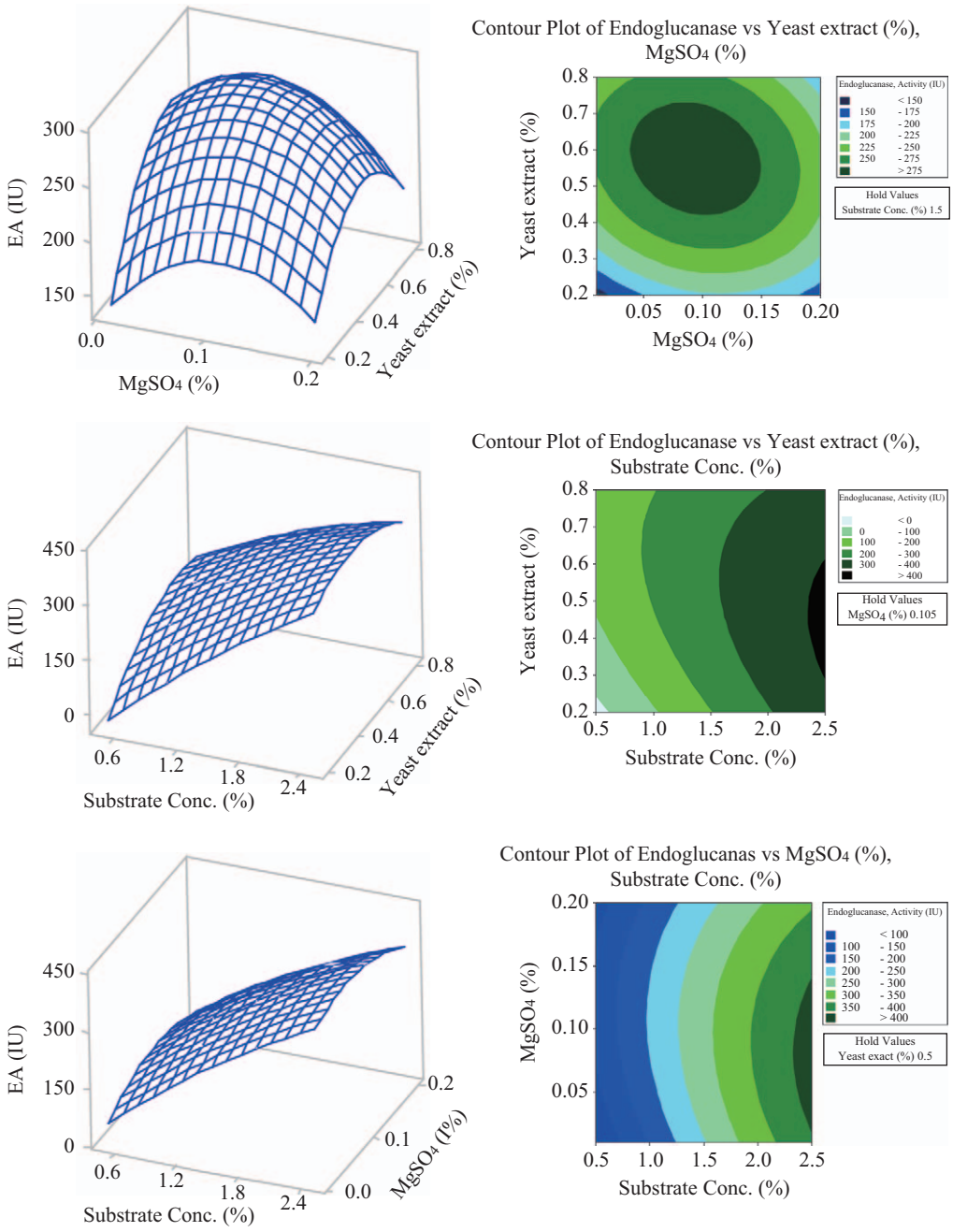

Fig. 3. Surface and contour plot of interactions of various parameters for endoglucanase production.

1. Optimization Using Box-Behnken Design (BBD)

On the basis of PBD results, the experiment was more broadened for optimization using BBD. The Plackett-Burman design identified the variables X_1 (Substrate concentration), X_3 ($MgSO_4$), and X_6 (yeast extract) as significant for the strong effects on the response. Therefore, the effect of these three significant parameters were studied by Box-Behnken design. The designed model for BBD (Box-Behnken design) and their predicted and observed values are given in Table 3. The run No. 5 showed higher cellulase activity in which 2.5 % (+1 level) of substrate concentration (X_1), 0.01 % (–1 level) of $MgSO_4$ (X_3), and 0.5 % (0 level) of yeast extract (X_6) were used. The lowest production was observed at run No. 10 where -1 level of substrate concentration (X_1), 0 level of $MgSO_4$ (X_3), and -1 level of yeast extract (X_6) was employed.

2. Interaction Effects of Significant Parameters

The interactive consequences of variables on the production of cellulase were examined by plotting 3-D surface curves across any two independent variables, while managing another variable constant or at it's central (0) level at the same time. The con-

Table 4. Analysis of variance for endoglucanase production.

Effect	df	SS	MS	F	p
Model	9	224012.6	24890.29	5.474730	0.054486
X_1	1	20736.66	20736.66	4.561120	0.094562
X_1^2	1	884.34	884.34	0.194516	0.689034
X_3	1	7441.34	7441.34	1.636757	0.290748
X_3^2	1	4612.49	4612.49	1.014538	0.388018
X_6	1	19239.11	19239.11	4.231729	0.090160
X_6^2	1	9290.20	9290.20	2.043420	0.248209
X_1*X_3	1	2715.96	2715.96	0.597388	0.495887
X_1*X_6	1	9424.36	9424.36	2.072931	0.245550
X_3*X_6	1	1495.54	1495.54	0.328951	0.606451
Error	4	13639.19	4546.40		

$R = 0.970880$, $R^2 = 0.942609$, Adj. $R^2 = 0.770434$

tour plots for response surfaces were plotted which depicted the combined effects of concentration of substrate (X_1) and $MgSO_4$ (X_3), concentration of substrate (X_1) and yeast extract (X_6), $MgSO_4$ (X_3) and yeast extract (X_6), respectively. The plotting 3-D surface curves of the estimated responses (cellulase activity) from the associations between the variables are shown in Fig. 3.

Fig. 3 illustrates the dependency of endoglucanase on substrate concentration. The endoglucanase production rises with increase in the substrate concentration up to about 3.5 % and after that endoglucanase production decreases with further increase in substrate concentration. Effect of $MgSO_4$ was observed in Fig. 3 which showed that endoglucanase production was high when $MgSO_4$ was used 0.3 % and a thereafter further increase in its concentration decreased endoglucanase production. The dependencies of endoglucanase production on yeast extract could be seen from Fig. 3. The endoglucanase production increases with the increase in concentration of yeast extract up to 1.2 % and further increase in the concentration of yeast extract resulted decreased production of endoglucanase. The smaller concentrations of both yeast extract and $MgSO_4$ increased the cellulase activity, whereas in Fig. 3, it has been shown that high substrate concentration with low concentrations of yeast extract and $MgSO_4$ produces high amounts of endoglucanase. It was proposed in an earlier study that yeast extract was a symbolic framework for the production of cellulase by *Aeromonas* sp which was isolated from the gut of *Labeo rohita* (Majeed et al., 2016). Recent study showed that substrate (potato peel) concentration of 2.0%, yeast extract concentration of 1.0% and 24 h of fermentation period yield maximum carboxymethyl cellulase production from *B. subtilis* K-18 (Irfan et al., 2017). Arooj et al. (2017) reported that 2% pretreated banana peduncle and 1% yeast extract gave better cellulase productivity by *B. subtilis* K-18 after 24 h of fermentation.

Enzyme production was positively affected by the interaction coefficients between temperature and pH, substrate concentration as well as basal medium contents and incubation period (Sarkar and Aikat, 2012). Pandey et al. (2001) also claimed that the yield and output of enzyme production can be affected by many factors such as, nature of strain, nature of the substrate, cultural conditions and availability of the nutrients. Another study reported that cellulase output come to be dependent upon a very composite relationship which involves a variety of variables such as pH value, inoculum size and temperature, existence of inducers, medium additives, growth time aeration and so on (Immanuel et al., 2006). Satisfactory optimization of microbial enzyme production becomes possible with the implication of the 3D plots for response surface which allows direct visualization of parameter interaction (Mullai et al., 2010).

3. Statistical Analysis and Model Fitting

The experimental data were analyzed by the regression procedure to fit the following second-order polynomial equation for calculation of endoglucanase production

$$\begin{aligned} Y_{activity} = & -461.26 + 324.46X_1 + 1699.37X_3 + 1120.02X_6 \\ & - 19.67X_1^2 - 4993.28X_3^2 - 708.37_6^2 - 74.10X_1X_3 \\ & - 161.80X_1X_6 - 677.99X_3X_6 \end{aligned} \quad (3)$$

where, Y is the cellulase activity, X_1, X_3, and X_6 are concentrations of substrate, $MgSO_4$ and yeast extract, respectively.

Statistical analysis of the data was also operated by analysis of variance (ANOVA) to determine the importance of significant parameters that were optimized using BBD. ANOVA of the different parameters (i.e., F value and p-value) have been given in Table 4. The F and p values essentially exhibit the individual and associated effects of the independent variables. This method of analysis was regulated for assessment of effects of the variables and their possibly occurring interactions. Coefficients of the full model were evaluated for their significance and the insignificant ones were knocked out from the model by backward elimination. The reduced model was regulated after all the insignificant variables (p-value > 0.05) were eliminated. Therefore, the higher the F-value and lower the p-value, the more sig-

nificant the analogous coefficient (Zambare and Christopher, 2011). The Fisher F-test with a very minor probability value indicated very high significance for the regression model (Long et al., 2009).

Results of ANOVA for the adjusted model are shown in Table 4. The p-value of the model (0.054486) indicates that the model was statistically compelling and accurate. In the current work, the linear effects of X_1, X_6 and the square effects of X_6^2 are significant model terms for endoglucanase production because these values were very near to adjusted model value which was equal to 0.054486. The coefficient of determination (R^2) for endoglucanase activity was determined as $R^2 = 0.942609$. The overall regression coefficient of the model ($R^2 = 0.942609$) showed a proximate relationship with adjusted $R^2 = 0.770434$. The vicinity of adjusted R^2 to model R^2 means a positive adjustment and fitting of the theoretical values with the experimental data by the model.

IV. CONCLUSION

The above results advised that isolated bacterium had cellulolytic potential and can easily be employed for industrial exploitation like saccharification of lignocellulose biomass for the production of biofuel and can also be used for nutritional purposes to enhance the bio assimilation of cellulosic substrates and commercialization of aquaculture by improving and promoting the growth of fishes.

REFERENCES

Arooj, A., M. Irfan, F. Tabssum, H. A. Shakir and J. I. Qazi (2017). Effect of dilute sulphuric acid pretreatment on cellulase production by *Bacillus subtilis* (K-18) through response surface methodology. Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 54 (1), 11-20.

Bhat, M. K. and S. Bhat (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advance 15,583-620.

Heck, J. X., P. F. Hertz and M. A. Z. Ayub (2002). Cellulase and xylanase production by isolated amazon Bacillus strains using soybean industrial residue based solid-state cultivation. Brazilian Journal of Microbiology 33, 213-218.

Immanuel, G., R. Dhanusha, P. Prema and A. Palavesam (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. International Journal of Environmental Science and Technology 3, 25-34.

Irfan, M., Q. Mushtaq, F. Tabssum, H. A. Shakir and J. I. Qazi (2017). Carboxymethyl cellulase production optimization from newly isolated thermophilic *Bacillus subtilis* K-18 for saccharification using response surface methodology. AMB Express 7, 29.

Kar, N. and K. Ghosh (2008). Enzyme Producing Bacteria in the Gastrointestinal Tracts of Labeo rohita (Hamilton. and Channa punctatus (Bloch). Turkish Journal of Fisheries and Aquatic Science 8, 115-120.

Lee, Y. J., B. K. Kim, B. H. Lee, K. I. Jo, N. K. Lee, C. H. Chung, Y. C. Lee and J. W. Lee (2008). Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. Bioresource Technology 99, 378-386.

Long, C., Y. Ou, P. Guo, Y. Li, J. Cui, M. Long and Z. Hu (2009). Cellulase production by solid state fermentation using bagasse with Penicillium decumbens L-06. Annals of Microbiology 59, 517-523.

Majeed, H. S., M. Irfan, H. A. Shakir and J. I. Qazi (2016). Filter Paper activity producing potential of *Aeromonas* species isolated from the Gut of Labeo rohita. Pakistan Journal of Zoology 48, 1317-1323.

Mullai, P., N. S. A. Fathima and E. R. Rene (2010). Statistical analysis of main and interaction effects to optimize xylanase production under submerged cultivation conditions. Journal of Agricultural Science 2, 144-153.

Murad, H. A. and H. H. Azzaz (2010). Cellulase and dairy animal feeding. Biotechnology 9, 238-256.

Plackett, R. L. and J. P. Burman (1946). The design of optimum multifactorial experiments. Biometrika 33, 305-325.

Pandey, A., C. Soccol, J. Rodriguez-Leon and P. Nigam (2001). Solid-state fermentation in biotechnology-fundamentals and applications, Asiatech Publishers Inc., New delhi 50, 225.

Ray, A. K., A. Bairagi, K. S. Ghosh and S. K. Sen (2007). Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* cy5 and *Bacillus circulans* tp3 isolated from fish gut. Acta Ichth et Piscatoria 37, 47-53.

Sadhu, S., P. Saha, S. K. Sen, S. Mayilraj and T. K. Maiti (2013). Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. Springer Plus 2, 10.

Sarkar, N. and K. Aikat (2012). Cellulase and xylanase production from rice straw by a locally isolated fungus *Aspergillus fumigatus* NITDGPKA3 under Solid State Fermentation-Statistical optimization by response surface methodology. Journal of Technology Innovation and Renewable Energy 1, 54-62.

Shahid, Z. S., M. Irfan, M. Nadeem, Q. Syed and J. I. Qazi (2016). Production, purification, and characterization of carboxymethyl cellulase from novel strain *Bacillus megaterium*. Environmental Progress & Sustainable Energy, 35, 1741-1749.

Shakir, H. A. (2013). Enteric bacterial and heavy metals load and health status of fishes from river ravi; Pakistan. Ph.D. Thesis; Department of zoology; University of the Punjab new campus Lahore.

Singh, A. (1999). Engineering enzyme properties. Indian Journal of Microbiology 39(2), 65-77.

Singh, A., R. C. Kuhad and O. P. Ward (2007). Industrial application of microbial cellulases. In, *Lignocellulose Biotechnology*, *Future Prospects*, (Eds. R. C. Kuhad and A. Singh., I.K) International Publishing House, New Delhi, India 345-358.

Siro, I. and D. Plackett (2010). Microfibrillated cellulose and new nano composite materials, a review. Cellulose 17, 459-494.

Sreeja, S.J., M. P. W. Jeba, S. F. R. Joseph, S. Tiburcius, G. Immanuel and A. Palavesam (2013). Optimization of cellulase production by *Bacillus altitudinis* APS MSU and Bacillus licheniformis APS2 MSU, gut isolates of fish *Etroplus suratensis*. International Journal of Advance in Research and Technology 2, 401-406.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar (2011). MEGA5, Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Molecular Biology and Evolution 28, 2731-2739.

Thompson, J. D., D. G. Higgins and T. J. Gibson (1994). Clustal-W improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuclic Acids Research 22, 4673-4680.

Watanabe, H. and G. Tokuda (2001). Animal Cellulases. Cell Molecular Life Science 58, 1167-1178.

Watanabe, H., H. Noda, G. Tokuda and N. Lo (1998). A cellulase gene of termite origin. Nature 394, 330-331.

Zambare, V. and L. Christopher (2011). Statistical analysis of cellulase production in *Bacillus amyloliquefacien* UNPDV-22. ELBA Bioflux 3, 38-45.