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Filter Paper Activity Producing Potential of *Aeromonas* Species Isolated from the Gut of *Labeo rohita*

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ABSTRACT

In this study different species of *Aeromonas* were isolated from gut of *Labeo rhoita* and identified by 16SrDNA gene sequencing technology. All isolated species were evaluated for exoglucanase production in submerged fermentation. Of all tested species, *Aeromonas bestiarum* was found best for maximum production of exoglucanase after 24 h of fermentation at 35 ˚C using sugarcane bagasse as substrate. To enhance exoglucanase production, various factors were screened by Plackettt-Burman designe and optimizations of significant parameters were carried out by Box-Behnken design of response surface methodology. Among nine parameters screened, substrate concentration, yeast extract and $(NH_4)_2SO_4$ concentration were found significant. The optimized levels of these parameters were. 2.5% sugarcane bagasse, 0.2% yeast extract and 0.6% $(NH_4)_2SO_4$ which yielded maximum (3.766 IU) exoglucanase production after 24h of fermentation period. These results suggested the potential utilization of this strain for nutritional purpose to promote the growth of fishes for commercialization of aquaculture.

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Authors' Contribution
JIQ and MI conceived and designed the study. HSM conducted experimental work. HAS isolated bacterial species. MI analyzed the data and wrote the article.

Key words
Aeromonas sp ,
16SrDNA,
Cellulase, RSM
Labeo rhoita

INTRODUCTION

In many plants, fungal, bacterial and yeast species, significant family of enzymes *i.e.*, cellulases (EC 3.2.1.4) are present. These enzymes degrade the β-1,4 glucosidic bonds connecting glucose units that contain the duplicate units of cellulose (Boschker and Cappenberg, 1994, Hilden and Johannson, 2004). Cellulases are produced abundantly in solution by some microbes such as bacteria and fungi (Lamed *et al.*, 1983). There are three types of cellulases namely exoglucanase or cellubiase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). The endoglucanase break the internal bonds of cellulose to produce glucan (chain of glucose) sequences of various lengths, exoglucanase act on the ends of chains of glucose to produce cellubiose and β glucosidase act on the cellubiose to produce monomers *i.e.*, glucose (Bayer *et al.*, 1998; Singh, 1999). Cellulases are used for many purposes, but these enzymes are mainly used in textile, chemical, food and fuel industries. Cellulases can also be used in protoplast production, medical industry, paper industry, genetic engineering, and pollution treatment (Coughlan, 1985; Mandels, 1985).

Most significant products, including enzymes production, are obtained through microbial processes for human use. Many enzymes are produced using diverse microorganisms in bioprocess technologies (Sukumaran *et al.*, 2005). In majority of reports, submerged fermentation is utilized for the microbial production of cellulases. On the other hand, cellulolysis and growth by aerobic microbes in nature have similarity with solid state fermentation as compared to liquid media (Hölker *et al.*, 2004; Zhu *et al.*, 2009).

The optimization of different parameters is obtained by using response surface methodology (RSM) (Balusu *et al.*, 2005). RSM is a statistical and mathematical approach to determine the effect of response function and independent variables (Balusu *et al.*, 2005; Wang *et al.*, 2008). RSM is also used for designing models, experiments, formulation, estimating the effects of several parameters and determining the optimal culture conditions for better responses and lowering the number of needed experiments (Coninck *et al.*, 2000). To optimize the development of microbes, RSM is a better adopted approach (Balusu *et al.*, 2005; Popa *et al.*, 2007; Wang *et al.*, 2008; Mei *et al.*, 2009). The present study was designed for isolation and identification of cellulase producing bacteria from fish gut contents following optimization of medium using response surface methodology.

MATERIALS AND METHODS

Isolation and molecular identification of bacterium

Four bacteria *Aeromonas hydrophila* (KF551976),

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A. allosaccharophila (KF625182), *A salmonicida* (KF551975) and *A. bestiarum* (KF625168) were isolated from gut of *Labeo rohita* and identified. The procedure followed as, from the intestine of freshwater fish, *Labeo rohita* caught from river Ravi, Pakistan, 1g of gut contents were squeezed out and placed in 9 ml autoclaved saline solution (0.9 %) in labeled glass tubes and stored at 4°C. For the isolation of cellulase producing bacteria, 100 µl of the gut content dilution was spread on carboxymethyl cellulose agar plates. The four isolates appeared on the agar plates after incubation at 37°C for 24 h. The genomic DNA was isolated from these four isolates and then 16S rDNA gene was amplified by using the universal primers,
27 forward 5'AGAGTTTGATCMTGGCTCAG3'
and
1492 reverse 5'TACGGYTACCTTGTTACGACTT3'
following the procedure according to Shakir (2013). The PCR reaction mixture (50 µL) contained DNA extract as template (5 µl), 10X PCR buffer (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). PCR amplification was carried out in a thermocycler (Eppendorf, Mastercycler Personel, Germany). PCR condition was as follows; 95° C for 5 min (initial denaturation), followed by 35 cycles of 94°C for 45 sec. (denaturation), 53°C for 45 sec (annealing), 72°C for 1 min (extension) and final extension 72°C for 7 min. Amplified products were checked on 1 % agarose gel electrophoresis and then the amplified DNA was purified using GenJET ™ kit. The purified amplicons were then sequenced commercially. The nucleotide sequences were then aligned using NCBI BLASTn and were then deposited in GeneBank.

The sequence obtained was aligned using CLUSTAL W 1.81 (Thompson *et al.,* 1994). The phylogenetic tree was constructed by Neighbor-Joining method in MEGA 5.0 (Molecular Evolutionary Genetics Analysis, version 5.0) software (Tamura *et al.,* 2011). After identification, the enzyme producing potential of each strain was assessed.

Enzyme production

For enzyme production self-designed fermentation medium comprising of (%) peptone 1.25, $(NH_4)_2SO_4$, K_2HPO_4 0.5 with 2.5g sugarcane bagasse as substrate was taken in 250ml capacity Erlenmeyer flask and autoclaved at 121ºC, for 15 minutes and 15 Psi pressure. After sterilization, 1ml of the 24h old vegetative cell culture was transferred aseptically to each of the fermentation flasks. After inoculation, the flasks were incubated at 50°C with agitation speed of 120 rpm for 24 h. After the termination of the fermentation period, the fermented broth was filtered through muslin cloth followed by centrifugation (Sigma 2-16PK) for 10 minutes at 10,000 rpm and 4ºC for the removal of cell mass and unwanted particles. The clear cell free extract obtained after centrifugation was used as a crude source of enzyme. Triplicate readings were taken for each of the experiment.

Filter paper assay

Filter paper activity was estimated as described in our earlier report (Irfan *et al.,* 2011). Reaction mixture containing 0.5 ml of crude enzyme solution and 0.5ml of 0.05M citrate buffer pH 5 containing 50 mg of filter paper was incubated at 50ºC for 30 minutes. After incubation, 1.5 ml of dinitrosalicylic acid (DNS) solution was added to stop the reaction and test tube was boiled for 10 minutes in a water bath. Absorbance was taken at 540 nm using spectrophotometer (Spectrophotometer Cecil, CE 2042). One unit (U) of enzyme activity was defined as the quantity of enzyme, which released 1µmol of glucose under standard assay conditions. After enzyme assay, the better one out of four isolated strains was employed to optimization of medium components using surface response methodology.

Experimental design

Plackett–Burman experimental design was used to screen out and evaluate the relative importance of different medium components as 12 runs experiment for FPase production in submerged fermentation. Each variable was designated and used with a high (+) and a low (−) concentration (Table I). The nutrient factors tested included substrate concentration, $MgSO_4$, yeast extract, NaCl, peptone and $(NH_4)_2SO_4$. Furthermore, the physical parameters like pH, inoculum size and fermentation period were included due to possible changes in the levels of these parameters in the presence of various nutrients.

Table I.- Range of parameters used for Plackett-Burman design.

Parameter	Label	Codes	
		+1	-1
Substrate conc. (%)	X_1	2.5	0.5
pH	X_2	8.0	5.5
$MgSO_4$ (%)	X_3	0.2	0.01
Inoculum size (%)	X_4	5	1
Fermentation time (h)	X_5	72	24
Yeast extract (%)	X_6	0.8	0.2
NaCl (%)	X_7	1.0	0.2
Peptone (%)	X_8	1.25	0.25
$(NH_4)_2SO_4$ (%)	X_9	1.0	0.2

Table II.- Plackett-Burman design for screening of parameters for exoglucanase production in submerged fermentation.

un No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	Exoglucanase activity (IU)		Residue
										Observed	Predicted	
1	2.5	8.0	0.2	5	72	0.8	1.0	1.25	1.0	2.1307	2.130700	0.000000
2	0.5	8.0	0.01	5	72	0.8	0.2	0.25	0.2	1.28885	1.166422	0.122428
3	0.5	5.5	0.2	1	72	0.8	1.0	0.25	0.2	1.20839	1.330818	-0.12242
4	2.5	5.5	0.01	5	24	0.8	1.0	1.25	0.2	2.682	2.682000	0.000000
5	0.5	8.0	0.01	1	72	0.2	1.0	1.25	1.0	0.79864	0.798640	0.000000
6	0.5	5.5	0.2	1	24	0.8	0.2	1.25	1.0	1.4006	1.278172	0.122428
7	0.5	5.5	0.01	5	24	0.2	1.0	0.25	1.0	0.57663	0.576630	0.000000
8	2.5	5.5	0.01	1	72	0.2	0.2	1.25	0.2	2.17838	2.178380	0.000000
9	2.5	8.0	0.01	1	24	0.8	0.2	0.25	1.0	1.96233	2.084758	-0.12242
10	2.5	8.0	0.2	1	24	0.2	1.0	0.25	0.2	2.10239	1.979962	0.122428
11	0.5	8.0	0.2	5	24	0.2	0.2	1.25	0.2	0.88655	1.008978	-0.12242
12	2.5	5.5	0.2	5	72	0.2	0.2	0.25	1.0	1.40507	1.405070	0.000000

Table III.- Box-Behnken design for exoglucanase production.

Run No.	X_1	X_6	X_9	Exoglucanase activity (IU)		Residue
				Observed	Predicted	
1	1.5	0.5	0.6	1.88038	1.880380	0.000000
2	2.5	0.5	1.0	1.98617	2.361464	-0.375294
3	2.5	0.8	0.6	2.09345	2.433915	-0.340465
4	2.5	0.5	0.2	1.97127	2.115614	-0.144344
5	2.5	0.2	0.6	3.76672	2.906618	0.860102
6	0.5	0.8	0.6	0.94019	1.800293	-0.860103
7	1.5	0.2	0.2	1.19498	1.910739	-0.715759
8	0.5	0.5	1.0	0.37995	0.235606	0.144344
9	1.5	0.8	1.0	3.47766	2.761901	0.715759
10	0.5	0.5	0.2	0.77927	0.403976	0.375294
11	0.5	0.2	0.6	0.04321	-0.297255	0.340465
12	1.5	0.2	1.0	1.19796	1.682769	-0.484809
13	1.5	0.8	0.2	2.94126	2.456451	0.484809

In order to optimize process conditions for cellulase production, Box-Behnken design (BBD) was used for optimization study. The independent variables used were substrate concentration (X_1), $MgSO_4$ (X_3) and yeast extract (X_6) and their levels are mentioned in Table III. This design is most suitable for quadratic response surface and generates second order polynomial regression model. The relation between actual and coded values was described by the following equation.

$$x_i = \frac{X_i - X_\circ}{\Delta X_i} \qquad \text{Eq. (1)}$$

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 ΔXi is the change of *Xi*. The response is calculated from the following equation using STATISTICA software (99th edition).

$$y = \beta_\circ + \sum_{i=1}^{k} + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_i \sum_j \beta_{1j} X_i X_j \qquad \text{Eq. (2)}$$

Where Y is the response, k is the number of variables, $ß_0$ is the intercept, Xi and Xj are independent variables, *ßi* is the *i*th linear coefficient, $ß_{ii}$ is the *i*th quadratic coefficient and $ß_{ij}$ is the interaction coefficient.

RESULTS AND DISCUSSION

In the present investigation, four species of *Aeromonas i.e., Aeromonas hydrophila* (KF551976), *A. allosaccharophila* (KF625182), *A. salmonicida* (KF551975) and *A. bestiarum* (KF625168) were isolated

from gut of *Labeo rohita* and identified. Identification of these strains was done by 16S rDNA gene sequencing technology. The sequencing results were subjected to BLAST analysis revealing 99% homology with *Aeromonas* species as shown in Figure 1.

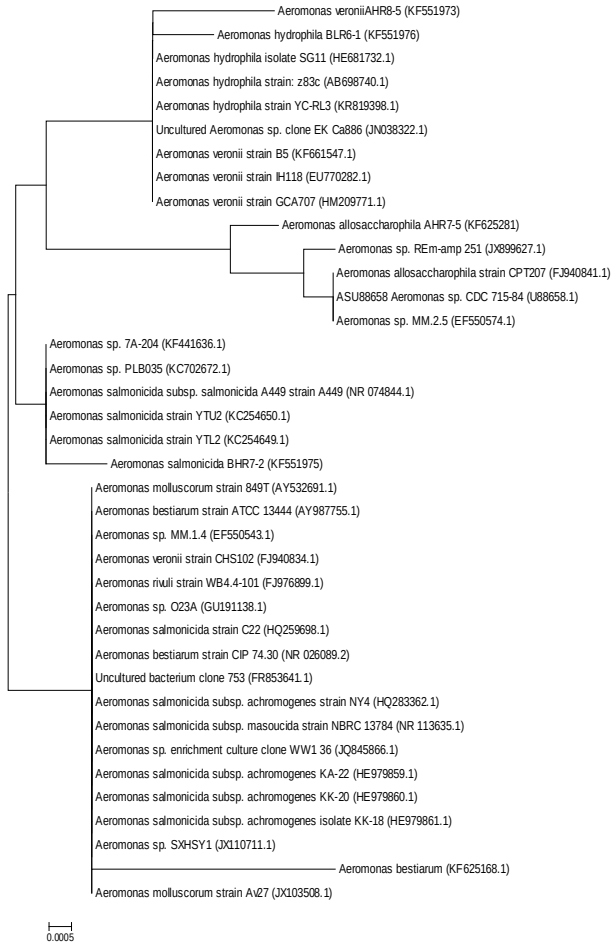

Fig. 1. Phylogenetic analysis of *Aeromonas bestiarum* isolated from gut of *Labeo rhoita*.

Selection of bacteria

These four *Aeromonas* species namely *Aeromonas veronii, Aeromonas hydrophila, Aeromonas bestiarum, Aeromonas salmonicidia,* and *Aeromonas allosaccharophila* were screened for exoglucanase production in submerged fermentation using sugarcane bagasse as substrate. Results (Fig. 2) revealed that *Aeromonas bestiarum* showed maximum exoglucanase production followed by *Aeromonas allosaccharophila, Aeromonas salmonicidia, Aeromonas veronii* and *Aeromonas hydrophila* after 24 h of fermentation at 37 ^{o}C, respectively. Further increase in fermentation period resulted decline in exoglucanase production. The best producer strain (*Aeromonas bestiarum*) was used for subsequent process optimization process for exoglucanase production. Among various bacteria isolated from gut of three phytophagus insect species, *Aeromonas* sp. exhibited positive for cellulase production (Shil *et al.,* 2014). Cellulolytic potential of *Aermonas* sp. has been reported earlier which effectively degrade the microalga for biogas production (Munoz *et al.,* 2014). Jiang *et al.* (2011) reported that *Aeromonas* sp were the dominant cellulase producing bacteria in the gut of the *Ctenopharyngodon idellus.*

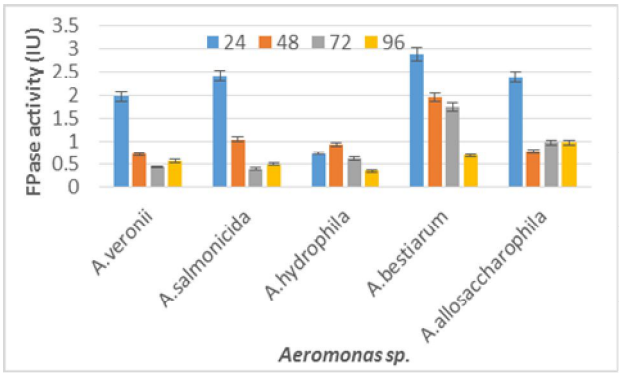

Fig. 2. Exoglucanase production potential of *Aeromonas* sp. isolated from gut of *Labeo rhoita* at different fermentation time.

Screening of most significant medium components by Plackett-Burman design

In comparison to the single-variable optimization approach, the use of RSM provides a more accurate determination of the parameters under optimization and reduces the time and number of experiments required. In most of the studies, scientists have used RSM technology for the production of cellulase enzymes by using different culture conditions and parameters.

Nine independent variables such as substrate concentration, pH, magnesium sulphate, inoculum size, fermentation time, yeast extract, sodium chloride, peptone and $(NH_4)_2SO_4$ were screened for exoglucanase production by Plackett-Burman design. Twelve runs were performed by different combinations of nutrient variables with minimum (-1) and maximum (+1) values as shown in Table I. Plackett-Burman experiments' results (Table II) showed a wide variation in exoglucanase production. This variation reflected the importance of optimization to attain higher productivity. Significant parameters were selected for further optimization of exoglucanase production. From the regression analysis, the variables which were significant at or above 95% level (P <0.05), were considered to have greater impact on cellulase

activity and were further optimized by Box -Behnken design.

Using Plackett-Burman design substrate concentration (bagasse), yeast extract and ammonium sulphate were found to be most significant variables, which significantly enhanced cellulase activity. Box-Behnken design was employed to optimize these selected nutrients. The optimal levels of components were obtained as substrate concentration (2.5%) yeast extract (0.2%) and ammonium sulphate (0.6%). Using the optimized conditions, the produced exoglucanase activity reached 3.766 IU in 24 h. The results showed a close agreement between the predicted and obtained activity levels and indicated that *A. bestiarum* DHR5-3 can be cultivated under submerged fermentation for the production of cellulolytic enzyme using sugarcane bagasse as carbon source.

Optimization by Box-Behnken design

Three significant variables such as substrate concentration (bagasse), yeast extract and $(NH_4)_2SO_4$ were selected for further optimization to attain a maximum production of exoglucanase. The levels of factors *i.e.*, bagasse, yeast extract and $(NH_4)_2$ SO_4 and the effect of their interactions on cellulase production were determined by Box-Behnken design of RSM. Experiments were performed at different combinations of the factors and predicted and observed values were compared and the residual obtained showed the relationships of variables (Table III). By optimizing with coded factor the maximum exoglucanase production observed by the model was 3.766 IU after 24h incubation which was close to the predicted value of 2.9066 IU that shows its validity. The optimized conditions were substrate concentration of 2.5%, yeast extract 0.2% and 0.6% of ammonium sulphate as medium components.

The response equation obtained (Eq. 3) for the critical medium components was as follows:

$$Y\ (\text{Exoglucanase activity, IU}) = -1.60985 + 3.51491X_1 - 0.29055X_6 - 0.48627X_9 - 0.54664X_1^2 + 4.19063X_6^2 - 0.34107X_9^2 - 2.14188\ X_1X_6 + 0.25889X_1X_9 + 1.11129X_6X_9 \quad \text{(Eq.3)}$$

Where *Y* is the predicted cellulase activity for critical medium components. X_1 – the coded value of substrate concentration. X_2 - the coded value of yeast extract and X_3 - the coded value of ammonium sulphate.

Interaction effects of significant variables

The response surface graphs are the graphical representations of the regression equation. A total of three response surfaces were obtained by considering all the possible combinations. Interaction effect of variables on cellulase production was studied by these response surface plots against any two independent variables, while keeping third variable at its central (0) level. The plotting 3D surface curves of the calculated responses (FPase activity) from the interactions between the variables are shown in Figure 3. The interaction effects of substrate concentration (X_1) and yeast extract (X_6), substrate concentration (X_1) and ammonium sulphate (X_9) and yeast extract (X_6) and ammonium sulphate (X_9) are mentioned in Figure 3A, B and C, respectively.

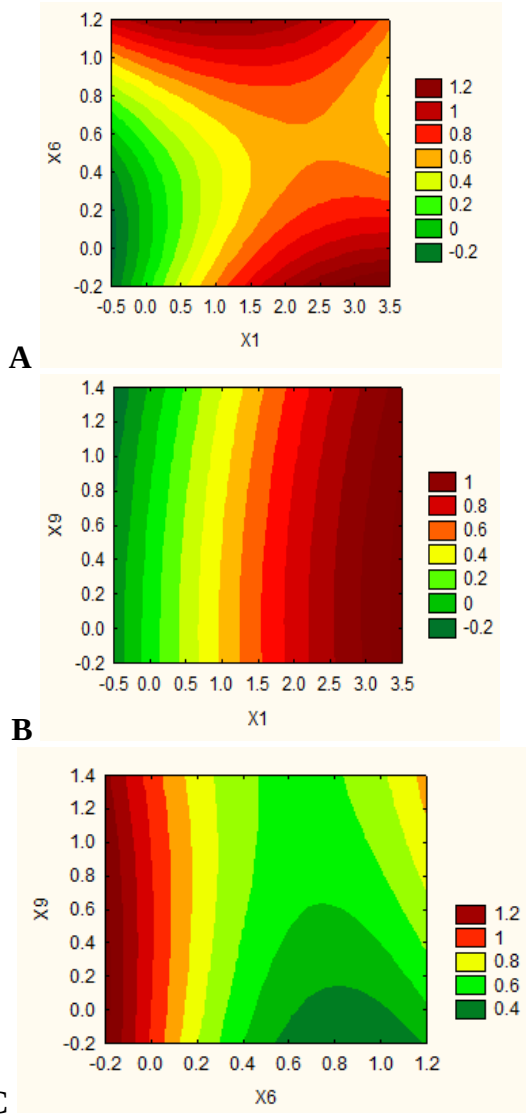

Fig. 3. Contour plots of exoglucanase production from *Aeromonas bestiarum* in submerged fermentation. (X_1, substrate concentration; X_6, yeast extract concentration; X_9, $(NH_4)_2$ SO_4 concentration).

Results showed that concentration of each factor had significant impact on exoglucanase production in submerged fermentation at 35ºC. This showed that exoglucanase production was mainly dependent on the substrate concentration because there was a direct relation between substrate concentration and the enzyme activity. Sethi *et al.* (2013) obtained highest yield of cellulase enzyme using ammonium sulphate as nitrogen source for bacteria isolated from soil. Lugani *et al.* (2015) reported that peptone was best nitrogen source for cellulase production by *Bacillus* sp. in submerged fermentation. A bacterium isolated from intestine of pig utilized 2% peptone and yeast extract as nitrogen source for maximum production of cellulase (Yang *et al.*, 2014).

The significant response for coefficients was found to be mainly dependent on the F-value and the resultant low p-value. Therefore, the greater the F-value (6.041) and lower the p-value (0.014), the more significant the corresponding coefficient. The Fisher F-test with a very low probability value demonstrated a very high significance for the regression model (Long *et al.*, 2009). The goodness of fit of model was verified by the determination coefficient (R=0.934) which indicated the validity of the model and showed that only 7% of the total variations could not be explained by the model. The value of the adjusted determination coefficient was also high (Adj R^2 =0.728), thus suggesting a high significance (p-value < 0.01) of the model (Table IV).

Table IV.- Univariate test of significance.

Effect	SS	df	MS	*F*	*P*
Model	9370434.3	9	9370434.3	6.04091	0.013518
X_1	75699.7	1	75699.70	1.974135	0.254656
X_1^2	22261.9	1	22261.93	0.580558	0.501520
X_6	40.5	1	40.52	0.001057	0.976109
X_6^2	10597.3	1	10597.33	0.276363	0.635478
X_9	231.8	1	231.81	0.006045	0.942921
X_9^2	221.9	1	221.86	0.005786	0.944156
X_1*X_6	53829.8	1	53829.80	1.403800	0.321398
X_1*X_9	1398.1	1	1398.09	0.036460	0.860760
X_6*X_9	2318.5	1	2318.52	0.060463	0.821628
Error	115037.3	3	38345.76		

R= 0.9342, R^2= 0.8834, Adj R^2 = 0.7279

Shankar and Isaiarasu (2012) reported the optimum parameters for cellulase production by *Bacillus pumilus* EWBCM1 using RSM based on Central Composite Design (CCD) model. Optimum conditions for cellulase production by *Bacillus pumilus* EWBCM1 were galactose 1.0 g/L, malt extract 0.5 g/L and incubation time 72h. By optimizing with coded factor the maximum cellulase production observed by the model was 0.5751 IU/ml.

CONCLUSION

Results of this study showed that *A. bestiarum* (KF625168) is potential candidate for exoglucanase production in submerged fermentation. The maximum exoglucanase production (3.766 IU) was achieved using substrate concentration of 2.5%, yeast extract concentration of 0.2% and ammonium sulphate concentration of 0.6% optimized through response surface methodology. These results suggested the potential utilization of this strain for nutritional purpose to promote growth of fishes for commercialization of aquaculture.

Statement of conflict of interest

Authors have declared no conflict of interest.

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