

Comparison and Optimization of Thermostable Xylanase Production by *Bacillus Pumilus* and *Bacillus Cereus* using Corn Husk

Ayishal Begam M¹, Annu A², Shameera Banu S³, Vishnu Priya D⁴

Department of Industrial Biotechnology, Government College of Technology, Coimbatore, Tamilnadu^{1,2,3,4}

Abstract: One of the most studied and widely used commercial Xylanase is endo-1, 4- β -xylanases. It is useful because the specific activity of Xylanase is higher than the other hydrolytic xylanases. Therefore, xylanases are commonly used in processes involved in degradation of plant materials, paper and pulp bleaching. The present study investigates the production and optimization of thermostable Xylanase by the bacteria *Bacillus pumilus* (MTCC 10209) and *Bacillus cereus* (MTCC 10202) under solid state fermentation using corn husk as carbon source since corn husk contains Xylan (28%) and Xylose (17.2%). Maximum production of Xylanase was observed to be 2.2019 (U/ml) and 2.5010 (U/ml) for *B. cereus* and *B. pumilus* respectively. The Response Surface Methodology revealed that the optimum temperature, pH and inoculum size for the enzyme activity were 65°C, pH 7.0 and 1 (%v/w) for *B. cereus*, whereas for *B. pumilus* it was 65°C, pH 7.0 and 1.50 (%v/w) respectively. The increasing concentration of metal ion (FeSO_4) ranging from 1mM-10mM increases the enzyme activity of *B. pumilus*. Meanwhile, the enzyme activity of *B. cereus* was strongly inhibited by increasing metal ion concentration.

Keywords: Thermostable Xylanase, solid state fermentation, *Bacillus pumilus*, *Bacillus cereus* and response surface methodology.

I. INTRODUCTION

Plant cell wall contains three major polymeric constituents (i) cellulose, an insoluble polymer composed of glucopyranosyl residues linked by glycosidic bonds (ii) hemicelluloses, a group of carbohydrates in which Xylan forms the major class (iii) lignin, a complex polyphenol, interconnected with the hemicelluloses. Due to the heterogeneity and complex chemical nature of plant Xylan, its complete breakdown is quite complex. But Xylanases (1,4- β -D-Xylan xylohydrolase; EC 3.2.1.8) are inducible enzymes involved in direct cleaving of the glycosidic bonds and liberate the xylooligosaccharides and its constituent sugar (xylose) [30]. The 3D structure of Xylanase (G11) from *Bacillus subtilis* was determined by X-ray crystallography. Molecular weight (MW) values ranging from 8.5 to 85 kDa. The aromatic residue on the surface of Xylanase forming an inter-molecular cluster could be responsible for the thermostability. The structure contains two twisted beta sheets forming a "jelly roll" fold. The individual beta strands form two domains like finger and palm

Cellulase free Xylanases active at high temperature and pH are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds. A treatment with Xylanases can improve the chemical extraction of lignin from pulp [34]. This results in significant saving of chemicals required for bleaching thereby reducing the release of toxic chlorine compounds into the environment. The specific activity of Xylanase is 2-3 times greater than the hydrolases of other polymers like crystalline cellulose [2]. Degradation of cellulose is the major problem associated with conventional pulping process, which invariably affects the cellulose fiber and

thus the quality of paper is also affected. Removal of Xylan from the cell walls by biotechnological methods leads to decrease in energy demand during bleaching. Therefore enzymatic treatments of pulp using Xylanases have better prospects in terms of both lower costs and improved fibre qualities than the chemical methods. Microbial Xylanases are preferred catalysis for Xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss and side product generation. Though fungal Xylanases are reported to be high yielding it is also associated with problems like low pH and temperature optima. Whereas some bacterial Xylanases were reported to be highly thermophilic and alkalophilic [33].

Industrial production of enzymes on large scale is associated mainly with substrate. The use of low-cost substrates for the production of industrial enzymes is a significant way to reduce the production cost. The technique of fermentation using solid state substrate has the greatest advantage over submerged fermentation due to absence or near absence of aqueous phase that provides natural habitat for growth of other microorganisms [22]. In consideration with all these facts, the present study aims to produce and optimize the thermostable xylanase by *Bacillus pumilus* (MTCC 10209) and *Bacillus cereus* (MTCC 10202) using corn husk as substrate under solid state fermentation. The production process was investigated by optimizing various process parameters such as pH, temperature, metal ion concentration and inoculum size by response surface methodology (RSM). The first step in the process of optimization is the screening of important variables. Following the initial

screening, the next step is optimizing the selected variables. The selected variables were reported to produce experimental design to check the interactive effect of the variables.

II. MATERIALS AND METHODS

A. Microorganism

The bacterial strain used in this comparative and optimization study was *Bacillus pumilus* (MTCC 10209) and *Bacillus cereus* (MTCC 10202).

B. Substrate preparation

Corn husk was obtained from local market in Coimbatore. The substrate was washed 2-3 times with distilled water. It was dried under room temperature for 24 h. Then the substrate was powdered and sieved between 400-600nm using sieve shakers.

C. Growth Conditions of Culture

The two bacterial strains were maintained in liquid medium as well as solid medium. The media contain beef extract

(1.0g/L), yeast extract (2.0g/L), peptone(5.0g/L), NaCl(5.0g/L) and Agar(20.0g/L). The media was inoculated with *B.pumilus* and incubated at 37°C for 12 h whereas *B.cereus* containing media was incubated at 37°C for 24 h and their growth curve characteristic was studied for every 2 h. At each time interval the solid media was suspended with 0.1% gelatin. The absorbance was read at 640nm using UV spectrometer.

D. Xylanase production by Solid State Fermentation

To produce the extracellular Xylanase under solid state fermentation corn husk was used as substrate. The bacterial strain was cultured in Erlenmeyer flasks (250 mL) containing 5 g of corn husk moistened with 10 mL of the basal salt solution. The media was inoculated with *B. pumilus* and *B.cereus* separately incubated at 37°C for 12 h and 24 h respectively. After incubation period of fermentation spent; 3g of the solid substrate was added to 30mL of 50mM of phosphate buffer. It was vortexed thoroughly to extract the enzyme. The sample was centrifuged at 5000 rpm for 10 min at 4°C. Centrifugation will remove Xylanase from substrate. Supernatant was filtered through Whatman No. 1 filter paper and the clear filtrate was used as crude Xylanase.

E. Xylanase assay

Xylanase activity was measured according to Rajashri et.al [27]. To 0.5mL of solubilised xylan solution 0.5mL of crude xylanase was added and left undisturbed for half an hour. To the mixture 2mL of distilled water and 3mL of DNSA was added and incubated at 50°C for 5 min in water bath. The absorbance was measured at 540nm using UV spectrometer. One unit of xylanase activity was defined as the amount of enzyme that liberates 1micromole of reducing sugars equivalent to xylose per minute under the assay conditions described.

F. RBB assay

This assay functions on the release of ethanol soluble RBB when the RBB-Xylan is cleaved by xylanase. RBB-xylan

was prepared by mixing 2.5g RBB dye and 2.5g Xylan powder (corn husk) to 60ml of water. 20ml of Acetate solution was added to RBB-Xylan solution. It was mixed thoroughly and added with 20ml Sodium hydroxide solution. The mixture was stirred for 90 min and two volumes of 96% ethanol were added to precipitate. It was filtered using Whatman filter paper and was washed using 1L wash solution containing 660ml ethanol, 330ml H₂O and 1.35g Sodium acetate. Again it was washed with 100ml 75% ethanol followed by 50ml Acetone. The precipitate was dried overnight at room temperature.

Remazol brilliant blue assay was done by dyed RBB-xylan in which 0.1M Acetate buffer contain 1.15g/ml of RBB-Xylan. To 250ml of cell culture 250ml of RBB-Xylan was added and incubated for 2 h at 30°C. 1ml of Ethanol was added to stop the reaction and RBB-Xylan was precipitated. Centrifugation was done at 5000 rpm for 1 min. the collected supernatant was measured at 595nm. The absorbance was compared to medium with no xylanase control.

G. Effect of temperature on enzyme activity

The optimum temperature for maximum xylanase activity was determined by varying the reaction temperature from 40 to 100°C. The optimum temperature was determined by plotting relative activity of the enzyme against temperature.

H. Effect of pH on enzyme activity

The effect of pH on enzyme activity was determined by incubating xylanase at various pH ranging from 6.0 to 9.0. The various buffers used were 50mM sodium phosphate (pH 6, 7), 50mM Tris HCl (pH 8, 9). The optimum pH was determined by plotting relative activity of the enzyme against pH.

I. Effect of metal ion on enzyme activity

Ferrous sulphate act as an inducer for maximum enzyme production. Hence, Fe⁺² ion at different concentrations were taken ranging from 1mM-10mM. The enzyme activity was measured using enzyme assay described above.

J. Effect of inoculum size on enzyme activity

Inoculum size is one of the important factor to be considered for a solid media under solid state fermentation. The media was inoculated with increasing inoculum concentration ranging from 0.5% to 2.5% in volumes.

I. Optimization of screened components by response surface methodology and statistical designs

RSM an empirical combination of mathematical and statistical technique is a quite powerful tool for modeling, improving and optimizing the processes. The significant medium components screened through OFAT were subjected to central composite design (CDD), a popular second-order experimental design for developing sequential experimentation and predicting the level of factors to get optimal response. Four variables such as pH, temperature, metal ion and inoculums size which have given significant yield from OFAT were selected and so 30 experiments were performed. The relationships

between the variables were determined by fitting the second order polynomial equation obtained from 30 trial experiments. The MINITAB of Version 17 was used to perform RSM. The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is provided in Table 4. The fitted equation was then expressed in the form of two-dimensional contour plots and response surface plots to illustrate the interactive effects of the independent variables on the dependent ones.

III. RESULTS AND DISCUSSION

A. Growth curve characteristics

The time course of microbial growth was studied in solid media under assay condition. Absorbance was measured for every 2 h of interval. The period of log phase for *B.cereus* has been found out to be between 6-20h meanwhile *B.pumilus* attained logarithmic phase at its 3rd h up to 12th h and it was shown in Fig. 1 and Fig. 2 respectively.

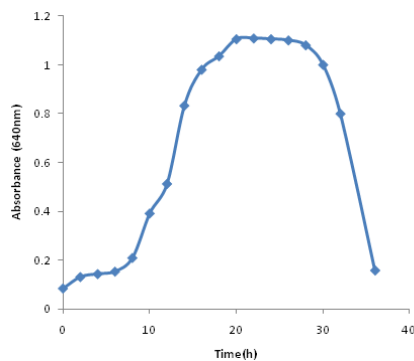


Fig.1. Growth curve pattern of *B.cereus*

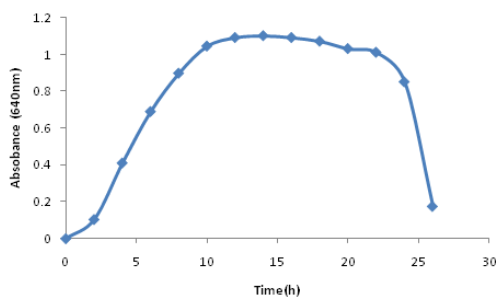


Fig.2. Growth pattern of *B.Pumilus*

B.RBB assay

The media with RBB-Xylan was compared with media containing no Xylanase. It was shown in Table I. The higher absorbance indicates more Xylanase activity.

TABLE I. RBB assay

Sample	Absorbance at 595nm
Control 1(without RBB-xylan)	0.066
<i>B.cereus</i>	1.550
Control 2 (without RBB-xylan)	0.723
<i>B.pumilus</i>	1.702

C. Effect of temperature on enzyme activity

The enzyme production was carried out at different temperatures ranging from 40-100°C. The maximum Xylanase production by *Bacillus pumilus* and *Bacillus cereus* was found to be 75°C and 65°C respectively and it was shown in Fig. 3. Optimum Xylanase production was reported by Ranganathan Kapilan et al., (2010) at 60°C using oats Xylan as substrate [28]. Anjum Banu et al., (2012) observed highest Xylanase activity at 30°C [2]. Miass E. Ahmed (2014) observed that the Xylanase exhibited greatest activity at 55°C. [17].

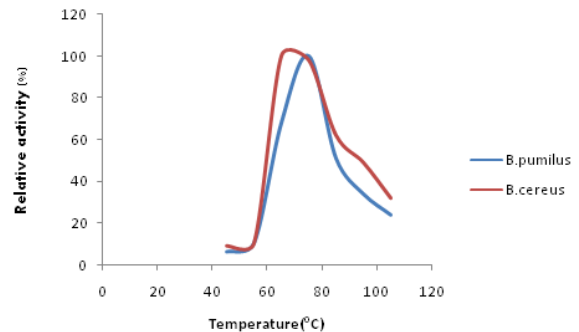


Fig.3. Effect of temperature on xylanase activity from *B.Pumilus* and *B.cereus*

D. Effect of pH on on enzyme activity

pH was the most important factor to characterize the enzyme. Maximum Xylanase from *B.pumilus* and *B.cereus* was observed at pH 8 and 7 respectively shown in Fig. 4. The production of Xylanase from *Bacillus pumilus* mtcc8964 at pH 6 was reported by Dinesh Kumar et al., [10]. AnjumBanu et al., observed maximum production of Xylanase by *Bacillus pumilus* at pH 7.0 [2]. They used substrates such as wheat straw, wheat bran, rice bran, banana stem, soybean flour and mustard oil cake. Roy et al., observed the Xylanase exhibited greatest activity at pH 6 produced by *Bacillus cereus* from soil.[30]

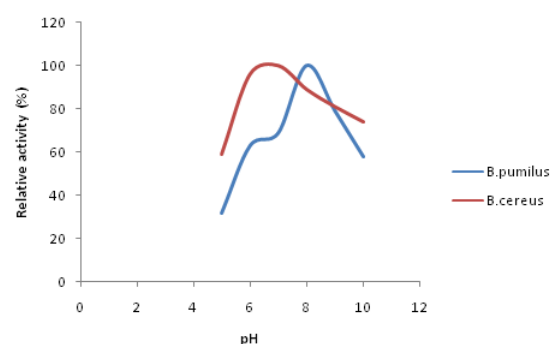


Fig.4. Effect of pH on xylanase activity from *B.Pumilus* and *B.cereus*

E. Effect of metal ion on enzyme activity

The enzyme production at different metal ion (FeSO_4) concentration values ranging from 1-10mM was carried out. As depicted in Fig. 5, the maximum Xylanase production by *B.pumilus* and *B.cereus* was found to be at 7mM and 1mM respectively. This indicates that the increasing concentration of metal ion (FeSO_4) increases the enzyme activity of *B.pumilus*. Whereas, the increasing

metal ion concentration strongly affects the enzyme activity of *B.cereus*. Such results were reported by Gessesse A [12]. They tested differential ions on Xylanase produced by *Bacillus* sp. and found significant effect on Xylanase activity.

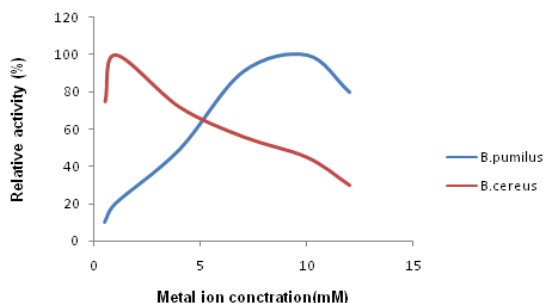


Fig.5. Effect of Meta ion concentration on Xylanase activity from *B.Pumilus* and *B.cereus*

F. Effect of inoculums size on enzyme activity

The maximum Xylanase production by *Bacillus pumilus* and *Bacillus cereus* was found to be at 1.5 % and 1% of inoculums size respectively. Similar studies were carried out as follows: 15% (w/v) of inoculums was used for the production of Xylanase from *B. licheniformis* [37]. 5% inoculums was sufficient for Xylanase production by *Streptomyces* sp. QG-11-3 whereas 10% inoculums led to good enzyme production by *Bacillus* sp. AR-009 [5] and Xylanase production was highest at inoculums size 1% (w/v) [2].

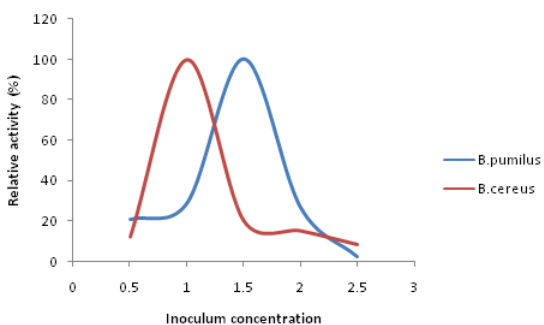


Fig.6. Effect of inoculums size on Xylanase activity from *Bacillus pumilus* and *Bacillus cereus*

G. Optimization of selected factor and their interactive effects

Optimum conditions of the above mentioned important variables and the result of their relations on xylanase activity were determined by response surface methodology. Table-II & III shows the information of the actual values worked in the Central composite design. The results obtained by central composite design were examined by standard analysis of variance and the predicted and observed response. The second order regression gave the stages of xylanase production as a function of initial values of pH, temperature, metal ion and inoculums size which can be predicted by the following equation. Equation 1 & 2 represents for *B.pumilus* and *B.cereus* respectively.

TABLEII. Range and levels of selected factors used in terms of actual values (*Bacillus cereus*)

Factors	Code	Low level	High level
Temperature(⁰ C)	A	65	75
pH	B	7	8
Metal ion(mM)	C	0.5	1.0
Inoculum concentration (%)	D	0.5	1.5

TABLE III. Range and levels of selected factors used in terms of actual values (*Bacillus pumilus*)

Factors	Code	Low level	High level
Temperature(⁰ C)	A	65	75
pH	B	6	7
Metal ion(mM)	C	9	10
Inoculum concentration (%)	D	1.0	1.5

$$\text{Enzyme Activity } Y = 4.3 - 0.603A + 4.15B - 4.85C + 56.59D - 0.00396A^2 - 0.491B^2 - 0.229C^2 + 1.936D^2 + 0.0228AB + 0.1245AC - 0.1796AD + 0.446BC - 2.005BD - 2.439CD \quad \text{----- (1)}$$

$$\text{Enzyme activity } Y = 4.3 + 0.149A - 0.83B + 8.09C + 5.27D + 0.00078A^2 + 0.271B^2 + 1.068C^2 + 1.030D^2 - 0.0333AB - 0.0842AC - 0.0745AD - 0.364BC - 0.022BD - 1.61CD \quad \text{----- (2)}$$

Furthermore, the results were analyzed by analysis of variance (ANOVA). This assisted in elucidating the main, squared and interaction effects among the process variables and their influence on the measured enzyme activity. All experiments were carried out in sequential order as specified by the design. In general the Fischer's variance ratio, the F value should be higher than the low probability, P values for the predictions to be significant [18]. In this study, the lowest P-value(0.001) and f-value(8.49) played a major role than the squared and interactive effect in the enzyme activity of *B.Pumilus*. While the p-value and f-value for the enzyme activity of *B.cereus* was p=0.001 and 12.52 respectively which indicated the significance of linear variables and it was shown in below Table IV & V.

TABLE IV. Anova for the quadratic regression model for enzyme activity of *B.pumilus*

Source	D F	Seq SS	Adj MS	F-value	P-value
Regression	14	7.490	0.535	4.85	0.004
Linear	4	4.079	1.019	8.49	0.001
Square	4	0.229	0.057	0.48	0.752
Interaction	6	3.181	0.530	4.41	0.010
Residual error	14	1.682	0.120	1.09	0.508
Lack-of-fit	10	1.232	0.123		
Pure error	4	0.450	0.112		
Total	28				

The determination coefficient R-sq 83.05% and 83.95% for *Bacillus pumilus* but the experimental data was acceptable. The linear, square and quadratic or interaction factors which have $p \text{ value} \leq 0.05$ have significant effect on enzyme activity.

TABLE V. Anova for the quadratic regression model for enzyme activity of *B.cereus*

Source	DF	Seq SS	Adj MS	F-value	P-value
Regression	15	5.6493	0.376	4.88	0.003
Linear	1	3.8640	0.966	12.52	0.000
Square	4	0.281	0.070	0.91	0.483
Interaction	6	0.621	0.103	1.34	0.303
Residual error	14	1.080	0.077		
Lack-of-fit	10	1.080	0.108		
Pure error	4	0.000	0.000		
Total	27				

Circular or elliptical shape contour plot indicate whether the reciprocal interactions between the factors are significant or not. Each contour plot showed an infinite number of combinations of the two test variables with the other variable maintained at a fixed level [18]. Circular contour plot indicates that the interactions between corresponding factors are negligible, while elliptical contour plot indicates that the interactions between corresponding factors are significant. Results of the present study showed that the contour plot which was elliptical in shape, indicates significant interaction effect between that two factors. The contour plots of enzyme activity are shown in the Figure 7 & 8. The peaks and curvature indicated the maximum enzyme activity in the RS plots. The shapes of the surfaces, circular (or) elliptical indicated whether the interactions among different variables were significant or not. In general, the RS plots can be dome shaped, inverted 'U' shaped, some with a saddle point and some do not show any regular variation with increase / decrease in variables. Fig. 9 & 10 illustrates the response surface of *B.pumilus* and *B.cereus* respectively.

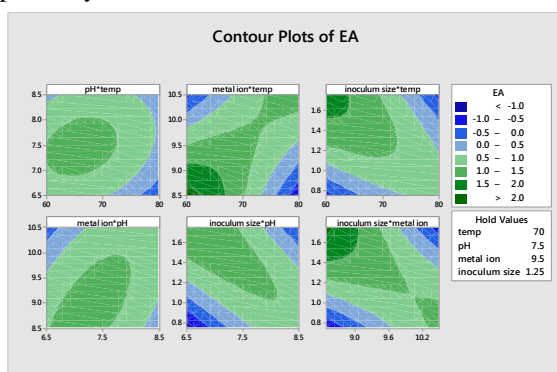


Fig.7 illustrating the contour plots of enzyme activity for *Bacillus pumilus*

In fig. 9.a) increase in the metal ion concentration with pH increase up to optima level increased the enzyme activity.

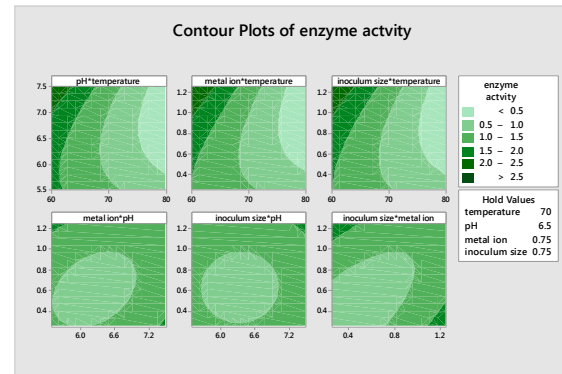


Fig.8 illustrating the contour plots of enzyme activity for *Bacillus cereus*

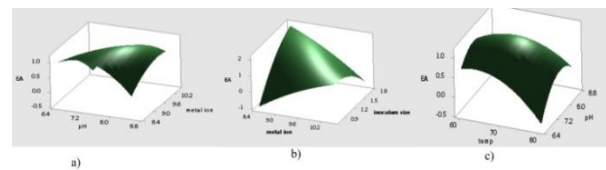


Fig. 9 Response surface plot of *B.pumilus* for enzyme activity at different ranges of a) pH and metal ion concentration b) metal ion and inoculum concentration c) temperature and pH

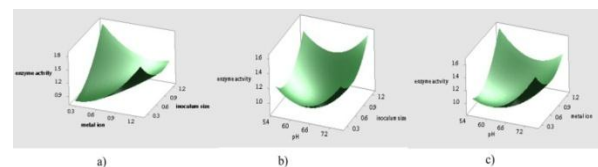


Fig. 10 Response surface plot of *B.cereus* for enzyme activity at different ranges of a) metal ion and inoculum concentration b) pH and inoculum concentration c) pH and metal ion concentration

The dome shaped surface (fig. 9.c) showed that the enzyme activity increased up to optimized temperature and pH level after that there is reverse trend on the surface. Meanwhile, the enzyme activity showed drastic decrease with increase in metal ion concentration. While, the inverted dome shaped (fig. 10.b) showed the increase/decrease in the variable with other two variable fixed at a position that is called hold values.

IV. CONCLUSION

The present study investigated the optimized level for xylanases production from *Bacillus cereus* and *Bacillus pumilus*. The production pattern was studied under solid state fermentation with corn husk as substrate. The analysis of enzyme production by *Bacillus cereus* revealed that the maximum production (2.2019 U/ml) was recorded in solid medium at pH 7, temperature 65°C, inoculum size 1%(v/w) and 1mM metal ion concentration. The analysis of enzyme production by *Bacillus pumilus* revealed that the maximum production (2.5010 U/ml) was recorded in solid medium at pH 7, temperature 65°C, inoculum size 1.5% (v/w) and 9 mM metal ion concentration. While Rajashri et.al reported that maximum Xylanase was observed at optimum level of 50°C and pH 8.0 [27]. The statistical analysis for the maximal

production of Xylanase was done using RSM. The interaction between independent variables having p-values less than 0.05 was found to be significant where the rest showed negative effect on the enzyme activity. The determination coefficient R-SQ 83.05%, and 85.95% were not too high, but the experimental data were acceptable. Further, statistical analysis will enhance the optimization of the Xylanase production.

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