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Statistical optimization of cost-effective lignocellulosic medium for maximum xylanase activity by *Massilia timonae* B2YR

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Abstract

Response surface methodology was used in the current study to optimise a lignocellulosic waste medium containing wheat bran (agro residue) and *Pistia* stratiotes (waterweed) for escalating xylanase activity using a bacterial isolate, *Massilia timonae* B2YR KY942185 screened from sawmill industry soil. After box behnken design (BBD) optimization, the medium composition was wheat bran 1.5 % w/v and *Pistia* 2 %w/v, demonstrating a 1.95-fold increase in xylanase activity. The xylanase enzyme was purified to 20.61-fold by Cellulose Diethylaminoethyl (DEAE) Chromatography with 32.60% recovery of enzyme activity. The molecular weight of xylanase was found to be 43 kiloDalton (kDa) by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Mn, a metal ion, was found to increase xylanase activity (MnCl). Simultaneously, the enzyme demonstrated remarkable stability at acidic pH (4), with a retention of 75% in relative activity after 2 h of incubation in the corresponding pH buffer. Thus, the ideal conditions for maximum xylanase activity were 28± 2°C and pH 7. The kinetic experiments revealed a Michaelis menten constant for enzyme activity (K_m)of 2.7 mg/mL and a maximum velocity of enzyme activity (V_{max}) of 178.3 mol/min/mg. A crude xylanase fraction treatment on a *Pistia* and wheat bran mixture yielded 20.66mg of xylooligosaccharides after 18 h.

Keywords: Lignocellulosic waste, Optimization, Xylanase, Xylooligosaccharides

1. Introduction

Lignocelluloses such as crop residues, inedible crops, and forest materials are the most readily and profusely available renewable biomass on earth. The waste generated from agricultural and agro-industry such as wheat bran, sugarcane bagasse, corn straw, and corncob has increased in confounding quantities all over the world as a result of industrialization. The critical issue is space for the disposal of these wastes which is causing environmental pollution. The growth of noxious water weeds like *Pistia stratiotes*, *Echornia* is a threat throughout the globe. The biomass of the *Pistia* species doubles every 10 to 15 days, depending on the nutrients and water temperature [1]. The hemicellulose fraction from these biomass can be used for the production of novel food products, with high value-added [2]. Hemicellulose is the second most abundantly available renewable resource composed of xylan [3]. Xylan which is a chain of xylose linked by β-1, 4 linkages is degraded by the enzyme xylanase [4]. Xylanase is classified as endo and exo xylanase. Endo xylanase designated as E.C 3.2.1.8, randomly cleaves xylan to xylooligosaccharides while Exo E.C.3.2.37, release xylose from the non-reducing end. Microbial xylanases are industrially important with many applications like improving the consistency of beer in brewing, raising dough in baking, in the paper industry for bleaching, clarification of fruit juices and improving the quality of livestock feed in the animal feed industry. Nowadays xylanase is also used for the production of xylooligosaccharides [5]. Many bacterial genera are reported to have xylanase production ability like *Bacillus, Microbacterium Arthrobacter Pseudoxanthomonas, and Staphylococcus* [6]. Fungal genera conveyed to produce

xylanases are *Aspergillus and Trichoderma*. The different types of raw substrate reported for the production of xylanase are wheat straw, corn cob, and sugarcane bagasse. Wheat straw was used by the organism *Bacillus lpuarvinder st. lpu002* which gave a yield of xylanase to 22.16 IU/mL [7]. Naz et al. used corn cob with the organism *Bacillus subtilis* which reported 265 U/mL of xylanase yield [8]. Xylan beechwood was degraded by xylanase produced by *Geobacillus stearothermophilus* KIBGE-IB29 in a study stated by Bibi et al. [9].

The current research emphasises maximizing the activity of xylanase by optimizing the growth parameters using both "Classical one-factor-at-a-time" (OFAT) and "Statistical response surface methodology" (RSM) methods. Any fermentation's economics depends on the medium composition used for the production. Hence, the commercial xylan is replaced by wheat bran and *Pistia* in this experiment. Wheat bran is composed mainly of arabinoxylan (19-25%), starch (17-29%), protein (14-18%) and in fewer amounts lignin (~3%) [10]. While *Pistia* leaves are composed of cellulose (27.55%), hemicellulose (29.71%), and lignin (3.4%) [11]. The low lignin percentage in both substrates makes them suitable for the production of xylooligosaccharides and xylanase.

2. Materials and methods

2.1 Chemicals and reagents

All the media, chemicals and reagents were analytical grade and procured from Hi media, Sigma Aldrich. The standard sugar xylose and Birchwood xylan were purchased from Hi media. The standards of xylobiose and xylotriose were ordered from Megazyme, Ireland. While thin-layer chromatography (TLC) Merck, Germany sheets were secured.

2.2 Substrates

Agro residue like wheat bran was collected from local houses in the district of Kolhapur, Maharashtra, India. The water weed *Pistia stratiotes* was collected from Takala quarry, Kolhapur, with the GPS location 16°41'56.1"N 74°14'58.7"E in properly labelled pouches. The substrates were bought to the laboratory washed with water, sundried, ground to powder and stored in a cool and dry place till their use.

2.3 Microorganism

The microorganism *Massilia timonae* B2YR KY942185 was isolated from soil collected from sawmill industries at Badhule, Kolhapur, Maharashtra on modified Horokoshi medium containing 0.5% Birchwood xylan as the sole carbon source. The organism was identified by using the 16s ribosomal ribonucleic acid (rRNA) technique. The gene sequence was submitted to national center for biotechnology information (NCBI) and accession number KY942185 was assigned to it [12]. The microorganism was preserved on a nutrient agar slant, with a frequent transfer at an interval of 7 days.

2.4 Production of xylanase enzyme

Production of xylanase was carried out using *Massilia timonae* B2YR KY942185 in modified Horokoshi Broth [13] using wheat bran and *Pistia stratiotes* and Birchwood xylan as the source of carbon in a concentration of 1% w/v, the production broth was inoculated with the suspension of an organism with a cell density of 1×10^8 cells/mL and incubated at a temperature of 28±2°C for 48 h.

2.5 Xylanase assay

The xylanase assay was carried out using the 3,5, Di nitro salicylic method [14]. The reaction mixture contained 900 µL of 0.1% w/v solubilised Birchwood xylan solution in 100 mM sodium phosphate buffer with 100 µL enzyme solution in a test tube. The reaction mixture was incubated for 10 min at 28-30°C. Then 2 mL Di nitro salicylic reagent was added and kept boiling for 10 min in the water bath. The absorbance was measured at 540 nm.

$$\text{Xylanase unit} = \frac{\text{xylose concentration} * 1000}{\text{amt of crude enzyme (mL)} * \text{time in min}} \quad (1)$$

2.6 Protein assay

Estimation of protein was done by performing the method described by Lowry [15].

2.7 Classical optimization

"One factor at a time," was the traditional method for optimising various growth factors. Wheat bran, *Pistia stratiotes*, and Commercial xylan from birch wood were employed as carbon sources at concentrations ranging from 0.5% to 2% w/v. Peptone was utilized as an organic nitrogen source, while potassium nitrate was used as an inorganic nitrogen source (0.125% w/w -0.5% w/v) to evaluate their effects. Temperatures ranging from 28°C to 45°C were investigated, while buffers with pH ranging from 4 to 10 were utilised to investigate the effect of pH. The medium was inoculated with 1% v/v of 1×10^8 cells/mL of *Massilia timonae* B2YR KY942185 culture suspension, then incubated for 48 hours at room temperature.

2.8 The response surface methodology

The Box Behnken design of response surface methodology was employed for the optimization of medium components [16]. The independent variable (factors) used in the study were wheat bran (A) (g/L), *Pistia stratiotes* (B) (g/L), and Potassium nitrate ($CKNO_3$) (g/L) their values and levels are represented in Table 1.

Table 1 Levels of independent variables used in the Box-Behnken design.

Factor	Name	Units	Minimum	Maximum	Coded-low	Coded-high	Coded-centre value
A	Wheat bran	gms	1.00	2.00	-1↔1.00	+1↔2.00	0↔1.50
B	*Pistia* sp	gms	1.00	2.00	-1↔1.00	+1↔2.00	0↔1.50
C	KNO3	gms	0.00	0.50	-1↔0.00	+1↔0.50	0↔0.250

The relation between actual and coded values is given as:

$$xi = \frac{Xi - xo}{\Delta Xi} \quad (2)$$

where xi and Xi represent the coded and actual values of an independent variable, the centre value of an actual independent variable is *xo* and ΔXi is the change of Xi. The relationship between A, B, and C independent variables to the response Y was calculated using the quadratic model Equation.

$$Y = \beta_0 + \beta_1 A + \beta_2 B_2 + \beta_3 C_3 + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (3)$$

where Y= Predicted response, β_0 = constant term, A= Wheat bran, B= *Pistia stratiotes* C = KNO_3, while $\beta_1, \beta_2, \beta_3$, were the linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}$ were square coefficients and $\beta_{12}, \beta_{13}, \beta_{23}$ were interactive coefficients [17].

The analysis of responses obtained through the experiments was done by using the Design Expert Version 12 [Stat. Ease Inc., Minneapolis, Minnesota, USA] a statistical tool. The analysis of variance (ANOVA) combined with the F- test was used to evaluate the given terms to have a significant effect ($p \leq 0.05$) for enhancing xylanase activity.

2.9 Purification of xylanase enzyme

The optimized medium was inoculated with *Massilia timonae* and incubated for 48 h for the production of xylanase. Ammonium sulphate saturation from 30-70% was carried out at 10°C in the cell-free extract obtained after centrifugation at 8000 rpm for 10 mins to precipitate xylanase enzyme, then the mixture was incubated at 4°C to enhance precipitation. The precipitate obtained was dissolved in 100 mM Sodium phosphate buffer (pH7) and dialysed at 10°C. The dialysed enzyme was loaded on a pre-equilibrated Cellulose Diethylaminoethyl (DEAE) Column with the same buffer. The enzyme was eluted by NaCl gradient with a flow rate of 5 mL/min. All fractions collected were checked for protein concentration and xylanase activity.

2.10 Molecular weight determination

Molecular weight was determined by performing Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) as defined by Laemmeli [18]. The molecular weight of the xylanase enzyme was resolved by SDS PAGE with protein molecular markers ranging from 18.5 kiloDalton (kDa) to 240 kDa the gel was stained with Coomassie brilliant blue.

2.11 Characterization of xylanase

The optimum temperature for maximum xylanase activity was determined between 10°C and 60°C. For determination of temperature stability, the enzyme was incubated at respective temperatures for 2 h at pH 7.0 and the relative activity was determined. The effect of metal ions, with final concentrations of (5 mmol and 10 mmol) on enzyme activity was determined by incubating the enzyme with respective compounds at room temperature and determination of residual activities under standard assay conditions. The optimal pH was determined by using different buffers ranging between 4.0 and 10.0 Citrate buffer (pH 4-6), Sodium Phosphate buffer (pH 7-8), and Glycine NaOH Buffer (pH 9-10). The stability of the enzyme at various pH was determined by incubating the enzyme in respective buffers for 1 h and 2 h followed by determining the residual activity.

2.12 Kinetic parameter

The rate of substrate hydrolysis, Km and Vmax values for Birchwood xylan were determined using the Michelis Menten equation. The kinetic constants of the enzyme were measured using Sigma XL v.14 software.

2.12 Study of hydrolysis of xylan

The crude fraction of the enzyme was incubated with 0.5%w/v *Pistia* and wheat bran mixture in distilled water for 12 h and 18 h. After incubation, the broth was centrifuged and the supernatant was added with two volumes of ice-cold isopropanol and refrigerated to remove the unused xylan as a precipitate by performing centrifugation at 10000 rpm for 10 min from the production broth [13]. The xylooligosaccharides in the supernatant was detected by performing TLC on silica gel 60 in a solvent system of Butanol: Acetic acid: Water (2:1:1) along with standards xylose, xylobiose, xylotriose. The standard xylooligosaccharide (XOS) was dissolved in Distilled water at a concentration of 1mg/mL before loading. The Chromatogram was developed by 0.2 %w/v orcinol in 1% H_2SO_4 solution in methanol [19]. The xylooligosaccharides produced were measured as reducing sugar [20].

2.13 Economics

The cost of production medium designed using response surface methodology, Wheat bran(1.5%), *Pistia sp* (2%) was compared with the Horokoshi medium KNO_3 2.5g/L, KH_2PO_4 1.0 g/L, $MgSO_4$ 0.1 g/L, $CaCl_2$ 0.1 g/L, NaCl 5.0%, Commercial xylan 10 g/L used for the production of xylanase enzyme [21]. The cost of both mediums was compared by analysing the cost of individual components in the Horokoshi medium as per the catalogue of Hi media [22].

3. Results

3.1 Classical optimization

3.1.1 Classical optimization of medium components and growth parameters

The microorganism *Massilia timonae* B2YR KY942185 showed the highest yield of xylanase 281.51 IU/mL at a concentration of 1.5% (w/v) of Wheat bran and pure xylan in 48 h. While for *Pistia stratiotes* maximum xylanase activity of 206.76 IU/mL was observed at 2% concentration in Figure 1A. A xylanase activity of 206.76 IU/mL at a concentration of 0.25% of potassium nitrate and 281.54 IU/mL at 0.5% of peptone was observed in Figure 1B. The highest xylanase activity of 188.06 IU/mL was observed at room temperature (27-28°C), while the activity was found decreasing with increasing temperature Figure 1C. The organisms *Massilia timonae* B2YR KY942185 showed the highest, 272.19 IU/mL xylanase activity at pH 7 Figure 1D, the salt concentration of 0.5% (w/v) and inoculum volume 3% (v/v).

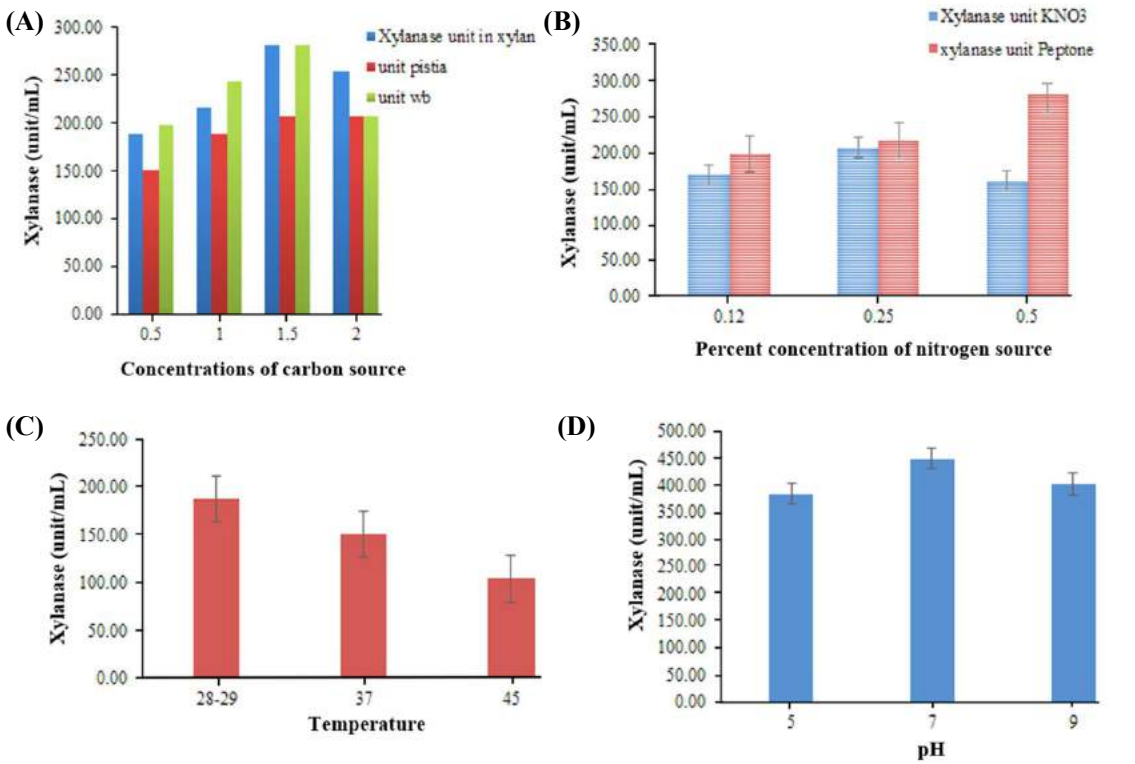

Figure1 Classical optimization of medium components and growth parameters (A) Optimization of carbon sources in the medium, (B) optimization of nitrogen sources in medium, (C) optimization of temperature, (D) optimization of pH.

3.2 Optimization of the medium by Response surface methodology for maximum xylanase activity

To study the effect of independent variables like Wheat bran (A) (g/L), *Pistia stratiotes* (B) (g/L), and Potassium nitrate ($CKNO_3$) (g/L), on enzyme activity a set of 15 experiments with 3 centre points were performed. The experimental design, xylanase activity and residual values are shown in Table 2. The xylanase production potential was the function of independent variables which was determined by the regression expression obtained after ANOVA, given by the Equation.

$$y = 160.017 + 12.8539 \times A + 19.8647 \times B + -109.841 \times C + -21.0337 \times AB + 14.0224 \times AC + 14.0224 \times BC + -10.5165 \times A2 + 8.17936 \times B2 + 122.695 \times C2 \quad (4)$$

Linear coefficients with the negative sign-109.841 (C) i.e., nitrogen source KNO_3 suggest that it negatively affects xylanase response, whereas wheat bran and *Pistia* have a positive effect. The negative effect means increasing the concentration of these variables will lead to a lowering of activity. The linear coefficients 12.8539 and 19.8647 for wheat bran and *Pistia stratiotes* respectively suggest that *Pistia stratiotes* have maximum effect in xylanase activity [23].

Table 2 The Box-Behnken experimental design with experimental and predicted values of xylanase production by *Massilia timonae* B2YR KY942185.

Std	Run	Factors			Enzyme units (IU/mL)	
		A	B	C	Actual	Predicted
1	1	1	1	0.25	122.624	103.93
12	2	1.5	2	0.5	216.105	214.94
14	3	1.5	1.5	0.25	178.713	160.02
7	4	1	1.5	0.5	131.972	135.48
15	5	1.5	1.5	0.25	178.713	160.02
9	6	1.5	1	0	393.722	394.89
10	7	1.5	2	0	421.765	406.57
2	8	2	1	0.25	169.365	171.70

Table 2 (continued) The Box-Behnken experimental design with experimental and predicted values of xylanase production by *Massilia timonae* B2YR KY942185.

Std	Run	Factors			Enzyme units (IU/mL)	
		A	B	C	Actual	Predicted
13	9	1.5	1.5	0.25	122.624	160.02
8	10	2	1.5	0.5	206.758	189.23
3	11	1	2	0.25	188.062	185.72
5	12	1	1.5	0	365.677	383.20
11	13	1.5	1	0.5	131.972	147.16

ANOVA for the quadratic regression model obtained for xylanase yield had a very low value of probability (0.0015) and a very high F value of 22.93 indicating that the model is very significant. A lack of fit was found to be insignificant with a *p*-value of 0.6843 displayed in Table 3.

The goodness of fit for the model was tested by the coefficient of determination value (R^2). The current study has shown an R^2 value of 0.9763, which is very close to 1, making the model accurate. The adjusted R^2 value of model 0.9338 is near to the value of R^2 in the present model [24]. The difference between adjusted R^2 (0.9338) and predicted R^2 (0.7960) is less than 0.2 which is in reasonable agreement. Adequate precision measures signal-to-noise ratio. A ratio greater than 4 is desirable. This model has a ratio of 13.254 indicating adequate precision.

Table 3 ANOVA of regression analysis.

Source	Sum of squares	df	Mean Square	F-value	*p*-value	Significant
Model	1.614E+05	9	17933.90	22.93	0.0015	
A-Wheat bran	1321.78	1	1321.78	1.69	0.2503	
B-*Pistia* sp	3156.85	1	3156.85	4.04	0.1008	
C-KNO3	96520.66	1	96520.66	123.41	0.0001	
AB	1769.66	1	1769.66	2.26	0.1929	
AC	786.51	1	786.51	1.01	0.3620	
BC	786.51	1	786.51	1.01	0.3620	
A^2	408.36	1	408.36	0.5221	0.5023	
B^2	247.02	1	247.02	0.3158	0.5984	
C^2	55584.03	1	55584.03	71.07	0.0004	
Residual	3910.68	5	782.14			
Lack of Fit	1813.32	3	604.44	0.5764	0.6843	Not significant
Pure Error	2097.37	2	1048.68	22.93		
Cor Total	1.653E+05	14				

Based on the model equation, the three-dimensional response plot and their corresponding contour plot were drawn to study the interaction among the variables and their concentration responsible for increasing yields of xylanase. The circular nature of the contour plot depicts no interaction between independent variables in Figures 2A and 2B [25]. The interaction between KNO_3 and wheat bran as observed in the Figure 2A depicts wheat bran as independent of nitrogen source. While similar interaction between *Pistia* and KNO_3 was also observed indicating an independent behaviour of *Pistia stratiotes* Figure 2B. The smallest elliptical in the contour plot at the centre shows the maximum predicted value and positive interaction between two independent variables wheat bran and *Pistia stratiotes* observed in Figure 2C [26]. The activity of 421.576 IU/mL of xylanase was observed at wheat bran 1.5% and *Pistia stratiotes* 2 % w/v concentration. The organism showed an increase in the activity of xylanase at zero concentration of nitrogen thus making the medium completely organic and cost-effective. After applying the Box Behnken design in this study the activity was found to increase by 1.09-fold, from 216.11 IU/mL to 421.756 IU/mL in 48 h.

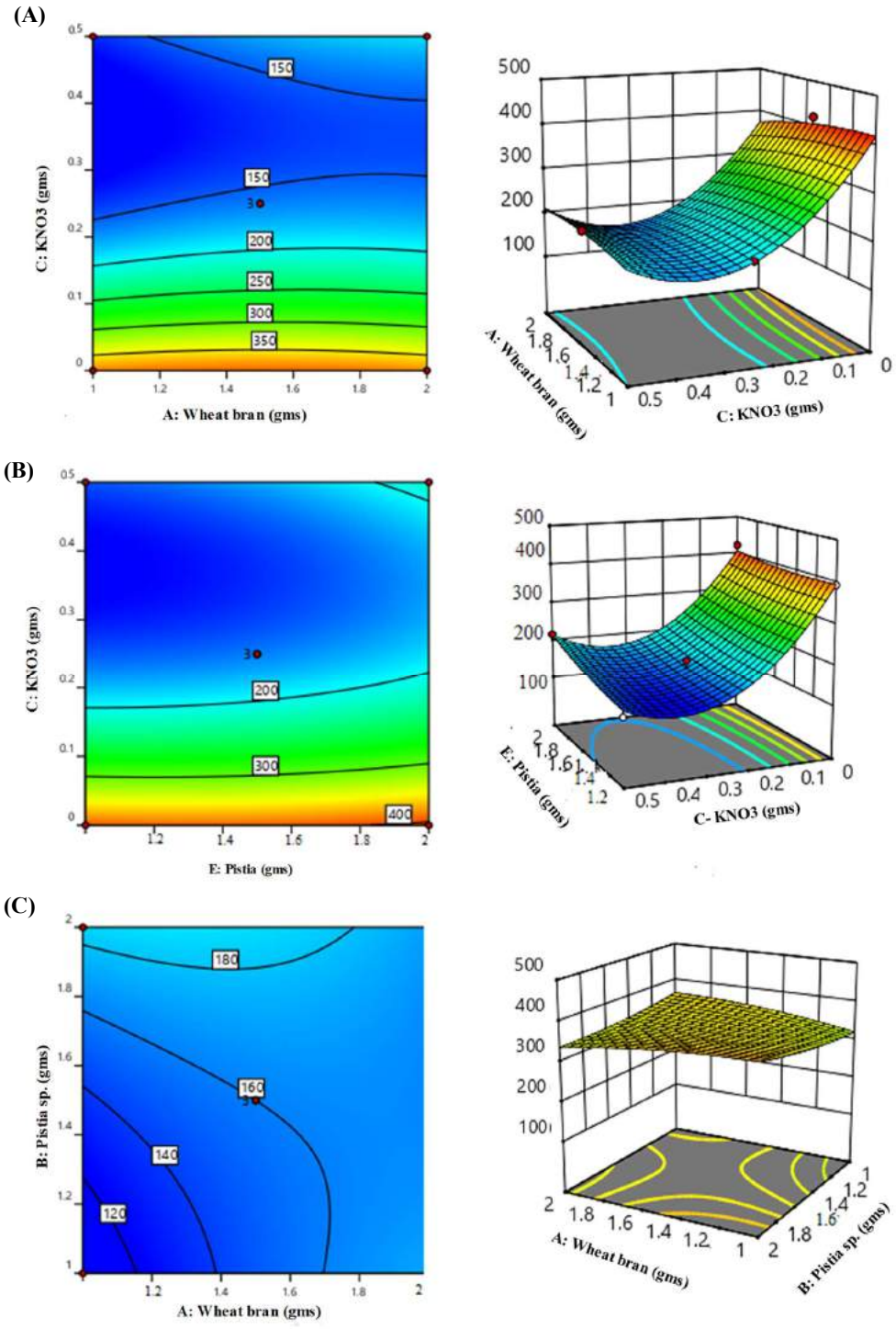

Figure 2 Optimization by Response surface methodology for maximum xylanase activity (A) Response surface and contour plot between potassium nitrate and wheat bran, (B) Response surface and contour plot between potassium nitrate and *Pistia stratiotes,* (C) Response surface and contour plot between wheat bran and *Pistia stratiotes*.

3.3 Purification of Xylanase enzyme

The purification of xylanase produced from optimized lignocellulosic medium *Massilia timonae* B2YR KY942185 was performed by ammonium sulphate precipitation. The purification processes of xylanase are

summarized in Table 4. Enzyme activity of 1126.85 units with the concentration of protein to 618.61 mg/mL was found in crude fraction. Ammonium precipitation at 30-50% saturation gave a yield of 83.40%. While enzyme fraction obtained after cellulose DEAE chromatography showed 32.60% recovery of enzyme activity with a purification fold of 20.61 and a specific activity of 938.46 was observed.

Table 4 Purification process of xylanase.

Purification step	Fraction mL	Enzyme units	International units	Protein concentration mg/L	Specific activity	Purification fold	Yield %
Crude enzyme	200	1126.17	187.70	618.61	45.53	1.00	100.00
50% ammonium sulphate	130	939.21	156.53	444.92	52.80	1.16	83.40
Dialysis Cellulose DEAE	3	755.98	126.00	196.80	96.08	2.11	67.13
	5	367.10	61.18	9.78	938.46	20.61	32.60

3.4 Molecular weight determination

The molecular weight of the purified fraction of enzyme was nearly 43 kDa as shown in Figure 3. Native gel electrophoresis was carried out to study the activity staining wherein agar gel containing 1% xylan was over layered. The zymogram studies revealed a single zone of clearance determining the activity of the enzyme.

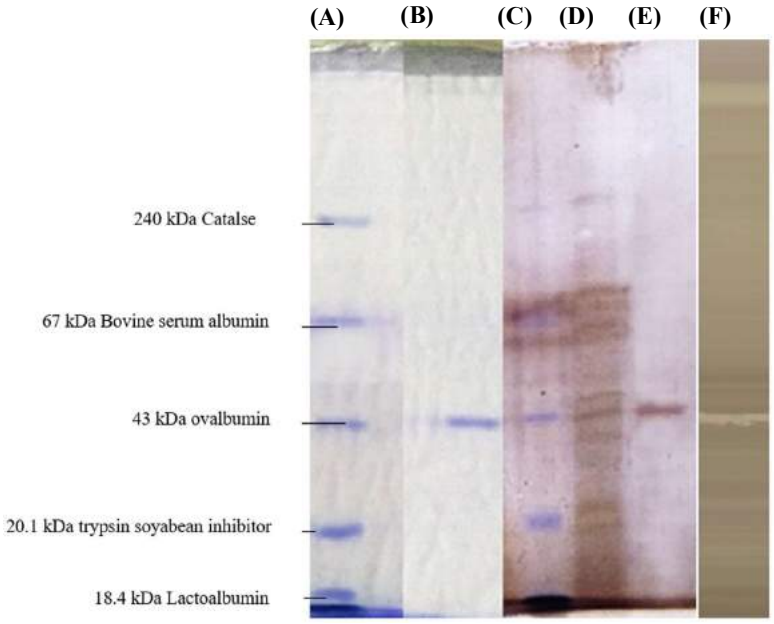

Figure 3 SDS-Page and Native gel electrophoresis studies of purified xylanase enzyme (A) pre-stained marker, (B) purified xylanase Stained by coomassie brilliant blue, (C) Pre-stained marker Silver staining, (D) ammonium saturation precipitation enzyme fraction, (E) Cellulose DEAE enzyme fraction visualized by silver staining, (F) zymogram study.

3.5 Characterization of xylanase

The maximum xylanase activity of purified enzyme from *Massilia timonae* was found to be at 28°±2°C Figure 4A. The effect of pH on enzyme purified from *Massilia timonae* exhibited optimum to 7 for maximum activity of 451.39 units/mL represented in Figure 4B. Residual activity of 17% was retained for pH 10 while for pH 8, 50 % activity was maintained after 2 h of incubation. After incubation of 1 and 2 h, 75% activity was educed at pH 4 and 5 as observed in Figure 4C. Most of the xylanase reported are stable in the alkaline range but the enzyme studied here is more stable in the acidic range. The relative activity of 100 % was retained after 1 h incubation at 28°C, 60% activity was retained at 37°C. The stability of the enzyme was checked at a wide range of pH from 4-

10 for 1 and 2 h and it was found to be stable at these pH ranges. The enzyme activity was enhanced by metals like $MnCl_2$, $MgCl_2$ and $MgSO_4$ at both concentrations 5mM and 10 mM Figure 4D, while $HgCl_2$, NaCl and KCl showed a negligible effect on enzyme activity. At 10 mM concentration (136%) more activity was observed for metal MnCl2, while KCl showed no activity at 10 mM concentration.

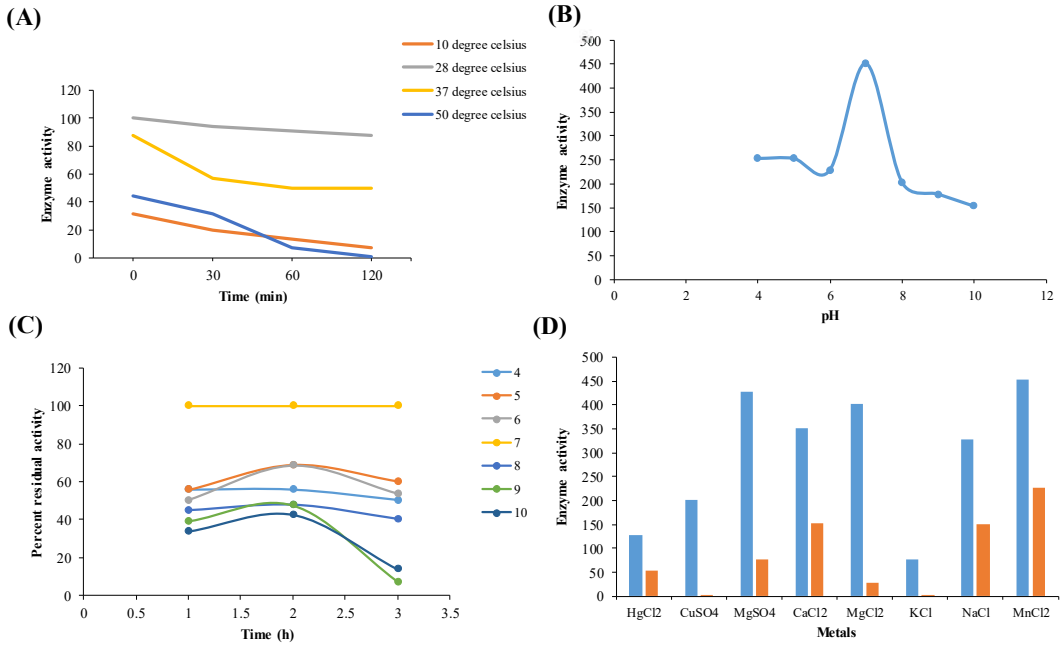

Figure 4 Characterization of xylanase enzyme produced by *Massilia timonae* (A) Relative activity of temperature on xylanase, (B) Effect of pH on xylanase activity, (C) Percent residual activity of the enzyme at different pH incubated for 2 h, (D) Effect of metals on xylanase activity.

3.6 Kinetic parameter

The effect of substrate concentration on enzyme activity showed that the enzyme obeys Michaelis Menten kinetics when Birchwood xylan was used as substrate Figure5. The line weaver Burk plot presented was constructed using Sigma plot software version 14.0. The enzyme showed a K_m of 2.7 mg/mL and V_{max} of 178.3 µmol/min/mg under the optimum operating conditions.

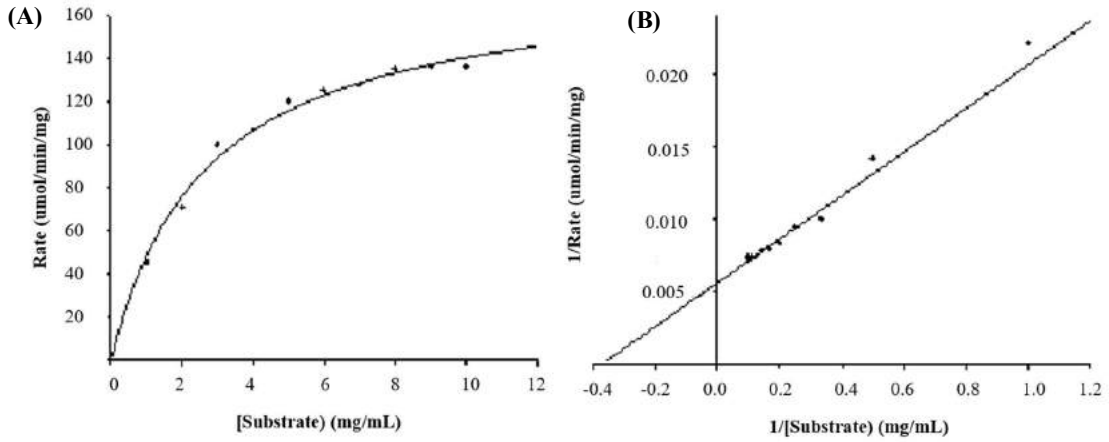

Figure 5 Kinetic parameters studies of xylanase enzyme produced *Massilia timonae* (A) Michaelis Menten plot for xylanase enzyme from *Massilia timonae* B2YR, (B) Line weaver Burk plot for xylanase enzyme of *Massilia timonae* B2YR.

3.7 Study of hydrolysis of xylan

The xylan was hydrolysed to xylooligosaccharides by crude xylanase enzyme obtained from *Massilia timonae*. These xylooligosaccharides were detected by performing TLC Figure 6. The Rf values of standards Glucose, xylose, xylobiose, xylotriose were 0.53, 0.58, 0.47, 0.41. On the lane loaded with hydrolysed fraction, two spots were developed their Rf values were 0.48 and 0.41which are near to standard xylobiose and xylotriose. Crude quantification was done and found that 20.66 mg of xylooligosaccharides per gram of xylan was produced after 18 h.

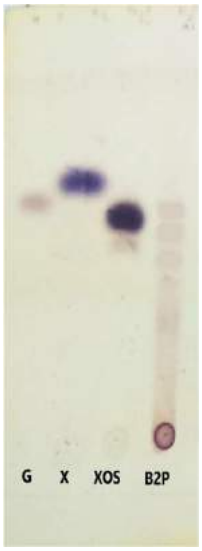

Figure 6 Hydrolysis of *Pistia* and wheat bran mixture after 18 h by xylanase produced by *Massilia timonae* B2YR.

3.8 Economics

The comparison of cost between Horokoshi medium and medium designed using the lignocellulosic waste wheat bran and *Pistia* by response surface method showed a substantial reduction. The final cost of Horokoshi medium with commercial xylan as a source of xylan was 106.91 USD for a litre, While the cost of a medium designed by RSM was 0.018 USD. A 99.98% cost-effective medium was developed. Thus, using such a designed medium can reduce the overall production cost of xylanase.

4. Discussion

The approach of using lignocellulosic waste for the production of xylanase and xylooligosaccharides can lower the cost. Wheat bran is the most often used substrate for the production of xylanase. *Massilia timonae* in the current study showed 281.51 IU/mL at a 1.5 % concentration of wheat bran. The microorganism *Sphingobacterium* SaH-05 was reported to produce 40 U/mL at 0.9% of wheat bran [27]. One more report for use of wheat bran as a substrate to produce xylanase was by the organisms *Bacillus subtilis* which yielded 15.55 IU/mL, [28]. But the use of *Pistia stratiotes* has not been reported yet, which at 2% concentration gave the highest yield of xylanase. The organism *Massilia* gave a maximum yield of xylanase at 0.5% peptone and potassium nitrate in 48 h, but *Arthrobacter sp* MTCC 6915 was used for xylanase production and gave xylanase activity of 170 IU/mL, at 1% peptone concentration with low activity at 1-4% potassium nitrate [29]. While *Trichoderma orientalis E*U7-22 showed 261.5 IU/mL xylanase activity at 0.5% of peptone after 72 h fermentation [30]. Most of the xylanase activity was shown at room temperature, pH-7 and inoculum size of 3% by the organism in the current work. *Bacillus mojavenjis* displayed enzyme activity equal to 290.76 IU/mL at room temperature [31]. The organism *Bacillus circulan*s reported maximum production of xylanase at pH 6.0 at 30°C [32]. *A. pullulans strain SN090* was studied by Nasr and demonstrated a xylanase activity of 2.55 IU/mL at 2% v/v of inoculum concentration [33].

Optimizing the variable using the Box Behnken design showed an increase in yield of xylanase by 1.95-fold to 421.65 IU/mL. Similar studies were reported for the use of Box Behnken design of response surface methodology for increasing xylanase yield by the *Trichoderma orientalis* EU7-22 from 107.6 to 269.4 IU/mL [30]. *Thermomyces lanuginosus* NCIM1374/ DSM28966 gave a yield of 102.60 IU/L at 1.8% wheat bran concentration [34], and a marked increase in xylanase activity was observed in *Aspergillus niger* AN-13 from

4.80 U/mL to 127.12 U/mL with wheat bran 54.2 g/L of medium [25]. Xylanase enzyme when purified from *Massilia* in the present work was purified with 20.61 purification fold and 32.60% yield recovery but xylanase purified from *Bacillus licheniformis* showed a yield of 6.2% with 7.9 purification fold [35]. The molecular weight of the enzyme xylanase in the current study is similar to the reported strain of *Enterobacter* sp. of 43 kDa [36]. The enzyme activity was enhanced in presence of a 5 mM concentration of $MnCl_2$ to 136% more for the enzyme produced by *Massilia timonae* in the present study, while *Trichoderma viridae* showed 102% activity at a 5 mM concentration [37]. One of the characteristic properties of the purified enzyme was its stability at acidic pH. A few examples of bacterial xylanase are known for their stability at acidic pH like *Bacillus subtilis subsp. subtilis* JJBS250 with showed optima at pH 4 [38]. A low Km value of enzyme shows its affinity towards the substrate which can be observed in the current study Km 2.7 mg/mL and Vmax for 178.3 µmol/min/mg. The K_m and V_{max} for enzyme produced by *Massilia sp* RBM26 investigated by Xu et al. [39] were 9.79 mg/mL and 65.79 µmol/min/mg. respectively. Sanghi et al. [40] reported a Km and Vmax of 3.33 mg/mL and 100 IU/mL towards birchwood xylan by enzyme xylanase of *Bacillus subtilis* (the strain of microbial isolate (ASH)). The cost of the product produced by the fermentation process depends on the production medium used. The raw materials used like, wheat bran, *Pistia* have a higher nutritive value. The wheat bran comprises 55% of the arabinoxylan fraction. While the xylan fraction in Pistia is 1.4% protein and 2.6% of carbohydrate. These substrates support the growth of microorganisms and also enhance the activity of xylanase [10].

5. Conclusion

The response surface methodology used during the current study showed a 1.09-fold increase in xylanase activity by *Massilia timonae*. The xylanase produced from lignocellulosic waste like wheat bran and *Pistia stratiotes* showed characteristics of stability at acidic pH (4) which makes it suitable for the production of animal feed. Such xylanase can also be used in the clarification of fruit juices where the pH is lower. The same xylanase can be used to produce xylooligosaccharides, which has many potential applications. Thus, replacing commercial xylan with raw lignocellulosic waste will reduce the production cost of both xylanase and xylooligosaccharides.

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