Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Enhancing the efficiency and functionality of xylanase from *Bacillus sp.* RTS11: Optimization, purification, characterization, and prospects in kraft pulp bleaching

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Article Info

Article history:

Received: October 21, 2023
Accepted: December 28, 2023
Published: January 31, 2024

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Abstract

Bacillus sp. RTS11, a xylanolytic strain, was isolated from the Algerian Sahara desert rocks. Genetic analysis revealed a remarkable 98.69% similarity to *Bacillus pumilus*. We harnessed optimization techniques, including Plackett-Burman screening and Box-Behnken optimization design to amplify xylanase production and activity. The outcome of these efforts was an optimized medium that yielded an impressive xylanase production titer of 448.89 U, a threefold increase compared to the non-optimized medium (146 U). The purification of xylanase was achieved through the three-phase partitioning technique, employing t-butanol and various chromatographic methods. Notably, anion exchange chromatography led to isolating a highly pure enzyme with a molecular weight of 60 kDa. The xylanase exhibited its peak activity at a temperature of 60°C and a pH of 9.0. When applied to pulp pretreatment, 20 U/g of xylanase demonstrated a substantial increase in the release of phenolic and chromophore compounds while reducing sugar content in the pulp. Furthermore, this versatile xylanase shows its ability to efficiently hydrolyze a variety of agro-industrial residues, including wheat bran, corn and grape waste, wheat straw, and sugarcane bagasse. These findings underscore the significant potential of this xylanase enzyme in biobleaching processes and the utilization of agro-industrial waste, opening up exciting avenues for sustainable and environmentally friendly industrial applications.

Keywords: *Bacillus sp.* RTS11, Xylanase, Statistical optimization, Kraft pulp treatment, Agricultural waste recycling

1. Introduction

Xylan is a biopolymer widely found in plant tissues and is a key constituent of the primary cell wall. It is a complex molecule composed of a highly branched hetero-polysaccharide with a linear backbone consisting of d-xylopyranose units linked together in a β-1,4 configuration [1]. In addition to this fundamental structure, xylan incorporates various side chains, including glucuronopyranosyl, 4-O-methyl-D-glucopyranosyl, a-L-arabinofuranosyl, and acetyl branches. Furthermore, it can contain feruloyl and coumaryl components of lignin, the proportions and distribution of which vary depending on the specific plant species [2]. To fully break down xylan, the collaborative action of different xylanolytic enzymes is required, as observed by Asha Poorna and Prema [3]. Enzymes involved in xylan degradation can be categorized based on the specific substrates they hydrolyze [4], including xylanase, β-D-xylosidase, α-L-arabinofuranosidase, α-L-glucuronidase, acetyl xylan esterase, ferulic acid esterase, and q-coumaric acid esterase [5]. Among bacteria, common producers of xylanase enzymes include *Bacillus*, *Clostridium*, *Cellulomonas*, *Acetovibrio*, *Streptomyces*, *Ruminococcus*, and *Aerobacter* [6]. *Bacillus* strains, in particular, are highly appealing due to their capacity to generate cellulase-free extracellular xylanases at substantial levels, and these enzymes remain stable even in high-temperature and alkaline pH conditions [7]. Xylanases have a wide range of biotechnological applications, including improving the digestibility and feed absorption of livestock when added to animal feed, facilitating dough handling and improving bakery product quality in baking, clarifying fruit juices using pectinase and cellulase, improving fiber quality in the textile industry, and decreasing chlorine consumption for pulp bleaching in the pulp and paper industry [8-10]. In summary, this investigation extensively explores xylanase production, purification, and characterization, along with

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.1.10

practical evaluations of its performance in critical industrial processes. This underscores the enzyme's significance in both biotechnological and environmental contexts.

2. Materials and Methods

2.1. Microorganism

The strain of *Bacillus sp.* RTS11 was isolated from rock fragments collected from the El-Hoggar mountain range, Tamanrasset, Algerian Sahara desert (22°47'37.8"N, 5°34'32.4"E).

2.2. Estimation of xylanase activity

The xylanase activity of RTS11 isolate was quantitatively evaluated using liquid medium (g/l): xylan, 5; yeast extract, 5; peptone, 5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.1. The culture is incubated at 30°C for 72 h with stirring (100 rpm). After incubation, the cultures were centrifuged at 12 000 rpm, 10 min at 4°C, and the supernatant was analyzed for xylanase activity.

2.3. Enzymatic assay and protein estimation

The xylanase enzyme's activity was assessed using the procedure outlined by Bailey *et al*. [11]. To determine this activity, we combined 0.5 ml of the enzymatic solution with 0.5 ml of a 1% xylan solution in a 0.05 M phosphate buffer at pH 7. The resulting mixture was incubated at 60°C for 15 minutes. Subsequently, we introduced 1.5 ml of DNS into the mix and set it at 90°C for 5 minutes. The quantification of xylanase activity was based on releasing one mole of xylose per minute. For protein analysis, we employed the method detailed by Lowry *et al*. [12].

2.4. Estimation of xylanase activity without cellulase

Cellulase activity was determined using 1% (w/v) carboxymethylcellulose (CMC) as substrate and glucose as standard. This test was performed to detect the presence or absence of cellulosic activity of the xylanase produced.

2.5. Biomass estimation

The bacterial cells in the fermentation broth were harvested by centrifugation (12000 rpm, 10 min, 4°C), washed with distilled water, and dried in an oven at 80 °C until a constant weight was reached. Biomass has been reported as dry cell mass (mg/l) [13].

2.6. DNA extraction and identification of the isolate RTS11

The strain's genomic DNA was prepared from LB-grown bacteria using a Wizard Genomic DNA Purification Kit. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using two primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (GGTTACCTTGTTACGACTT-3'). Amplification was performed using HOT FIREPol® (Solis Biodyne, Estonia) according to the supplier's recommendations. Cycling conditions were as follows: activation at 95°C for 10 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 mn. The sequencing was done using an ABI 3730 XL gene analyzer (Applied Biosystem, ThermoFisher, USA). The obtained 16S rRNA gene sequence data was analyzed using an advanced BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST. The nucleotide sequence data were deposited to the gene bank for accession number assignation. Phylogenetic analysis was performed using a neighbor-joining method in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms [14].

2.7. The effect of specific mono and polysaccharides on enzyme production

Carbon sources were used to supplement the basal medium to determine their effectiveness in inducing the production of xylanase by the *Bacillus sp*. strain following the protocol [83]. For this purpose, 50 ml of mineral salt broth was prepared and added with 0.3% (w/v) of one of the following carbon sources: birch xylan, spelled oat xylan, corncob, xylose, glucose, starch, and carboxymethylcellulose and 0.2% birch xylan, for a total of 0.5% carbon source. The cultures were incubated for 48 h. The broth was centrifuged and analyzed for xylanase activity.

2.8. Plackett-Burman experimental design

The Plackett-Burman experimental design was used to screen and evaluate essential factors that influence the yield of xylanase produced by *Bacillus sp.* RTS11 and rank factors according to their importance. Fifteen variables were selected as possible factors affecting xylanase production (Xylan, Yeast extract, Peptone, KNO_3, Urea, $MgSO_4$, NaCl, $CaCl_2$, Tween 80, $MnSO_4$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, Temperature, $(NH_4)_2SO_4$). Sixteen experimental tests were conducted, and Design Expert 11 software (Stat Ease, Inc., Minneapolis, USA) was used to generate the design table and analyze the data.

2.9. Box-Behnken Experimental design

Plackett-Burman's design allowed us to select Xylan for its significant effect on xylanase production. Five variables of A (*Inoculum* Age), B (*Inoculum* Rate), C (pH), D (Agitation), and E (Xylan) were chosen for further study based on the Box-Behnken design.

2.10. Enzymatic Purification

2.10.1. Purification by the TPP technique

After recovery of the crude extract by centrifugation of the culture broth at 12 000 rpm, 4°C, and 15 min, The TPP experiments were performed following the protocol described by Gagaoua *et al*. [15]. The interfacial phase containing the enzyme xylanase was carefully recovered, dissolved in Tris-HCl buffer 50 mM (pH 7.5), and dialyzed overnight at 4°C.

2.10.2. Purification by chromatographic technique

The crude extract of xylanase enzyme was subjected to fractional precipitation of 70% ammonium salt with stirring at 4°C overnight and centrifuged at 12 000 rpm for 10 min. The pellet obtained was dialyzed against a sodium phosphate buffer of 50 mM at 4°C. The dialysate was applied to a gel filtration column (Sephadex-G50), and protein elution was performed with the same dialysis buffer. Fractions were analyzed for xylanase activity, and absorbance at 280 nm was used for protein measurements. The fractions with xylanase activity were collected and concentrated by ultrafiltration with an Amicon membrane (Millipore). The resulting filtrate was loaded onto a DE-AE-Sepharose (Sigma-Aldrich) anion exchange column. The unbound proteins were removed with sodium phosphate buffer 50 mM, pH 7.5, and the enzymes were eluted with a gradient of 0 to 0.5 M NaCl. The fractions were

collected with a volume of 4 ml. The fractions collected were used for the determination of proteins and xylanase activity.

2.11. Determination of molecular weight by SDS-PAGE

The molecular weight of xylanase was determined by SDS-PAGE electrophoresis (12 %) using the Laemmli method [16], using bright blue from Coomassie R-250 to stain the gel and molecular weight markers from 20.0 to 120.0 kDa (Thermo Scientific).

2.12. Biochemical characterization of purified xylanase

To determine the effect of temperature and pH on purified xylanase activity, the enzyme was maintained at different temperatures (30 to 80°C) and pH (6 to 12) using phosphate buffers (pH 6 to 8), glycine-NaOH buffer (pH 9 to 12). The effect of many 5 mM metal ions (Hg^{+2}, Cu^{+2}, Zn^{+2}, Mg^{+2}, Co^{+2}, Na^{+}, Fe^{+2}, Mn^{+2}, Ca^{+2}, K^{+}), as well as the effect of Tween 20, Tween 80 and Triton-X on purified xylanase activity, were also studied.

2.13. Application of xylanase

2.13.1. Preparation of the pulp

Cardboard boxes were cut into small pieces, soaked in distilled water, and kiln-dried to remove the absorbed water until it reached a constant weight.

2.13.2. Parameter optimization

Different amounts of xylanases (5, 10, 15, and 20 U) were used to treat one gram of pulp, the reaction mixture was incubated for 1 h at 60°C, and the amount of reducing sugars was analyzed by the DNS method. The pulp's consistency was optimized using different concentrations of oven-dried dough (3.0%, 5%, and 10%), and the reaction medium was maintained at 60°C for 1 h. The optimal pulp treatment time with xylanase was also optimized, incubating the enzyme with the pulp at varying time intervals (1 to 4 h).

2.13.3. Bleaching of xylanase pulp

The reaction medium containing 20 U xylanase/g pulp in 20 ml glycine buffer (pH 9.0, 0.05 M) was incubated for 4 h with stirring (100 rpm). After incubation, the water was removed by filtration, and the leaves were pressed and dried at 60°C. The filtrate was analyzed for the release of phenolic and chromophore compounds from the pulp, measuring absorbance at 280 and 465 nm, respectively, and reducing sugar release according to the DNS method.

2.13.4. Hydrolysis of agro-industrial residues

To evaluate the capacity of xylanase to hydrolyze specific agro-industrial residues, reaction mixtures containing 20 U of purified enzymatic solution and 50 mg dry weight of agro-industrial residues (wheat bran, wheat straw, apricot kernel, grape, corn, and tomato waste, sugar cane bagasse, corn cob, and sawdust). The enzyme and each substrate were suspended in glycine buffer (0.05 M, pH 9.0) and incubated for 24 h at 60°C. Reducing sugars were quantified by the dinitrosalicylic acid method.

2.14. Statistical analysis

Data are presented as mean ± standard error (SE). The significance of the difference between the different groups was tested using ANOVA analysis on GraphPad prism 10 software. Significance was determined at the $p < 0.05$ level.

3. Results

3.1. Estimation of xylanase activity

RTS11 isolate was tested for its ability to produce xylanase in a non-optimized culture broth; maximum enzyme activity (174.17 U) was observed after 24 h of incubation.

3.2. Molecular identification

The BLAST analysis of the RTS11 isolate's partial 16S rRNA sequence demonstrated high similarity with organisms belonging to the *Bacillus* genus. A sequence alignment was conducted to explore this relationship further, comparing the 16S rRNA gene of the RTS11 isolate with a select group of representative members from both the *Bacillus* genus and *Staphylococcus aureus*. The resulting data was then visualized as a phylogenetic tree (Figure 1). Notably, the partial molecular sequencing of the 16S rRNA gene revealed a striking 98.69% similarity with the *Bacillus pumilus* strain. As a testament to these findings, the RTS11 strain has been officially deposited in the database and can be referenced using the identifier OL989329.

3.3. The effect of certain sugars on stimulating or inhibiting xylanase activity

The influence of nine individually supplemented carbon sources in the fermentation medium on xylanase production was studied. Low xylanasic activity was observed in the presence of xylose, sucrose, starch, and CMC. Of the carbon sources tested, xylan in all its forms was found to be the best source of carbon, while in the medium containing glucose and xylan, the enzyme activity was zero, indicating that glucose acts as a catabolic repressor of xylanase. However, maximum xylanase production was achieved in a birch xylan crop, followed by oat-spelled xylan and corn cob xylan as the second and third-best carbon sources. This result indicated that xylan-containing substrates could induce xylanase production by the *Bacillus sp* strain RTS11.

3.4. Temporal and growth kinetic evolution for xylanase production by *Bacillus sp.* RTS11

The temporal evolution of xylanase production was monitored at regular intervals during 72 h of a culture of the strain in the optimized medium and under static conditions (Figure 2). After inoculation, the crop began to grow until 32 h, when the highest biomass was reached, remained almost constant until 40 h, and decreased afterward. The production of xylanases by strain RTS11 appears to be partially associated with growth. The enzyme synthesis begins at the beginning of fermentation but in

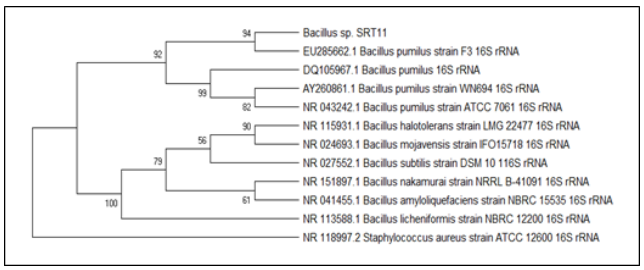

Fig. 1. Phylogenetic tree of strain RTS11 showing the similarity with *Bacillus pumilus* strains.

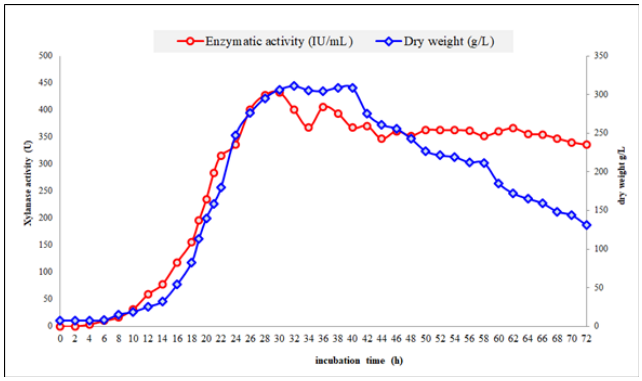

Fig. 2. The profile of growth and production of xylanase by the strain of *Bacillus sp.* RTS11.

small quantities, corresponding to a logarithmic growth phase. On the other hand, optimal xylanase production was achieved at 30 h (432.83 U) in the late logarithmic growth phase. Xylanase activity decreased after reaching a maximum value, possibly due to proteolysis or depletion of nutrients available for the isolate.

3.5. Estimation of xylanase activity without cellulase

In general, xylanase secretion is accompanied by cellulase production of varying degrees. It was for this reason that the cellulase activity of xylanase was determined. our xylanase showed showed very low cellulase activity (1.87 U).

3.6. Plackett-Burman design

The Plackett-Burman plan was used to screen crop parameters to select the most significant ones capable of increasing xylanase production. The Plackett-Burman experiments are presented in Table 1. They showed a wide variation in xylanase production (from 27.03 U to 251.41 U), which reflects the importance of optimizing the medium to achieve a better yield.

The analysis of variance (ANOVA) of the model demonstrates that the model is highly significant, with an *F* value of 12.17, *p* of 0.0134, and R^2 of 0.9786. The variables, which were significant at the 95% level ($p<0.05$), were considered to impact xylanase production. From Table 2, Xylan and $CoCl_2$ were determined as the two significant factors, with *p* values corresponding to 0.0007 and 0.0014, respectively. The other variables were found to have insignificant effects on xylanase production and were not studied. Xylanase production was expressed in terms of the following regression equation:

Activity = 127.277 + 46.09 A + 3.53 B - 6.06 C+ 0.45 D - 11.09 E+ 9.74 F - 3.54 G -1.15 H -10.88 J + 8.89 K+ 8.28 L – 38 M - 10.234 N - 5.17 O - 7.52 P;

Where A, B, C, E, F, G, H, J, K, L, M, N, O, P represents xylan, yeast extract, peptone, KNO_3, urea, $MgSO_4$, NaCl, $CaCl_2$, Tw80, $MnSO_4$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, T°, $(NH_4)_2SO_4$.

3.7. Box Behnken design

This design indicates the statistical optimization of xylanase production. The Plackett-Burman plan allowed us to select xylan as a component affecting xylanase production. In addition to xylan, four other factors were included in the optimization (*inoculum* rate, *inoculum* age, agitation, and pH). A set of 46 experimental tests are presented in Table 3. The second-order polynomial equation is shown as follows:

$Y = 386.04 + 39.54\,A + 6.03\,B + 12.83\,C - 88.68\,D - 6.90\,E - 21.55\,AB + 40.24\,AC + 27.89\,AD + 10.03\,AE + 32.28\,BC + 11.85\,BD + 6.61\,BE + 2.17\,CD - 0.3400\,EC + 4.26\,A^2 - 2.10\,B^2 - 43.44\,C^2 - 48.48\,D^2 + 1.49\,E^2$

Where Y is the xylanase activity (U), A, B, C, D, and E are *inoculum* age, *inoculum* rate, pH, agitation, and xylan, respectively.

Analysis of variance (ANOVA) for the quadratic response surface model is given in Table 4. Model values *F* and *p* and R^2 were calculated as 18.90, 0.0001, and 0.9380, respectively, indicating the model's meaning. The linear effect of A and D, the interactive effects of AC, AD, and

Table 1. Plackett-Burman experimental design matrix for screening factors affecting xylanase production by *Bacillus sp.* RTS11.

Run	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	R_1	R_2
1	10	10	5	1	1	0.1	1	0.02	0.1	0.005	0.005	0.02	0.02	30	1	124.94	115.12
2	10	10	1	1	1	0.1	0.1	0.3	0.3	0.02	0.02	0.001	0.02	30	3	237.12	225.15
3	3	10	1	1	1	0.5	1	0.3	0.1	0.02	0.005	0.02	0.001	30	1	80.67	90.49
4	10	10	5	5	1	0.5	1	0.3	0.3	0.02	0.02	0.02	0.02	37	3	128.01	139.98
5	3	10	5	5	1	0.1	1	0.02	0.3	0.005	0.005	0.001	0.001	37	3	100.13	92.76
6	3	1	5	5	0.05	0.1	1	0.02	0.1	0.02	0.02	0.02	0.02	30	3	53.03	58.25
7	10	1	1	1	0.05	0.5	1	0.02	0.1	0.02	0.005	0.001	0.02	37	3	231.66	230.25
8	10	10	5	5	0.05	0.5	0.1	0.3	0.1	0.005	0.005	0.001	0.02	37	1	222.9	228.12
9	3	1	5	1	0.05	0.5	1	0.3	0.3	0.005	0.02	0.001	0.02	30	1	156.82	145.63
10	10	1	5	1	0.05	0.1	0.1	0.3	0.3	0.005	0.005	0.02	0.001	30	3	122.5	138.29
11	3	10	5	1	0.05	0.5	0.1	0.02	0.3	0.02	0.02	0.02	0.001	37	1	119.98	113.98
12	3	1	1	5	1	0.1	0.1	0.3	0.1	0.005	0.02	0.02	0.02	37	1	41.32	39.91
13	3	10	1	5	0.05	0.1	0.1	0.02	0.3	0.02	0.005	0.001	0.02	30	1	144.85	156.82
14	3	1	1	1	1	0.5	0.1	0.02	0.3	0.005	0.005	0.02	0.02	37	3	25.62	27.03
15	10	1	1	5	1	0.5	1	0.02	0.3	0.005	0.02	0.001	0.001	30	1	240.22	251.41
16	3	10	1	1	0.05	0.1	1	0.3	0.1	0.005	0.02	0.001	0.001	37	3	135.23	142.60
17	10	10	1	5	0.05	0.5	0.1	0.02	0.1	0.005	0.02	0.02	0.001	30	3	210.06	198.87
18	10	1	1	5	0.05	0.1	1	0.3	0.3	0.02	0.005	0.02	0.001	37	1	182.48	166.69
19	3	1	5	5	1	0.5	0.1	0.3	0.1	0.02	0.005	0.001	0.001	30	3	150.07	140.25
20	10	1	5	1	1	0.1	0.1	0.02	0.1	0.02	0.02	0.001	0.001	37	1	229.61	235.61

A, xylan (; B, yeast extract; C, peptone; D, KNO_3; E, urea; F, $MgSO_4$; G, NaCl; H, $CaCl_2$; J, Tw80; K, $MnSO_4$; L, $FeSO_4$; M, $CoCl_2$; N, $ZnSO_4$; O, T°; P, $(NH_4)_2SO_4$; R_1, Activity (U); R_2, Predicted activity (U).

Table 2. Estimates of xylanase production effects from Plackett-Burman design results.

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Estimation
Model	83719.37	15	5581.29	12.17	0.0134	
A-Xylan	42483.92	1	42483.92	92.63	0.0007	46.09
B-Yeast extract	248.94	1	248.94	0.5428	0.5022	3.53
C-Peptone	734.96	1	734.96	1.60	0.2743	-6.06
D-KNO_3	3.98	1	3.98	0.0087	0.9303	0.4460
E-Urea	2459.76	1	2459.76	5.36	0.0815	-11.09
F-$MgSO_4$	1897.35	1	1897.35	4.14	0.1117	9.74
G-NaCl	250.92	1	250.92	0.5471	0.5006	-3.54
H-$CaCl_2$	26.40	1	26.40	0.0576	0.8222	-1.15
J-Tween 80	23.67	1	23.67	0.0516	0.8314	-10.88
K-$MnSO_4$	1579.58	1	1579.58	3.44	0.1371	8.89
L-$FeSO_4$	1370.84	1	1370.84	2.99	0.1589	8.28
M-$CoCl_2$	28880.00	1	28880.00	62.97	0.0014	-38.00
N-$ZnSO_4$	2094.70	1	2094.70	4.57	0.0994	-10.23
O-Temperature	533.96	1	533.96	1.16	0.3413	-5.17
P-$(NH_4)_2SO_4$	1130.41	1	1130.41	2.46	0.1915	-7.52
Residual	1834.56	4	458.64			
Cor Total	85553.93	19				

Table 3. Box-Behnken design matrix with experimental and predicted values of xylanase activity.

Run	*Inoculum* age (h)	*Inoculum* size (%)	pH	Agitation (rpm)	Xylan (g/l)	Activity (U)	Predicted Activity (U)
1	5.5	1	5	100	12.5	375	352.17
2	5.5	3	5	100	10	358.11	341.56
3	5.5	5	7.5	200	12.5	254.46	263.96
4	8	3	7.5	0	12.5	416.81	404.65
5	5.5	3	10	0	12.5	395.52	396.71
6	5.5	5	10	100	12.5	379.13	388.83
7	5.5	3	5	100	15	325.45	313.96
8	5.5	3	7.5	100	12.5	407.42	389.38
9	5.5	3	10	100	15	352.1	347.55
10	5.5	3	5	200	12.5	172.19	189.82
11	5.5	3	7.5	200	10	305.44	264.08
12	8	3	5	100	12.5	376.16	378.92
13	5.5	1	7.5	100	10	384.57	401.63
14	3	5	7.5	100	12.5	345.48	322.02
15	5.5	1	7.5	200	12.5	201.88	231.95
16	5.5	1	10	100	12.5	346.78	316.06
17	3	3	5	100	12.5	184.27	208.11
18	8	1	7.5	100	12.5	401.18	404.03
19	5.5	3	7.5	100	12.5	401.12	398.89
20	3	1	7.5	100	12.5	292.64	290.60
21	8	3	7.5	100	15	374.53	389.38
22	5.5	1	7.5	0	12.5	415.61	434.63
23	5.5	3	7.5	100	12.5	377.12	389.38
24	5.5	3	7.5	0	10	429.02	439.08
25	3	3	7.5	100	15	265.01	288.49
26	5.5	3	7.5	200	15	266.57	238.18
27	8	3	10	100	12.5	335.42	326.35
28	3	3	10	100	12.5	304.48	316.49
29	5.5	3	7.5	100	12.5	363.69	389.38
30	8	5	7.5	100	12.5	407.83	389.26
31	5.5	3	10	100	10	373.4	363.78
32	5.5	5	7.5	100	15	400.52	388.04
33	5.5	5	5	100	12.5	278.25	296.48
34	8	3	7.5	100	10	389.37	400.74
35	8	3	7.5	200	12.5	299.98	298.43
36	3	3	7.5	0	12.5	414.56	387.09
37	5.5	5	7.5	0	12.5	420.8	419.26
38	5.5	3	7.5	0	15	398.12	421.15
39	3	3	7.5	200	12.5	152.19	135.33
40	5.5	3	7.5	100	12.5	396.57	398.38
41	5.5	3	5	0	12.5	381.28	369.15
42	5.5	1	7.5	100	15	375.45	361.50
43	5.5	3	7.5	100	12.5	390.33	389.38
44	3	3	7.5	100	10	319.97	330.47
45	5.5	3	10	200	12.5	187.12	218.07
46	5.5	5	7.5	100	10	373.2	391.73

Table 4. Analysis of variance (ANOVA) for the fitted quadratic model of xylanase activity as a function of independent variables.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Estimation des coefficients
Model	2.254E+05	20	11270.96	18.90	< 0.0001	
A-Age	32641.65	1	32641.65	54.74	< 0.0001	45.17
B-Size	276.89	1	276.89	0.4643	0.5019	4.16
C-pH	3114.76	1	3114.76	5.22	0.0310	13.95
D- agitation	1.281E+05	1	1.281E+05	214.90	< 0.0001	-89.49
E-xylan	1921.29	1	1921.29	3.22	0.0848	-10.96
AB	533.38	1	533.38	0.8945	0.3533	-11.55
AC	6476.23	1	6476.23	10.86	0.0029	-40.24
AD	5295.47	1	5295.47	8.88	0.0063	36.38
AE	402.40	1	402.40	0.6748	0.4191	10.03
BC	4166.70	1	4166.70	6.99	0.0140	32.28
BD	561.45	1	561.45	0.9415	0.3412	11.85
BE	331.97	1	331.97	0.5567	0.4625	9.11
CD	0.1190	1	0.1190	0.0002	0.9888	0.1725
CE	32.26	1	32.26	0.0541	0.8180	2.84
DE	15.88	1	15.88	0.0266	0.8717	-1.99
A^2	10379.23	1	10379.23	17.41	0.0003	-34.49
B^2	101.54	1	101.54	0.1703	0.6834	-3.41
C^2	19625.50	1	19625.50	32.91	< 0.0001	-47.42
D^2	20540.19	1	20540.19	34.45	< 0.0001	-48.51
E^2	0.5036	1	0.5036	0.0008	0.9770	-0.2402
Residual	14907.70	25	596.31			
Lack of Fit	13581.55	20	679.08	2.56	0.1506	not significant
Pure Error	1326.15	5	265.23			
Cor Total	2.403E+05	45				

BC, and the square effects of D^2 are significant model terms. The pH and age of the inoculum significantly affect xylanase production.

The pH and age of the inoculum significantly affect xylanase production. The effect of pH and age of *inoculum* on chitinase activity is shown in Figure 3 (AC). Initially, an increase in pH (7-8) and *inoculum* age (5-6 h) indicated a rise in xylanase activity, a remarkable decrease after increasing the latter above the previous interval. Figure 3 (AD) shows the effect of agitation on xylanase activity. Xylanase activity levels were not significantly influenced by crop agitation. As can be seen in the figure, maximum xylanase activity was obtained due to a lack of agitation, but increased agitation resulted in decreased activity. Figure 3 (DE) shows the dependence of xylanase activity on xylan concentration. Maximum xylanase activity was observed at lower xylan values (<10.5 g/l), and xylanase activity decreased with a further increase in xylanase. Figure 3 (BC) shows the effect of *inoculum* level on xylanase activity. It can be deduced that the maximum synthesis of xylanase occurred when the *inoculum* level was maintained at intermediate levels (2-3%).

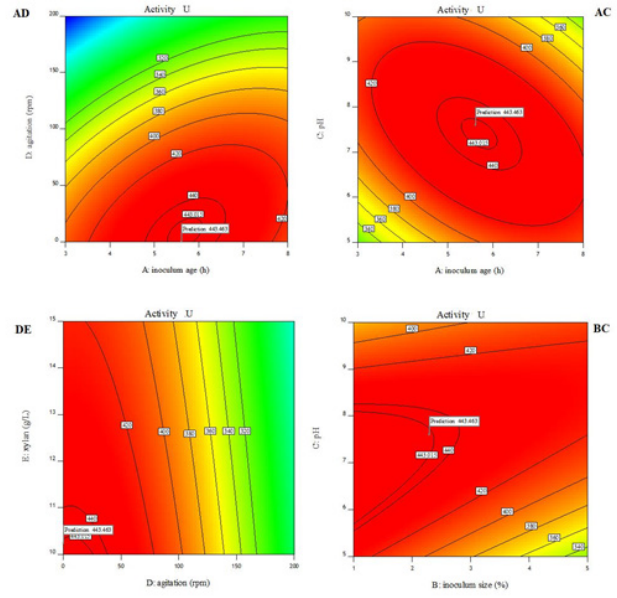

Fig. 3. Response surface curves of xylanase production by Bacillus sp. RTS11 shows the interactions between pH and *inoculum* age (AC), agitation and *inoculum* age (AD), pH and *inoculum* rate (BC), and between Xylan and agitation (DE).

Model validation

The experiment was conducted under optimized conditions predicted by the statistical analysis of the Box-Behnken: xylan (10.24 g/l), agitation (0.36 rpm), pH (7.60), *inoculum* age (5.60 h), *inoculum* level (2.28%). The experimental result (448.89 IU) was close to the model's predicted value (443.46 IU). After optimization, the excellent correlation between predicted and experimental values justified the model's validity.

3.8. Purification of xylanase produced by the strain of *Bacillus sp.* RTS11

To purify the xylanase of *Bacillus sp.* RTS11, The purification technique included t-butanol, and the concentration of ammonium sulfate, t-butanol, and pH was optimized to facilitate enzymatic purification. Purification involved precipitation of the enzyme extract by 70% ammonium sulfate at pH 9 and an enzyme extract ratio: t-butanol (v/v) (1/1.75). A purification factor of 0.34 and a recovery efficiency of 22.93% were achieved. On the SDS page, different protein bands probably indicate the absence of significant purification.

In a second step, a xylanase enzyme purification test was carried out by a three-step chromatographic procedure: precipitation with ammonium sulfate, gel filtration chromatography, and anion exchange chromatography.

Table 5. Summary of purification profile of the enzyme xylanase secreted by *Bacillus sp.* RTS11.

Purification steps	Enzymatic activity (U)	Proteins (mg)	Specific activity (U/mg)	Purification fold	Purification yield (%)
Culture supernatant	466.61	0.930	501.73	1	100
Ammonium sulfate precipitation	391.46	0.636	615.30	1.22	83.89
Sephadex G-50	302.57	0.245	1234.97	2.46	64.84
DEAE-Sepharose	278.45	0.067	4155.97	8.28	59.67
TPP	107	0.613	174.55	0.34	22.93

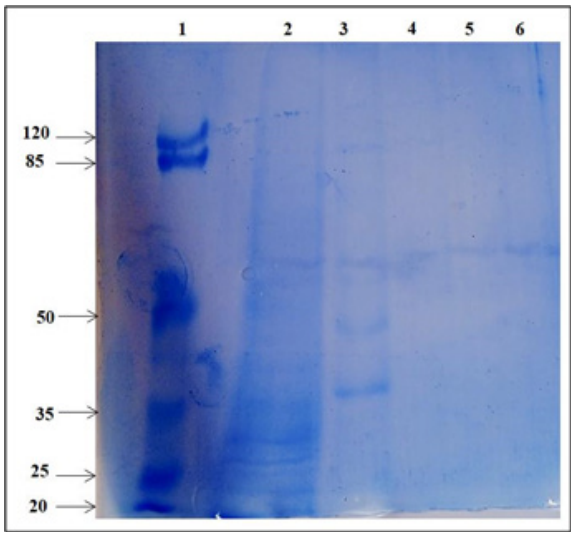

Fig. 4. SDS-PAGE analysis of the purified xylanase from *Bacillus sp.* RTS11. Lane 1. Molecular weight standards. Lane 2. Sample of the crude extract. Lane 3. Ammonium sulfate fractionated protein. Lane 4. Proteins of gel filtration chromatography. Lane 5-6. Proteins are purified by ion exchange chromatography.

The different steps of xylanase purification are presented in Table 5. The culture filtrate had a total xylanase activity of 466.61 U. After the three purification steps. Xylanase was purified 8.28 fold with a yield of 59.67% and a specific activity of 4155.97 U/mg.

3.9. Characterization of purified xylanase

3.9.1. Effect of pH and temperature on xylanase activity

The enzyme was incubated at different pH values (6 to 12) at 30°C, and residual activity was measured (Figure 5a). Purified xylanase showed optimal enzyme activity at pH 9, with decreased activity at pH 6-11, while at pH 12, no activity was recorded.

The effect of various temperatures (30-80°C) on the enzymatic activity of purified xylanase is shown in Figure 5b. The optimum temperature of the enzyme turned out to be 60°C. When the temperature rose above 60°C, the enzyme activity was reduced.

3.9.2. Effect of metal ions and Surfactants on xylanase activity

The effect of inhibitors and enzyme activators on the activity of purified xylanase is shown in Figure 5c. It can be concluded that the xylanase activity of *Bacillus sp.* RTS11 was slightly strengthened in the presence of Fe^{+2}, Mn^{+2}, and Mg^{+2}, and decreased in the presence of Zn^{+2}, Co^{+2}, Cu^{+2} and was inhibited in the presence of Hg^{+2}. The ions Ca^{+2}, Na^{+}, and K^{+} are inert and do not affect this enzyme's activity. The addition of surfactants, such as Triton X-100, Tw 20, and Tw 80, slightly increased xylanase activity.

3.10. Biobleaching of pulp

The xylanase dose, pulp consistency, and incubation time of the enzyme-substrate for pulp biobleaching at 60°C were optimized. The release of reducing sugars was increased with increasing enzyme dose (Figure 6a) and treatment time (Figure 6c). As well as the reducing sugars released were 0.165 mg/ml, 0.211 mg/ml, 0.177 mg/ml, and 0.164 mg/ml at 3%, 5%, 10%, and 20% consistency, respectively (Figure 6b).

It can be concluded that the effectiveness of biobleaching of xylanase from *Bacillus sp.* RTS11 was maximum after 4 h of Treatment at 60°C, pH 9 using a xylanase dose of 20 U/g pulp, at a pulp consistency of 5 %. Details on the release of reducing sugars, phenolic compounds, and other hydrophobic compounds are presented in Table 6. Enzymatic treatment increased the amount of reducing sugars released into the medium compared to the control, indicating the effective action of xylanase on the pulp.

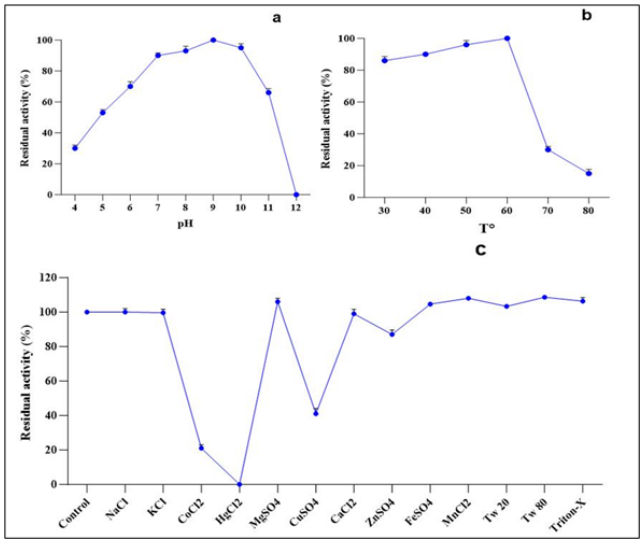

Fig. 5. Effect of pH (a), Temperature (b), metal ions, and surfactants (c) on the activity of xylanase.

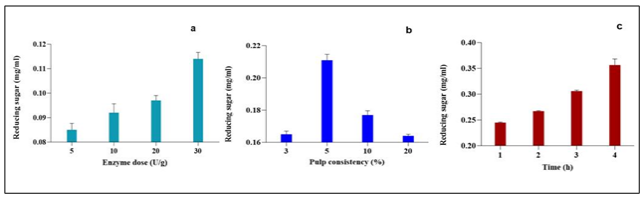

Fig. 6. Effect of xylanase dose (a), pulp consistency (b), and incubation time (c) on pulp bleaching.

Table 6. Effect of xylanase treatment on bleaching of paper pulp at 60 °C, pH 9.0, after 4h.

Parameter	Control	Xylanase
Hydrophobic compounds (A_{465})	0.11	0.39
Chromophoric compounds (A_{237})	0.37	1.106
Reducing sugar (mg/ml)	0.09	0.323

3.11. Hydrolytic capabilities of purified xylanase

Hydrolysis experiments of specific agro-industrial residues were conducted under optimal conditions (60°C and pH 9.0) using purified xylanase and birch xylan as control (1.79 mg/ml of reducing sugars). The results were expressed in Figure 7, 0.908 mg/ml of reducing sugars were released from wheat bran, 0.88 mg/ml from corn cob, 0.833 mg/ml grape waste, 0.674 mg/ml wheat straw, 0.626 mg/ml sugarcane bagasse, and 0.422 mg/ml sawdust. However, a small amount of sugar was released in the case of apricot kernels (0.19 mg/ml) and tomato waste (0.093 mg/ml).

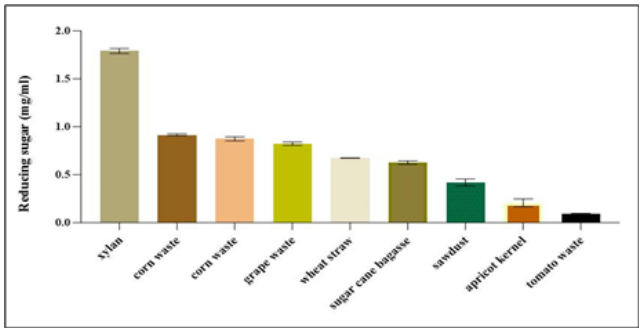

Fig. 7. Hydrolysis of agricultural waste by purified xylanase.

4. Discussion

Xylan is one of the most common hemicellulose components in soft and hard woods. Xylanases are the hydrolytic enzymes responsible for degrading this polymer [84]. Many micro-organisms, including bacteria, fungi, yeasts, are capable of producing xylanase. Although fungi generally produce large quantities of xylanase, the enzymes produced by bacteria are more stable [85]. *Bacillus* are interesting industrial organisms because of their rapid growth rate, which enables a short fermentation cycle, and their ability to secrete various enzymes into the external environment, including xylanase [72]. Carbon sources are essential in cellular metabolism and xylanase synthesis [17]. Xylan, generally considered the best inducer, can induce xylanase enzymes, while easily assimilated sugars such as glucose, lactose and xylose have a repressive effect on xylanase [30]. Xylan is not the direct inducer since it cannot penetrate directly into cells. The products resulted from its hydrolysis under the action of xylanases such as xylobiose and xylotriose, which can play the role of inducer [18]. Kapilan *et al.* [19] reported that higher xylanase production by *B. pumilus* was achieved with arabinose in corn cob media. While xylan induces the highest level of xylanase production by strains of *B. pumilus* [20], *B. pumilus* SV-205 [21], *Bacillus circulans* AB 16 [22], and *Jonesia denitrificans* [23]. Xylose increased xylanase production by *Bacillus megaterium* [24]. The fermentation time required to achieve optimal xylanolytic activity depends on the growth rate of the microorganism and its enzyme production pattern. It varies from one strain to another and from one fermentation medium to another [23]. A literature review revealed the highest enzyme production from *B. pumilus* SV-85S after 36 h [18], *B. pumilus* MK001 after 24 h [25], and *B. pumilus* SILB-X MTCC 8964 after 48 h during the late logarithmic phase [26].

During xylanase production, it became important to know what effect a specific nutritional variable had on hydrolytic enzyme activities when different factors were modified at the same time. Several statistical models have been developed to optimize fermentation conditions for xylanase production, including the Plackett-Burman and Box-Behnken designs. An optimal *inoculum* level is necessary to balance biomass and available nutrients to achieve maximum enzyme yield. Lower enzyme yield at a higher *inoculum* level may result from faster nutrient intake [28]. Also, a higher concentration of *inoculum* is not preferable in industrial fermentations [29]. Other researchers recorded observations for age and *inoculum* levels, and a maximum xylanase yield of the *B. pumilus* strain ML001 was obtained with 1.25% (v/v) of a culture aged 2h with OD_{600} nm = 0.2 [27]. Xylanase titer was highest when 2% (v/v) of a 24 h (18×10^7 CFU/ml) *B. pumilus SV-205 inoculum* was used for xylanase production [21].

The production of xylanase by various bacteria and fungi is highly pH-dependent. The acidic pH (4–6) generally favors fungal xylanases, while the higher pH favors bacterial xylanases [30]. Initial pH influences many enzyme systems and the transport of several enzyme species across the cell membrane [31]. Similar results to our result were obtained. An optimal pH of 7.5 was reported for xylanase production from *B. pumilus* B20 [5], with a pH of 7.0 *for B. pumilus* AB-1 [32]. Other results indicate that among a wide range of initial pH of the production medium tested, pH 8 was optimal for xylanase production by *B. pumilus* [33].

It is well known that microbial growth and the production of their metabolites are strongly influenced by the components of the medium and physical factors, such as agitation and aeration [34]. Our result shows maximum xylanase activity under stationary conditions (0 rpm). Other reports indicate that activity is higher under stirring conditions than under stationary conditions, an agitation speed of 140 rpm is required for *B. pumilus* RXAIII-5 [4].

The presence of xylan in the culture medium can induce the synthesis of xylanolytic enzymes. 1.0% (w/v) birch xylan was sufficient to achieve maximum xylanase activity by *Bacillus amyloliquefaciens* [35], *Anoxybacillus kamchatkensis* NASTPD1 [36]. A base medium containing 1.5% birch xylan is preferable for the *Bacillus tequilensis* ARMATI strain [37], *Bacillus megaterium* BM07 [38]. This implies that the carbon source can act as a limiting substrate, and little change in their concentration will affect the level of enzyme synthesis [39].

Triphasic partitioning involves precipitating biomolecules present in an aqueous solution by means of a mixture of a crude protein extract with a solid salt, essentially ammonium sulfate $(NH_4)_2SO_4$, and an organic solvent, typically tert-butanol. The organic solvent added in the

presence of the salt forces the protein out of solution and forms an interfacial precipitate layer between the lower aqueous layer and the upper organic layer [70]. In our study, this method failed to purify xylanase properly, but it has been used effectively in other research. it has been used to purify in a single step the exo-polygalacturonase of *Aspergillus sojae* ATCC 20235 [40], α-galactosidase of *Aspergillus oryzae* [41], while partially purified xylanase [42] and pectinase [43] of *Aspergillus niger,* laccase of *Ganoderma sp.* WR-1 [44]. Our xylanase was harvested and purified to absolute homogeneity by a chromatographic procedure including gel filtration chromatography and anion exchange chromatography. Other *Bacillus* xylanase has also been purified by anion exchange chromatography, such as *B. pumilus* VLK-1 xylanase [17], *B. pumilus* B20 [45], *B. pumilus* GESF1 [46], *Bacillus arseniciselenatis* DSM 15340 [47], *Bacillus subtilis* ASH [48]. Our enzyme had a molecular mass of 60 kDa, it is similar to those to those observed for *B. pumilus* [49], other xylanases of *B. pumilus* [50], *B. pumilus* B20 [45], *Bacillus circulans* [51], *Bacillus* sp. ASX42 [52] had molecular weights of 55.4 kDa, 85 kDa, 59 kDa, and 66 kDa.

Our research aims to produce an alkaline xylanase, an enzyme that performs well in alkaline conditions. This quality is crucial when selecting xylanases for bleaching, as industrial pulp processing typically occurs in an alkaline environment [81]. Early studies on bleaching primarily utilized xylanases derived from fungi. However, a notable limitation of these fungal xylanases is that they function optimally at acidic pH levels [82]. Xylanases produced by thermophilic bacterial systems offer distinct advantages. They can withstand higher temperatures and exhibit a broader range of optimal pH values, which makes these enzymes stable and highly suitable for various industrial applications [80]. As a result, *Bacillus* strains have garnered attention as attractive candidates for producing substantial quantities of extracellular xylanases that remain active under high temperatures and alkaline pH conditions [68]. Our xylanase enzyme demonstrated its most increased activity in alkaline pH environments, specifically at pH 9 and 10 and at a temperature of 60°C. These characteristics suggest that our xylanase could potentially play a valuable role in the bleaching process, given its ability to perform effectively under the conditions typically encountered in industrial pulp bleaching. The optimum pH observed for xylanase from *Bacillus sp.* RTS11 corresponds to those of *Bacillus sp.* AR-009 [53], *Anoxybacillus kamchatkensis* NASTPD13 [36] and *B. pumilus* 13_a [27], Its optimum temperature is equivalent to those obtained for xylanases produced by *Bacillus subtilis* [54], *B. tequilensis* BT21 [55], *B. subtilis* cho40 [56] and *B. licheniformis* [57]. Concerning metal ions, our result is similar to those reported by other research on the effect of Mg^{+2} [58], Co^{+2}, Cu^{+2} [59], Mn^{+2} [60], Mn^{+2}, Fe^{+2} [61], Cu^{+2}, Mn^{+2} [62], Zn^{+2}, Co^{+2}, Cu^{+2} [63], Na^{+} [64], K^{+} [65]. Inhibition by Hg^{+2} ions is a common feature of xylanases due to cysteine thiol groups near or in the enzyme's active site [21]. The addition of surfactants slightly increased xylanase activity, and a similar effect was observed for xylanase from *Streptomyces sp.* [66].

In paper industries, contamination of xylanase preparations with cellulase is a problem in the bleaching process. Treatment with xylanases facilitates the extraction of lignin from the pulp. The positive effect of xylanase is generally attributed to the degradation of Xylan, breaking the bond between cellulose and lignin. Once the lignin is detached from the cellulose, it is easily reduced in subsequent bleaching steps. Still, the xylanases used must be free of cellulase activity to avoid damage to the cellulosic pulp [27,76,77]. *Bacillus sp.* RTS11 xylanase has low cellulase activity (1.87 U). According to Subramaniyan and Prema [78]. Negligible amounts of cellulases do not prevent xylanases from producing good quality pulps with minimal cellulose damage, and this activity can be attributed to the presence of cellulose-binding domains in xylanases (CBD). Different species of *Bacillus* are recognized for their ability to secrete xylanases devoid of cellulase activity such as *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus polymyxa*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus stearothermophilus*, *Bacillus aerophilus* [79]. Mushrooms are particularly interesting producers of xylanases; they produce considerable levels of xylanases, but generally, this production is accompanied by high cellulase activity, which deteriorates the quality of the fibers [80].

The application of xylanases for the improvement of pulp bleaching has been reported by several researchers using *B. pumilus* ASH xylanase with a xylanase dose of 5 IU/g dried paste, pH 7, 60C°, and 180 min of Treatment [67], *B. pumilus* SV-85S xylanase with an enzymatic amount of 10 IU/g of oven-dried paste, pH 9.0 and 120 min incubation at 55°C [68]. Several other reports also support xylanase pretreatment in pulp biotechnology. They have exploited various bacterial and fungal xylanases, among them *Bacillus cereus* xylanase [69], *Bacillus licheniformis* Alk-1 [59], *B. pumilus* SV-205 [71]. The release of phenolic and hydrophobic compounds and reducing sugars suggest dissociating the lignin-carbohydrate complex (LCC) from pulp fibers by enzymatic action [60]. Kamble and Jadhav [47] reported that chromophore release is well correlated with total sugar release, which can be considered a simple method to determine the effectiveness of enzyme therapy.

Recycling lignocellulosic waste receives a lot of attention because it is inexpensive. Its richness in hemicelluloses makes it the primary substrate for the production of valuable biomolecules, including chemicals (polyphenol, organic acids) and organic solvents (acetone, butanol), as well as the enzymatic degradation of these wastes via cellulases and hemicellulases is essential for their conversion into fuel [73, 74]. As well as industrial production of xylanases recognizing obstacles and limitations due to the high costs of using pure Xylan, agricultural waste could be an inexpensive alternative substrate for xylanase production because of its hemicellulose richness [75].

5. Conclusions

In this study, we successfully identified and harnessed xylanase activity from *Bacillus sp*. RTS11. Through the application of statistical methodologies such as Plackett-Burman and Box-Behnken, we not only optimized but significantly increased the enzyme's activity, achieving an impressive titer of 448.89 U. This enhancement was achieved while maintaining the enzyme's efficacy under non-optimized conditions, highlighting its robust performance. Moreover, we pursued the purification of this enzyme, resulting in a purification factor of 8.28 and an impressive yield of 59.67%. Characterization of the en-

zyme revealed a molecular weight of approximately 60 kDa, underscoring its suitability for various industrial applications. Furthermore, this xylanase demonstrated its versatility by displaying catalytic activity towards various agricultural waste substrates, showcasing its potential for sustainable waste utilization. Our findings suggest that this enzyme can play a pivotal role in the paper biobleaching process, contributing to more eco-friendly and efficient practices. In conclusion, our study not only identified and optimized xylanase activity from *Bacillus sp.* RTS11, but also highlighted its purification, characterization, and diverse catalytic capabilities. These results emphasize the enzyme's potential in various biotechnological and industrial applications, further advancing the field of enzymology and sustainable practices.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution

SS, BY: writing original draft manuscript; SS, BY, AM, HLH, AB: methodology; SS, BY, HLH: analysis and investigation; SS, AM reviewed the statistical optimization section; MA, YB, KH: review and editing; BY: supervision and funding acquisition. All authors read and approved the final manuscript.

Funding

This research was funded by the Algerian Ministry of Higher Education and Scientific Research (MESRS)'s Directorate-General of Scientific Research and Technological Development (DGRSDT). The authors are thankful to the Researchers Supporting Project (RSP2023R113), King Saud University, Riyadh, Saudi Arabia.

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