Egypt. J. Chem. **Vol. 67,** No. 6 pp. 373 - 385 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Characterization and Purification of Xylanase Derived from Marine *Bacillus subtilis* AKM1 and its potential applications

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Abstract

The synthesis, enhancement, and impact of the marine bacterial enzyme xylanase on agricultural waste was studied in order to prepare it for use in biotechnological applications. *Bacillus subtilis* AKM1 bacterial strain was obtained from the local coastal Red Sea in Hurghada, has accession number (MK377251) was identified using 16S rRNA as a perfect strain produced the enzyme. The optimal conditions for the production of xylanase were determined as follows: 48 hrs. incubation, pH 7.0, 50 rpm, and 40°C. The maximum enzyme production was obtained with wheat bran and KNO_3 as carbon and nitrogen sources. After purification using gel filtration and ion exchange chromatography methods, xylanase exhibited its highest level of activity and stability at pH 7.0 and 50 °C. The residual activity enhanced by the presence of metal ions, specifically Mn^{2+}, Ca^{2+}, and Fe^{2+}, proportionally with their concentrations increase. Conversely, the activity was entirely suppressed at higher concentrations of Ag^{+}, SDS, and EDTA (2-10 mM). The molecular weight was 34 KDa, and the kinetic studies using a Lineweaver-Burk plot, with *Km and Vmax* 0.044 mM, and 22.22 U/ml respectively. Xylooligosaccharides generated through enzymatic hydrolysis utilizing pure xylanase demonstrated antibacterial efficacy against all tested organisms except *E. coli*, antioxidant activity using DPPH with Ic50 of 121 µg/ml, and shown anti-cancer efficacy versus breast cancer cells and colon of cancer cells as well.

Keywords: Characterization; Xylanase; *Bacillus Subtilis*; Xylooligosaccharide; biotechnological applications

1. Introduction

Xylan is considered to be the second most prevalent polysaccharide, constitutes approximately a third of the Earth's renewable organic carbon [1], and is hemicellulose's primary component [2]. The compound possesses a xylose's backbone and exhibits characteristics of a polysaccharide with intricate composition, namely a residue composed of a 5-carbon sugar. These residues are connected through β-1,4-glycosidic linkages. The structure of xylan, which is the primary component of hemicellulose, varies among plant species due to the substitution of the homopolymeric chain consisting of 1,4-linked D-xylopyranosyl units [3], [4], due to the diverse composition and intricate chemical structure of plant xylan. The process of xylan degradation involves the hydrolysis of xylan by a complex of xylanolytic enzymes, which includes β-1,4-endoxylanase. These enzymes work together to fully break down xylan into its sugar components [5], [6].

In the realm of enzymology, xylanase stands out as a highly significant enzyme, finding extensive utilization in both industrial and biotechnological sectors for hydrolyzing xylan [7]. Although wide variety of organisms produce xylanases, microbial sources, specifically notably bacteria, fungi and yeast are undertaken as a majority of microbial resources for use in commercial applications [8]. The marine ecology, particularly mangrove forests, is widely recognized as a significant supplier of xylan [9] and seaweeds (macroalgae) [10]–[12], therefore it was an excellent source for xylanases. There are significant differences observed in the enzymes generated by marine microorganisms compared to those derived from terrestrial sources, because of the elevated levels of salt concentration, increased pressure, reduced temperature, and highly variable brightness of the marine environment [13], so marine enzymes were therefore favored from the terrestrial one.

The features of xylanase enzymes exhibit variability based on their respective sources, hence rendering them valuable for diverse applications. Several Bacilli, namely *Bacillus amyloliquefaciens*, *Bacillus circulans*, *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus halodurans*, and *Bacillus pumilus*, and, have been identified as potential candidates for the production of xylanases [14]–[17], where others determined that these sources exhibit noteworthy xylanolytic activity.

Xylanases are widely recognized for their environmentally friendly properties in industrial applications, particularly in the processing of xylan. One notable application is their application in the medical field, whereby they are essential in the production of xylooligosaccharides (XOS). XOS is an oligomer that is generated through the catalytic action of xylanase on xylan [18], the efficiency of bleaching in the paper and pulp sector, while minimizing using the chlorine as a harsh chemical [19]–[21], food industry [22] and generation of biofuel [23]. Therefore, the objective of this study is to refine and clarify the enzymatic properties of exogenous xylanase derived from a hitherto undiscovered marine bacterium, as well as investigate its potential uses in the field of biotechnology.

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Receive Date: 19 November 2023, Revise Date: 10 December 2023, Accept Date: 20 December 2023
DOI: 10.21608/EJCHEM.2023.249877.8895

2.Experimental

All chemicals utilized in this study were procured from the Sigma Chemical firm.

2.1 Isolation of bacteria

Various types of marine debris were used for isolation of bacteria from the Red Sea (Sharm el-Sheikh 27°54'56.95" N 34°19'47.82" E – Hurghada 27°15'26.57" N 33°48'46.48" E) and the Mediterranean Sea (Alexandria 31°11'52.80" N 29°55'9.12" E – Marsa-Matrouh 31°19'60.00" N 27°12'60.00" E). Sediments were collected at a depth of 5 cm from the shoreline with the serial dilution technique developed by Hayakawa and Nonomura [24]. Three distinct forms of media utilized for isolation [25]–[27] .

2.2. Method for rapid assay plates

A qualitative screening process was used to determine the bacterial isolates' capacity to produce the enzyme xylanase via a culture composed of beech wood xylan, 10 g; yeast extract, 2.0 g; peptone,5.0 g; $MgSO_4$, 0.5 g; NaCl, 0.5 g; $CaCl_2$, 0.15 g; agar, 20 g; distilled water, 500 ml, and seawater, 500 ml; pH 7.0. The medium was subjected to incubation at a temperature of 35ºC for 48 hours. Subsequently, the plates were subjected to staining using Gram's Iodine, which consisted of a solution containing 0.25% w/v of Iodine (I_2) and Potassium Iodide (KI). The dye was allowed to remain on the plates for 5 minutes, after which it was washed off using distilled water. As a result of xylan degradation, a yellow-opaque zone became visible. This phenomenon has been previously seen and documented by Cordeiro et al. [28].

2.3 Xylanase Assay

The measurement of xylanase activity is conducted by the utilization of the di-nitrosalicylic acid (DNS) method [29], using the crude enzyme solution from the liquid culture of isolates including: wheat bran weighing 20.0 g; peptone weighing 5.0 g; KNO_3 weighing 5.0 g; yeast extract weighing 5.0 g; KH_2PO_4 weighing 1.0 g; $MgSO_4$ weighing 1.0 g; distilled water (500 ml), and seawater (500 ml); pH, 7.0 and temperature 35 ºC for 48 hrs. [30], [31]. The quantification of the released reducing sugars was performed by utilizing a standard curve for D-xylose. The unit of measurement for the level of activity was formally established and delineated as the quantity of enzyme necessary to liberate 1 µmol of reducing sugar per minute under the specified parameters.

2.4 Identification of Extracellular Protein Level

The amount of protein in the filtrate obtained from the produced crude was determined using the Bradford reagent [32] with bovine serum albumin serving as a standard protein.

2.5 The identification of the isolate exhibiting promising characteristics

2.5.1 Biochemical identification

The strain of bacteria with the greatest xylanase induction was identified using morphological, physiological, and biochemical tests as outlined in Bergey's manual of systematic bacteriology [33]. These tests included assessing spore production, motility, gram staining, catalase activity, nitrate reduction, indole production, Simmons citrate utilization, Voges-Proskauer reaction, NaCl tolerance, and starch hydrolysis.

2.5.2 Molecular identification

A bacterium that is being referred to the most significant induction of xylanase was determined by the use of 16S rRNA sequencing. The process of extracting chromosomal DNA was conducted, followed by the amplification of the 16S rRNA gene using universal primers F (5′-GTG CCAGCAGCCGCGGTA-3′) and R (5′-TTGTAGCAC GTGTGTAGCCC-3′) [34] (Manfred Kroger, Institute of Microbiology and molecular biology, at the University of Gießenbei). To exclude any impurities, the PCR product obtained from the 16S rRNA underwent a purification process. Following this, the purified product underwent sequencing and subsequently underwent a comparison with pre-existing 16S rRNA sequences of bacterial species that are accessible in the NCBI databases. This comparison was carried out using the BLAST program [35] (http://www.ncbi.nlm.nih.gov). The sequences that exhibited the greatest resemblance to the 16S rRNA sequences of the bacterial isolate were chosen, aligned, and subsequently applied in the creation of a phylogenetic tree. The study presented the phylogenetic tree of the bacterial species sequence, which was subsequently submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases.

2.6. Optimization of the medium for the synthesis of xylanase

To achieve optimal generation of xylanase by a bacterial strain, along with an examination of the components of the growth medium, they were subjected to optimization through the investigation of the impact of varying pH values (4, 5, 6, 7, 8, 9, and 10 adjusted with 0.1 M HCl or 0.1 M NaOH), temperatures (25, 30, 35, 40 and 45 ℃), incubation periods (24, 48, 72, 96 and 120 hrs.), and agitation speeds (0, 50, 100, 150, 200 rpm) [36] .

Various nitrogen sources, namely sodium nitrate, potassium nitrate, and ammonium sulphate, varied amounts of these nitrogen sources (2, 4, 5, 6 and 8 g/l), and varied amount of wheat bran (0, 5, 10, 15, 20, 25 and 30 g/l) were also used for optimization process.

2.7 Purification of Xylanase

Protein precipitation was achieved by employing ammonium sulfate saturation ranging from 0% to 80% as per the methodology outlined by Dixon [37]. The fraction exhibiting the greatest level of activity of xylanase was subjected to centrifugation using a SIGMA 3-18 KS cooling centrifuge (manufactured in Germany) at a speed of 10,000 rpm for 30 minutes. The resulting supernatant was then dissolved in a small quantity of 50 mM phosphate buffer with a pH of 7.0. Subsequently, the solution was dialyzed overnight at a temperature of 4 ℃ opposed to the same buffer. Subsequently, the dialyzate was introduced onto a (1.5 x 60 cm) Sephadex G-100 column prepared and equilibrated using a 50 mM phosphate buffer at pH 7. Elution was carried out using one liter of the same buffer at a flow rate of 0.5 ml/min. During elution, fractions of 5 ml were collected to quantify absorbance at a wavelength of 28, and assessing enzyme activity [31]. The fractions that demonstrated activity were combined and underwent dialysis against the same buffer solution, followed by their application onto a DEAE-cellulose column. The absorption

at a specific wavelength: of 280 nm was determined for the fractions that got eluted, via the PC spectrophotometer UV/VIS-2401, manufactured by Shimadzu in Kyoto, Japan. Subsequently, the most active fractions were assessed for xylanase action, as outlined by Fatokun [31], and protein content following the method outlined by Bradford [32].

2.8. The Characterization and features of the pure Xylanase

2.8.1 Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The pure protein produced using DEAE-cellulose was analyzed using SDS-PAGE in order to determine its molecular weight [38].

2.8.2 The relation between Xylanase activity and stability with the temperature

The ideal thermal state for the xylanase that has undergone purification was found by subjecting mixture of the reaction to several temperatures, spanning within the temperature range of 30 to 90 degrees Celsius, in a 50 mM phosphate buffer solution [39]. The thermal stability degree of the isolated enzyme was assessed by subjecting solution-containing enzymes to pre-incubation within a time frame ranging from 10 to 60 minutes. at different temperatures (ranging from 30 ℃ - 90 ℃) without the presence of substrate. Then, the aliquots were extracted and allowed to cool [9]. The residual activity had been purified and quantified using a standardized method described before, and the non-incubated enzyme was referred to as a control (100 %).

2.8.3 The impact of pH on the activity and stability of Xylanase

The pH optimization of purified xylanase was done by utilizing three distinct buffers employing varying pH values including a 50 mM quantity of citrate phosphate buffer within the pH (4.0-5.0), phosphate buffer with pH range typically between 6.0-7.0 [40], and Tris-HCl pH was adjusted to be within 8.0 and 10.0 [41]. Subsequently, the xylanase activity was assessed during a 1-hour incubation period at 50 ℃. To assess the xylanase's solution pH stability, it was subjected to pre-incubation at various pH levels (4.0 to 10.0) for a 1 hour at 4 ℃. Subsequently, the pH values were recalibrated to a neutral pH of 7.0. Following this, residual enzyme activity was assessed using the previously established standard methodology. The enzyme that was not subjected to the incubation period was used as the control variable (100%).

2.8.4 The impact of metal ions and inhibitors on xylanase activity

The impact of metal ions Mn^{2+} ($MnCl_2$), Ca^{2+} ($CaCl_2$), Fe^{2+} ($FeSO_4$), Ag^{+} ($AgNO_3$), Cu^{2+} ($CuSO_4$), EDTA and SDS at various doses (2, 4, 6, 8, and 10 mM) on the xylanase's activity was assessed by subjecting the enzyme solution to incubation with each chemical for 20 min prior to the substrate addition. Following a period of pre-incubation, the enzymatic activity was assessed using the optimal assay conditions [31] and the non-incubated enzyme was referred to as a control (100 %).

2.8.5 Concentrations of substrate

Various quantities of xylan used in this study were 0.05 mM, 0.1 mM, 0.15 mM, 0.216 mM, 0.25 mM, and 0.3 mM which were employed in order to ascertain the apparent Michaelis-Menten constant (*Km*) value of isolated and purified enzyme. This was accomplished by conducting experiments that examined the relationship between substrate concentrations and reaction velocities. The values of apparent *Km* and *Vmax* were obtained through Lineweaver-Burke plots [42].

2.9 Applications

2.9.1 Xylooligosaccharides (XOS) production

Xylan was derived through the process of wheat bran's alkali extraction process. The material that had undergone delignification was extracted using a 15% NaOH solution (1 M) at a 1:20 (w/v) solid-to-liquid ratio for a duration of 90 minutes at a temperature of 90 ℃. Following this, the extracted material was precipitated by adding three ethanol volumes and subsequently separated through filtration using filter paper. The precipitate "solid substance" was solubilized using distilled water, subjected to dialysis, and then freeze-dried "lyophilized". The xylan-containing hemicelluloses that had been purified were dissolved in a sodium acetate buffer with a concentration of 50 mM and a pH of 4. This was done in 50-ml conical flasks, and the concentration of the solution was adapted to 2% (w/v). The addition of purified xylanase was performed. The hydrolysis process was conducted at 55 ℃, with agitation at a speed of 100 rpm for 24 hours.

"Following the necessary incubation period, liquids containing XOS were extracted from the incubation mixture and subjected to centrifugation at a speed of 6,000 for 5 minutes. The process of XOS synthesis was repeated multiple times in order to get adequate quantity of liquor containing XOS. Following the addition of three volumes of ethanol, the hemicelluloses that have not undergone hydrolysis underwent precipitation, whereas the xylooligosaccharides (XOS) remained in the filtrate. The ethanol present in the filtrate was eliminated using the process of rotational evaporation which was performed under reduced pressure conditions at a temperature of 45 ℃. Subsequently, the remaining solid portion was subjected to freeze-drying [43].

2.9.2 Quantification of XOS through the phenol-H_2SO_4 assay

The quantification of XOS was conducted by employing the phenol- H_2SO_4 method to determine the total carbohydrate content [44]. To one milliliter of the sample, phenol was added in 1 ml (5% w/v) then 5 ml of concentrated H_2SO_4. Sample tubes were stored in ice with adding H_2SO_4. The combine ingredients subjected to incubation at ambient temperature for a duration of 20 minutes, after which the absorbance was conducted at a certain wavelength: 490 nm via a UV/VIS-2401 PC spectrophotometer by (Shimadzu, Kyoto, Japan). A standard range of glucose concentrations ranging from 0 to 100 µg was employed, with a stock solution of 1 mg/ml. A standard graph was constructed by plotting the absorbance at a wavelength of 490 nm versus the level of glucose concentration. Also, a blank sample was created using the same procedure.

2.9.3 Activity of XOS as a radical scavenger of the DPPH radical

The estimation of XOS's ability to neutralize free radicals was conducted using the approach outlined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) [45]. A volume of 5 ml of DPPH dissolved in ethanol was introduced into a solution containing 1 ml of pure XOS at concentrations ranging from

50 to 300 mg/ml. The combined ingredients were subjected to incubation in a dark environment for 30 minutes. Subsequently, the measurement was performed at a wavelength of 517 nm by a UV-Visible spectrophotometer model 2401PC (Shimadzu, Japan).

The absorbance level of DPPH decreases in proportion to the strong scavenging free radical's capacity. The scavenging capability of the free radical was determined by utilizing the subsequent formula:

$$\text{Scavenging ability } (\%) = \frac{A_{517 \text{ of control}} - A_{517 \text{ of sample}}}{A_{517 \text{ of control}}} \times 100$$

The IC50 value is described as the concentration (expressed in mg) of XOS that results in a 50% reduction in DPPH absorbance.

2.9.4 Antimicrobial activity

The antimicrobial efficacy of xylooligosaccharides, derived from the utilization of xylanase obtained from *Bacillus subtilis* AKM1, was evaluated against a diverse range among microbes, encompassing Gram-positive bacteria (*Staphylococcus aureus* NRRL B-767 and *Bacillus thuringiensis*), Gram-negative bacteria (*Escherichia coli* NRR-B 210 and *Pseudomonas aeruginosa* NRRL B-23), yeast (*Candida albicans* NRRL Y-477 and *Saccharomyces cerevisiae* Y-2034), and fungi (*Fusarium oxysporum* NRRL 26406 and *Aspergillus niger* NRRL-3). The inhibitory zones induced by varying the xylooligosaccharide concentrations (50 and 100 lg/disc). Rimactane, administered at a specific concentration of 200 lg/disc, was utilized as an antibacterial agent, while flucoral, also at a specific concentration of 200 lg/disc, served as an antifungal control [46]- [47].

2.9.5 The assessment of cytotoxic effects on several cell lines

The cytotoxic effect of the generated XOS was evaluated on two distinct cell cultures, namely MCF-7 cells (the human breast cancer cell line), in addition to HCT-116 cells (the colon carcinoma cell line) in the study obtained from the VACSERA Tissue Culture Unit [48]- [49].

3. Results

3.1 Quantitative and qualitative screening to identify the synthesis of xylanase

The ability of fifty-seven marine bacterial strains to synthesize the xylanase enzyme which isolated from different locations. A total of 13 isolates exhibited a distinct zone following the use of the gram's iodine degradation technique, indicating their capacity to manufacture extracellular xylanase. The three most effective isolates, which exhibited the highest level of activity, were identified quantitatively by using the DNS approach outlined in the procedure section. The measurement of the enzyme's activity was conducted for each isolate, and was discovered the strain identified as H14 which was obtained from Red Sea (Hurghada) exhibited the highest efficacy in producing extracellular xylanase with a recorded activity level of 2.06±0.001 U/ml after 48 hrs. of incubation, pH7, 50 rpm and 37℃ temperature.

3.2 Identification of the isolate having promising characteristics

The morphological, physiological, and biochemical examinations were conducted on the isolate H14, revealing motility and spore production when observed when using the microscopy, as illustrated in Table 1.

Table 1. Characteristics of *Bacillus subtilis* sp.

Characteristics	*Bacillus subtilis*
Motility	+
Spore formation	+
Gram stain reaction	+
Catalase	+
Nitrate reduction	+
Indole	-
Simmons citrate	+
Voges-Proskauer	+
7.5% NaCl	+
Starch hydrolysis	+

The sequence of nucleotides derived from molecular identification of 16s rRNA was subjected to comparative analysis with pre-existing sequences available in the databases. (Huddleston et al., 1997). The 16S rRNA sequencing of the isolate exhibited a high degree of similarity (99%) with *Bacillus subtilis* sp. The findings were consistent with the results obtained from the morphological, physiological, and biochemical analysis, as described in Bergy's manual of systematic bacteriology (Bergey & Holt, 1994). Based on the preceding outcomes, the aforementioned strain has been designated as *Bacillus subtilis* AKM1 and has been assigned (MK377251) as an accession number. Figure 1 displays the outcome of the 16S rRNA analysis as illustrated in a phylogenetic tree.

Fig. 1 A Phylogenetic tree of *Bacillus subtilis AKM1*

3.3 The impact of physical and cultural factors on xylanase's production via *Bacillus subtilis* AKM1

3.3.1 Incubation period

The highest level of xylanase induction was found after incubation for 48-hours, resulting in an activity of 2.03±0.346 U/ml. Subsequently, there was a progressive drop in the synthesis of xylanase with increasing incubation length, as depicted in Figure 2a.

3.3.2 Initial pH

Effects of pH on the microbial induction of xylanase were investigated in this work, with a focus on the pH range of 4 to 10. Various buffers were employed to analyse this relationship. The optimum pH for xylanase synthesis was determined to be 7, resulting in a maximum yield of (2.08±0.130 U/ml) activity then a lower activity (1.62±0.073

U/ml) was noted at a pH level of 8 which was afterward followed by an activity measurement (1.28U±0.165 U/ml) at pH 6 and no measurable activity observed below a pH of 5 and above a pH of 9, as shown in Figure 2b.

3.3.3 Agitation speed

The largest peak of induction for xylanase occurs at 50 rpm, with an activity level degree (2.00±0.170 U/ml) as illustrated in Figure 2c.

3.3.4 Incubation temperature

The experiment focused on the impact of temperature on xylanase production. The highest yield of xylanase, with an activity of (2.23±0.234 U/ml), was seen at a temperature of 40 ℃. Subsequently, a temperature of 35 °C resulted in an activity of (2.15±0.443 U/ml), as illustrated in figure 2d.

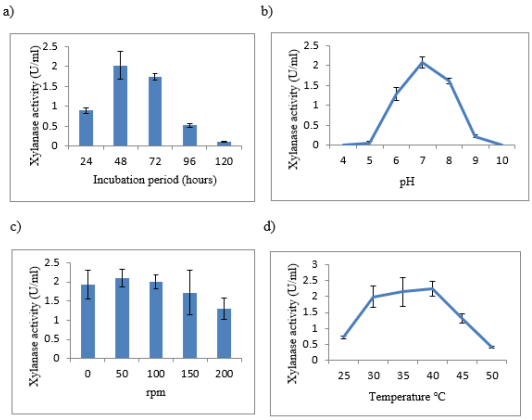

Fig. 2 Impact of physical and cultural factors on xylanase production from marine *B. subtilis* AKM1 a) Incubation period, b) pH, c) Agitation speed (rpm), and d) Incubation temperature.

3.3.5 Effect of Nitrogen Sources

In the context of inorganic nitrogen sources, an investigation was conducted to assess the impact of three distinct nitrogen sources on the production of xylanase $(NH_4)_2SO_4$, $NaNO_3$, and KNO_3. The results of this investigation demonstrate that KNO_3 has the highest activity for xylanase induction, with a recorded value of 2.12±0.259 U/ml. Following KNO_3, $NaNO_3$ shows an activity of 1.84±0.471 U/ml, as shown in Figure 3.

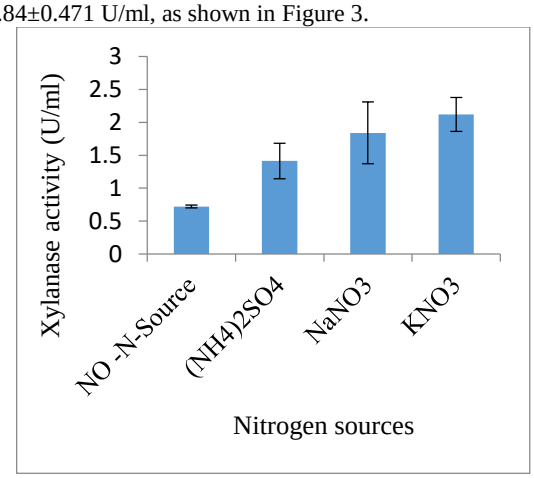

Fig. 3 The impact of different nitrogen sources on xylanase production by marine *Bacillus subtilis* AKM1

3.3.6 Effects of the Various Concentrations of KNO_3

The results of this experiment indicate that KNO_3 is the most effective nitrogen source for achieving the highest xylanase activity, with a recorded value of (2.12±0.259 U/ml). Regarding the impact of varying concentrations, it was observed that the production of xylanase reached its peak at a specific concentration: 5 g/l, exhibiting an activity level: (2.30±0.226 U/ml). This was subsequently an activity level of (2.09±0.096 U/ml) at a specific concentration: 6 g/l. However, beyond and below these concentrations, the enzyme activity decreased, as depicted in Figure 4

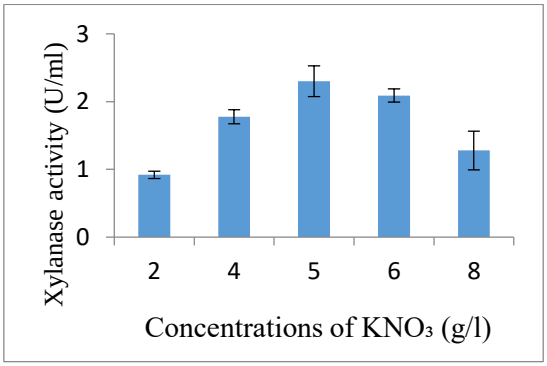

Fig. 4 The impact of different KNO_3 concentrations on the production of xylanase by *Bacillus subtilis AKM1*

3.3.7 Different concentrations of wheat bran

The highest level of xylanase induction was seen when the concentration of wheat bran was 10 g/l. Subsequently, the enzyme activity exhibited a progressive decline both above and below this concentration, as depicted in Figure 5.

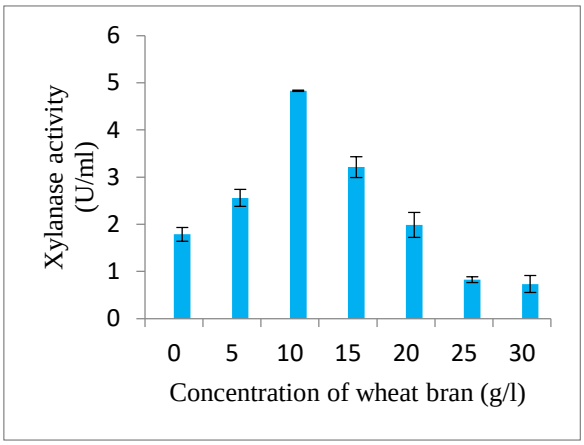

Fig. 5 The impact of different concentrations of wheat bran on xylanase production by *Bacillus subtilis* AKM1

3.4 Xylanase purification

The outcomes of the purification process are succinctly presented in Table 2. The xylanase enzyme, following partial purification using 40% ammonium sulfate fractionation, exhibits a significantly high specific activity of 32.75 U/mg protein, and the purification fold of 9.27 was observed when comparing the purified xylanase with the crude form. Subsequently, the purified xylanase was subjected to a second purification step using Sephadex G-100. The active fractions obtained from this step were then put onto a DEAE-Cellulose column where the specific activity was 73.48 U/mg protein and purification fold 20.81 with yield 5.56%.

3.5 Characterization and properties of the purified Xylanase

3.5.1 Molecular weight determination by SDS-PAGE

Xylanase's molecular weight was 34 KDa showing a single band as seen in Figure 6.

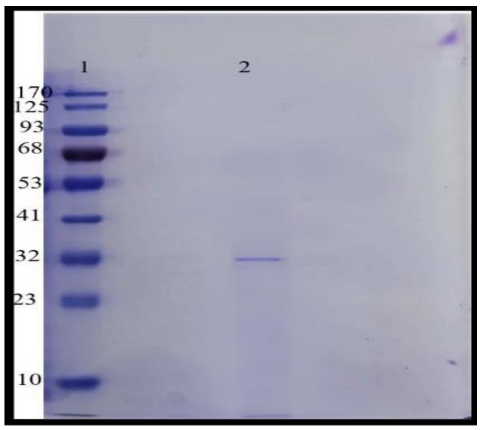

Fig. 6 SDS-PAGE of the xylanase enzyme produced by the marine Bacillus subtilis AKM1. From left to right: Lane 1, standard molecular weight markers and Lane 2, final purified xylanase obtained by DEAE-cellulose.

3.5.2 Influence of temperature on xylanase activity and stability

The experiment observed an ideal condition temperature of 50 ℃, at which the activity of the system achieved its highest value of (13.37±0.256 U/ml). As the temperature went beyond this optimum, the activity steadily declined until it reached 70 ℃. At higher temperatures, the activity was found to be weak, as depicted in Figure 7.

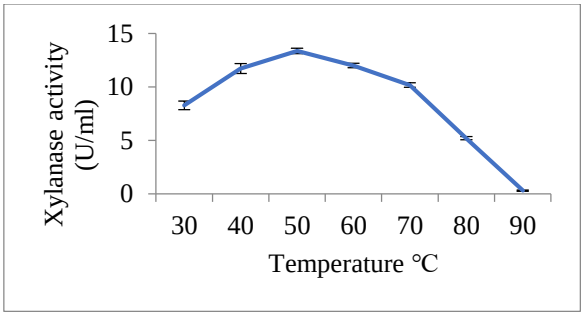

Fig. 7 Impact of temperature on purified xylanase activity

The evaluation of temperature stability revealed that the xylanase enzyme exhibited constancy throughout the range of 30 to 50 ℃ under different time restrictions. A decline in residual activity is observed throughout time and temperature within the range of 60-90 ℃. Table 3 illustrates the total suppression of xylanase activity after 30 minutes at a temperature of 80 ℃, and after 10 minutes at a temperature of 90 ℃.

Table 2. Summary of marine *Bacillus subtilis AKM1* xylanase purification

Purifica-tion steps	Total xylanase activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	8407.80±0.03	2377.80±0.41	3.53	1	100
Ammonium- sulfate 40%	197.53±0.13	6.03±0.0.22	32.75	9.27	2.34
Sephadex G-100	557.20±0.23	10.99±0.004	50.70	14.36	6.62
DEAE-Cellulose	468.13±0.003	6.37±0.02	73.48	20.81	5.56

Values represent means of triplicate determinations ± standard deviations.

Table 3. Impact of temperature on the stability of purified xylanase from marine *Bacillus subtilis AKM1*.

Time (min)	30-50℃	60 ℃	70 ℃	80 ℃	90 ℃
10	100	89.70±1.68	76.07±0.324	38.96±0.141	2.33±0.847
20	100	82.23±2.15	63.40±0.849	13.21±0.156	0
30	100	75.71±2.46	50.62±0.141	7.80±0.156	0
40	100	70.23±0.317	23.73±0.043	0	0
50	100	63.10±0.417	10.21±0.148	0	0
60	100	60.22±1.71	0	0	0

Values of residual xylanase activities (%) represent means of triplicate determinations ± standard deviations. 100% (control 13.37 U/ml).

3.5.3 The Influence of pH on xylanase Efficiency and Stability

The highest recorded xylanase action (13.37±0.186 U/ml) was noted when the pH was7.0, as depicted in Figure 8. The activity exhibited a small drop at pH levels of 6 and 8, while a significant decline in activity was observed outside the range of pH: 6.0-8.0.

The highest residual efficiency of the extracted xylanase was observed at pH 7, as determined through an evaluation of pH stability. Within the pH range of 6.0-8.0, the enzyme exhibited a retention of over 70% of its activity. The observed decrease in residual efficiency was noted at pH levels of 6.0 and 8.0, as shown in Figure 9.

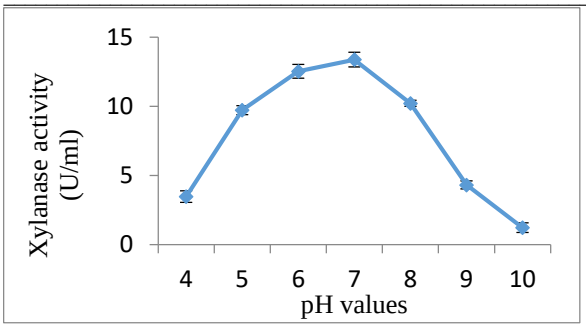

Fig. 8 Effect of pH on the purified xylanase activity from marine *Bacillus subtilis AKM1*.

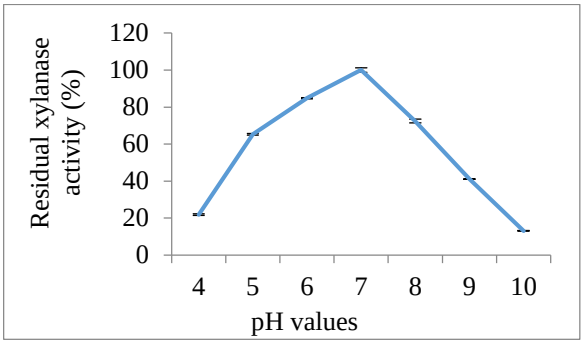

Fig. 9 pH stability of purified xylanase from marine *Bacillus subtilis* AKM1

3.5.4 The impact of metal ions on the activity of xylanase

Metal ions are frequently required by enzymes as activators to enhance their catalytic activity. In light of the results obtained from this investigation, it was seen that the activity of xylanase residual exhibited a raise when concentrations were raised within the range of (2 – 10 mM) for Mn^{2+}, Ca^{2+}, and Fe^{2+}.

Conversely, a progressive drop-in activity was noticed for Cu^{2+}. The activity of Ag^{+} was shown to be rather low at doses of 2 and 4 mM, and after that totally suppressed at higher concentrations. The quantities of EDTA and SDS were increased from 2 to 8 mM, resulting in a decrease in residual xylanase activity. Complete inhibition of the xylanase activity was observed at a dosage of 10 mM, as indicated in Table 4.

Table 4. Impact of metal ions on marine *Bacillus subtilis* AKM1's purified xylanase.

Concentration (mM)	Residual xylanase activity (%)						
	Mn^{2+}	Ca^{2+}	Fe^{2+}	Cu^{2+}	Ag^{+}	EDTA	SDS
2	119.24± 0.932	112.17± 0.136	104.21± 0.297	73.12± 0.141	31.21± 1.511	64.24± 0.241	54.22± 0.141
4	126.41± 0.841	117.07± 0.099	108.71± 0.276	64.71± 0.085	9.02± 0.424	51.31± 0.990	37.89± 0.156
6	132.11± 1.649	120.13± 0.183	111.02± 0.248	53.21± 0.459	0	22.17± 0.240	13.17± 0.886
8	141.78± 2.531	122.14± 0.099	114.11± 1.273	48.08± 0.026	0	9.43± 0.424	3.21± 0.297
10	148.21± 0.219	124.11± 0.283	119.92± 0.112	34.50± 0.707	0	0	0

Values of residual xylanase activities (%) represent means of triplicate determinations ± standard deviations. 100% (control 13.37 U/ml)

3.5.5 Effect of xylan concentration on xylanase activity

The activity of xylanase exhibited a rising pattern as the concentration of xylan was augmented, reaching its peak at 0.16 M with a value of 15.84 U/ml.

Subsequently, the increase in activity became low, indicating a state of near stability.

The Michaelis constant (*Km*) was determined to have a specific concentration: 0.044 mM, whereas the maximal velocity *Vmax* was found to be 22.22 U/ml.

3.6 Applications

Xylooligosaccharides (XOS) are generated through the utilization of a purified xylanase enzyme, which finds diverse applications.

3.6.1 Evaluation of the DPPH scavenging process

The DPPH assay was employed to evaluate the ability of xylo-capacity oligosaccharides to effectively neutralize free radicals.

The process of scavenging demonstrates an increase as the concentration of xylooligosaccharides is elevated, reaching its peak at 84.92% when the concentration is set at 250.0 µg/ml. Subsequently, the IC50 value is determined to be 121 µg/ml, as shown in Figure 10.

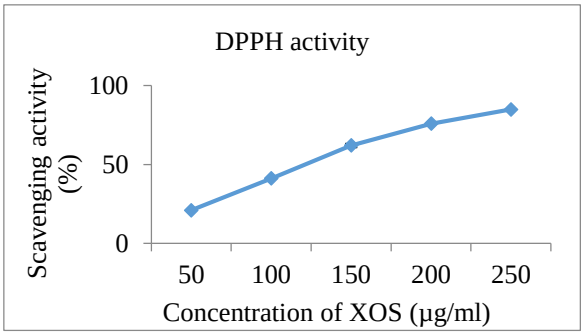

Fig. 10 Effects of marine *Bacillus subtilis AKM1* xylooligosaccharides on scavenging free radicals.

3.6.2 Antimicrobial activities

The antibacterial capabilities of xylooligosaccharides were observed in all examined microorganisms, except for *E. coli*, as indicated in Table 5.

The rise in the concentration of xylooligosaccharides correlated with the observed antimicrobial effects.

The zone of inhibition exhibited an increase in size, expanding from 5 mm to greater than 15 mm.

The antimicrobial efficacy was assessed by the measurement of the clear zone's diameter that corresponded to growth suppression.

3.6.3 In vitro antitumor effectiveness opposing colon and breast cancer cells

The colon cancer cell line HCT-116 and the breast cancer cell line MCF-7 were subjected to treatment with varying doses of XOS, specifically 12.5, 25, 50, and 100 g/ml. Both the MCF-7 and HCT-116 cancer cell lines demonstrate a progressive decrease in the viability of tumour cells as the concentration of XOS increases. Specifically, at a specific concentration level: 12.5 µg/ml of XOS, the tumour cell viability is recorded at 92%, which subsequently decreases to 71% at a specific concentration: 100 µg/ml. The

IC50 value for the breast cancer cell line is determined to be 182.51 µg/ml, as depicted in Figure 11a. In the case of the colon cancer cell line HCT-116, it was observed that the viability of tumour cells decreased from 92% to 60% when exposed to XOS at doses of 12.5 µg/ml and 100 µg/ml, accordingly. IC50 or half-maximum inhibitory concentration was determined to be 123.71 µg/ml, as depicted in Figure 11b.

Table 5. Inhibition zones of microbial growth by different concentrations of XOS from marine *Bacillus subtilis* AKM1.

Concentrations of XOS (µg/disk)	Gram +ve bacteria		Gram–ve bacteria		Yeast		Fungi	
	B. thuringiensis	*St. aureus*	*E. coli.*	*P. aeruginosa*	*S. cerevisiae*	*C. albicans*	*A. niger*	*F. oxysporum*
	Inhibition zone (mm)							
50	+	++	-	++	+	+	+	++
100	+++	+++	-	++	+	+	++	+++
Rimactane	+++	+++	+++	+++	-	-	-	-
Flucoral	-	-	-	-	+++	+++	+++	+++

Values represent means of triplicate determinations ± standard deviations. Inhibition zones: +++: >15 mm, ++: 5>15 mm, +: <5 mm, and−: no activity

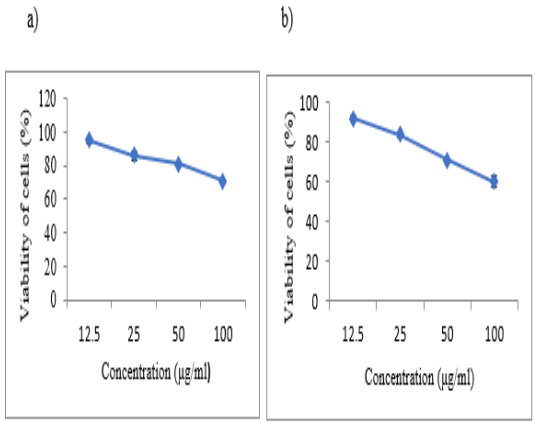

Fig. 11 Antitumor activities against a) MCF -7 breast cell line b) HCT-116 colon cancer cell line of the purified XOS from marine *Bacillus subtilis* AKM1.

4. Discussion

Most of bacterial isolates were isolated from Red Sea sediments where, many scientists mentioned that many bioactive compounds were secreted from marine bacteria, because of the wide pH range, varieties of nutrients, high salinity and wide temperature range,[51]–[53] . The different features of xylanases from different sources make them suitable for various applications. As a result, many studies were done to determine the ideal circumstances for maximal xylanase synthesis during the submerged fermentation process. Several studies matched to our findings demonstrated the identical optimal fermentation duration of xylanase enzyme is synthesized by the bacterium *Bacillus subtilis BS04* [54], *Streptomyces* strains [31]**,** and alkalophilic *Bacillus licheniformis*[55]. Against this, alternative study indicated that the optimal peak of xylanase activity is achieved following a 72-hour incubation period for *Bacillus pumilus* SV-205 [20] and *Streptomyces olivaceus (MSU3)* [39],while being at 36 hrs. for *Pseudomonas mohini* [56]. In order to ascertain the optimal value of pH for the xylanase enzyme when utilizing xylan as the substrate, some studies found a neutral pH (6.5-7.0) for xylanase produced by *Bacillus tequilensis strain* ARMATI [57] and *Streptomyces olivaceus* (MSU3) [39]. According to Shakir et al., [58] the xylanase generated by *Bacillus licheniformis* had an acidic at pH of 5 and 6, and Malhotra & Chapadgaonkar [59] respectively, while pH for *Micrococcus sp.* SAMRC-UFH3 was recorded as 10 [36]. Previous research has documented that the most favourable enzymatic activity of xylanase in alkalophilic *Bacillus licheniformis* occurs at a pH level of 9 [55] whereas a pH level of 8 is ideal for the strain *Bacillus tequilensis* UD-3 [19]. Agitation is commonly employed in order to satisfy the oxygen requirements and facilitate the homogenous distribution of nutrients throughout the fermentation process for xylanase induction by different bacterial species , the outcome of the present investigation was different from other studies related to different strains as for xylanase enzyme production by strain of *Streptomyces,* the peak was recorded at 150 rpm [31]. Previous research has demonstrated that the highest level of induction for *Aureobasidium pullulans* Y-2311-1 was observed at a rotational speed of 200 rpm [60] and *Micrococcus sp.* SAMRC-UFH3 [36].

Temperature is one of the key factors that significantly influences both the production and the biochemical characteristics of the enzyme [61]. The maximum output occurred at a temperature of 40 ℃, resulting in an activity level of (2.23±0.234 U/ml). This finding matches with other research that also identified the same optimal xylanase incubation temperature production generated by *Pseudomonas Mohini* [56] and *Streptomyces olivaceus* (MSU3) [39], while being extremely similar to other investigations that found that *Bacillus licheniformis*-produced xylanases were observed at 35 °C [59], *Bacillus subtilis* BS04 [54] and *Bacillus tequilensis strain ARMATI* [57]. But still, 50 °C was the optimal xylanase's temperature for its production by *Bacillus tequilensis* strain UD-3 produced [19].

Investigating the most effective nitrogen source followed by its perfect concentration, KNO_3 was the best nitrogen source recording activity of (2.30±0.226 U/ml) at a specific concentration: 5 g/l. Additional research revealed that $(NH_4)_2SO_4$ ranked as the top source of inorganic nitrogen for both *Bacillus subtilis* BS04 [54] and *Streptomyces olivaceus* (MSU3) [39]. Although KNO_3 has shown superior performance in promoting the growth of *Bacillus megaterium* BM07 throughout the process of submerged fermentation [54]. The output of xylanase was further augmented by incorporating an appropriate supplementary carbon source into the fermentation medium.

Agroindustrial residues such as wheat bran have the potential to serve as a substitute for commercial pure xylan due to their low cost and they have been found to support xylanase production. It was shown that wheat bran has the potential to serve as an alternative for the pure commercial xylan as a source of carbon in the induction of xylanase. Subsequent research confirmed this finding, as it was determined that wheat bran has the highest level of xylanase's induction among several sources of agro-industrial carbon for *Stenotrophomonas maltophilia* [62] and *Bacillus subtilis* ASH [63].

Following the previously mentioned purification steps, SDS-PAGE was performed for molecular weight determination and it was displayed at 34 KDa. These results were so close matched to extracted xylanase from *Bacillus sp.* in soil termite abdomen [64] and *Paecilomyces variotii* xylanase [18] at which the molecular weight of the substances were 30 KDa and 32 KDa respectively. In the case of xylanases isolated from *Streptomyces olivaceus* (MSU3), [39], *Streptomyces griseorubens* LH-3 [65], *Caulobacter crescentus* [66], and *Rhodothermaceae bacterium* RA [67]**.** The molecular weights were determined to be 45, 45.5, 43, and 43.1 KDa, respectively. In contrast, the xylanase generated by *Paenibaillus barengoltzii* had a molecular weight of 109 KDa [68].

The optimal temperature was observed at 50 ℃, where the activity reached its peak, it was shown that the enzyme showed stability within the temperature level range: of 30 to 50 ℃ under different time restrictions. The xylanase enzyme, which was extracted from *Bacillus sp*. found in the abdomen of soil termites, exhibited stability within the temperature range of 30 to 40 ℃. This stability was seen after a pre-incubation period of 80 minutes, during which the enzyme retained over 50% of its residual activity [64], even though the purified xylanase from *Caulobacter crescentus* exhibited thermal stability, as it remained stable at a temperature of 60 ℃, keeping around 66% of its enzymatic activity even when exposed to a higher temperature of 90 ℃, without undergoing denaturation [66]. A separate study investigated that the xylanase enzyme exhibited its maximum efficiency within a specific temperature range: 20-50 ℃. Furthermore, it kept over 85% of its initial activity even after being pre-incubated for 24 hours. This xylanase enzyme was obtained from the strain *Pediococcus acidilactici* GC25 [22]. For *Paenibaillus barengoltzii* [68], and in the case of *Streptomyces griseorubens* LH-3, it was noted that the best temperature for enzyme activity was 60 ℃. Conversely, for *Streptomyces griseorubens* LH-3, the enzyme retained around 80% of its activity within a temperature range of 30-50 ℃ for 1 hour [65].

Based on an evaluation of pH stability, it was observed that the residual efficiency of the purified xylanase exhibited its maximum level at a pH value of 7. The greatest activity of xylanase, which was extracted from *Paenibaillus barengoltzii*, was reported at a pH of 6.5 [68]. The enzyme isolated from *Paecilomyces variotii* demonstrated maximum activity at a specific pH (5), indicating an acidic environment. Furthermore, the enzyme demonstrated stability across a pH range of 5.0 to 9.0 [18], the optimal efficacy for *xynBT*, which was obtained from *Bacillus sp*. in the soil termite abdomen, is observed at a pH of 5.5. Furthermore, it retains over 83% of its original activity at a pH of 5.0 [64]. The optimal pH for *Caulobacter crescentus* was determined to be 8, with the bacteria demonstrating stability at alkaline pH for 24 hours during incubation [66] and Rhodothermaceae bacterium RA exhibits a wide pH tolerance, ranging from 6.5 to 9.5, and is capable of maintaining over 50% of its maximum activity within this pH range [67]**.**

Various metal ions and chemicals have been investigated extensively as either enhancing or suppressing xylanase activities. The residual xylanase activity was increased in the case of Mn^{2+}, $Ca^{2+,}$ and Fe^{2+}.Other studies which investigated multiple strains showed that Ca^{+2}, and Mn^{+2} promote xylanase activity obtained from *Streptomyces olivaceus* (MSU3) [39], where those metal ions also exhibit a stimulatory effect on the xylanase activity that has been isolated from *Bacillus amyloliquefaciens* [69]. The presence of Fe^{2+}, together with Ca^{2+} and Mn^{2+}, enhances the xylanase activities generated by the alkaliphilic *Bacillus licheniformis* [55]. While slightly enhancing the activity for *Streptomyces griseorubens* LH-3 [65]. In contrast, it was shown that the presence of Fe^{2+} had a suppressing action on the activity of the isolated xylanase from *Paecilomyces variotii*, as previously described [18]. The inhibitory effect of Cu^{2+} on the xylanase enzyme, which was obtained from *Bacillus sp*., was observed [64] Conversely, the activity of xylanase generated from *Paecilomyces variotii* was shown to be stimulated [18]. The xylanase activities extracted from *Paecilomyces variotii* was found to be decreased by SDS, as reported by Abdella et al., [18]. Additionally, the xylanase activity produced by the genus *Massilia* was fully inhibited by Ag^{+}, as seen in the research conducted by [70].

The impact of free radical scavenging activity was a concentration-dependent manner with xylo-oligosaccharides. XOS antioxidant activity was also estimated by different investigations, where the scavenging action of XOS generated through enzymatic hydrolysis, exhibited a notable efficacy of 87.36% when tested at a dosage of 5 mg/ml [71], while the scavenging efficacy of XOS derived from the finger millet seed coat exceeded 70% at a specific concentration: 2 mg/ml [72]. The antioxidant ability of products derived from the hydrolysis of xylo-oligosaccharide obtained from maize cob hemicellulose can be attributed to their IC50 values, which are below 100 ppm [18], Yamani etal., [73] recording a maximum scavenging percentage of (70.57%) at a dosage of 2 mg/ml.

Antibiotics potency assessment for the produced XOS was performed against a wide group of microorganisms. The xylanase derived from *Bacillus mojavensis* UEB-FK, after undergoing purification, was found to generate xylooligosaccharides (XOS) from xylan obtained from garlic straw. Furthermore, this purified xylanase exhibited the characteristics that exhibit efficacy against both Gram-negative and Gram-positive bacteria. The antibacterial activity of the substance demonstrates high efficacy against *Klebsiella pneumonia*, followed by *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Bacillus thuringiensis*. *Escherichia coli*, *Bacillus thuringiensis*, *Listeria monocytogenes, and Staphylococcus aureus* exhibited minor levels of activity [43]. The production of acidic xylooligosaccharides (XOS), specifically aldotetrauronic acid and aldopentauronic acid, was achieved by subjecting birchwood xylan to endoxylanases derived from the *Thermoascus aurantiacus* family 10 and the *Sporotrichum thermophile* family 11. Both endoxylanases showed a mild impact on the Gram-positive *Micrococcus flavus* and *Bacillus cereus*. In contrast, aldopentauronic acid exhibited a similar effect on *Staphylococcus aureus*. Neither of the compounds had any

effect against *Proteus mirabilis* and *Pseudomonas aeruginosa*. The findings of the study indicate that the aforementioned substances had anti-*Helicobacter pylori* properties, with aldopentauronic acid exhibiting the highest level of activity [74], Ratnadewi et al [75] proved the prebiotic action of XOS, as it was observed to positively influence the growth of the probiotic strain *Lactobacillus acidophilus*.

The investigation of cytotoxic effects using different cell lines where displayed in other investigations at which they found that the viability of human breast cancer MCF-7 cells was dose-dependently decreased by XOS derived from -1,3-xylan [76]. While, in the case of the HCT-116 colon cancer cell line, a tumor cell viability of 92% was seen when exposed to a specific concentration: 12.5 µg/ml of XOS. However, this viability decreased to 60% when the concentration was increased to 100 µg/ml. IC50 or the half-maximal inhibitory concentration for XOS in this cell line was determined to be 123.71 µg/ml. In a study conducted by Aachary, it was shown that the consumption of dietary Xylooligosaccharides (XOS) had a notable effect on the post-initiation phase of colon malignancy [77]. The research indicated that dietary XOS potentially offers protection against colon cancer produced by dimethyl hydrazine in rats. Another study discovered that XOS exhibits anticancer effects on many types of cells, including lung fibroblasts, colon adenocarcinoma, and histiocytic lymphoma [78].

5. Conclusion

The xylanase enzyme, derived via *Bacillus Subtilis* AKM1 and obtained regarding the marine environment of the Red Sea (specifically, Hurghada), possesses a molecular weight of 34 KDa. It exhibits a pH of 7.0 and demonstrates stability at a temperature of 50 °C. considering these characteristics, this enzyme holds significant potential for utilization in various biotechnological applications on a wide scale where, purified xylanase was used to generate xylooligosaccharides, which exhibited antibacterial action against all assessed organisms except *E. coli*, the antioxidant activity as measured by the IC50 value of 121 g/ml, and anti-tumor potency against breast cancer cells and colon cancer cells.

6. Conflicts of interest

The authors declare that they have no competing interests.

7. Formatting of funding sources

Not applicable

8. Acknowledgments

Not applicable

9. References

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