Egyptian Journal of Chemistry

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

Statistical optimization of xylanase production using Box-Behnken Design and its application in paper bleaching

Mohamed M. Bakry, Salem S. Salem*, Hossam M. Atta, Mamdouh S. El-Gamal, Amr Fouda

Botany and Microbiology Department; Faculty of Science; AL-Azhar University; Nasr City; Cairo-11884; Egypt

Abstract

Xylanase play important role in many industries because reduce using of chemicals and reduce the environment pollution, that caused by chemical compounds. Xylanase in this study produced by Bacillus tequilensis strain, which chosen from forty bacterial isolates isolated from Ain-Helwan spring water (hot) and soil. The Bacillus tequilensis strain was the most potent isolate that give large amount of xylanase so subjected to identification by 16s rRNA sequence analysis and more studies for detection of the optimum factors that make it give the highest xylanase production. To improve the production of xylanase, the optimum factors subjected to studying with Box-Behnken design resulted in the xylanase activity becoming (51.51 U/mL). The Bacillus tequilensis xylanase extracted from broth culture by precipitation with ammonium sulphate (60 %), then the enzyme enhanced with dialysis and purified with sephadex G-100 column chromatography. Purified xylanases had a 12-fold enrichment, with a specific activity of 76.83 U/mg and a molecular weight approximately 359.0 KDa. The amino acids content of xylanase obtained from Bacillus tequilensis were 16 amino acids, started with aspartic acid and finished with proline, from these amino acids found two amino acids with highest concentration arginine 1410 mg/L and glycine 900 mg/L. Xylanase produced by B. tequilensis strain used in biobleaching, where it gives the highest brightness (61.4 %), in case the control sample was (48 %), with increasing 13.4 % about the control sample. This xylanase used to bioleaching the waste paper as alternative of chemical compounds that used in the bleaching process.

Keywords: Xylanase; Bacillus tequilensis; Box-Behnken design; purification; bio-bleaching

1- Introduction

Enzymes, a biological substance or biological macromolecule that catalysts a particular biochemical reaction, are capable of being produced by living organisms [1, 2]. Due to their capacity for most industrial processes, these enzymes are commonly referred to as "Biocatalysts" since they serve to accelerate biological/biochemical reactions both within and outside of cells, comparable to a chemical catalyst in a chemical reaction [3-6]. Xylanases are group of hemicelluloses enzymes which are the best interest area for researchers on their insightful investigation and prospective of industrial applications, especially in the production of fermentable sugars through biomass degradation [7]. Xylanases have been used in the feed industry to reduce the viscosity of food and improve the absorption of nutriment in the digestive tract of animals [8, 9], and they play important roles in many applications such as biobleaching of paper pulp and production of starch, coffee and plant oil clarification of fruit juices and wines [10, 11]. Xylooligo-saccharides (XOS) are a material that has been discovered and used in a number of industrial processes, particularly in the chemical and biofuel production industries [12, 13]. Whereas the full degradation of xylan releases the XOS, the process requires interaction between a number of enzymes, namely endoxylanases and β--xylosidase, as well as accessory enzymes [14, 15]. In some reactions, xylanases act as a catalyst. In one such reaction, it can catalyze the chemical reaction of the glycosidic linkage -1,4 of xylosides, producing an organic sugar compound and the corresponding free aglycone, a non-sugar compound that remains after the organic compound is replaced by an atom [16, 17]. Xylooligosaccharides and xylose were produced by depolymerization action of b-$_D$- xylosidase (1,4-b-xylan xylohydrolase; EC 3.2.1.37) and endo-1,4-xylanases (1,4-b-xylan xylanohydrolase; EC 3.2.1.8) through changing in the polymeric substance [18, 19]. The shape of xylan is extended ribbon with twofold that bind with intra-chain hydrogen bonding which is stated to be springier than the twofold helix of b-(1–4) cellulose. A large variety of xylanases which become a major group of industrial enzymes produced by microorganisms and are capable to degrade xylan to renewable fuels and chemicals [20, 21], in addition to their use in food, paper and pulp industries [22-24]. However, because of their significant negative impacts

Correspondence: Salem S Salem: salemsalahsalem@azhar.edu.eg, ORCID: 0000-0003-2898-6708

Receive Date: 19 July 2022, **Revise Date:** 10 August 2022, **Accept Date:** 15 August 2022, **First Publish Date:** 15 August 2022
DOI: 10.21608/EJCHEM.2022.151198.6545

on the environment and public health, developed countries now work to recycle solid wastes, particularly paper and paperboard products. Using the microbial enzymes decrease the using the chemicals which are harmful for human and environment. Microbial xylanases were discovered to be more beneficial than others [25, 26]. Extracellular xylanase production has several advantages over intracellular xylanase production, including ease of enzyme recovery and purification [27]. As a result, the aim of this paper was to isolate the thermotolerant bacteria from hot springs and producing xylanase from these bacteria. Additionally, the most potent factors for the best xylanase production and improving xylanase production utilizing Box-Behnken Design, which was defined by a number of characteristics. Finally, xylanase was added to the waste paper pulp to bio-bleaching.

2- Materials & Methods

1.1. Collection and Storage of Samples

From water and soil of springs located in Ain Helwan spring lie in an area east of the Nile River and 25 Km South of Cairo between El-Maasara in the North and El-Tabbin in the south with Latitude N29°51', and Longitude E31°19', about 1 cm a top layer removed from the surface of soils during collection of soils samples. Samples were collected in sterilized falcon tubes and immediately transferred to the lab. The temperature and pH of these samples when measured the temperature was between 30 – 40 °C and the pH was 6 – 7 according to the site of location. The samples stored at 4 °C for conducting further experiments.

1.2. Isolation and identification of xylanolytic bacteria

From soil samples was measured aseptically one gram and transferred to 100-ml distilled water in 250-ml flask and covered with aluminum foil. Was mad dilution for sample until the dilution became 10^{-5} or 10^{-6}, where these dilutions done by transferring one- milliliter of suspension stock solution was transferred into 9-ml sterilized distilled water in sterilized test tube and suspension was serially diluted until obtained dilution dilutions [28]. Inoculums used from dilution 10^{-5} or 10^{-6} for pour plating performed in a laminar air flow by using corn cob xylan-enriched (Solarbio Company with CAS NO: 9014-63-5) plat count agar supplemented with 2 g xylan powder. The pH was adjusted, at 7.4 the medium was sterilized by autoclaving at 120 °C (1.5 psi) for 20 min. Then the inoculated media incubated for 24 - 48 h at 50 °C. After 48 h incubation, the colonies showing clear zones upon flooding with Logule's iodine solution were confirmed as xylanase producers.

1.2.1. Determination of bacterial xylanase activity and protein content determination

Inculcation the xylanase production media which consist of (g/L): 5g $NaNO_3$, 1g KH_2PO_4, 2g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.1g KCL, 0.01g $CaCl_2$, 0.02g $FeSO_4.7H_2O$ and supplemented with 2 g/L xylan powder, the pH of this media was adjusted to 7.4 and inoculated with 0.5 ml of an overnight and incubation at 50 °C for 24 h after sterilizing this medium by autoclaving at 120 °C (1.5 psi) for 20 min. Before assay the cells after incubation the inoculating media for 24 h at 50 °C, were separated by centrifugation at 5000 rpm, the clear supernatant used as crude enzyme. Then the supernatant separated carefully by using pipettes from pelts and used for preparing the mixture of the reaction for enzyme assay. The substrate solution prepared by 1 % of corn cob xylan with phosphate buffer (0.1M) at pH 7, then take 1ml of substrate solution in test tube and add 1 ml of crude enzyme. The assaying tube containing mixture substance incubated for 15 min at 55 °C, then transferred 1 ml of mixture into test tube and added 1 ml DNS and boiled the test tube at 100 °C for 5 min. The amount of reducing sugar produced from hydrolysis by bacterial supernatant was measured by UV-VIS Spectrophotometer at an absorbance value of 540 nm. Where 1 µMol of xylose liberated per milliliter of enzyme per minute equal one unite (IU) of xylanase enzyme. The enzyme activity assayed according to the reaction of control sample which prepared without bacterial supernatant (crude enzyme) and was measured according to DNS protocol for comparison with other samples. The soluble protein content was determined by Bradford method [29], where this procedure depend upon the using the protein reagent Coomassie Brilliant Blue G-250 that prepared by dissolving the CBB G-250 in 50 ml of 95 % ethanol. 100 ml of o-phosphoric acid was added later and the whole reagent was diluted to 200 ml to make 5X concentrated dye, where the CBB G-250 bind to protein and the color of reaction change from light green to blue. The absorbance was read off a spectrophotometer at wavelength of 595 nm.

1.2.2. Phenotypic characterization of xylanase-degrading bacteria

The morphological and biochemical studies were taken for phenotypic characterization. The morphological studies were carried through making Gram's staining for the xylanolytic bacterium *Bacillus tequilensis* strain. The bacterium observed after staining and drying under a phase contrast microscope (× 100 objectives) for determination it's size, shape and response to Gram stain (Gram-positive or Gram-negative).

1.2.3. Biochemical characterization of xylanolytic bacteria

This study depends upon biochemical tests as per Bergey's Manual of Systematic Bacteriology. A number of biochemical tests such as catalase test, coagulase test, citrate utilization test, oxidase test, oxidation/fermentation test, methyl red test, carbohydrate metabolism test urease test, hydrogen sulfide (H_2S) test, indole test, gelatin hydrolysis test, and voges-prausker test were performed.

1.2.4. Molecular identification of xylanolytic bacteria

The pure most potent xylanolytic strain *Bacillus tequilensis* strain was done by specific method that called 16s rRNA gene sequencing. In the first the *Bacillus tequilensis* strain DNA was extracted according to standard methods [30]. The 16s rRNA gene was amplified by PCR using universal primers**.** By using the BLAST database available at the GenBank database of the NCBI server was done compression *Bacillus tequilensis* strain 16s rRNA gene sequences and another homolog 16s rRNA gene sequences [31].

1.3. Optimization factors for xylanase production

According to the standard composition of media which used for xylanase production from *Bacillus tequilensis* strain. Where the media composed of (g/L): 1 g KH_2PO_4, 2 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g KCL, 0.01 g $CaCl_2$, 0.02 g $FeSO_4.7H_2O$, where this media was (50 ml), was poured in conical flasks separately and was inoculated with 0.5 ml of an overnight culture at 50 °C for 24 h after sterilizing this medium by autoclaving at 120 °C (1.5 psi) for 20 min [32]. The different parameters used in this studying for obtaining the best xylanase production, were temperature (30 °C, 35 °C, 40 °C, 45 °C and 50 °C), pH(6, 7, 8, 9 and 10), inoculum size (1 %, 2 %, 3 % and 4 %), nitrogen source (NH_4NO_3, $NH_4H_2PO_4$, NH_4CL, Yeast extract, Urea and Peptone), incubation period (6 h, 12 h, 18 h, 24 h and 30 h) and xylan concentration (1 g/L, 2 g/L, 3 g/L and 4 g/L). After checking the optimum time and temperature the other parameters were incubated with static conditions accordingly. Finally, after incubation the inoculated media were centrifuged at 5000 rpm at 4 °C for 10 min. The supernatant which produced from centrifugation used for determination of xylanase activity by DNS method that measured the amount of reducing sugars produced from reaction.

1.4. Optimization of xylanase enzyme production by Box-Behnken design

In this study was used a Box-Behnken design for investigation of the combined effect of six variables such as temperature, pH, inoculum size, xylan concentration, bacterial incubation time and different nitrogen source. Table (1) show the three levels (- 1, 0, + 1) were used in the experimental design. The Box-Behnken design was used to get up higher- order response surfaces using fewer required runs than a normal factorial technique [33]. Also the Box-Behnken design used with widely shape for preparation of quadratic response surfaces and a second-degree polynomial model to analyze the pattern of enzyme production. According to the second-degree polynomial model the optimization process was carried out a set of experimental runs. To maintain the higher-order surface definition, the Box-Behnken design essentially suppresses selected runs in an attempt. This technique is (version 12, Stat-Ease Inc., Minneapolis, USA), where this design with statistical software used to obtain the best xylanase activity. The experimental design depend upon this equation $N = k^2 + k + C_p$ for seting up a number of runs(N), where k and C_p are the factor number and replications number in the present study. Through the second-degree polynomial equation, the predicted model using were 54 different experimental runs in the current work. In this study the factors that formed the Box-Behnken design, were six factors vis. Temperature (A) [30 °C, 35 °C, 40 °C, 45 °C and 50 °C], pH (B) [6, 7, 8, 9 and 10], inoculum size (C) [1 %, 2 %, 3 % and 4 %], nitrogen source (D) [NH_4NO_3, $NH_4H_2PO_4$, NH_4CL, yeast extract, urea and peptone], incubation period (E) [6 h, 12 h, 18 h, 24 h and 30 h] and xylan concentration (F) [1 g/L, 2 g/L, 3 g/L and 4 g/L] further production of xylanase enzyme from xylanolytic bacteria *Bacillus tequilensis* strain.

Table (1): Actual levels for the six variables Box-Behnken design in response surface methodology of the xylanase enzyme extracted from the bacterium *Bacillus tequilensis* strain

Factor Name	Symbol	Units	-1	0	+1
Temperature	A	°C	43	45	47
pH	B	-	6.5	7	7.5
Inoculum size	C	%	0.5	1	1.5
Peptone	D	g/L	3	5	7
Incubation period	E	h	20	24	28
Xylan	F	g/L	2	3	4

Xylanase extraction and purification and protein content detection

The cultures after incubation were harvested and cells removed from crude enzymes through centrifugation at 5000 rpm for 10 min at 4 °C. Then made precipitation for enzyme by addition ammonium sulfate with concentration 60 %, where the ammonium sulfate added to crude enzyme and

stirred continuously for 2 h until ammonium sulfate completely dissolved to aid precipitation of enzyme, then the precipitated enzyme dissolved in buffer phosphate at pH 7, then the enzyme undergone for further purification.

1.5. Partially purification with Dialysis process

In this study the enzyme purified partially by dialysis into dialysis bag, where the precipitated enzyme that produced from purification with ammonium sulfate against distilled water for 3 h, then the dialysis bag was introduced against phosphate buffer at pH 7 overnight. The obtained xyllanase was concentrated by putting dialysis bag against crystals of sucrose and kept in the refrigerator at 5 °C for further purification.

1.6. Purification with Sephadex G-100 Chromatography

The completely purification of xylanase enzyme in the current study done by column matrix. The method was carried by using a gel filtration column. The Sephadex G-100 column material was soaked in phosphate buffer and add sodium azid to prevent sodium growth of microorganism, the soaking done for overnight at 4 °C. After soaking the fines were taken off and it was loaded into a column carefully to avoid any air bubble. The column was permitted to settle and by 50 ml of phosphate buffer of pH 7, made washing away. The dialyzed enzyme which was 3 ml, it was loaded in the Sephadex column pre-equilibrated with the extraction buffer. The purified enzyme collected as fractions each fraction was 5ml. The collected fraction examined for the eluted protein by measuring it's absorbance at 595 nm, the xylanase activity by measuring the reducing sugars with DNS at 540 nm and the specific activity calculated though the values of protein content and xylanase activity.

1.7. Detection of molecular weight by TLC mass

In the current study the molecular weight of xylanase enzyme measured by Thin-Layer Chromatography/Mass Spectrometry, where this system is a planar chromatographic technique used to separate the components of a mixture by employing a thin stationary phase supported by an inert backing. It can used to monitor the progress of a reaction on the analytical scale. The technique is low cost, simplicity, relatively good sensitivity and speed of separation so it is widely used. TLC spotes are observed by UV or fluorescence light or by the use of chromogenic sprays that enhance detection. First make adjustment parameters with mode of fragmentation: typical. It's mass range from 100 to 1200, with mass type (ESI). N.B. Standard sulphadiazine (Mol. Wt = 250) was injected to assure the quality of analysis. A 2 µL aliquot of the enzyme reaction mixture from the stirred round – bottom flask was spotted onto TLC plate at a position 1 cm above the bottom of the plate. Thirty milliliters of benzene was added to the developing chamber of a TLC plate at a depth of 0.5 cm. The spotted TLC plate was placed into the developing chamber and sealed with the glass lid. Separation was concluded when the development solvent front reached a position 1 cm from the top of the TLC plate. Then putting the TLC plate in oven at 80 °C for 5 min until drying. Under the UV light at 254 nm the separated spots were observed on TLC plate.

1.8. Determination of amino acids sequencing of xylanase enzyme

The amino acids of xylanase enzyme in this study were detected by Sykam Amino Acid Analyzer (Sykam GmbH, Germany) equipped with Solvent Delivery System S 2100 (Quaternary pump with flow range 0.01 to 10.00 kl/min and maximum pressure up to 400 bar), Autosampler S 5200, Amino Acid Reaction Module S 4300 (with built-in dual filter photometer between 440 and 570 nm with costant signal output and signal summary option) and Refrigerated Reagent Organizer S4130. Standard preparation, where the stock solution contains 18 amino acids (aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histadine, lysine, ammonia, arginine) all amino acids concentration are 2.5 µMol/ml, except Cystine 1.25 µMol/ml, then dilute 60 µl in 1.5 ml vial with sample dilution buffer then filtered using 0.22 µm syringe filter then 100 µl was injected. Sample preparation; 1 gm of sample was mixed with 5ml hexane. The mixture was allowed to macerate for 24 h. Then, the mixture was filtered on whatman no. 1 filter paper and the residue was transferred into a test tube where it was incubated in an oven with 10 mL 6N HCl for 24 h at 110 °C. After the incubation, the sample was filtered on whatman no. 1 filter paper, evaporated on rotary evaporator and dissolved completely in 20 ml dilution buffer, filtered using 0.22 µm syringe filter and 100 µl was injected.

Instrument parameters;

Column: LCA k06/ Na
Mobile phase: Buffer A, Buffer B and Regeneration solution
Mode of elution: Gradient
Flow rate: 0.45 ml/min
Temperature: Gradient 57 °C -74 °C
Wavelength: 440 and 570 nm

Table (2): Buffers and solutions preparation

Solutions preparation	Buffer A	Buffer B	Column Regeneration Solution	Sample Dilution buffer
pH Value	3.45	10.85	-	2.20
Normality	0.12	0.20	0.50	0.12
Tri-sodium citrare dihydrate	11.8g	19.6g	-	11.8g
NaOH	-	3.1g	20.0g	-
Citric acid	6.0g	-	-	6.0g
Boric acid	-	5.0g	-	-
Methanol	65 ml	-	-	-
Thiodiglycol	-	-	-	14 ml
Hydrochloric acid 32%	6.5 ml	-	-	12 ml
EDTA	-	0.2 g	-	-
Phenol	0.5 g	-	-	2.0 g
Final volume	1L	1L	1L	1L

1.9. Application of xylanase on waste Paper

This study explain the bleaching of paper by xylanase enzyme through taking two oldest whatman filter papers were similar in the physical properties and shape, one of them was sample test and another was control. The sample filter paper treated with 3 ml of xylanase enzyme and incubated under optimal conditions at pH 7, temperature 45 °C, for 1.5 h. and the control filter paper treated with distilled water. The sample and the control after incubation investigated by TMI device for softness measurement, and Technobright device for measurement brightness and darkness.

1.10. Statically analysis for optimal conation by Tukey HSD test

All results presented in this study are the means of three independent replicates. Data were subjected to analysis of variance One-way ANOVA by a statistical package Minitab v19. The mean difference comparison between the treatments was analyzed by the Tukey HSD at $p<0.05$.

2. Results & Discussion

2.1. Isolation and identification of xylanolytic bacteria

All collected samples undergone for serially dilution and screened for isolation of pure xylanase producing bacteria. Forty isolates grown on screening medium, from them 21 isolates give clear zone around the culture, and it took the isolate with the highest clear zone and selected as xylanase producers. The selected isolate was purified using repeated subculture by using plate count agar medium and made stock culture through maintaining in plate count agar slant [34]. Then the pure isolate was identified through studying the physiological, morphological, and biochemical characters as showing in Table (3). From this study the bacterial isolate has rod shape and was gram positive, spore former, facultative anaerobic, cannot grow at 20 °C, give heavy growth, and give weakly growth at 55 °C - 60 °C. Then the isolate identified by 16s rRNA as shown in Figure (1). The isolate after 16s rRNA test was *Bacillus tequilensis* strain, but Paul, et al. isolated *Pseudomonas mohnii* which identified by 16s rRNA, where this isolated was xylanlytic bacteria.

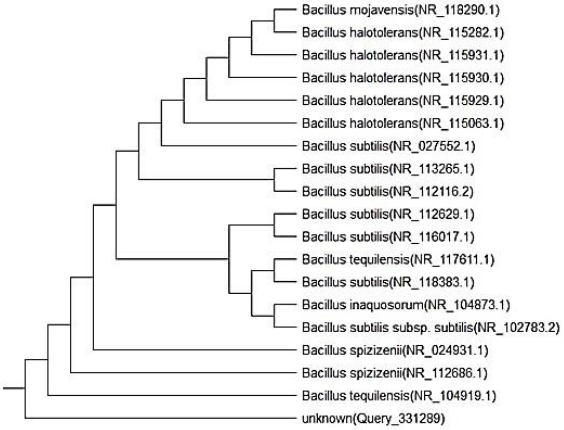

Fig. (1): Phylogenetic analysis of the isolate *Bacillus tequilensis* strain.

2.2. Effect of different parameters on xylanase production

In this study, it was studied six factors to identify the best factor through it the *Bacillus tequilensis* strain has ability to produce the best xylanase production. In the first temperature was studied through incubation the *Bacillus tequilensis* strain at different temperature (30 °C, 35 °C, 40 °C, 45 °C and 50 °C), and found the best temperature at it, the strain gives the best production was 45 ºC. Where at this temperature By Tukey Method for temperature factor, the (45 °C) take symbol A that indicate the optimum pH as in Fig. (2), at it, the enzyme activity 16.12 U/mL.

When the temperature increases or decrease the enzyme activity decreased. The optimum temperature of 45 °C found in this study is comparable to experiments conducted by Lawrence, et al., who found that 50 °C was the optimum temperature for producing xylanase from Bacillus pumilus. At temperatures above or below 50 °C, enzyme activity decreased [35]. Then the *Bacillus tequilensis* strain undergone for different pH for detection the optimum pH which at it, the *Bacillus tequilensis* strain give the best xylanase production, where the different carried out in this study were (6, 7, 8, 9 and 10). From this the optimum pH was 7. By Tukey Method for this factor the pH 7 take symbol A that indicate the optimum pH as in Fig. (2), at it, the enzyme activity 16.0367 U/mL.

Table (3): A comparative study for the morphological, physiological and biochemical test of the most potent isolate, *Bacillus tequilensis* strain.

Test	*Bacillus tequilensis* strain		Test	*Bacillus tequilensis* strain	
Colour	Yellowish		Growth at different pH	6	+
Surface	Smooth			7	++
Cell Shape	rod			8	+
Gram stain	G +ve			9	+
Motility	+			10	+
KOH test	-		Hydrolysis	Pectin	+
Anaerobic growth	-			Xylan	+
Methyl red	+			Starch	+
Nitrate Reduction	+			Lipid	+
Indole test	+			Cellulose	+
Vogase- proskauer	-			Gelatin	+
Citrate utilization	+			Casein	+
Fermentation	Glucose	+		Catalase	+
	Galactose	+		Oxidase	+
	Fructose	+	Growth at different Temperatures	15-25	-
	Xylose	+		30-40	+
	Arabinose	+		40-50	+
	Manitol	-		50-60	+
	Lactose	-		60-70	-
	Maltose	+	Oxidation fermentation (O/F)	(O/F)	
	Mannose	+			
	Starch	+			

Symbols: (+) Positive; (-) Negative; (W) Weak reaction. (O/F) Oxidative fermentation.

Above this pH 7 or below the xylanase activity. Then in the current study *Bacillus tequilensis* strain undergone to another parameter which is inoculum size, where in this parameter four different inoculum size (1 %, 2 %, 3 % and 4 %) used to detection the best inoculum size which through it the *Bacillus tequilensis* strain give the best xylanase production. By applying Tukey Statistical Method on this factor, found the optimum inoculum size take Symbol (A), through it the enzyme activity was (41.19 U/mL) as in Fig. (2). When the inoculum size became 2 %, 3 % and 4 % the enzyme activity decreased gradually. Comparable to the work carried out by Marimuthu, et al., the optimum inoculum size was used in the current investigation.

They observed that the various initial inoculum concentrations play a crucial role in determining the enzyme yield in production media. They examined different inoculum sizes from 1.5 % to 4.5 %. In order to get the best xylanase output from *Bacillus subtilis*, they determined the optimum inoculum size, which is 3.5 % [36].

In case of studying the best nitrogen source in the present study and with applying Tukey Statistical Method on the results obtained during studying the different nitrogen source effect on xylanase production from *Bacillus tequilensis* strain. From statistical Tukey method found the optimum nitrogen source that marked with symbol (A), was peptone where the enzyme activity at this factor was 51.50 U/mL as in Fig. (2). When applying another nitrogen source such as (NH_4NO_3, NH_4CL, $NH_4H_2PO_4$, urea and beef extract) the xylanase activity decreased. According to Pual et al., the urea nitrogen source produced the most xylanase from *Pseudomonus mohnii* and had a maximum enzyme activity of 21.72 IU/ml [37].

Additionally, Lawrence et al. reported that beef extract was the best nitrogen source for Bacillus pumilus to produce xylanase [35]. In applying Tukey Statistical Method on results obtained through studying the effect the of incubation period, found the optimum incubation period was 24 h at this factor the *Bacillus tequilensis* strain give the highest xylanase activity (47.173 U/mL) as in Fig. (2), when the incubation period was 30 h or 18 h the xylanase activity decreased, so the factor 24 h take symbol (A) by Tukey method. Another study was conducted by Kumar et al., who looked at various incubation times for the synthesis of xylanase by *Bacillus subtilis*. They discovered that 72 h was the ideal incubation time, at which time the enzyme activity was 2.89 µg/ml [38].

Finally, studying the effect of xylan concentration on xylanase production from *Bacillus tequilensis* strain, where the xylan concentration used in this study 1 g/L, 2 g/L, 3 g/L, and 4 g/L. By applying the Tukey Statistical Method on obtained results from this study.

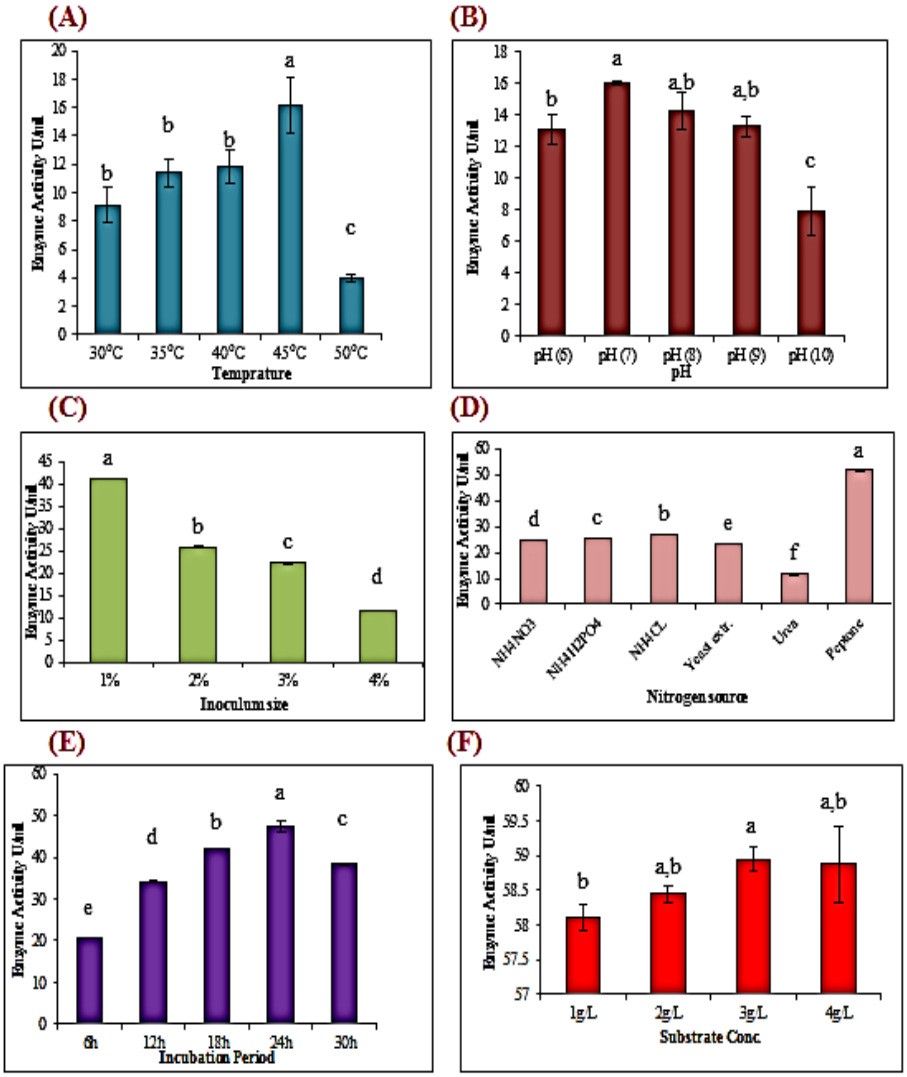

Fig. (2): show the effect of different parameters that effect on xylanase production by *Bacillus tequilensis* strain, where (A) refer to temperature, (B) pH, (C) inoculum size, (D) nitrogen source, (E) incubation period and (F) refer to xylan concentration.

Tukey give symbol (A) for the concentration 3 g/L, where this concentration was the optimum concentration, at it the *Bacillus tequilensis* strain give the highest xylanase production and the xylanase activity was at this concentration (58.943 U/mL) as showed in Fig. (2). When using another concentration such as 4 g/L, 2 g/L or 1 g/L the enzyme activity decreased. Another investigation on the impact of substrate concentration on xylanase production was conducted by Lee and Choi, who looked at various xylan concentrations in order to produce xylanase from Bacillus species. According to the results of this investigation, the ideal xylan concentration was 0.5 %, with a xylanase activity of 21.4 U/mL [39].

2.3. Optimization of growth factors through Box-Behnken

In the present study the optimum growth factors that obtained from pervious studying **(**effect of different parameters on xylanase production**)**, the results obtained from this test undergone into studying with Box-Behnken design. Where the number of runs obtained from Box-Behnken design were 54 runs used to determine the best xylanase production from *Bacillus tequilensis* strain. There are six independent variables factors such as temperature, pH, inoculum size, nitrogen source, incubation period and substrate concentration based on which the responses were calculated.

The full experimental plan as per the designed parameters along with the response values are given in Table (4), and Fig. (3) Using multiple regression analyses, a second-order polynomial equation was developed which represents the relationship between enzyme activity, temperature, substrate concentration, incubation time, nitrogen source, pH, and inoculum size. The regression equation in uncoded units.

Bacillus tequilensis strain xylanase 1= (-2631 + 87.1 temperature +152.8 pH + 8.8 peptone + 3.6 inoculum size + 11.2 xylan conc. +15.67 incubation period – 0.940 temperature *temperature – 6.68 pH*pH + 0.058 peptone*peptone – 9.72 inoculum size*inoculum size – 1.834 xylan conc.*xylan conc. – 0.4717 incubation period*incubation period – 1.089 temperature *pH – 0.090 temperature *peptone – 0.449 temperature *inoculum size + 0.063 temperature *xylan + 0.175 temperature *incubation period – 0.687 pH*peptone + 3.52 pH*inoculum size – 1.04 pH*xylan conc. – 0.212 pH*incubation period – 0.560 peptone*inoculum size + 0.182 peptone*xylan conc. – 0.0499 peptone*incubation period + 3.68 inoculum size*xylan conc. + 0.159 inoculum size*incubation period – 0.015 xylan conc.*incubation period).

The significance of the second-order polynomial equation for xylanase activity was measured by the analysis of variance (ANOVA) [37]. According to this study, the ideal conditions for producing the most xylanase from the *Bacillus tequilensis* strain are 45 °C, pH 7, peptone 5 g/L, 1% inoculum size, 3 g/L of xylan, and a 24-hour incubation time. At these conditions, the xylanase activity was 51.51 U/mL. However, Pual et al. discovered that when they used the Box-Behnken design to determine the best conditions for producing xylanase from Pseudomonus mohnii, the best conditions were 42.5 °C, pH 6, 0.5 % xylan, and a 36-hour incubation period. At these conditions, the xylanase activity was 152 U/mL [37].

2.4. Purification of xylanase enzyme

From broth media which inoculated with *Bacillus tequilensis* strain and after *incubation* at 45 °C for 24 h, the enzyme extracted from this media through centrifugation the broth media at 5000 rpm for 15min. then after that the xylanase enzyme precipitated by using ammonium sulphate $(NH_4)_2SO_4$, but the concentration of ammonium sulphate which used for precipitation xylanase was the concentration that give the best xylanase precipitation through studying different 10 % - 80 % concentration of ammonium sulphate.

The best concentration that used was at 60 % ammonium sulphate, at this concentration the enzyme activity was the highest enzyme activity (62.33 U/mL) as in Fig. (4), when increasing the concentration of $(NH_4)_2SO_4$ the xylanase activity decreased. But the protein content increased with increasing the concentration of $(NH_4)_2SO_4$, where the protein precipitated when the concentration of ammonium sulphate 80 %, was 2.98 mg/ml. Sharma and Chand [40], studied the different concentration of ammonium sulphate from 10 % - 100 % for xylanase production, where they found the enzyme activity increased with increasing the concentration of ammonium sulphate until to 80 %, they found the highest enzyme activity (31.3 U/mL). After that the precipitated xylanase enzyme subjected to dialysis with dialysis page against sucrose. The xylanase activity increased to be (73.22 U/mL).

The *concentrated* xylanase subjected to completely purification by using Sephadex G-100, where the obtained xaylanase from dialysis loaded on Sephadex G-100 resulted purified xylanase collected as fractions each fraction 5 ml, where each fraction subjected to total protein and xylanase activity measurements as showed in Table (5). Gessesse and Mamo [41] reported, the xylanase enzyme which produced by *Micrococcus sp* AR-135, the xylanase activity after DEAE-cellulose chromatography was (500 U), the specific activity (42.5 U/mg), and the protein content was (13.5 mg), also they reported the specific activity after purification with Gel filtration was (111.7 U/mg), and the total protein (3.3 mg).

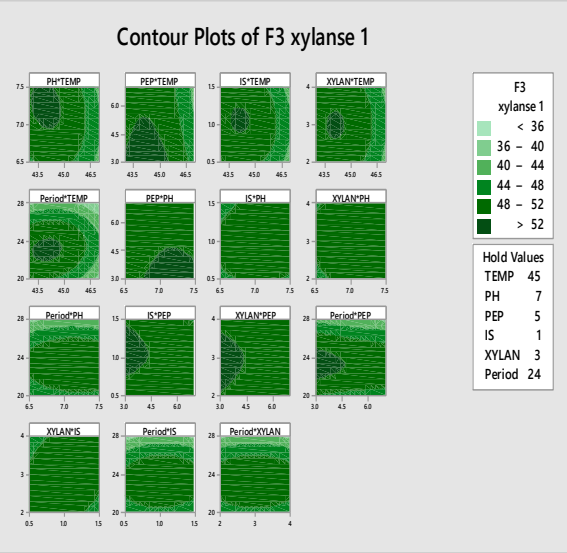

Fig. (3): Contour plots showing interactive effect of six factors with six levels on xylanase production by *Bacillus tequilensis* strain.

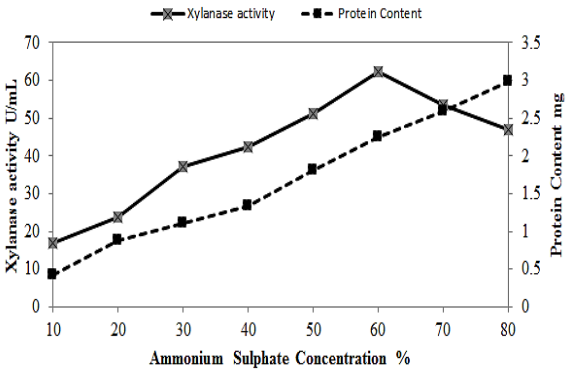

Fig. (4): Show effect of concentration of $(NH_4)_2SO_4$ on xylanase activity and protein content that obtained *Bacillus tequilensis* strain

Table (4) Result of Box-Behnken design for xylanase production by *Bacillus tequilensis* strain

Run	Temp	PH	PEP	IS	Xylan	Period	Enzyme activity (U/mL)	Predicted
1	45	7	3	1.5	3	20	43.21	46.2019
2	47	6.5	5	0.5	3	24	36.54	41.3137
3	43	7	3	1	3	20	47.99	48.6319
4	43	7	5	0.5	2	24	49.19	48.0321
5	47	7	3	1	3	28	37.30	37.7756
6	45	7.5	5	1	2	28	37.37	38.9031
7	45	7	5	1	3	24	51.51	51.5100
8	45	7	5	1	3	24	51.51	51.5100
9	45	7.5	3	1	4	24	50.14	50.4419
10	45	7.5	7	1	2	24	49.45	47.5048
11	47	7.5	5	1.5	3	24	38.83	41.1321
12	47	7	7	1	3	20	38.64	37.2765
13	43	7.5	5	0.5	3	24	46.54	47.4696
14	45	6.5	5	1	4	28	36.75	37.3048
15	45	7	7	0.5	3	28	37.88	37.0144
16	47	7.5	5	0.5	3	24	39.49	39.5704
17	43	7	5	1.5	4	24	49.66	49.2421
18	45	7	3	0.5	3	20	46.61	45.0202
19	45	7.5	5	1	2	20	45.76	45.3865
20	43	7	7	1	3	28	37.37	38.0610
21	47	7	5	0.5	4	24	39.54	38.4962
22	45	7	5	1	3	24	51.51	51.5100
23	43	7	7	1	3	20	45.76	47.4106
24	45	7.5	5	1	4	20	43.65	44.3285
25	43	7.5	5	1.5	3	24	56.82	50.8262
26	45	7	3	1.5	3	28	45.94	41.8823
27	45	6.5	5	1	2	20	43.47	41.2985
28	45	6.5	5	1	2	28	37.37	36.5102
29	45	7.5	7	1	4	24	48.07	47.0594
30	45	7	5	1	3	24	51.51	51.5100
31	45	7	5	1	3	24	51.51	51.5100
32	47	6.5	5	1.5	3	24	39.06	39.3504
33	45	6.5	3	1	4	24	46.15	47.9140
34	43	6.5	5	1.5	3	24	45.99	44.6896
35	45	7	7	0.5	3	20	42.27	44.2015
36	45	7	7	1.5	3	20	41.37	43.1431
37	47	7	5	0.5	2	24	40.42	42.0579
38	45	6.5	3	1	2	24	46.54	47.7319
39	45	7.5	3	1	2	24	50.84	52.3473
40	45	7	3	0.5	3	28	43.33	39.4306
41	47	7	5	1.5	4	24	42.04	41.9779
42	47	7	5	1.5	2	24	39.68	38.1746
43	43	7	5	1.5	2	24	43.68	45.9437
44	43	6.5	5	0.5	3	24	45.94	44.8579
45	47	7	3	1	3	20	42.75	39.9327
46	43	7	5	0.5	4	24	43.68	43.9654
47	45	6.5	7	1	2	24	45.76	45.6394
48	43	7	3	1	3	28	37.39	40.8798
49	45	6.5	5	1	4	20	43.68	42.3281
50	45	7	5	1	3	24	51.51	51.5100
51	45	6.5	7	1	4	24	48.97	47.2815
52	45	7.5	5	1	4	28	35.62	37.6102
53	47	7	7	1	3	28	36.29	33.5219
54	45	7	7	1.5	3	28	33.51	37.2260

2.5. Molecular weight and amino acids contents of xylanase

The molecular weight of xylanase obtained from *Bacillus tequilensis* strain in the present study was 359.0 KDa Fig. (5A). This molecular weight is comparable with the molecular weight that obtained by Amoresano, et al. [42], where they reported that the xylanase obtained from psychrophilic yeast by mass

spectrometry was 39508.4 KDa. In the present study, the amino acids sequencing of xylanase produced by *Bacillus tequilensis* strain were 16 amino acids started with aspartic acid forward with Serine, glutamic, proline, glycine, cystine, valine, methionine, isoleucine, lucine, tyrosine, phenyalanine, histidine, lysine, arginine and finally proline as Fig. (5B). In another study done by Rashad, et al. [43], they studied the amino acid composition of *Bacillius amyloliquefaciens* pure xylanase enzyme was 17 amino acids consistently rich in glutamic acid (31.54 %) followed by tyrosine (12.83 %), alanine (9.54 %), glycine (9.36 %), serine (5.85 %), phenylalanine (5.06 %) and threonine (4.27 %). Leucine, lysine, cystine, histidine and aspartic acid represented (2.45 – 3.80 %).

2.6. Bio-bleaching of waste paper by xylanase enzyme

In some countries have more than 81 paper and industries. Every year these industries in these countries continues to increase, where this increasing can cause more pollution that affected on the enviromentes in these countries because of the unavoidable use of chemicales [44]. Removing of lignin from pulp and paper by using of chemicales is one of the steps to obtain pulp with a more perfect brightness. The bleaching process is usually carried out in stage: the chlorination, extraction and addition of chlorine dioxide. Because of diffeculty degredation of these compoundes chemically or biologically so that they can last long on the earth's surface and have serious impact on the enviroment through possibly can enter into food and will accumlate in the human body or animals that are carcinogenic [45]. So found alternative method used for bleaching by using enzymes to reduce impact caused by the use of chlorine, and increase the strength of pulp and paper [46]. One enzyme that is used in biobleaching of pepar and pulp, where it used in the early stages of bleaching paper pulp.

Table (5): Show the xylanase activity, protein content and specific enzyme activity of purification profile fractions of xylanase obtained *Bacillus tequilensis* strain.

Fraction No.	Xylanase activity (U/mL)	Protein Content(mg/mL)	Specific enzyme activity (U/mg)
1	0	0	0
2	3.05	11.13	0.27
3	19.34	6.34	3.05
4	50.21	3.09	16.25
5	89.18	1.25	71.34
6	100.13	0.88	113.78
7	70.11	0.69	101.61
8	29.57	0.42	70.41
9	8.04	0.21	38.29
10	1.15	0.14	8.21

Xylanase produced by *Bacillus tequilensis* strain used in biobleaching, where it give the highest brightness (61.4 %), in case the control sample was (48 %), with increasing 13.4 % about the control sample as Table (6). Isabela, et al. [47] when they studied the brightness on paper pulp after treatment with xylanase obtained *Bacillus sp* TC-DT13. They found the increasing amount of brightness was about 5 %.

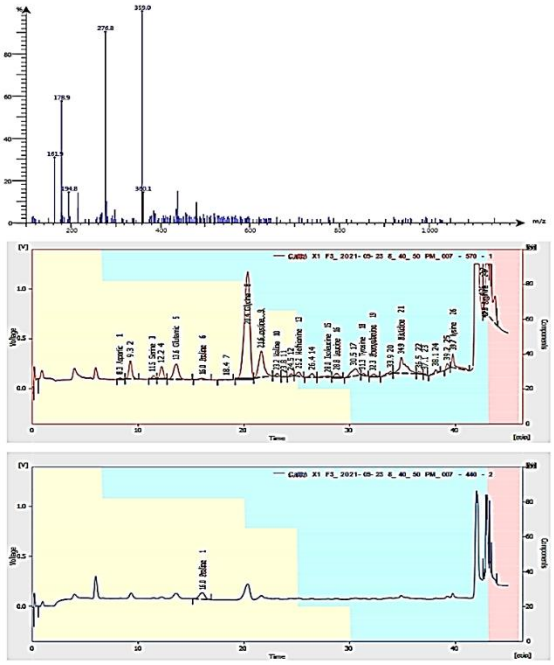

Fig. (5): Molecular weight of xylanase (A) and amino acids peaks (B) from *Bacillus tequilensis* strain.

Table 6. Physical properties of wastepaper after treatment with thermotolerant xylanase enzyme as compared with untreated sample (control).

Physical properties	Unit	Wastepaper treated with xylanase	Untreated wastepaper (control)
Whiteness	%	61.4	48
Whiteness index	%	27.92	-
Darkness	%	93.6	86.9
Softness	m/s	1669	1416

3. Conclusion

In the current, *Bacillus tequilensis* strain isolated from Ain-Helwan springs and identified by biochemical test and by 16s rRNA. *Bacillus tequilensis* strain have ability to produce extracellular xylanase through making clear zone around colony growth. Then the *Bacillus tequilensis* strain subjected to optimization for detection of the optimum parameters that give the best xylanase production. The optimum parameters were temperature 40 °C, the incubation period was 24 h, and the xylan concentration used for xylanase production 3 g/L, inoculum size 1 %, the best nitrogen source was peptone and the best pH 7. After that these

results subjected to Tukey HSD at $p < 0.05$. Also in this study the Box-Behnken design was utilized to find the best conditions for producing huge amounts of the xylanase enzyme. The results of this investigation can be used to improve settings for future fermenter scale-up experiments and to determine the economization of the xylanase enzyme manufacturing process. The obtained xylanase have 16 amino acids and molecular weight 359.0 KDa through characterization by amino acids analyzer and TLC mass. The xylanase in this study have the ability to brightness the waste paper, where the amount of increasing in brightness about 13.4 % about control sample resulting in improvement the quality of paper. This suggests that xylanase is appropriate for industrial usage, particularly in the bleaching process, because it lowers the use of toxic chemicals and is economically safe.

Acknowledgments
Authors are greatly thankful to Dr. Amr H. Hashem for his support and statistical analysis.

Conflict of Interest: The authors declare no competing interests.

5. References

[1] Patel, A. K., Singhania, R. R., Pandey, A., Production, purification, and application of microbial enzymes, *Biotechnology of Microbial Enzymes*, Elsevier 2017, pp. 13-41.

[2] Bhardwaj, N., Kumar, B., Verma, P., A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresources and Bioprocessing* 2019, *6*, 1-36.

[3] Chakdar, H., Kumar, M., Pandiyan, K., Singh, A., *et al.*, Bacterial xylanases: biology to biotechnology. *3 Biotech* 2016, *6*, 1-15.

[4] Singh, B., Production, characteristics, and biotechnological applications of microbial xylanases. *Applied microbiology and biotechnology* 2019, *103*, 8763-8784.

[5] Ahmed, N. E., Salem, S. S., Hashem, A. H., Statistical optimization, partial purification, and characterization of phytase produced from talaromyces purpureogenus nsa20 using potato peel waste and its application in dyes de-colorization. *Biointerface Research in Applied Chemistry* 2022, *12*, 4417-4431.

[6] Jabborova, D., Choudhary, R., Azimov, A., Jabbarov, Z., *et al.*, Composition of Zingiber officinale Roscoe (Ginger), Soil Properties and Soil Enzyme Activities Grown in Different Concentration of Mineral Fertilizers. *Horticulturae* 2022, *8*, 43.

[7] Basit, A., Liu, J., Rahim, K., Jiang, W., Lou, H., Thermophilic xylanases: from bench to bottle. *Critical reviews in biotechnology* 2018, *38*, 989-1002.

[8] Dhaver, P., Pletschke, B., Sithole, B., Govinden, R., Isolation, screening, preliminary optimisation and characterisation of thermostable xylanase production under submerged fermentation by fungi in Durban, South Africa. *Mycology* 2022, 1-22.

[9] Duarte, M. E., Zhou, F. X., Dutra, W. M., Kim, S. W., Dietary supplementation of xylanase and protease on growth performance, digesta viscosity, nutrient digestibility, immune and oxidative stress status, and gut health of newly weaned pigs. *Animal Nutrition* 2019, *5*, 351-358.

[10] Dhevagi, P., Ramya, A., Priyatharshini, S., Geetha Thanuja, K., *et al.*, Industrially important fungal enzymes: productions and applications, *Recent Trends in Mycological Research*, Springer 2021, pp. 263-309.

[11] Ahmed, S., Riaz, S., Jamil, A., Molecular cloning of fungal xylanases: an overview. *Applied Microbiology and Biotechnology* 2009, *84*, 19-35.

[12] Pinales-Márquez, C. D., Rodríguez-Jasso, R. M., Araújo, R. G., Loredo-Trevino, A., *et al.*, Circular bioeconomy and integrated biorefinery in the production of xylooligosaccharides from lignocellulosic biomass: A review. *Industrial Crops and Products* 2021, *162*, 113274.

[13] Samanta, A. K., Jayapal, N., Kolte, A. P., Senani, S., *et al.*, Enzymatic production of xylooligosaccharides from alkali solubilized xylan of natural grass (Sehima nervosum). *Bioresource Technology* 2012, *112*, 199-205.

[14] Gaurav, N., Sivasankari, S., Kiran, G. S., Ninawe, A., Selvin, J., Utilization of bioresources for sustainable biofuels: A Review. *Renewable and Sustainable Energy Reviews* 2017, *73*, 205-214.

[15] Malgas, S., Mafa, M. S., Mkabayi, L., Pletschke, B. I., A mini review of xylanolytic enzymes with regards to their synergistic interactions during hetero-xylan degradation. *World Journal of Microbiology and Biotechnology* 2019, *35*, 1-13.

[16] Chadha, B. S., Kaur, B., Basotra, N., Tsang, A., Pandey, A., Thermostable xylanases from thermophilic fungi and bacteria: current perspective. *Bioresource technology* 2019, *277*, 195-203.

[17] Cai, Z.-W., Ge, H.-H., Yi, Z.-W., Zeng, R.-Y., Zhang, G.-Y., Characterization of a novel psychrophilic and halophilic β-1, 3-xylanase from deep-sea bacterium, Flammeovirga pacifica strain WPAGA1. *International journal of biological macromolecules* 2018, *118*, 2176-2184.

[18] Monteiro, C. R., Avila, P. F., Pereira, M. A. F., Pereira, G. N., *et al.*, Hydrothermal treatment on depolymerization of hemicellulose of mango seed shell for the production of

xylooligosaccharides. *Carbohydrate Polymers* 2021, *253*, 117274.
[19] Kiran, E. U., Akpinar, O., Bakir, U., Improvement of enzymatic xylooligosaccharides production by the co-utilization of xylans from different origins. *Food and Bioproducts Processing* 2013, *91*, 565-574.
[20] Hatanaka, K., Incorporation of Fluorous Glycosides to Cell Membrane and Saccharide Chain Elongation by Cellular Enzymes, in: Horváth, I. T. (Ed.), *Fluorous Chemistry*, Springer Berlin Heidelberg, Berlin, Heidelberg 2012, pp. 291-306.
[21] Eid, A. M., Fouda, A., Abdel-Rahman, M. A., Salem, S. S., *et al.*, Harnessing Bacterial Endophytes for Promotion of Plant Growth and Biotechnological Applications: An Overview. *Plants (Basel, Switzerland)* 2021, *10*.
[22] Goluguri, B. R., Thulluri, C., Cherupally, M., Nidadavolu, N., *et al.*, Potential of Thermo and Alkali Stable Xylanases from Thielaviopsis basicola (MTCC-1467) in Biobleaching of Wood Kraft Pulp. *Applied Biochemistry and Biotechnology* 2012, *167*, 2369-2380.
[23] Singh, V., Pandey, V. C., Agrawal, S., Potential of Laceyella sacchari strain B42 crude xylanase in biobleaching of kraft pulp. *African Journal of Biotechnology* 2013, *12*.
[24] Walia, A., Guleria, S., Mehta, P., Chauhan, A., Parkash, J., Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. *3 Biotech* 2017, *7*, 1-12.
[25] Kumar, V., Marín-Navarro, J., Shukla, P., Thermostable microbial xylanases for pulp and paper industries: trends, applications and further perspectives. *World Journal of Microbiology and Biotechnology* 2016, *32*, 1-10.
[26] Bhardwaj, N., Verma, P., Microbial Xylanases: A Helping Module for the Enzyme Biorefinery Platform. *Bioenergy Research: Evaluating Strategies for Commercialization and Sustainability* 2021, 129-152.
[27] Contreras, F., Amenabar, M. J., Blamey, J. M., Purification and characterization of two thermostable xylanases from a halotolerant Bacillus sp. Asc6BA isolated from Salar de Ascotán, Atacama Desert. *Extremophiles* 2021, *25*, 51-59.
[28] El-Sersawy, M. M., Hassan, S. E., El-Ghamry, A. A., El-Gwad, A. M. A., Fouda, A., Implication of plant growth-promoting rhizobacteria of Bacillus spp. as biocontrol agents against wilt disease caused by Fusarium oxysporum Schlecht. in Vicia faba L. *Biomolecular concepts* 2021, *12*, 197-214.
[29] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 1976, *72*, 248-254.
[30] Ismail, M. A., Amin, M. A., Eid, A. M., Hassan, S. E., *et al.*, Comparative Study between Exogenously Applied Plant Growth Hormones versus Metabolites of Microbial Endophytes as Plant Growth-Promoting for Phaseolus vulgaris L. *Cells* 2021, *10*.
[31] Fouda, A., Eid, A. M., Elsaied, A., El-Belely, E. F., *et al.*, Plant Growth-Promoting Endophytic Bacterial Community Inhabiting the Leaves of Pulicaria incisa (Lam.) DC Inherent to Arid Regions. *Plants (Basel, Switzerland)* 2021, *10*.
[32] Bakry, M. M., Salem, S. S., Atta, H. M., El-Gamal, M. S., Fouda, A., Xylanase from thermotolerant Bacillus haynesii strain, synthesis, characterization, optimization using Box-Behnken Design, and biobleaching activity. *Biomass Conversion and Biorefinery* 2022.
[33] Shreif, O., Shehabeldine, A. M., Abu-Elghait, M., Hamed, A. A., Desouky, S. E., Statistical optimization of chromium (VI) reduction using response surface methodology (RSM) by newly isolated Stenotrophomonas sp. (a novel strain). *BioMetals* 2022, *35*, 99-114.
[34] Abdel-Hamid, M. S., Fouda, A., El-Ela, H. K. A., El-Ghamry, A. A., Hassan, S. E., Plant growth-promoting properties of bacterial endophytes isolated from roots of Thymus vulgaris L. and investigate their role as biofertilizers to enhance the essential oil contents. *Biomolecular concepts* 2021, *12*, 175-196.
[35] Lawrence, R., Kumar, Y., Singh, A. K., Singh, S., Production and optimization of xylanase by thermophilic Bacillus sp. isolated from soil. *J Pure Appl Microbiol* 2013, *9*, 1117-1128.
[36] Marimuthu, M., Sorimuthu, A., Muruganantham, S., Production and optimization of xylanase enzyme from Bacillus subtilis using agricultural wastes by solid state fermentation. *International Journal of Pharmaceutical Investigation* 2019, *9*, 169-173.
[37] Paul, M., Nayak, D. P., Thatoi, H., Optimization of xylanase from Pseudomonas mohnii isolated from Simlipal Biosphere Reserve, Odisha, using response surface methodology. *Journal of Genetic Engineering and Biotechnology* 2020, *18*, 81.
[38] Kumar, S. S., Abrar, A., Jai, G., Raj, M. D., Partial Purification and Characterization of Xylan Degrading Alkaline Xylanase from Bacillus Subtilis'. *Research Journal of Chemistry and Environment* 2019, *23*.
[39] Lee, J.-H., Choi, S.-H., Xylanase production by Bacillus sp. A-6 isolated from rice bran. *Journal of microbiology and biotechnology* 2006, *16*, 1856-1861.

[40] Sharma, P. K., Chand, D., Purification and Characterization of Thermostable Cellulase Free Xylanase from <i>Pseudomonas</i> Sp. XPB-6. *Advances in Microbiology* 2012, *Vol.02No.01*, 9.

[41] Gessesse, A., Mamo, G., Purification and characterization of an alkaline xylanase from alkaliphilic Micrococcus sp AR-135. *Journal of Industrial Microbiology and Biotechnology* 1998, *20*, 210-214.

[42] Amoresano, A., Andolfo, A., Corsaro, M. M., Zocchi, I., *et al.*, Structural characterization of a xylanase from psychrophilic yeast by mass spectrometry. *Glycobiology* 2000, *10*, 451-458.

[43] Rashad, M. M., Mahmoud, A. E., Nooman, M. U., El-Torky, A., *et al.*, Purification and characterization of a new thermoalkali-stable xylanase produced from Bacillus amyloliquifaciensNRRL B-14393 by solid-state fermentation of water hyacinth. *Res J Pharm BiolChemSci, 2015b* 2015, *6*, 467-478.

[44] Söderholm, P., Bergquist, A.-K., Söderholm, K., Environmental Regulation in the Pulp and Paper Industry: Impacts and Challenges. *Current Forestry Reports* 2019, *5*, 185-198.

[45] Valchev, I., Tsekova, P., Xylanase post-treatment as a progress in bleaching processes. *Appita: Technology, Innovation, Manufacturing, Environment* 2010, *63*, 53-57.

[46] Cheng, X., Chen, G., Huang, S., Liang, Z., Biobleaching effects of crude xylanase from Streptomyces griseorubens LH-3 on eucalyptus kraft pulp. *BioResources* 2013, *8*, 6424-6433.

[47] Rodrigues, I. d. S. V., e Silva, C. G. S., da Silva, R. S., Dolabella, S. S., *et al.*, Screening of bacterial extracellular xylanase producers with potential for cellulose pulp biobleaching. *Acta Scientiarum. Biological Sciences* 2019, *41*, 42101.