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Growth and xylanase production by bacteria Isolated from Agricultural Soil samples

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Abstract
Xylanase research has noticeably increased due to its potential applications in pulping and bleaching, textile, and waste treatment. The present study aimed to isolate and characterize the xylan degrading bacteria from the soil. Many bacteria degrade xylan through production of xylanase. Twenty bacterial isolates were obtained from soil samples of different areas in Jeddah and studied to detect xylanase activity. Nine bacterial isolates showed good Xylanase activities and the most active isolates was isolate TM16 which was selected for detail studies. The isolate was belong to the genus Bacillus and identified as B. amyloliquefaciens TM 16, based on morphological and physiological characters. B. amyloliquefaciens TM 16 produced xylanase extracellularly in liquid medium containing xylan as carbon source. The optimum temperature and pH for maximum xylanase production were determined to be 50◦C and 6.0, respectively after 4 days of growth at 120 rpm in xylan broth medium which contained xylan as a carbon source. In conclusion, bacteria from soil can be used for xylan degradation in agricultural wastes and optimization of growth conditions enhanced degradation activities.

Key words: ***xylanases, xylan, Bacillus amyloliquefaciens, enzyme activity***

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I. Introduction

Hemicellulose is the second most abundant natural polysaccharide after cellulose; its degradation can generate low-cost raw materials for industrial applications. Xylan is the major hemicellulose component of the plant cell wall, usually accounting for 20 to 30 % of its total dry mass (Collins *et al*., 2005). Also, Xylan is a major component of hemicelluloses in plants and is made up of a polymer of xylose molecules, which can hold the cell walls together. Xylan is a linear of β-(1, 4)-D-xylose backbone and substituted with different side chains especially α-L-arabinosyl and α-D-glucuronosyl units (Khusro *et al*., 2016). Xylan was degraded mainly by Xylanase which identified as an extracellular enzyme that is responsible for xylan hydrolysis forming usable products such as xylooligosaccharides (xylose, xylobiose) (Ahmed *et al*., 2016). Studies have shown that xylanases are produced by a variety of sources, including bacteria, fungi, yeast, algae (Mandal, 2015). However, fungal and bacterial xylanases enzymes have different characteristics. Therefore, xylanases produced by bacteria and actinomycetes like the genera *Bacillus*, *Pseudomonas* and Streptomyces are efficient in a broader pH range of 5 to 9 and temperature of 35-60◦C (Motta., 2013, Mandal, 2015, Binod et al., 2019) being useful in different industries due to their alkali tolerance and thermostability, like the pulp and paper industry. Fungal xylanases from *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. are effective at a pH range of 4 to 6 and temperature below 50◦C (Mandal, 2015), thus being used in limited industrial applications. However, bacterial and fungal xylanases with higher activity are important in wastes degradation in soil and the presence of cellulase enhanced the degradation process in soil. This study aimed to isolation and identification of some bacterial isolates from soil or agriculture wastes, active in xylan degradation and studying factors affecting the degradation process

II. Materials and Methods

Different soil samples were collected from the agricultural area in Jeddah, Saudi Arabia, by sharp spade at 5 cm deep, kept in sterile plastic bags, brought to the science laboratory, king Abdulaziz University, and processed within two hours.

Isolation of xylanase producing bacteria

Approximately one gram of each soil sample was weighed and suspended in 100 ml of sterile distilled water. Then serial dilution was done up to ten and 0.1 ml of each dilution plated on a xylan agar medium. All

plates were incubated at 37°C for 2-3 days. To obtain pure colonies, morphologically distinct colonies were collected and streaked onto the same medium.

Screening of xylanase producing bacteria

To detect the xylanase production ability, microbial strains were inoculated on 0.1 % xylan agar medium (Composition: g/l; yeast extract 3.0, peptone 1.5, NaCl 3.5, $NaNO_3$ 1.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.3 Agar 20, and 0.1 % xylan) plates. Plates were incubated at 37°C for 72 hrs. All the plates were stained with 0.5 % Congo red dye for about half an hour and were then destained using 1 M NaCl solution at room temperature. Zones of clearance were observed for the presence of xylanase activity (Kalim and Ali, 2016).

Xylanase activity assay

The xylanase activity was examined by the 3,5-dinitro salicylic acid (DNS) method by measuring the amount of reducing sugars liberated from xylan and using a calibration curve for D-xylose. Xylose was used as the standard. Assay mixture (0.5% xylan - 0.5 ml, phosphate buffer - 50 mM, pH 7.0, and enzyme - 1.5 ml) was incubated at 50°C for 15 min, then add 1.5 ml of DNS reagent was added to terminate the reaction. Test tubes containing reaction mixture were stoppered and kept in a boiling water bath for 10 minutes and then cooled to room temperature. The absorbance was recorded against the reagent as blank at 575 nm, keeping the enzyme as control. One unit of xylanase activity is defined as the amount of enzyme required to produce one µM of xylose released in 1 min (Kumar *et al.*, 2018).

Effect of incubation temperature

About 2 ml of the preculture of the selected isolates containing (4×10^4 CFU/ml) was inoculated in the 250 ml Erlenmeyer flasks containing 48 ml of xylanase production medium (Torkashvand *et al*., 2020). After that, the flasks were incubated at different temperatures such as 20**,** 30, 37, 40, 45, 50 and 60°C for 3 days with agitation at 120 rpm. At the end of the growth period the growth of inoculated bacteria and enzyme assay were measured as described before.

Effect of pH

The xylanase production medium was prepared with different pH as following 5.5, 6.0, 7.0, and 8.0 by adjusted the medium with NaOH or HCl. Then, about 2 ml of the preculture of the selected isolates containing (4×10^4 CFU/ml) was inoculated in the 250 ml Erlenmeyer flasks containing 48 ml of xylanase production medium (Torkashvand *et al*., 2020) and incubated flasks at 37°C with agitation at 120 rpm for 3 days. Then, the growth of inoculated bacteria and enzyme assay were measured as described before.

Effect of incubation period

About 2 ml of the preculture of the selected isolates containing (4×10^4 CFU/ml) was inoculated in the 250 ml Erlenmeyer flasks containing 48 ml of xylanase production medium (Torkashvand *et al*., 2020). After that, the flasks were incubated at 37°C and 120 rpm for different time periods ranging from 1 to 6 days. At the end of the growth period the growth of inoculated bacteria and enzyme assay were measured as described before.

III. Results

About four different agriculture soil samples were collected and used for bacterial isolation on nutrient agar medium at 37ºC for two days. The obtained bacterial isolates were purified on the same medium and screened on xylan agar medium which contain xylan as a carbon source at 37ºC for 3 days to detect the active isolates.

Out of 20 bacterial isolates, 9 isolates were active in xylanase production. The active isolates showed degradation activity for xylan using Congo red as indicator. Isolates TM16, TM33, and TM37 showed excellent activity (+++) while isolates TM5, TM9, TM11, TM36, TM38, and TM48 recorded moderate activity (Table 1). These isolates had different shapes and colors on nutrient agar medium. They grow well on both nutrient agar and xylan agar medium as reported in Table 1.

Table1. The growth of some bacterial isolates obtained from the soil on nutrient agar (N.A) and xylan agar (X.A

Bacteria Samples	Source	Colony color	Colony elevation	Colony surface	Growth on N.A	Growth on X.A
TM5	Soil 1	Creamy	Convex	Smooth	+++	++
TM9	Soil 1	White	Convex	Rough	+++	++
TM11	Soil 3	Creamy	Flat	Smooth	+++	++

TM16	Soil 3	Creamy	Flat	Rough	+++	+++
TM33	Soil 1	Creamy	Convex	Smooth	+++	++
TM36	Soil 3	Creamy	Convex	Rough	+++	++
TM37	Soil 4	Creamy	Flat	Rough	+++	++++
TM38	Soil 2	Creamy	Flat	Smooth	+++	+++
TM48	Soil 4	Light yellow	Convex	Smooth	+++	++

All the selected isolates were grown in xylan production broth medium for 3 days and growth and enzyme production of the 9 selected bacterial isolates were determined and compared. The enzyme activity was detected from a standard curve of Xylose. The lowest growth was for isolates TM5, TM16, and TM38 while the highest growth was recorded for isolates TM11, TM36, and TM37 on the other hand; lower enzyme activity was recorded for TM5, TM33, and TM36 while moderate activity was recorded by TM9, TM37, and TM48. The highest xylanase production was recorded by the isolate TM16 (Table 2).

Table 2. Growth and enzyme production of the nine selected bacterial isolates

Bacterial isolates	Growth ($A_{600\ nm}$)	Enzyme assay ($A_{575\ nm}$)	U/ml
TM5	1.551± 0.21	0.784	6.27±0.30*
TM9	1.899± 0.31*	1.605	12.84±2.5*
TM11	1.997± 0.31*	1.464	11.71±1.5*
TM16 (control)	1.611± 0.41	1.999	15.99
TM33	1.970± 0.61*	0.883	7.06±0.90*
TM36	1.999± 0.16*	0.612	4.90±0.12*
TM37	1.990± 0.18*	1.879	15.03±0.33
TM38	1.416± 0.13	1.317	10.54±0.89*
TM48	1.987± 0.01*	1.729	13.83±0.23

***: significant results at p≤ 0.5**

Morphological and biochemical characters of the selected bacterial isolate were shown in Table 3. The colonies of the isolate TM16 have Creamy color, Rough surface and Round shape. The diameter of the cell was 0.5-1.5 µm and the cells were gram positive bacilli. The isolate showed positive results for Catalase test, Motility, Hemolysis, Gelatin liquefaction, Citrate utilization, and Methyl red and negative results for Oxidase test, Indole, Urease, and Voges-proskauer. The isolate TM16 being sensitive to Nalidixic Acid, Nitrofurantoin, Norfloxacin, Ceftazidime, Imipenem, Piperacillin, and Ciprofloxacin and resistance to Cephalothin, Ampicillin, and Cotrimoxazole (Table 3).

Table3. Morphological and biochemical characters of the selected bacterial isolate TM16

Morphological characteristic	**Results**	**Biochemical test**	**Results**	**Antibiotics**	**Results**
Colony color	Creamy	Catalase test	+	Nalidixic Acid	Sensitive
Surface texture	Rough	Oxidase test	-	Nitrofurantoin	Sensitive
Colony elevation	Convex	Motility	+	Cephalothin	Resistance
Form	Round	Hemolysis	+	Ampicillin	Resistance
Margin	Entire	Indole	-	Cotrimoxazole	Resistance
Cell diameter	0.5-1.5 µm	Urease	-	Norfloxacin	Sensitive
Shape	Bacilli	Gelatin liquefaction	+	Ceftazidime	Sensitive
Spore	+	Citrate utilization	+	Imipenem	Sensitive
Capsule	-	Methyl red	+	Piperacillin	Sensitive
Gram stain	+	Voges-proskauer	-	Ciprofloxacin	Sensitive

+: Positive results -: Negative results

Effect of incubation temperature on growth and enzyme production of the selected bacterial isolate TM16. The best growth was recorded at 45ºC while the lowest was at 20, 50, and 60ºC. Moderate growth was recorded at 30, 37, and 40ºC. The best enzyme production was recorded at 50ºC while the lowest was at 20, 30ºC. Enzyme production recorded at 37, 40, and 45 ºC showed no significant differences compared to control.

Effect of incubation period on growth and enzyme production of the selected bacterial isolate TM16. The best growth was recorded after 4 and 5 days while the lowest was after 1 and 2 days. Moderate growth was recorded after 3 and 6 days. The best enzyme production was recorded after 4 days while the lowest after 6 days. Moderate enzyme production was recorded after 2, 4, 5 and 6 days.

Effect of pH on growth and enzyme production of the selected bacterial isolate TM16 was showed in Table 5. The best growth was recorded at pH 6 and 7 while the lowest was at 5.5. Moderate growth was recorded at pH 8. The best enzyme production was recorded at pH 6 and pH 7 while the lowest was at pH 8. Moderate enzyme production was recorded at pH 5.5.

Table 4. Effect of incubation temperature on growth and enzyme production of the selected bacterial isolate TM16

Temperature °C	Growth ($A_{600\ nm}$)	Enzyme assay ($A_{575\ nm}$)	Enzyme activity (U/ml)
20	1.055±0.22	1.191	9.53±1.54*
30	1.396±0.25	1.466	11,73±0.34*
37 (control)	1.594±0.5	1.818	14.54±0.33
40	1.70±0.12	1.850	14.80±0.32
45	1.79±0.10	1.938	15.51±1.11
50	1.306±0.3	1.985	15.88±1.04
60	0.378±0.4	1.623	12.98±1.21*

***: significant results at p≤ 0.5**

Table 5. Effect of incubation period on growth and enzyme production of the selected bacterial isolate TM16

Period (days)	Growth ($A_{600\ nm}$)	Enzyme assay ($A_{575\ nm}$)	Enzyme activity (U/ml)
1.0	1.185±0.15*	1.523	12.18±0.11
2.0	1.399±0.17*	1.643	13.14±0.22
3.0 (control)	1.463±0.19	1.785	14.28±0.31
4.0	1.794±0.12*	1.985	15.88±0.13*
5.0	1.707±0.01*	1.687	13.50±0.15
6.0	1.587±0.10*	0.800	6.40±0.25*

***: significant results at p≤ 0.5**

Table 6. Effect of pH on growth and enzyme production of the selected bacterial isolate TM16

pH	Growth (600 nm)	Enzyme assay ($A_{575\ nm}$)	Enzyme activity (U/ml)
5.5	0.345±0.11	1.737	13.90±0.12
6.0	1.678±0.19	1.989	15.81±0.13
7.0	1.714±0.51	1.985	15.88±0.23
8.0	1.532±0.35	1.469	11.7±0.125

***: significant results at p≤ 0.5**

IV. Discussion

Microbial enzyme market increased up to 3.7 billion dollars by 2019 and the growth rate was increased by more than 8.0% each year during the period of 2013 to 2018. Moreover, 70% of the these enzymes had many applications in paper, medicine and food industries (Butt et al. 2008). Xylanases, cellulases, mannanases, and pectinases are the main useful microbial enzymes that hydrolyze agriculture wastes (Gusakov et al. 2011, Torkashvand et al., 2020). Depolymerization of xylan was observed mainly by a hydrolytic enzyme called Xylanase (Rajoka 2007).

Xylanases are widely used in vast industrial processes like preparation of animal feed (Bhat 2000), juice and dough indicteries (Bajpai 1999; Jia et al. 2011, Qaseem et al., 2021). Using submerged and solid state fermentation, xylanases can be produced from actinomycetes, bacteria (Azeri et al. 2010), mold (Cao et al. 2008; Chapla et al. 2010, Thomas et al. 2013, 2014), and yeast (Xin and He 2013)

In this study, the more extensively used method, SmF, was used due to good enzyme yield, low time and simple fermentation methods (Renge 2012). From different soil bacterial genera, the isolate TM16 was the most active to grow and degradation of xylan. It was characterized using different morphological and biochemical methods and identified as species of the genus *Bacillus*. Monoculture *of Aspergillus species, Bacillus species* or *Kluyveromyces* species are wildly used for xylanase production on contrast Gutierrez-Correa and Tengerdy (1998) reported that about 45% higher xylanase production was recorded using cocultures of *Trichoderma reesei* with either *Aspergillus niger* or *A. phoenicis* while Garcia-Kirchner et al. (2002) recorded higher xylanase production using a mixed cultue of both *Penicillium* sp. and *A. terreus.*

In this study, the growth of the isolate TM16 and xylanase productions were directly proportional with time and maximum growth using xylan as a carbon source was at 50◦C, pH 7 and after 4 days while the highest xylanase production was recorded at 50◦C, pH 6-7 after 4 days then, a decrease was observed in enzyme production**.** Similar to our results, Ling Ho and Heng (2015) detected the highest xylanase activity (11.968±1.419 U/ml with *B. subtilis* in SmF using wheat bran and soybean hull after 48 hrs. Thus, microbial strain and carbon sources can affect the timing of maximal xylanase enzyme production. Our results showed that xylanase production is growth dependent at the first but the enzyme reduction when growth is good may due to

the presence of inhibitory metabolites (Niladevi and Prema 2008). On contrary, Bocchini et al. (2002) used *Bacillus circulans* for maximum xylanase production after 24 hrs of incubation. Xylanase production by single and co-cultures of *B. subtilis* and *K. marxianus* was evaluated at35 °C and, pH 6.5 after 24 hrs. When fermentation medium The study of Garcia-Kirchner et al. (2002), reported that *Penicillium* sp. and *Aspergillus terreus* produced lower xylanase activities while the co-culture of both supports the results and the best enzyme was obtained after 24 hrs but the biomass increased up to 72 hrs, then decreased slowly.

In conclusion, soil are rich in agricultural wastes that contain xylan and different bacteria used xylan as a carbon source to produce energy. Genus *Bacillis* was found in soil and can used xylan effectively. Physical factors like temperature, incubation period and medium pH affected both growth and xylanase production. Thus, bacteria can be used for production of xylanases for many biotechnological applications.

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