XYLANASE PRODUCTION FROM LOCAL BACTERIAL ISOLATE

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ABSTRACT

Seventeen local isolates of *Bacillus* were isolated from soil to produce extracellular xylanase under submerged fermentation process by using xylan as carbon sole source. All isolates were subjected to quantitative scanning to select the most efficient one. The highest activity of xylanase (2680u/ml) was obtained from isolate *Bacillus* sp RS1. The isolate identified by 16SrRNA gene sequence of *Bacillus subtilis* (accuracy of 99%)which was matched with sequence of *Bacillus subtilis* VBN25 that recorded in Genebank under the Accession Number of MG027675.1.Extracted xylan from agricultural waste by acidic method(papyrus, sun flower stalks, Ibaa Wheat type, Furat wheat type and Abo Ghraib wheat type)were used as the substrate for xylanase production from *Bacillus.* The results showed that the papyrus gave the highest amount of xylan (187.6µ g/ml) as compared with that of the sun flower stalks, Ibaa Wheat type, Furat wheat type and Abo Ghraib wheat type(161.3, 161.6, 157.6, 157.2) µ g/ml respectively. The results indicated that the highest xylanase activity was 2800 u/ml produced by *Bacillus subtilus* when Papyrus xylan was used.

Keywords: *Bacillus subtilis*, 16S rRNA , Xylan hydrolyzing enzymes, hemicelluloses.
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انتاج الزايلينيز من عزلة بكتريا محلية

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المستخلص

تم الحصول على سبعة عشر عزلة محلية لبكتريا ***Bacillus*** منتجة لانزيم الزايلنيز **Xylanase** باستعمال نظام تخمرات المزارع المغمورة بأستعمال الزايلان **Xylan** مصدرا وحيدا للكاربون، تم الحصول عليها من التربة. خضعت جميع العزلات للغربلة الكمية لاختيار الاكثر كفاءة في انتاج الانزيم، اعلى فعالية لانزيم الزايلنيز بلغت 2680 وحدة /مل ، تم الحصول عليها من العزلة ***Bacillus* sp RS1** . شخصت العزلة جزيئيا بالاعتماد على تتابعات القواعد النتروجينية للجين **16S rRNA**على انها ***Bacillus subtilus*** وبنسبة تطابق 99%بالمقارنة مع تتابعات القواعد النتروجينية للعزلة العالمية ***Bacillus subtilis*** **VBN25** في بنك الجينات **NCBI** بالرقم MG027675.1. استعمل الزايلان المستخلص من الفضلات الزراعية(البردي وسيقان زهرة الشمس والحنطة اصناف اباء وفرات وابو غريب) .اكدت النتائج ان اعلى نسبة من الزايلان تم الحصول عليها من البردي (187.6 µ g/ml) بالمقارنة مع سيقان زهرة الشمس والحنطة اصناف (اباء وفرات وابو غريب) حيث بلغت كمية الزايلان(161.3, 161.6, 157.6, 157.2) مايكروغرام/مل على التوالي. اكدت النتائج ان اعلى فعالية لانزيم الزايلنيز تم الحصول عليها من زايلان القصب حيث بلغت 2800 وحدة/مل باستعمال بكتريا ***Bacillus subtilis*** .

الكلمات المفتاحية: الانزيمات المحللة للزايلان، الهيميسيليلوز.16S rRNA، *Bacillus subtilis*
بحث مستل من اطروحة دكتوراه للباحث الاول

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INTRODUCTION

Many bacterial genera including *Bacillus*, *Cellulomonas*, *Clostridium*, *Rumminococcus*, *Acetivibrio*, *Bacteriodes*, and *Alteromonas* can produce xylanas (17).*Bacillus subtilis* is nonpathogenic and nontoxigenic bacterium that has been used a source of enzymes (19).

Xylanase (E.C 3.2.1.8) is a type of hydrolytic enzyme, degrades *β*-1, 4 xylan, to produce xylose and xylooligosaccharides like xylobiose, Xylan is substrate of Xylanase, renewable organic materials and a major component of hemicellulose in plant (9),(12). Biodegradation of xylan, is a complex process that requires action of several enzymes, among which xylanase (1,4-β-D-xylan, EC 3.2.1.8) play a key role and β-xylosidase, ά-glucuronidase, furanosidase,ά-arabinose and esterase (13), (16). Xylanase has many applications in the food, feed, pulp and paper industry because of its bleach boosting properties, improvement of nutritional properties of cereal diets in poultry, improvement of bakery products, clarification of fruit juice and biofuel (1),(11). The aim of this study is to get *Bacillus* isolate from local sources which produce a large amounts of xylanase enzyme by using agricultural waste.

MATERIALS AND METHODS

Bacterial isolates sources

The sample of Bacterial isolates were collected at depth of 5-7cm from different zones of Baghdad university soils. The samples kept in sterile plastic back until time for use.

Culture media

Isolate media: The isolate media was prepared according to (17). Xylan 0.5g, yeast extract 0.5g, peptone 0.5g, KH_2PO_4 0.1g, $MgSO_4.{}_7H_2O$ 0.02g, Congo red 0.015g, $Ca_2CO_3$0.01g, Agar 2g The media was autoclaved at 121^o C and 15 pound/inch^2 for 15 min.

Isolation of *Bacillus* species

Each soil sample (20g) was suspended in sterile distilled water in a steril bottle(total volume of 20 ml, vortexed and placed in water bath at 100 $^{\circ}$C for 5 min with shaking. Heat treated soil suspensions were incubated at room temperature for 2h and serially diluted from 10^{-1} to 10^{-7}, 1ml from each dilute was transferred to petri dish followed by adding above mentioned media. All petri dishes were incubated at 35°C for 48h. The transferring process was repeated for several times for colonies which have clear zone to get pure isolates.

Identification of Bacillus isolates

Study of cultural and morphological characteristics of Bacterial isolates

The cultural characteristics of bacterial isolates on NA media and morphological characteristics, was carried out by using Gram and malachite stains according to method of (7). The shape of bacterial cells and the presence of spores were examid under microscopic field.

Catalase test

Small amount of maintend colonies was transferred to the surface of a clean dry glass slide, a drop of 3% $H_2O_2$was placed on the top of colonies for testing oxygen bubbles formation (7).

Quantitative screening

Production media

The media was used for quantitative screening was prepared according (5) contained Xylan 0.5g، Peptone 0.5g، NaCl 0.1g ،K_2HPO_4 0.2g ، $CaCl_2$ 0.01g، $MgSO_4.{}_7H_2O$ 0.01g، and Yeast extract 0.1g were dissolved in 100 ml distilled water, and pH was adjusted to 7. Media was autoclaved and used for screening xylanase production isolates.

Screening the isolates for xylanase production

Production media was placed in to 250 ml Erlenmeyer flask, autoclaved at 121c$^{\circ}$ for 15 min, Flasks were inoculated with 1ml cell suspension at adensity of 10^7 cell /ml media.The fermentation was carried out in ashaking incubater at aspeed of 150 rpm at 37 c$^{\circ}$ for 48 hr (5). After the fermentation process was finished, the supernatant was separated by centerfuge at aspeed 12000 rpm for 10 min at 4c^{o} (13).The supernatant was used as the source of crude enzyme for xylanase assay.

Enzyme activity assay

Crude xylanase activity was assay in supernatant according to (10). The reaction mixture contained 0.9 mL substrate (prepared by dissolve 1g xylan in 100 ml phosphate buffer, 50 mM, pH7), 0.1 mL of enzyme sample. The assay mixture was incubated at 50 °C/5min. one mL DNS reagent was added to the reaction mixture, boiled for 5 min. Then,

the mixture cooled down. The absorbant read by spectrophotometer at a wavelength of 540 nm. The amount of xylose produced by enzymes function was determined using xylose standard curve. One unit of xylanase enzyme was defined as the amount enzyme required to release 1 µm of xylose per minute in under assay condition.

Molecular identification

DNA Extraction: The local *Bacillus* sp. isolate (RS1) was cultured on NB at 37°C on rotary shaker at 150 rpm for 24 hr. 3 ml of the broth media culture was centrifuged at 13000 x g for 1 min. (the supernatant was discarded). 100-500 mg of precipitated biomass cells was taken and smashed under liquid nitrogen by using a mortar. The smashed sample was transferred to a 1.5 ml Eppendorf tube. A molecular biology kit (from Presto Mini g DNA Bacteria Kit, Tiwan) was used to extract the DNA from the isolate according to the kit extraction method. DNA purification was estimated by Nano drop

Table 1. The sequence of used primers

Primer	Sequence
Forward	5'-CGGGTGAGTAACACGTG-3'
Reverse	5'-CGGTGTGTACAAGCCC-3'

Polymerase chain reaction (PCR)

PCR was used to amplify the 16S rRNA of the rRNA gene. The forward primer 5'-CGGGTGAGTAACACGTG-3' and reverse primer 5'-CGGTGTGTACAAGCCC-3' were used in PCR table (1) (14). The concentration of the primer was 10 pecomole and the numbers of nitrogen bases were 17 in forward and reverse primer. The amplification was done in 20 µl which then added to the master mix that was supplied by the Bioneer Company Table (2).

Table 2. Master mix amplification compounds

Compounds in the master mix	Vol.(µl)
10 picomole Forward primer	2
10 picomole Reverse primer	2
DNA extract	4
Deionized water	12
Total volume	20

The master mixture was mixed for few seconds using vortex. The tube was placed in PCR thermo cycler. The device was programmed according to table (3) and the amplification was taken place to amplify the extracted DNA. By the end of the reaction time, 5 µl of 16S rRNA amplifying product was withdrawn for electrophoresis assy.

Table 3. The components of the PCR master mixture

No.	Step	Temperature	Time	Cycles
1	Initial Denaturation	94°C	4 min.	One cycle
2	Denaturation	94°C	40 sec.	
3	Annealing	53°C	50 sec.	
4	Elongation-1	72°C	80 sec.	35 cycle
5	Elongation -2	72°C	8 min.	One cycle
6	Holding	4°C	∞	

The Electrophoresis of DNA amplification products on agarose gel

The PCR products were loaded on 1.5% agarose gel using a horizontal electrophoresis. 5 µl of PCR products and 2 µl of loading buffer for every each 5 µl of DNA extract. The mixture was mixed well and located into the sample gel wells. The sample was subjected to electrophoresis assay for 1 hour, 5 volt\cm and 70 milliamps in order to initiate the movement toward negative and positive poles. The DNA bands were detected by using UV light Tran's illuminator device.

Determination of nitrogen bases sequence

The gene amplifying products have been sent to Korean Macrogen Company in order to determine the nitrogen bases (DNA sequencing) for the pure isolate. The sequencing was compared with the available information on that gene with NCBI website using BLAST Nucleotides software to identify the species of the chosen isolates.

xylanase Production from agricultures waste

Xylan extraction

Xylan was prepared from three different agriculture wastes by dilute acid according to (20),wheat stalks,sun flower stalks and papyrus were soaked in sulfuric acid diluted(0.01M) for 12hr at 60C°, , the biomass was collected, washed with distilled water until pH 7 and oven-drying. Biomass was mixed with water (1:3 w/v) then autoclaved at 121C° for 60 min. Biomass was drided and grinded for xylan getting.

Quantification of extracted xylan

Extracted xylan was determined by H_2SO_4 - Phenol method as describe by (3). The absorbance was measured at 490 nm by using the xylose standard curve.

RESULTS AND DISCUSSION

Isolation of Bacteria

Seventeen local bacterial isolates were obtained from soil samples, have been survived heat treatments at 100c° for 5 min and that was due to their spore's resistance to high temperature (4),(15). All isolates tested under the microscope were Gram-positive, rod-shaped and contains oval endospores at inside the cell when they stained by Malachite green stain. Colonies are spreading and irregularly-shaped and **Catalase-** positive The (Fig.1) shows all isolates were able to form clear zone around their grown colonies on isolation which contain congo red dye. Formation of clear zones by isolates as indication of xylanase production. Obtaind isolates were identified as *Bacillus* according to the cultural and morphological characteristics table (4). Jernejc (8) reported that the isolation process for any microorganism need to known the most important characteristic which represents the main target of this process. Generally, the desired characteristic is a restricted factor to isolate the microorganism from its natural media (soil, water, air, etc).

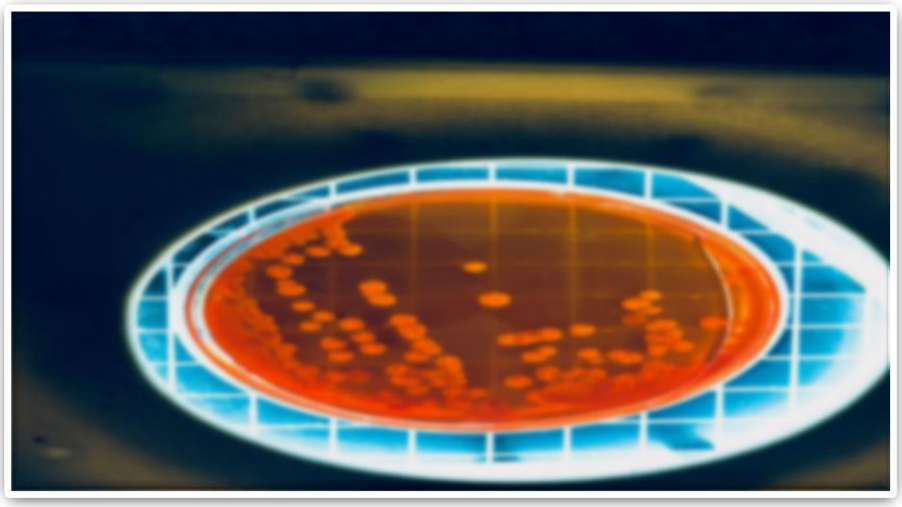

Fig. 1. clear zone on isolate media which contain congo red dye

Table 4. The most important cultural and morphological characteristics of the local *Bacillus* sp. Isolates

Characteristic	Observations
Growth on the solid medium	Fairly regular and widespread with a smooth surface
Colonical color	Opaque
Colonial edges	Circular with irregular or cut edges
Shap of cells	Long rod,straight or slightly curved
Response for gram's stain	Positive for gram's stain
Spores forming	Central or sub-central spores
Spore shape	Elliptical

Screening of *Bacillus* isolates

The seventeen isolates were subjected to the quantitative screening. Despite that the isolates varied capabilities to produce the xylanase enzyme, isolate *Bacillus* sp.RS1was the most distinguished one among others by giving enzymatic activity with value reached 2680 unit/ml (Fig.2), this isolate was selected to be used in the experiments of this research study.

Several studies have been conducted about bacteria producing xylanase, Yasinok (21) reported that *Bacillus pumilus* M1and *Bacillus pumilus* M2 which were isolated from zea mays, had ability to give enzymatic activity (188.0 and 5.6) U/ml repectivly. Chaturvedi (2) have found that *Bacillus licheniformis* had high ability to give enzymatic activity as compared with 18 isolates of *Bacillus* which were isolated from decomposed wood.

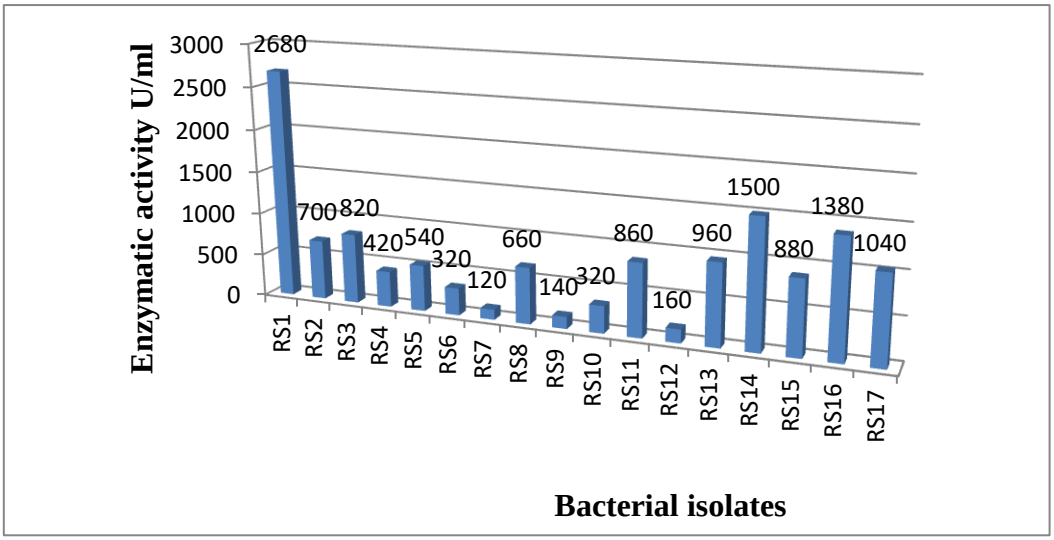

Fig. 2. A screening for 17 *Bacillus* isolates in production media depending on the total xylanase activity

Molecular Identification

DNA extraction: The DNA was extracted from *Bacillus* sp**.** RS1 and the purity of DNA was examined by Nano Drop with a purity of 1.95 which is adequate for Polymerase Chain Reaction (PCR) process. Green (6) reported that the PCR did not need a large quantity of DNA which may instead produce unlimited amplifying products. On other hand, an adequate quantity of DNA may reduce the accuracy.

Polymerase Chain Reaction (PCR)

A PCR for the local *Bacillus sp*. RS1 for 16S rRNA gene was carried out. The electrophoresis on 1.5% agarose show (by using U.V detector), that there was a clear band represents the genes amplifications (Fig.3). The molecular size of gene amplification band was between 1000-1500 bp comparing with ladder size at the same conditions, which refers to the prime binding to the complete sequence in DNA pattern.

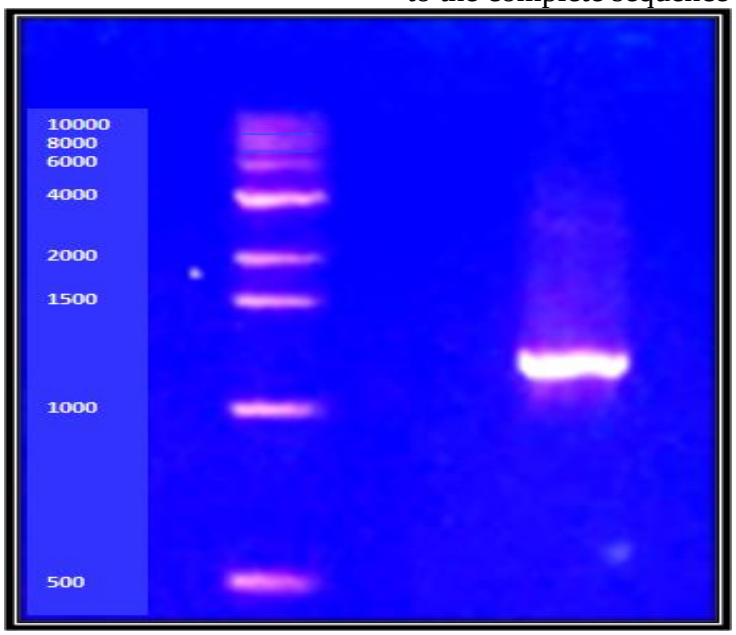

Fig. 3. Electrophoresis for the local *Bacillus sp*. RS1 for 16S rRNA gene on agarose gel.

Sequence analysis of amplification products

The sequence of nitrogen bases, of the16S-rRNA gene, for the local mold isolate (*Bacillus* sp. RS1) was studied by sending the amplification products to the Korean company Macrogen (Fig.4). The nitrogen bases sequence (1429 base-pair) which was taken from the local isolate sequence (of the present study) is shown in (Fig.5). The PLAST program has been used to find out the

similarity of gene with the bank information (NCBI). The results showed that there is a match between isolation and 99% with global isolation sequences global ID: MG027675.1 Registered on the NCBI website and registered in the United States of America Which belong to *Bacillus subtilus* strain VBN25. (Fig.6).

GGGAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGG
CTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGT
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGA
AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGT
GGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTC
TCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACG
CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATC
CTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCA
GCATTCAGTTGGGCACTCTAAGGTGACTGGCCGGTGACAAACCGGAAGAAAGGTGGGGGATGAAC
GTCAAATCATCATGCCCCC

Fig. 4. Sequence for the local *Bacillus* sp. RS1 for the 16S rRNA

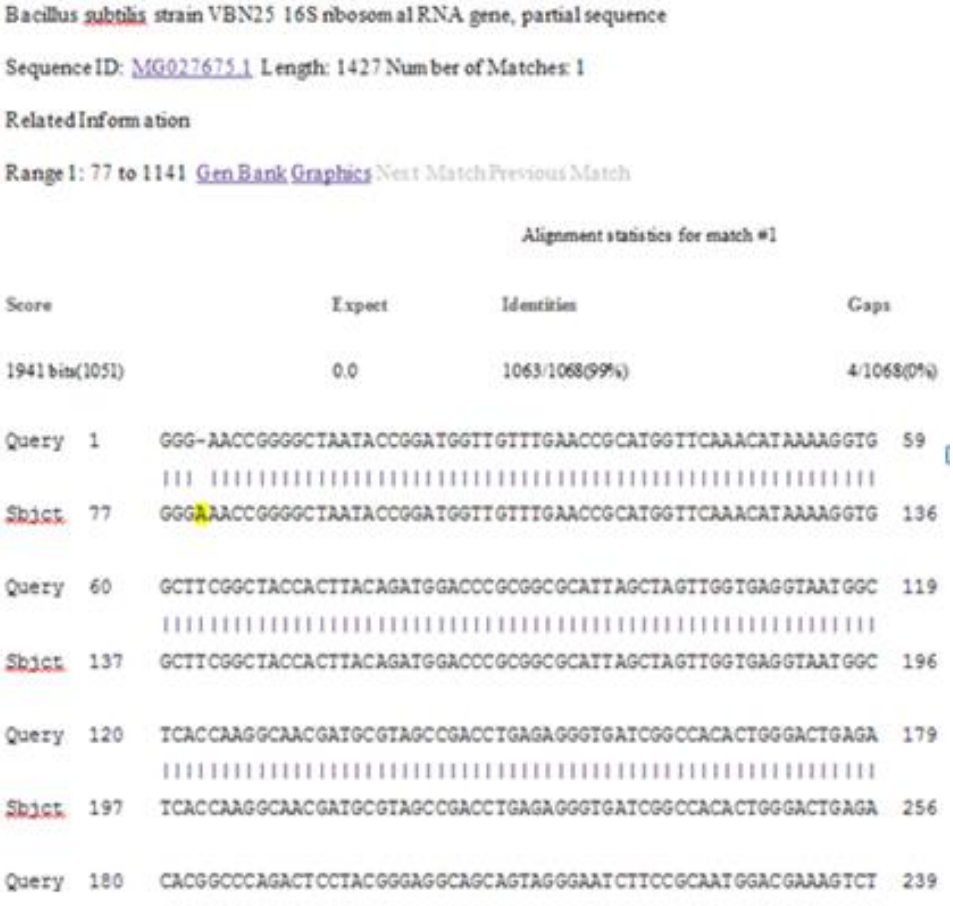

Fig. 5. Match the sequence of nitrogen bases for 16S rRNA for the local *Bacillus* sp.RS1 **with global isolate *Bacillus subtilus* strain VBN25**

Shows phylogenetic tree for the local isolate with the other strain in NCBI, the results shows clear convergence between a local isolate *Bacillus subtilus* RS1 and standerd strains in genes bank NCBI

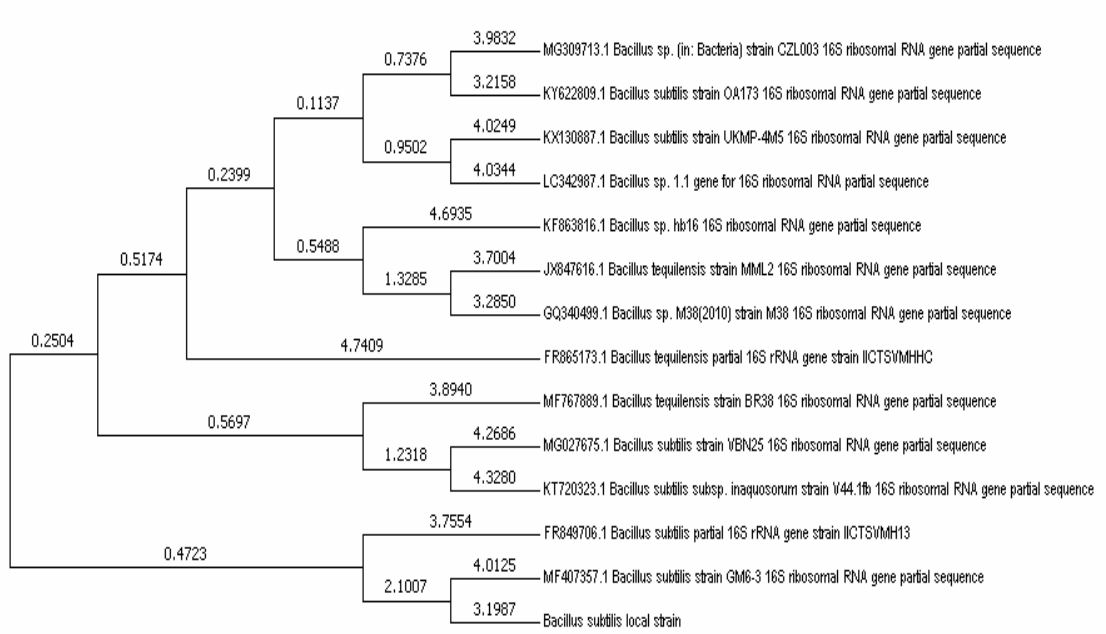

Fig. 6. phylogenetic tree for the local isolate with the other strain in genes bank NCBI

Quantification of extracted xylan

Table (5) has shown that the papyrus gave the highest amount of xylan (187.6µ g/ml) as compared with that of the sun flower stalks, Ibaa Wheat type, Furat wheat type and Abo Ghraib wheat type(161.3, 161.6, 157.6, 157.2) µ g/ml respectively.

Production of xylanase from agricultural wastes by *Bacillus subtilus*

Fig (7) shows the results of the enzymatic activity for the product xylinase by *Bacillus subtilus* by using extracted xylan from different agriculture waste. The results

Table 5. Quantity of xylan in variety agricultur waste

Quantity of xylan µ g/ml	Agricultur waste
157.6	**Furat wheat type**
157.2	**Abo Ghraib wheat type wheat**
161.6	**Ibaa Wheat type**
187.6	**Papyrus**
161.3	**sun flower stalks**

indicated that the highest activity was 2800 u/ml produced by *Bacillus subtilus* when used Papyrus xylan, and the enzymatic activity by using Ibaa Wheat type, sun flower stalks , Furat wheat type and Abo Ghraib wheat type were(2411،2407،2352،2346) u/ml respectivily, these results could be attributed to the difference in xylan soures**.**

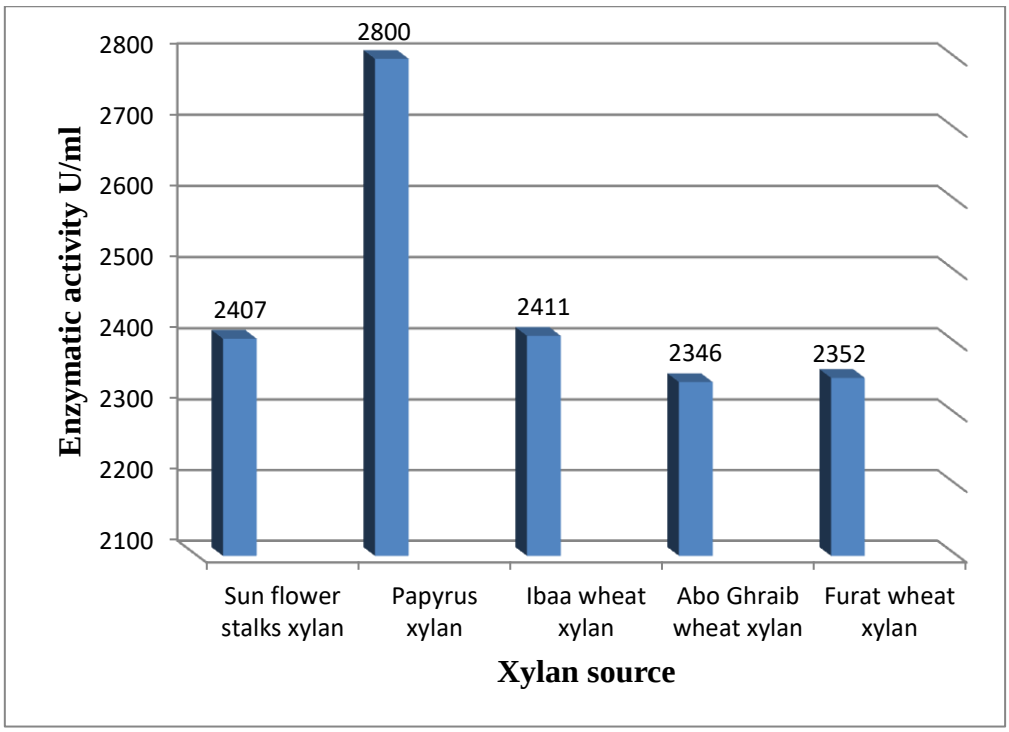

Fig. 7.Effect of xylan soures in xylanase production by *Bacillus subtilus*

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