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Detection of local *Bacillus subtilis* producing *Endo-β-1,4-glucanase* Of Thi Qar province

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Abstract:

The study included isolating and diagnosing of *Bacillus subtilis* different soils at Thi Qar province. The ability of bacterial isolates to produce *Endo-β-1,4-glucanase* was determined after growing on carboxymethyl cellulose CMC medium, Using Congo red and NaCl. Enzyme *Endo-β-1,4-glucanase* isolates were identified using biochemical tests and VITEK2 as *B. subtilis*. Isolates were identified using 16S rRNA tests after extraction of DNA from isolates and amplified by PCR using 27F primers (Forward) and 1492R (Reverse) . All isolates in the current study were positive for this gene and the size of the gene for all isolates was at 1500 pb. After identifying the gene sequences and comparing them with the data available in the Gen Bank, NCBI data showed that they were new strains of *B. subtilis* strain m1 (MF449304) and *B. subtilis* strain M2 (MF449461) bacteria. Isolates were recorded in NCBI GenBank and were design for each evolutionary tree isolation by Software MEGA6. After the diagnosis of bacterial isolates, the ideal conditions for the production of *Endo-β-1,4-glucanase* were changed for incubation period, temperature, pH , Incubator Shake, carbon and nitrogen sources. Enzymatic efficacy was determined using of dinitro salicylic acid DNS detector to detect the glucose releasing glucose molecules *Endo-β-1,4-glucanase*. The enzyme was produced after growing of bacterial isolates of plant culture containing plant and cardboard residues as natural sources of carbon and alternative to costly industrial sources as a source of carbon at a concentration of 1% at 45 ° C for 48 hours and pH (6 - 5) The highest wheat bran and millet bran has superior efficacy over other natural and industrial sources were used as an alternative to the industrial nitrogen source of peptone with a concentration of 1%. The malt extract as the best nitrogen source for all isolates in the present study was superior to all other nitrogen sources. Other sources came from either banana Peel that showed no growth for all isolates.

The study aimed to isolate and diagnose *B. subtilis* producing an *Endoglucanases* using cheap natural sources as alternatives source to reduce to costly carbon cost of and nitrogen sources and benefiting from biological treatment of agricultural and industrial waste.

Keywords: *B. subtilis* strain m1, M2 , Produce *Endo-β-1,4-glucanase*, carbon source , nitrogen source and DNS Reagent.

1- Introduction:

B. subtilis, Soil bacteria, a positive gram stain represents a fruitful model for important research on bacteria in the future (Mirouze and Dubnau , 2016) . Christian Gottfried was Ehrenberg in 1835 , it was named *Vibrio subtilis*, Ferdinand Cohen renamed *Bacillus subtilis* in 1872 (Pokhrel *et al*., 2014), and reongised as Positive for catalysts and chemoheterotrophic (Bais *et al*., 2004) . which are aerobic microbes, despite the evidence that they are facultative aerobic , rod shaped and constitute protective spores that allow them to withstand the adverse environmental conditions of heat , dryness , many chemicals and radiation; They are not harmful to mammals, including humans, they are industrially , commercially important, producing high and varying amounts through the metabolic process, including antibiotics, chemicals, heterogeneous proteins, antigens, vaccines and enzymes (Sorokulova *et al*., 2013 ;Olmos SJ, 2014).

The external enzymes produced cellulase enzyme, which was classified in bacteria and fungi

according to its analytical capacity into three categories: *Endoglucanases* (EGs), (also known as *Endo-cellulase*, carboxymethyl cellulose, and *Endoglucanase*), It has a high rate of degradation of the thickness of cellulose layers and reduce the intensity of adhesion with each other and breaks the internal link of cellulose randomly, so many sugars have different lengths and create new ends (Pandey *et al*., 2014; Guan *et al*., 2017). *Exoglucanases*, (also called *Cellobiohydrolases* and *Exocellulase*),and *Carboxymethyl cellulase* (*β-glucosidases*), synonym cellobiase (Kim *et al*. 2008, Yang *et al*., 2014). *Endoglucanase* enzyme acts on a breakdown of internal glycosidic bonds between the molecules of glucose in cellulose chains (Yennamalli *et al*. 2011). Release of glucose units that play an important role in industrial applications (Nakari, S.T.1995). The biological transformation of cellulosic materials depends to a great extent on the nature of cellulose and sources of the cellulose enzyme and the optimal conditions for catalytic activity (Alam *et al*., 2004).

2- MATERIALS AND METHODS:

2-1- Bacteria isolation:

Soil samples were collected from different areas of Thi-Qar province and took at depth of 5-10 cm , and was collected 100 g from each site to 1g of sample was added 9 ml of distilled water, and made a sierial dilution, then 100 µL of each dilution was spread on carboxymethyl cellulose agar CMC dishes were Incubated for 48 hours at 37°C (Chelab and Faisal, 2016).

2-2- Screening of Microorganisms:

After incubation period, dishes were flooded with 1% Congo red for 15 minutes at room temperature, Observe clear zones of hydrolysis around the bacterial colonies was indicated cellulose analysis in the nutrient dishes, which represent the cellulose enzyme production, clear zones varied among strain of isolated bacteria. The desired and produced isolates of the enzyme are purified and stored at 4 ° C.

2-3- Identification of Bacteria:

Morphological characteristics and biochemical tests were used according to electronic microscopy and Bergey's Manual of Bacteriological Methodology (Bergey, 2009). Gram stain, endospore stain and Physiological tests was used. The rod-shaped cells with peritrichous flagella and colonies were variable and irregular in shape, with wet to dry, mucus or butyrous, with uneven, undulating edges. While colors, varied from yellow, orange, pink and red, to brown or black.

Biochemical tests were determind using Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Gelatin test, Motility test, starch hydrolysis and VITEK2.

2-4- Identification of Bacteria by VITEK2:

VITEK2 device was used to diagnose some bacterial isolates with high accuracy, by Bio Merieux, Elite Specialized Medical Labs Baghdad

The bacteria were cultured on the center of the nutrient agar for 18-24 hours at 37 ° C; Single colonies were selected and diluted in 3 ml normal saline in a sterile tube and turbidity was measured by VITECH2 dinschic to 1.8 – 2.2, then transferred to the VITEK2 device and the results emerged 14 hours after isolation (Pincus, 2006).

2-5- Cellulase Enzyme Production Medium Detection of *Endo-ß-1,4glucanase:*

Enzyme activity was determined by preparing a medium CMC broth (containing 5g CMC , 5g Pepton, 1g KH_2PO_4 , 2g $(NH_4)_2SO_4$, 0.25g $MgSO_4$) in 500 ml distilled water. Cultures were incubated for 48 hours at 37°C. The tubes were centrifuged at 10000 rpm for 10 minutes supernatant were taken as crude enzyme source, cellulase enzyme activity was Measured using Spectrophotometer and a dinitro salicylic acid (DNS) (Miller, 1959).

Activity was measured using a reaction mixture containing 1ml of CMC as substrate (1% w/v) with 1ml of crude enzyme and 1 ml citrate buffer (pH 4.8). This mixture was incubated in a water bath for 30 min at 50 ° C then 2 mL of 3,5-dinitro salicylic acid (DNS) solution was added. The treated samples were heated at 100 °C in a water bath for 15 min.finally the samples were left at room temperature; The optical absorbance was examined at 540 nm, by using a spectrophotometer (Choudhary, 2013 ; Abedin, 2015).

2-6-Molecular Identification of Bacterial Isolates:

Detection 16S rRNA gene was done throught DNA extraction from bacterial isolates according to for the boiling

Two primers were used in this study and synthesized by Bioneer company (Korea), first universal bacterial primers, Which consists of a sequence complement at 5 and 3' end of the 16S rRNA were used: 27F (Forward) (5–AGAGTTTGATCCTGGCTCAG -3) and second 1492R (Reverse) (5–GGTTACCTTGTTACGACTT -3) (Chelab and Faisal, 2016). Amplification was performed in 20µl, solution contains 5 µm Master MIX, 1 µm Forward Primer, 1µm Reverse Primer, 8µm of distilled water and 5 µm of isolated DNA .

PCR was performed in The T100 thermal cycler (Biometra, Germany). Multiplication products were detected by electrophoreses of samples on the agarose gel, 16S rRNA genes were detected using a leader 2000 bp.

PCR results (Contains 20µl from PCR products, 17µl 27F and 17µl 1492R) were sent to Korea for sequencing. The nucleotide sequence was then compared to available data in GenBank (http://blast.ncbi.nlm.nih.gov/ Blast.cgi).

3- RESULTS:

3-1- Sample collection and identification of Bacteria by VITEK2:

Soil samples were collected from Thi-Qar province, samples were identified using morphological tests depend on the shape, color, size of colonies, electronic microscopy and biochemical tests. Most of the results indicated that isolates belonged to *B.subtilis*, were studied as shown in figure1 and table1.

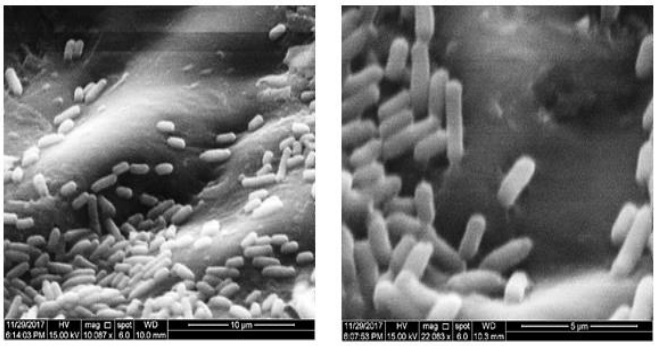

Figure (1) Vegetative cells after 24 hours under electronic microscope

Table 1 Biochemical characteristics for the identification of isolates

Morphological and Bio-chemical Test	Organism	
	***B.subtilis* strain m1**	***B.subtilis* strain M2**
Gram stain	+	+
Endospore stain	+	+
Motility	+	+
Colonial diameter	1.2cm	1.6cm
Analysis diameter	3cm	4.4cm
shape	Rod	Rod
starch test	+	+
Blood haemolysis	+	+
Catalase test	+	+
Gelatin	-	-
Oxiduase	-	_
Citrate	+	+
Mannitole	+	+
Klikler Iron test	A/A	A/A
Indole	-	-
Methyl	+	-
Voges	-	+

Table (2) shows VITEK2 results

Analysis Time hours	Confidence	Probability Identification	Selected Organism	Sample number
14.00			Unidentified Organism	m1
14.25	Very good	93%	*B.subtilis*	M2

3-2-Screening of *Endo-ß-1, 4-glucanase* activity:

Endo-β-1,4-glucanase activity was used to examine the rate of enzymatic activity using 1% Congo red and sodium chloride (NaCl 5.85g), As in figure 2.

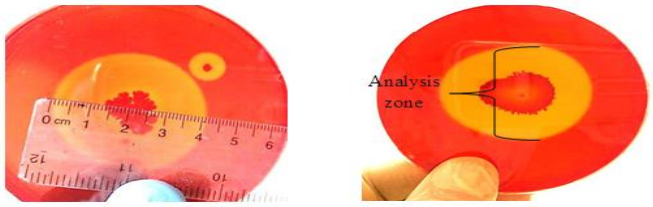

Figure 2: shows of CMC agar dishes

3-3- Molecular Identification and Genotypic characterization:

Results of the gel electrophoresis were shown figure3 DNA extraction and gene 16SrRNA assay, current results of gene sequence were compared with models which recorded in GenBank, using the BLASTN tool, isolate m1 showed identity with isolation MF092720 ratio 95% and isolate M2

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symmetry with isolation MF356876 ratio 99% as showed in figure3 and table 3.

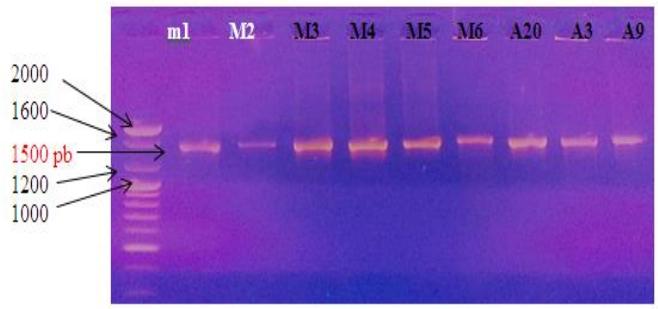

Figure (3): gene 16SrRNA assay

Table (3): Proportion of parity of isolates recorded in NCBI

Deposited Accession numbers	Match%	Match with existing Accession numbers
m1 = MF449304	95%	MF092720.1
M2 = MF449461	99%	MF356876.1

3-4- Phylogenetic analysis:

The optimal tree was drawn for all studied isolates. The evolutionary distances were calculated using the number of basic differences in each sequence. All codes containing gaps and incomplete data were eliminated. The MEGA6 program was used to design the evolutionary tree for each isolation after comparing it with the nearest six isolates similar to each isolation of isolates *B.subtilis*. As in figure 4 and 5.

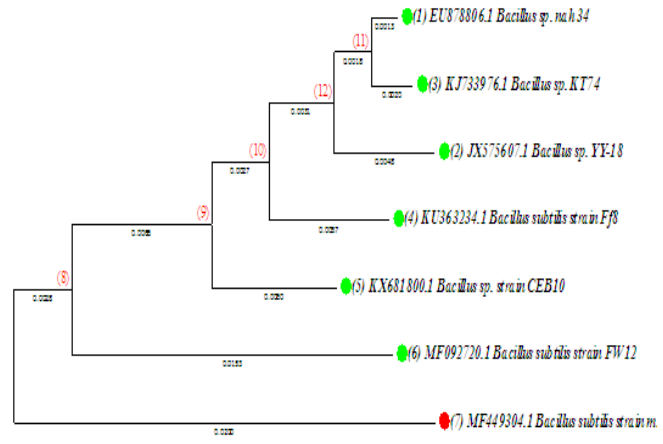

Figure (4): Evolutionary tree *Bacillus subtilis* strain m1

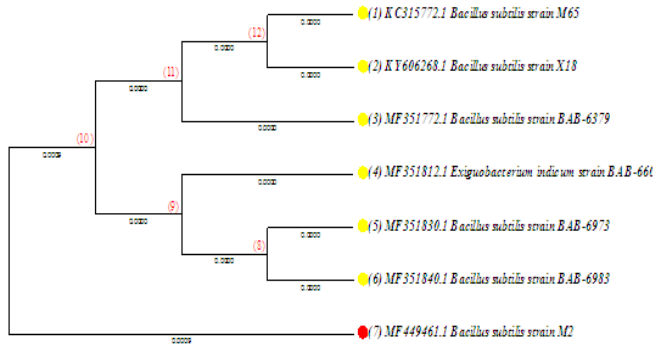

Figure (5): Evolutionary tree *Bacillus subtilis* strain M2

3-5- Glucose standard curve:

This method was used to detect C = O group in glucose sugar and this sugar was reduced when a detector was added DNS (3, 5-dinitro salicylic acid) Which is reduced to 3-amino, 5-nitrosalicylic acid, 0.35% glucose was prepared in 100 ml distilled water and make different concentrations (0 , 0.1 , 0.2 , 0.3 , 0.4 , 0.5 to 1 ml) and a diluted DNS acid solution 2 mL was added, Absorption was then measured using a spectrometer at a wavelength of 540 nm as shown in figure 6

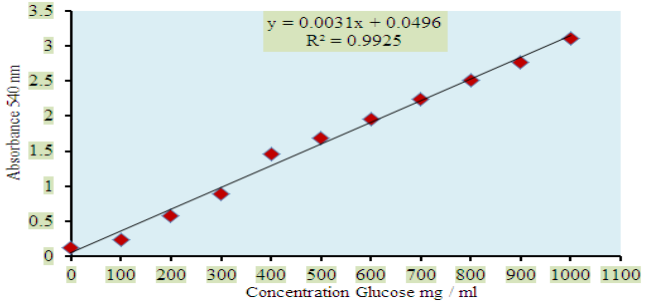

Figure (6): Glucose Calibration Curve

3-6Determine optimal conditions for endoglucanase activity:

B. subtilis strain m1 and *B. subtilis* strain M2 Isolates were selected to test optimal conditions for *Endo-β-1,4-glucanase* enzyme production.

3-6-1- Incubation Period:

Samples were incubated at different times (24, 48, 72, 96, 120) at 37 ° C, the optimum time for the production of *Endo-β-1,4-glucanase* enzyme was 48 hours as shown in figure 7.

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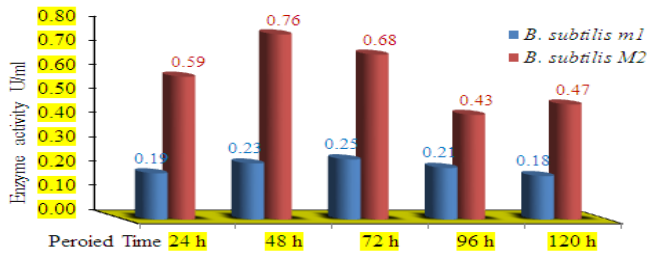

Figure (7): Incubation Period *B. subtilis* strain m1and M2

3-6-2- Effect of Temperature:

A 48-hour incubation period was selected for the highest enzymatic production value, and isolates were incubated at different temperatures (37° - 40° - 45°- 50° and 55°). The resultes were showen that 37° and 45° was the best produced as shown in figure 8.

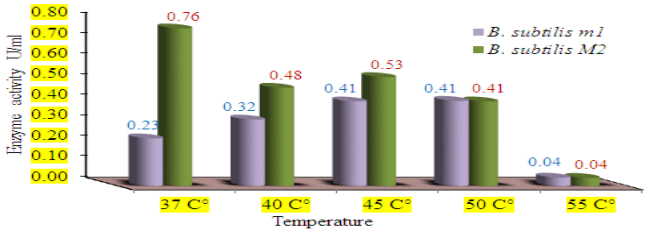

Figure (8): Effect of Temperature *B. subtilis* strain m1and M2

3-6-3- Effect of Shaking:

Isolates were incubated in the incubator and in the shaking incubator 150 rpm / minute for 48 hours at 45 ° C. Results were almost identical between isolates as shown in figure 9.

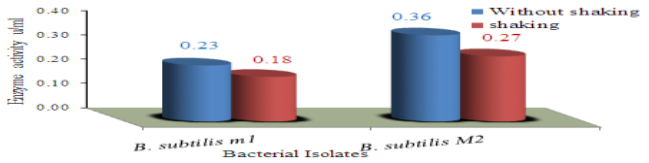

Figure (9): Effect of Shaking *B. subtilis* strain m1and M2

3-6-4- Effect of pH:

B.subtilis strain m1 and *B. subtilis* strain M2 isolates were incubated at 45° for 48 houre at different, pH ranged (5, 6, 7, 8 and 9). The appropriate concentration was between 5- 6 as shown in figure 10.

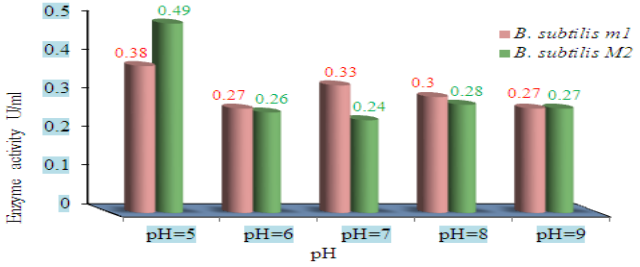

Figure (10): Effect of pH *B. subtilis* strain m1and M2

3-6-5- Effect of Concentration of Substrate:

Different concentrations of the main materials were used(2.5- 2.5- 1.5- 1- 0.5)%. The best enzymatic activity of isolates was at 1% concentration and enzymatic efficacy was 0.38 U / ml in isolation of *B. subtilis* strain m1 and 0.76 U / ml for *B. subtilis* strain M2 isolation as shown in figure 11.

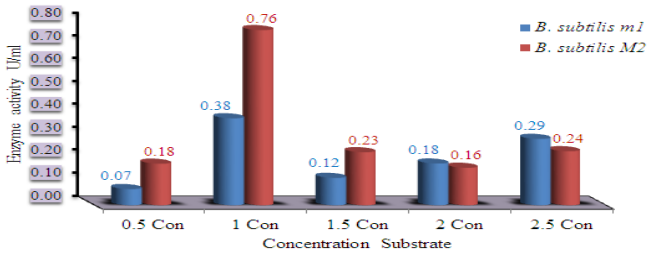

Figure (11): Effect Substrate Concentration *B. subtilis* strain m1and M2

3-6-6- Effect of carbon Source:

A variety of Natural materials were used as a carbon source (wheat bran, millet bran, cotton bran seeds, cardboard, corn stover, sawdust and pumpkin seeds). A 1%. Wheat bran was recorded as the best enzymatic activity of alternative carbon sources with enzymatic activity ranging from 0.45 U/ml in isolation *B. subtilis* strain m1 and 0.58 U/ml in isolation at 1% *B. subtilis* strain M2. Millet bran seeds were recorded as effective 0.34 U/ml in in isolation *B. subtilis* strain m1 and 0.32 U.ml In isolation *B. subtilis* strain M2 as shown in figure 12 .

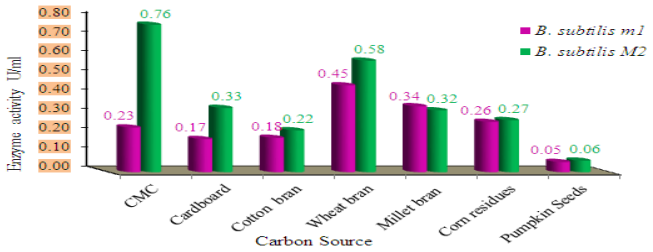

Figure (12): Effect of carbon Source *B. subtilis* strain m1and M2

3-6-7- Effect of Nitrogen Source:

Natural sources was from of pepton which used as altrrnative source of nitrogen and 1% (Malt extract, Yeast extract, Soybean, Cotton seeds and Chicken bones). Malt extract was the highest in terms of enzymatic activity compared to peptone. Enzymatic efficacy *B. subtilis* strain m1 0.91U/ml and *B. subtilis* strain M2 0.93U/ml . Cotton seeds and chicken bone powder came as the best source of natural nitrogen. As in the following figure 13.

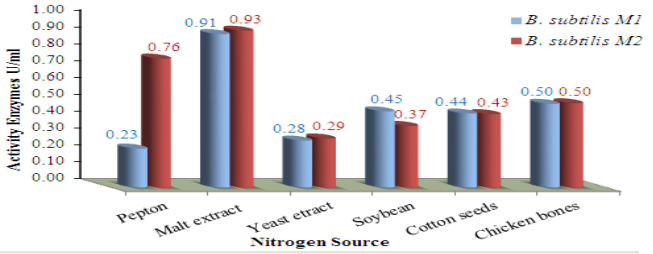

Figure (13): Effect of Nitrogen Source *B. subtilis* strain m1and M2

3-6-8- Media domestic production:

Results of the natural sources used in the production of wheat bran as an alternative to CMC and chicken bone powder as an alternative to industrial pepton source. The enzymatic efficiency of all isolates ranged between 0.32 U / ml to 0.52 U / ml and figure 13 showed results of enzymatic efficacy of isolates as in the following figure 14.

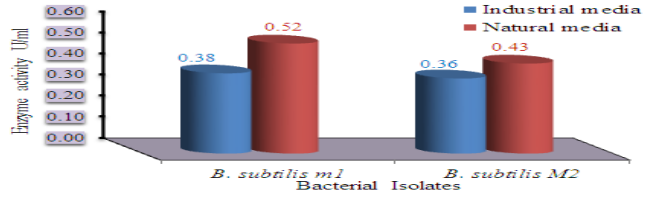

Figure (14): Media domestic production *B. subtilis* strain m1and

4- DISCUSSION:

Soil samples were collected from different areas of Thi-Qar province and The study was conducted in microbiology laboratory, College of Education for Pure Sciences / Department of Biology Thi-Qar University, Iraq. The current study showed that after growing of bacterial isolates on the CMC agar medium under optimal conditions at 37 ° C for 48 hours and 5 - 6 pH, a transparent halo between 4.5 cm - 3 cm around colonies of bacteria after using Congo red stain and NaCl. The ability to decompose the medium through the production of Endo-β-1,4-glucanase and matched these results with the statements of both Reddy (2016) and Vijayaraghavan (2012).

The phenotypic examination was showed that avariation colony size between round or irregular shape, with uneven edges, from wavy to linear, turning into ridged edges as the growth period progresses, they may become opaque or dark, and colonies resemble a concentric ring. Their surfaces are wrinkled from the center and they have white or creamy color and in some isolates tend to brown. Its strength varies from rough to crusty due to drought to a mucous or buttery diameter of 4 cm - 1.5 cm. While electron microscopy has rod-shaped of isolates. These characteristics described by Bergey (2009). Biochemical results were identical as described by Al-Attar (2016).

Magnification products detected by agarose gel electrophoresis at 1500 pb. DNA sequencing ranged from 352pb to 736pb, making it sufficient to diagnose isolates and were identical with Vimal (2016). New sequences were recorded in the Genbank of *B. subtilis* strain m1 (MF449304) and *B. subtilis* strain M2 (MF449461), after comparing them with other strains data and Software MEGA6 was used to design the evolutionary tree for each isolate after comparing it to the nearest isolates similar to each isolate.

Endo-β-1,4-glucanase production were shown to be based mainly on carbon and nitrogen sources used in growing of isolates. For example, some alternative sources of carbon have been used which used by researchers such as wheat bran, corn and cardboard residues and those results similar to Ma (2015) and Kumar (2017). Millet bran has been used as a new alternative source and has shown good results in the enzymatic effectiveness of all isolates.

Sources nitrogen are industrial. These sources such as the barley and yeast extract, exceeded the main source of nitrogen, these results were identical to Biosci (2015) and Rathnan (2016).

5- Conclusion:

The natural alternatives of nitrogen results and successful results, a matter which makes them cheap substitutes, such as chicken bones and cotton seeds, hence all isolates have grown up naturally debending on these sources and were enzyme producing.

In conclusion, the current study revealed that natural resources can be used as sources of carbon and nitrogen as successful and inexpensive alternatives in the production of enzymes, including Endo-β-1,4-glucanase by *B. subtilis* bacteria.

6- Recommendation:

1- Use of new natural sources as carbon and nitrogen for growth bacterial isolates and determine optimal conditions for the production of enzymes.
2- Identify the important genes the Endo-β-1,4-glucanase enzyme and its extracellular reproduction and transfer them to new species to improve the production of important enzymes.

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