ISOLATION AND MOLECULAR CHARACTERIZATION OF POTENTIAL CELLULOLYTIC *BACILLUS* SPECIES ISOLATED FROM SOIL SAMPLES

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ABSTRACT

The twenty-two bacterial isolates were screened for cellulolytic activity using Congo red stain on Carboxymethylcellulose (CMC) agar plates inoculated with the isolates. All the isolates were found to hydrolyze Carboxymethylcellulose. Five bacterial isolates (FAY88, FAY103, FAY117. FAY136 and FAY182) were selected according to their higher production of carboxymethyl cellulase (CMCase). A Gram stain test was carried out to identify five isolates as Gram-positive rods, all associated mainly with members of the *Bacillus*. Their CMCase activities were 1.49, 1.26, 1.21, 1.21 and 1.24 U/ml, respectively. Genetic distances for the five selected strains with high cellulase activity based on the 79 bands obtained from the results of RAPD revealed the similarity according to the morphological characterization agrees with genetic distance. The SDS-PAGE analysis was revealed that, the molecular weight of crude cellulase isolated from *Bacillus* isolates was found approximately of 54 kDa.

Keywords: *Bacillus*, Cellulase activity, morphological characterization, RAPD-PCR.

1. INTRODUCTION

Biotechnological conversion of cellulosic biomass is potentially sustainable approach to develop novel bioprocesses and products. Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications. Cellulases are composed of independently folding, structurally and functionally discrete units called domains or modules, making cellulases module (**Henrissat *et al*.,1998**). Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials. Basic and applied studies on cellulolytic enzymes have demonstrated their biotechnological potential in various industries including food, animal feed, agriculture, biomass refining, pulp and paper, textile, and laundry.

The search for extremophiles organisms is one of the means for obtaining enzymes with properties suitable for industrial applications. There are quite a few advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes (**Kristjansson, 1989**). The main advantage is that as the temperature of the process is increased, the rate of reaction increases. A 10 ~ increase in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed (**Haki and Rakish, 2003**). The thermostable enzymes are also able to tolerate higher temperatures, which give a

longer half-life to the enzyme. Thermostable enzymes are also believed to be more resistant to other denaturing factors. This correlation however does not hold for denaturing processes that do not depend on the folding stability of a protein e.g. oxidation of surface residues (**D'Amico *et al.,* 2006**). Cellulase can be used in waste and pollution management through degradation of agricultural and food wastes containing cellulosic wastes and converting these materials into valuable products like glucose. The use of some biological agents like nonpathogenic bacteria and cellulases in degradation of lignocellulose biomass for production of liquid biofuels and other valuable products is preferred to chemical treatments because the biological treatments are less hazardous, ecofriendly and more economic (**Lynd *et al.,* 2017; Liu *et al.,* 2019)**. This study was carried out to isolate and identify the potential cellulolytic *Bacillus* isolates using traditional microbiological and molecular techniques.

2. MATERIALS AND METHODS

2.1. Isolation of cellulolytic bacteria

For isolating cellulolytic bacteria, one gram of soil samples was first mixed with 9 ml of distilled water in a test tube and heated for 15 minutes at 65°C in a hot water bath. Serial dilutions from 10^{-1} to 10^{-10} were prepared using sterilized saline solution. An aliquot of 100 μL of each dilution was spread on CMC agar plates, pH 7.0 and incubated at 37°C for 24-48 hours (**Pandey *et al*., 2013; Potprommanee *et al.,* 2017)**. Morphologically different bacterial colonies were purified by repeated streaking. The isolated single cells were preserved in 40% glycerol solution and kept at -80°C for cellulase production screening and strains characterization.

2.2. Qualitative CMCase screening of cellulolytic bacteria activity

Congo red staining method was used for qualitative screening cellulolytic bacteria. Morphologically dissimilar and discrete colonies were picked from different dilution plates and streaked on separate CMC plate and incubated at 37°C for 96h. The replica plates were also prepared separately for staining. The replica plates were flooded with 0.3% Congo red for 20 min. The stain was poured off and the plates were washed with 1M NaCl. After washing, only the isolates showing clear and prominent zones around the colonies indicating cellulase production were selected and picked from master plate and for the enzyme production in liquid medium (**Potprommanee *et al.,* 2017**). The selected cultures were maintained on nutrient agar slants and stored at 4°C.

2.3. Quantitative CMCase screening of Cellulolytic Bacterial activity

The potential bacterial isolates selected by primary qualitative screening were evaluated for enzyme production and used for quantitative screening of extracellular CMCase activity. The selected isolates were grown in 50 mL culture medium containing the following components (g/L): CMC (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3 \cdot 6H_2O$ (0.05), $CaCl_2$ (0.02),

and yeast extract (5.0) at pH 7. Then, 50 mL medium containing 2% inoculum was prepared in 250 mL Erlenmeyer flask and incubated at 37°C at 180 rpm for 5 days. The culture was centrifuged at 10000 rpm for 10 min at 4°C (**Pandey *et al*., 2013**). The cell free culture containing the crude enzyme was used for estimation of CMCase activity. Isolates displaying the highest cellulase activity were selected for further characterization and enzyme production

2.4. Morphological characterizations of the isolates

Bacterial isolates producing significant clear zone on CMC agar were identified based on cultural and morphological characteristics. For this, a series of conventional tests were carried out for the identification of the genus of bacterial isolates according to the Bergey's manual of systemic bacteriology (**Boone *et al*., 2001**). The cell morphology of the selected isolate was observed under a light microscope. Gram staining and endospore staining were done as per standard protocol (**Cappuccino and Sherman, 2004**).

2.5. Genomic DNA isolation from *Bacillus* isolates

Genomic DNA was extracted from the isolates according the method described by **Tillett & Neilan (2000).** The quantified DNA were electrophoresed on a 1% agarose gel in 1x TAE buffer containing 0.5 µg/mL of ethidium bromide and visualized under UV light and stored at -20°C until further processing of PCR amplification of RAPD-PCR analysis.

2.6. Random Amplified polymorphic DNA isolated from of *Bacillus spp*

A total number of six primers (OPA-1-OPA-6) were used in present study to identify the five strains of *Bacillus spp*. The sequence of the six primers was presented in Table (1). PCR conditions were as follows; one cycle of initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation for 1 min at 94 °C, annealing primer for1 min at 37 °C and extension for 1 min at 72 °C, and one cycle for final extension step at 72 °C for 10 min using thermal cycler 2720 (Applied Biosystems, USA).

RAPD-PCR fingerprinting patterns were carried out with Computer assisted analysis using RAPD software package, version 1.4**.** Similarity of the band profiles was based on Excoffier matrix **(Excoffier *et al*., 1992)**. The correlation coefficient was used to compare the number of the DNA patterns obtained. The clustering of the strains was determined by the UN weighted Pair Group Method using Arithmetic Average (UPGMA).

Table (1) Nucleotide sequence of random primers used for RAPD analysis of *Bacillus spp.*

No.	Primer code	Primers sequence {5'-3'}
1	OPA- 1	-5′ CAGGCCCTTC 3′-
2	OPA -2	-5′ TGCCGAGCTG 3′-
3	OPA -3	-5′ AGTCAGCCAC 3′-
4	OPA -4	-5′ AATCGGGCTG 3′-
5	OPA -5	-5′ AGGGGTCTTG3′-
6	OPA -6	-5′ GCTCCCTGAC 3′-

2.7. SDS-PAGE electrophoresis

Sodium deodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to **Laemmli (1970)** which used for identification the genetic background for the studied bacterial strains by their total protein fingerprints. Total protein of the *Bacillus* strains was extracted according to **Von-Tersch and Gonzalez (1994)**.

3- RESULTS

3.1. Isolation and qualitative screening of thermophilic cellulase-producing bacteria.

A total number of 182 bacterial isolates were obtained from different agricultural soil and irrigation water samples, which collected from various locations in Fayoum Governorate, Egypt. These isolates were screened for their cellulase producing ability using CMC as a source of carbon and Congo red dye as an indicator to check zone of inhibition, produced by the hydrolysis of cellulose. Only 50 isolates out of 182 isolates showed positive test for cellulase production on CMC agar plates containing Congo red dye. From the previous experiment, 22 isolates exhibiting cellulose give clearer zone and five isolates (FAY88, FAY103, FAY117, FAY136 and FAY182) which exhibited highest cellulolytic activity as revealed by the formation of a clear zone on the screening medium and with highest ratio of clear zone diameter to colony diameter on Congo red agar plates were selected. These five isolates were found to have cellulolytic activity as shown in Figure (1).

Figure (1). Screening for cellulolytic bacteria by covering the petri dishes with Congo red dye. A zone of clearance surrounding the colonies is indicative of carboxymethyl cellulose (CMC) hydrolysis by secreted CMCase.

3.2. Quantitative CMCase screening

The 22 selective bacterial isolates exhibiting cellulase-positive were checked for quantitative CMCase production and the isolates FAY088, FAY0103, FAY0117, FAY0136 and FAY0182 showed the highest CMCase production with corresponding activities 1.49, 1.26, 1.21, 1.21 and 1.24 U/ml, respectively (Fig.2). Isolate FAY088 was expressed the highest production of cellulases as compared to the other isolates. These five isolates were used for morphological and molecular characterization.

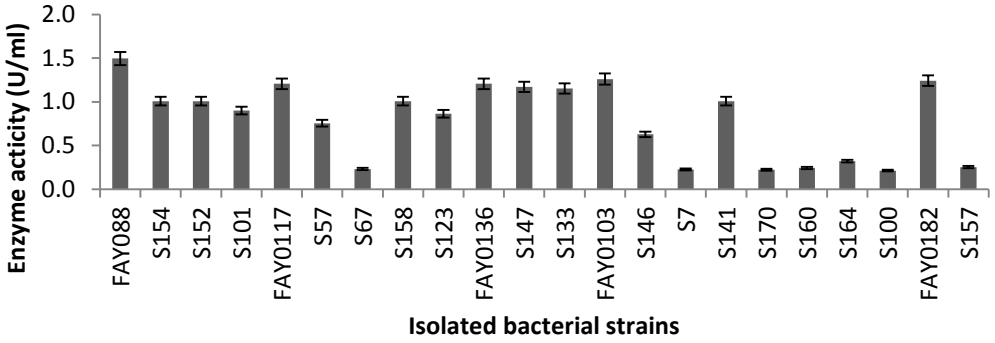

Figure (2).Quantitative CMCase activity for 22 selected *Bacillus* isolates.

3.3. Morphological characterizations of the isolates

Morphological and cultural characterizations of the five selected isolates were studied by the examination of single creamy colonies. All the five isolates exhibited a smooth and sticky surface, protruding shape, and irregular edge colony morphology. Colonies were large and vary in shape, from circular to irregular; it has granular texture and moist colonies. Microscopic observation showed the cells of the

isolates appeared as straight rods with rounded ends, arranged singly or in chains, and were motile and endospore-forming. The isolates cells appeared purple after Gram staining, indicating the isolates are Gram positive. The isolates were aerobic and facultative anaerobic, grew at pH 5-9.0 with an optimal pH of 7.0 and at 30°C-60°C with an optimal temperature of 50 °C. Oval spores are central or paracentral in sporangia. These characteristics have been summarized in Table (2).

Table (2) Morphological characteristics of cellulase producing bacterial isolates

Morphological characterization	***Bacillus* isolates**				
	FAY88	**FAY 103**	**FAY 117**	**FAY136**	**FAY182**
Colony shape	smooth and sticky surface	smooth and sticky surface	smooth and sticky surface	smooth and sticky surface	Smooth and sticky surface
Cell shape	straight rods with rounded ends arranged singly or in chains	straight rods with rounded ends arranged singly or in chains	straight rods with rounded ends arranged singly or in chains	straight rods with rounded ends arranged singly or in chains	straight rods with rounded ends arranged singly or in chains
Gram stain	+	+	+	+	+
Motile	+	+	+	+	+
endospore-forming	+	+	+	+	+

3.4. Molecular characterization of bacterial isolates

The quality of genomic DNA extracted from the bacterial isolates was analyzed on a 0.8% agarose gel stained with ethidium bromide. Figure (3) shows gel photographs of genomic DNA from the five bacteria isolates.

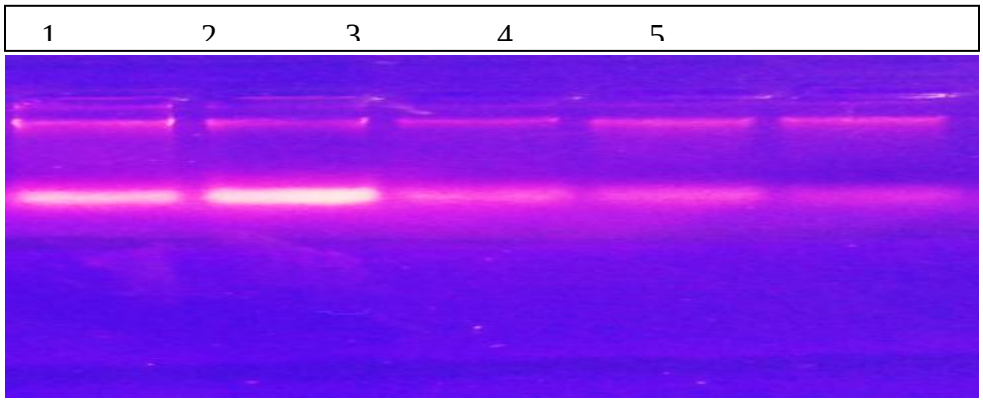

Figure (3). Agarose gel analysis of genomic DNA from *Bacillus* isolates.

3.5. RAPD-PCR for selected *bacillus* strains with high cellulase activity

The results of RAPD were analyzed to construct dendrogram. These primers were generated reproducible and easily securable RAPD profiles (Figure 4) with a number of amplified DNA fragments ranging from 9 to 20 amplicons per primer. Genetic distances for the Five selected strains with high cellulase activity based on the 79 bands obtained from the results of RAPD revealed two groups, among which isolate FAY136 and FAY182 were most close to each other (Figure 5).

The Dendrogram was constructed considering all bands generated by sex primers and suggested two primary genetic cluster, the first cluster consisted of two sub-clusters that include *bacillus* isolates FAY136 and FAY182 and the other sub-cluster include the isolate FAY١١٧ . The second cluster includes the other two isolatesFAY103 and FAY٨٨. The two isolated (FAY136 and FAY182) were closely related in one linage, while the isolates (FAY103 and FAY117) were highly distantly.

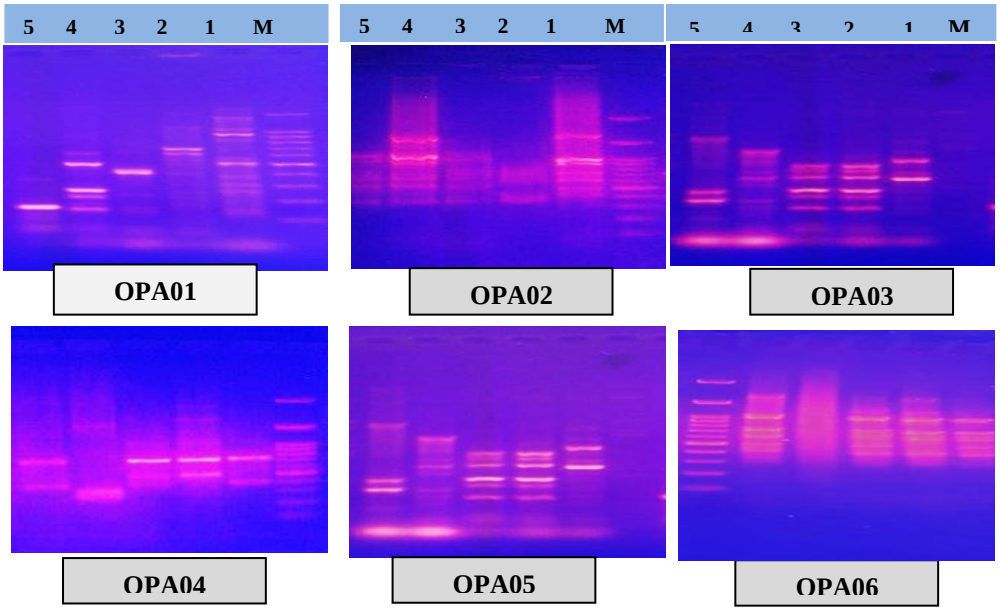

Figure (4): Photograph of RAPD profiles of the five different *Bacillus* strains amplified with RAPD primer, OPA01 to OPA7. M: 100 bp ladder DNA marker. Lanes from 1 to 5 represent: FAY182, FAY 136, FAY 88, FAY 103 and FAY 117, respectively.

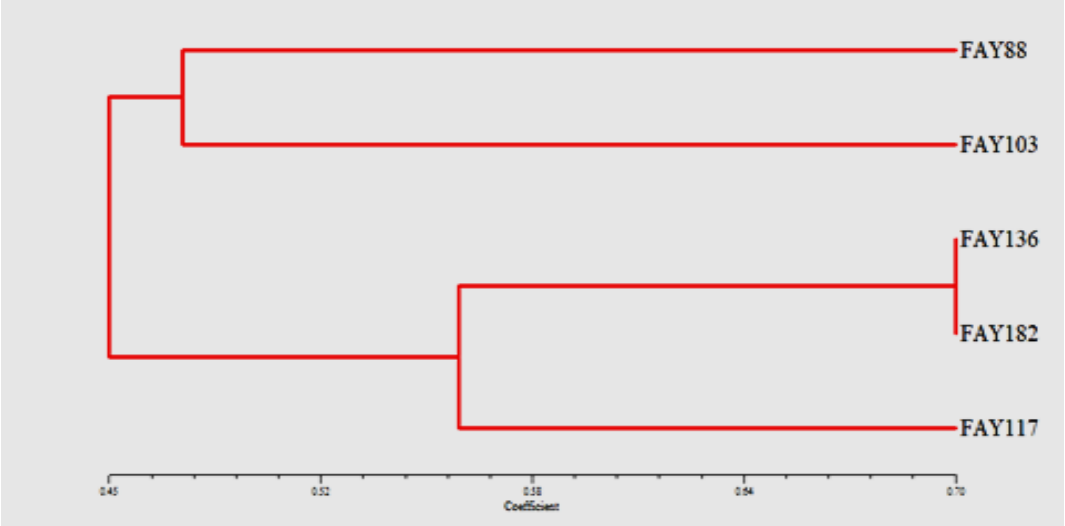

Figure (5): Dendrogram showing genetic relatedness of *Bacillus* isolates constructed considering all bands using UPGMA and similarity matrices.

3.6. SDS polyacrylamide gel electrophoresis

Total protein extracted from each of the *Bacillus* strains was run on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The SDS-PAGE analysis was revealed that, the molecular weight of crude cellulase isolated from *Bacillus* isolates was found approximately of 54 kDa (Fig.6).

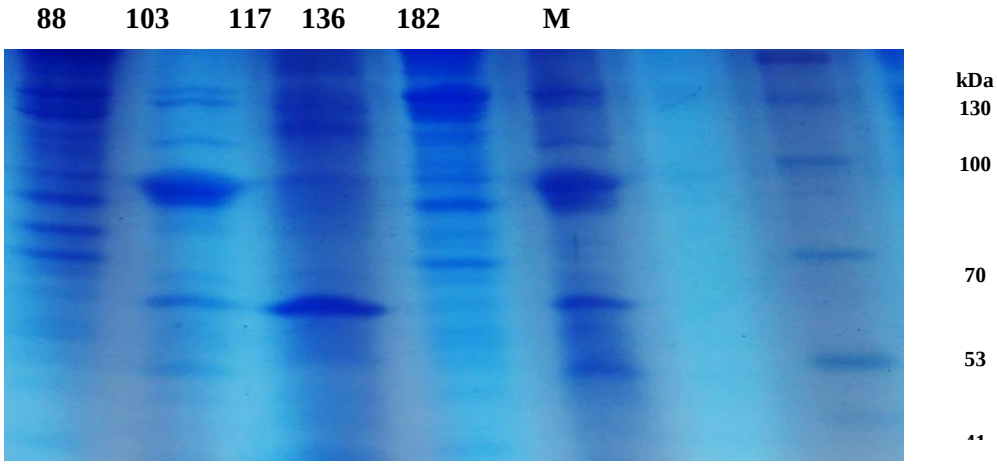

Figure (6). SDS-Polyacralamide gel electrophoresis for cellulase production by five selected isolates.

4. DISCUSSION

Cellulolytic bacteria have been isolated from diverse environments, *Bacillus* spp, isolated from cow dung, was found to possess good potential for endoglucanase production (**Sadhu *et al*., 2013)**. These microorganisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes.

It was reported that using CMC and yeast extract resulted in high CMCase production in *Bacillus* sp. **(Pandey *et al.*, 2013; Sreena and Sebastian, 2018)**. Considering carbon source from agro industrial wastes, it was found that corn Stover induced higher CMCase production by *B. subtilis* strain BY-3 when compared to CMC, rice straw and wheat bran and starch (**Meng *et al.*, 2014**).

Morphological identification of the bacterial isolates was carried out using the Gram stain procedure. All isolates were found to be Gram-positive rods hence were likely to belong to the *Bacilli* genus. Gram-positive bacteria have a thick mesh like cell wall comprising of 50-90% peptidoglycan. Classical taxonomy relies on a set of morphological characteristics (**Lynd *et al*., 2002**), this classification however, is limited by the large diversity and abundance of microorganisms bearing cellulolytic activity. The molecular techniques are more significant for the characterization of the new isolates, allowing grouping the strains. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation (**Rahna *et al.*, 2013**).

Microbial cellulases from different sources have been found to have optimum temperature of approximately 35-50 ^{o}C (**Aygan *et al*., 2011; Bakare *et al*., 2005).** Enzymes work rapidly at their optimum temperature, under below the optimum temperature; an increase in temperature increases the kinetic energy of the system thus increasing the rate of reaction. Consequently, the number of collisions between the substrate and the active site are increased. At elevated temperatures many enzymes become partly unfolded and inactivated thus rendering them unable to perform their desired tasks (**Eijsink *et al.*, 2005).**

Ibrahim and El-diwany (2007) were isolated three isolates of thermophilic cellulases producing bacteria (EHP1, EHP2 and EHP3) from an Egyptian hot spring by enrichment of the water and soil samples with cellulose for 3 weeks at 70 °C. Also they reported that, the optimum temperature and pH for the crude enzyme activity was 75 °C and 7.5, respectively.

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عزل وتعريف بكتيريا الباسيلس ذات الكفاءة العالية في انتاج إنزيم السليلولاز من عينات التربة

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تم اجراء هذه الدراسة بهدف عزل سلالات بكتيرية من مصادر مختلفة من التربة الزراعية لإستخدامها في إنتاج إنزيم السيليولاز، فقد تم الحصول على ١٨٢ عزلة بكتيرية وأظهرت ٥٠ عزلة فقط من أصل ١٨٢عزلة نتيجة ايجابية لاختبارإنتاج السليولاز من خلال قياس التغير في لون صبغة Congo red. أظهرت ٢٢ عزلة من ال ٥٠ عزلة المختبرة انتاج عالي من الانزيم وتم اختيار افضل ٥ عزلات(FAY88 ، FAY103 ، FAY117 ، FAY136وFAY182) أعلى إنتاج لانزيم CMCase حيث انتجت ١.٤٩ ، ١.٢٦ ، ١.٢١ و ١.٢٤ / U مل من انزيم CMCase على التوالي. ومن نتائج الاختبارات المورفولوجيه كانت جميع العزلات الخمسة تتبع جنس الباسيلس وباستخدام نتائج التضاعف العشوائي لحزم الDNA تم الحصول على ٧٩ حزمة مختلفة من نتائج RAPD . وبعمل شجرة القرابة باستخدام برنامج NSTY تم تقسيم السلالات الخمسة الي مجموعتين رئيسيتين وكانت العزلتين FAY136 وFAY182 الأقرب إلى بعضها البعض.وشملت المجموعة الأولى تحت مجموعتين فرعيتين احدهما تشمل السلالتين FAY136 و FAY١٨٢ والثانية تشمل السلالة FAY117. تتضمن المجموعة الثانية السلالتين FAY103 و FAY 88. كانت السلالتين (FAY136 و FAY182) مرتبطتين ارتباطًا وثيقًا في مجموعة واحدة ، في حين كانت العزلات (FAY103 و FAY117) بعيدة جدًا.

الكلمات الدالة: انزيم السيليولاز- بكتيريا الباسيلس- التعريف المورفولوجي- تفاعل البلمرة المتسلسل العشوائي.