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IDENTIFICATION OF SOME *BACILLUS* ISOLATES PRODUCING EXO-ENZYMES USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (PCR)

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Abstract

The basic goal of this study is the isolation of some *Bacillus* isolates from different sources for production of bioactive products such as detection of cellulose(s) and pectinase(s) and identification of these isolates using quantitative real-time PCR (qRT PCR) depending on 16S rRNA gene in each isolate. Nine, six and two isolates were obtained from municipal sewage water, composted rice straw and expired pharmaceutical drugs (Capozide), respectively. Out of nine isolates, only four *Bacilli* (SW2, SW3, SW6 and SW9) were selected and examined for cellulose(s) and pectinase(s) production, and revealed that only SW2 and SW9 exhibited maximum pectate lyase (PL) and polygalacturonase (PGase) viz. 465.55 and 222.53 (U/mg), respectively. Out of six *Bacilli* isolates (RS1, RS3 and RS5), only RS3 showed maximum productivity of carboxymethylcellulase (CMCase) viz. 657.21 (U/mg). On the other hand, only one *Bacillus* isolate out of two was selected ECC2 and exhibited the maximum Avicelase productivity viz. 533.32 (U/mg). TaqMan qRT PCR method was used to detect and quantify the 16S rRNA genes of *Bacillus* isolates tested. Forward primer Bsub5 F and the reverse primer Bsub3 R were used for the amplification of 16S rRNA of the "*Bacillus subtilis* group", it is also successful to demonstrate the most similarities of the *Bacillus* isolates in this study and they are closely related to "*Bacillus subtilis* group".

Keywords: *Polygalacturonase, Pectin Lyase, Avicelase, Carboxymethylcellulase, Quantitative Real-Time PCR.*

Introduction

REAL-TIME POLYMERASE CHAIN REACTION **(PCR)** is based on the revolutionary method of PCR, developed by Kary Mullis in the 1980s, which allows researchers to amplify specific pieces of DNA more than a billion-fold (Mullis, 1990; Mullis and Faloona, 1987; Saiki *et al.,* 1985). Real –time PCR represents yet another technological leap forward that has opened up new and powerful applications for researchers throughout the world (Valasek and Repa, 2005). Higuchi *et al.,* (1992); Higuchi *et al.,* (1993) at Roche Molecular Systems and Chiron accomplished the first demonstration of real-time PCR. By including a common fluorescent dye called ethidium bromide (EtBr) in the PCR and running the reaction under ultraviolet light, which causes EtBr to fluoresce, they could visualize and record the accumulation of DNA with a video camera.

The basic goal of real-time PCR is to precisely distinguish and measure specific nucleic acid sequences in a sample even if there is only a very small quantity. Real-time PCR amplifies a specific target sequence in a sample then monitors the amplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification (Valasek and Repa, 2005).

Recent applications of molecular techniques using 16S rRNA genes to environmental samples have shown the remarkable possibility of studying microbial populations in the absence of culturing (Akarsubasi *et al.,* 2005; Boon *et al.,* 2002; Godon *et al.,* 1997; Leclerc *et al.,* 2004; Sekiguchi *et al.,* 1998).

A real-time quantitative PCR (QPCR) technique (Heid *et al.,* 1996) using a fluorogenic probe allows rapid detection and quantification of various environmental microorganisms (Beller *et al.,* 2002; Harms *et al.,* 2003; Lebuhn *et al.,* 2003; Yu *et al.,* 2005). The QPCR assay with TaqMan system is highly specific and sensitive due to the use of three oligonucleotide sequences (i.e. two primers, a forward and reverse, and a fluorescent probe) complementary to the target DNA, and can be used for a large number of samples because of the advantages of PCR (Harms *et al.,* 2003). For subsequent discussions, a "primer and probe set" denotes the three oligonucleotides of two primers, a forward and reverse, and a dually labeled fluorescent TaqMan probe (Livak *et al.,* 1995).

Microorganisms mediate decomposition by utilization a wide variety of organic compounds under diverse environmental conditions, extracting energy from organic compounds by fermentation, anaerobic and aerobic respiration (Cunha-Santino and Bianchini, 2007). Microbial microorganisms release enzymes into the surrounding environment in order to degrade macromolecular and insoluble organic matter prior to cell uptake and metabolism (Chróst, 1990).

Cellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals. Enzymatic hydrolysis is an economic process in the conversion of cellulose to easily fermentable low cost sugars (Muthuvelayudham and Viruthagiri, 2006). Cellulase was identified as one of the key enzyme degrading cellulose (Kotchoni *et al.,* 2003). A cellulosic enzyme system consists of three major components: exo-1,4-β-D-glucan cellobiohydrolases, which cleave cellobiosyl units from the ends of cellulose chains; endo-1,4-β-D-glucases which cleave internal glucosidic bonds and 1,4-β-D-glucosidase, which cleaves glucose units from

cellooligosaccharides (Jorgensen *et al.,* 2003; Muthuvelayudham and Viruthagiri, 2006). Cellulose activities have seen in many nitrogen fixing bacteria such as *Sinorhizobium fredii* (Chen *et al.,* 2004; and Mateos *et al.,* 1992), *Bacillus spharricus* (Singh *et al.,* 2004), *Bacillus circulans* (Baird *et al.,* 1990), *Paenibacillus azotofixans* (Rosado *et al.,* 1998). Although with the change of *Bacillus* classification, all nitrogen-fixing Bacillus strains *Bacillus polymyxa, Bacillus macerans, Bacillus azotofixans* are now assigned to *Paenibacillus* (Rosado *et al.,* 1998; Gorska *et al.,*2001; Hossain *et al.,*1999; Kashem *et al.,* 2004; Mawadza *et al.,* 2000; Shawky, 1983; Krieg and Holt, 1984).

Pectinases are enzymatic biocomposites widely used in the industries because of their capacity to degrade pectic substances as fiber deguming (Baracat *et al.,* 1989), citric fruit peeling, industrial residues from the process of coffe, cocoa, tobacco, tea (Martins *et al.,* 2002), wine clarification, juice extraction, fruit and pectin hydrolyses (Kashyap *et al.,* 2003). Pectinase synthesis occurs in many microbial groups, however, for the industrial Pectinase production, fungi are the most used group due to their high enzymatic excretion capacity (Solis *et al.,* 1990). Pectinolytic enzymes are classified according to their way of attack on the galacturonan part of the pectin molecule. They can be distinguished between pectin methylesterases (EC 3.1.11.1) that deesterify pectins to low methoxyl pectins or pectic acid, and pectin depolymerases, which split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases split glycosidic linkage next to free carboxyl groups by hydrolysis while pectate lyase split glycosidic linkages next to free carboxyl groups by β-elimination. Both endo types of PGs and PALs (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known by splitting randomly the pectin chain.

Exo-PGs (EC 3.2.1.67) release monomers or dimmers from the non-reducing end of the chain, whereas exo-PALs (EC 4.2.2.9) release unsaturated dimmers from the reducing end. Highly methylated pectins are degraded by endo-pectin lyases (PL; EC 4.2.2.10) and also by a combination of PE with PG or PAL (Sarkanen, 1991; Pilnik and Voragen, 1993; Martin *et al.,* 2004).

Materials And Methods

All chemicals used were of analytical grade. Dinitrosalicylic acid (DNS), pectin, polygalacturonic acid (PGA), carboxymethylcellulose (CMC), Avicel was obtained from Sigma Chemical Co., USA.

Sampling collection: Samples were collected from different sources such as sewage water (Jeonnong-dong, Dongdaemun-gu, Seoul, Korea), rice straw (El-Bohira governorate, Egypt) and household disposal expired pharmaceuticals (expired Capozide compound), this hazard expired pharmaceutical only 0.1 g/100ml was dispersed in distilled water then filtered using 0.45 µm GHP cellulose acetate (Hydrophilic).

Cultivation: Serial dilutions for various purposes, particularly for estimation of the numbers of viable bacteria in a sample, and it is of obvious benefit to be able to assess the uncertainty at any step of such a series and to be able to construct confidence intervals (CLs) both for the dilution and for any count based on them (Hedges, 2002). The serial dilutions of samples were prepared to obtain "colony forming unit" (CFU) by pipetting 1ml of sample to a tube containing 9ml distilled water to obtain 10^{-1} dilution tube then vortex to mix completely, repeat and continue serial dilution until 10^{-9} dilution tube. Transfer 0.1ml dilution to each of three agar plates containing nutrient agar media (Difco^{TM}) (Atlas, 2005) and distribute evenly with Drigalski spatula. The agar plates were incubated upside down at 37ºC for 1-3 days. The plates with 30-300 colonies were counted and CFU in each plate was calculated. Proteins were estimated by the method of (Bradford, 1976) using bovine serum albumin as a standard.

Polygalacturonase (EC 3.2.1.15) activity

Polygalacturonase (PGase) (EC 3.2.1.15) activity was assayed by the colorimetric method. Briefly, 100µl of suitably diluted cell-free supernatant was incubated with 100µl of substrate (PGA, 1.0%, w/v) at 40ºC for 10 min under static conditions. Reducing sugar was determined using 3,5-dinitrosalicylic acid (DNS) reagent (Ghose, 1987 ; Thygesen *et al.,* 2003), the substrate (900µl) was equilibrated at 50ºC, 100µl sample was added and after 10 min 1500 µl DNS reagent was added. Mixture was finally diluted to 5ml with deionized water (4.4ml). The absorbance of the color developed was measured at 530nm. For the standards, 100µl glucose (2.5-10mM) was added to 900µl substrate and 1500µl DNS reagent (Thygesen *et al.,* 2003). The samples and standards were boiled (100ºC) for 5 min and cooled on ice. One unit of enzyme was defined as the amount of enzyme which catalyses the formation of 1µmol of galacturonic acid/min at fixed pH.

Pectin Lyase (EC 4.2.2.10) activity

Pectin Lyase (PL) (EC 4.2.2.10) activity of the given samples was assayed by the method of (Pitt, 1988), modified by (Kashyap *et al.,* 2000). Briefly, 1ml of suitably

diluted enzyme sample was added to 5.0ml of pectin solution (1% w/v). The volume of the test samples was adjusted to 10ml with distilled water. The samples were incubated at 40ºC for 2hr. This was followed by the addition of zinc sulphate (0.6ml, 9.0% w/v) and sodium hydroxide (0.6ml, 0.5M). The samples were centrifuged (3000 xg, 10 min) and 5.0ml of the clear supernatant was added to a mixture of thiobarbituric acid (3.0ml, 0.04M), HCl (2.5ml, 0.1M) and distilled water (0.5ml). The mixture was heated in a boiling water bath for 30 min, cooled to room temperature and the absorbance of the colored solution was measured at 550nm. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.01 under the condition of the assay.

Avicelase (EC 3.2.1.91) activity

Avicelase (EC 3.2.1.91) activity was assayed by incubation for 24h at 80ºC in a shaking incubator. Reaction mixture (1ml) contained a 1% (w/v) suspension of microcrystalline cellulose (Avicel) in (0.1M) Na-succinate pH 6.0. After removal of solids by centrifugation, aliquots of the supernatants were assayed for the release of reducing sugars. One unit of enzyme corresponds to the release of 1µmol of glucose equivalent per minute (Bronnenmeier and Staudenbauer, 1990).

Carboxymethylcellulase (EC 3.2.1.4) activity

Carboxymethylcellulase (CMCase) (EC 3.2.1.4) activity was assayed by incubation for 60 min at 80ºC in a 0.5% (w/v) solution of carboxymethylcellulose (CMC) in (0.1M) Na-succinate, at pH 6.0. Enzyme and reagent blanks were also simultaneously incubated with the test samples, and the rate of production of reducing sugar was determined. One international unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1µmol reducing sugar from CMC per minute using glucose as standard.

Isolation of DNA Extraction from Bacterial Cultures

DNA extraction was isolated from bacterial isolates using (Genomic DNA from Tissue, User Manual, NucleoSpin® Tissue, April 2007/Rev. 06, MACHEREY-ANGEL GmbH & Co. KG, Germany). The DNA was checked on 1.5% agarose gels and measured by photometry at 260 and 280 nm (Stephan *et al.*, 2004).

Primer design for Real-Time PCR

The primers used in this study hybridize to conserved regions on the 16S rRNA gene. The forward primer Bsub5 F (5`-AAGTCGAGCGGACAGATGG -3`) and the

reverse primer Bsub3 R (5`-CCAGTTTCCAATGACCCTCCCC -3`) were used for the qRT-PCR amplification of a 595-bp fragment corresponding to an internal protein of the "*bacillus subtilis* group" 16S rRNA. TaqMan polymerase was used with the buffer supplied by the manufacturer, with a final primer concentration of 0.4µM (Wattiau *et al.,* 2001).

Real-Time PCR

Amplification and detection were carried out in 48-well optical plates on MJ Mini (BIO-RAD) personal thermal cycler, with TaqMan Universal PCR 2x master mix (Applied Biosystem), a 0.4 µM concentration of each primer, a 0.2 µM concentration of each probe and 1 to 2 ng of sample DNA in a final volume of 20 µl per reaction. The whole 16S rDNA sequence was amplified for quantitative PCR with incubation at 95ºC for 3 min and a hold of 95ºC for 15 sec to activate DNA polymerase (TaqMan polymerase) and annealing incubation at 60ºC for 1 min, followed by 45 cycles of 95ºC for 15 sec, and 30ºC for 1 min. The specific fluorescent probe was labeled at the 5` end with the reporter dye 6-carboxyfluorescin (FAM).

Results and Discussion

At the end of incubation time the bacterial counts of CFU were differ according to the source of samples Table (1). All the samples plates were counted and stained using Gram reaction to select only the bacilli organisms obtained according to the primer specification used in our study also for determine the isolate characterization. Only four bacilli isolates out of nine were selected from sewage water samples after 24h incubation period. Three isolates out of six and only one out of two isolates were selected from rice straw and expired Capozide compound samples respectively Table (2).

Table (1): Determination of CFU and bacterial count of isolates from three sources.

Source of sample	CFU* (10^5/ml)	Incubation period (h)
Sewage water	16.33	24
Rice straw	21.00	48
Expired Capozide compound	8.33	72

* Colony forming unit.

The bacterial isolated from various sources were screened for pectinases and cellulases enzymes productivity as industrially important by-products by these bacilli using spectrophotometric assay methods. As it is shown in Table (3), SW9 isolate had maximum PGase enzyme specific activity 222.53 IU/mg, maximum pectin lyase specific activity 465.55 IU/mg was observed by SW2 isolate. However, at ECC2 and RS3 isolates exhibited the maximum Avicelase and CMCase specific activities 533.32 IU/mg and 657.21 IU/mg, respectively. On the other hand, Emtiazi *et al.,* (2007) showed that *Paenibacillus* strain E was isolated from soil and exhibited CMCase 4 Uml^{-1} when it was grown on CMC as the only sources of carbon.

Table (2): Characterization of bacterial isolates and symbols.

Type of sample	Isolate symbol	Isolate characterization
Sewage water	SW1	G- [1], cocci
	SW2	G+ [2], rods
	SW3	G+, rods, ENS [3]
	SW4	G+, cocci
	SW5	G-, cocci
	SW6	G+, rods
	SW7	G-, cocci
	SW8	G-, cocci
	SW9	G+, rods, ENS
Rice straw	RS1	G+, rods, ENS
	RS2	G-, cocci
	RS3	G+, rods
	RS4	G-, cocci
	RS5	G+, rods
	RS6	G-, cocci
Expired Capozide compound	ECC1	G+, cocci
	ECC2	G+, rods

[1]: Gram negative; [2]: Gram positive; [3]: Endo-spore former.

According to large multiple of *Bacillus* 16S rRNA sequences, it was noticed that group-specific consensus motifs were present in the variable domains I and II. Two PCR primers, Bsub5 F and Bsub3 R, were chosen that were predicted to specifically amplify a 595-bp DNA fragment of the "*Bacillus subtilis* group" 16S rRNA (Wattian *et al.,* 2001). These primers respectively encompassed nucleotides 59-79 (variable domain I) and 625-646 (variable domain II) relative to the *E. coli* 16S rRNA numbering. Nucleotide database comparisons using BLASTN (Altschul *et al.*, 1998). Given the considerable number of *Bacillus* species established to date, our

choice was restricted to species that were representative of the different *Bacillus* groups (Ash *et al.,* 1991) or that were seen to be either identical or very similar in the 16S rRNA multiple alignment.

Table (3): Production of pectinase(s) and cellulase(s) by selected bacterial isolates.

Source & symbol of isolate	PGase assay			PL assay			Avicelase assay			CMCase assay		
	Total activity (IU/ml)	Protein content (mg/ml)	Sp. act. (IU/mg)	Total activity (IU/ml)	Protein content (mg/ml)	Sp. act. (IU/mg)	Total activity (IU/ml)	Protein content (mg/ml)	Sp. act. (IU/mg)	Total activity (IU/ml)	Protein content (mg/ml)	Sp. act. (IU/mg)
Sewage water												
SW2	284.61	1.4	203.29	512.11	1.1	**465.55**	350.01	1.9	184.21	460.36	1.1	418.51
SW3	340.10	4.2	80.97	399.22	1.4	285.16	390.22	1.8	216.79	566.61	0.96	590.22
SW6	285.77	1.3	219.82	315.61	2.1	150.29	341.39	1.6	213.37	455.39	0.81	562.21
SW9	422.81	1.9	**222.53**	411.00	1.2	342.50	451.34	2.3	196.23	496.01	1.3	381.55
Rice straw												
RS1	270.11	1.3	207.78	408.12	1.9	214.74	492.99	1.0	492.99	421.62	1.4	301.16
RS3	320.21	3.2	100.06	320.21	2.2	145.55	510.20	1.2	425.17	611.21	0.93	**657.21**
RS5	280.39	1.4	200.28	500.31	1.3	384.58	503.31	1.4	359.51	365.50	1.1	332.27
Expired Capozide compound												
ECC2	103.33	2.3	44.93	109.21	2.4	45.50	533.32	1.0	**533.32**	375.67	2.2	170.76

The two PCR primers, Bsub5 F and Bsub3 R and the probe TaqMan polymerase were highly specific for the target group of microorganisms and did not detect a control without DNA template. For SW2, SW3 and SW6 isolates as shown in Table (4), were successfully amplified and closely identical for the *Bacillus* strains *Bacillus atrophaeus, Bacillus pumilus* and *Bacillus licheniformis* respectively. However, RS1 and RS3 isolates in this study were amplified and closely related to *Bacillus amyloliquefaciens* and *Bacillus subtilis* respectively. On the other hand, SW9, RS5 and ECC2 isolates were not amplified using RT-PCR and not closely related to any of *Bacillus subtilis* group and the primers were not specific for the target group of these *Bacillus* isolates.

Bacillus isolates and qRT-PCR were generated by adding PCR primers specific for *Bacillus subtilis* group and running a RT-PCR reaction. Cycle threshold indicates the cycle at which the fluorescence detected by the instrument passed a predetermined threshold, indicating the presence of a PCR product. Melting temperature (Tm) is the temperature at which the amplified DNA double helix denatured into single strands. This is sequence specific. The isolate samples in this study and tested for quantification real-time PCR were have approximately the same melting point of *Bacillus subtilis* group except SW9, RS5 and ECC2 isolates were not determined any similarities related to *Bacillus cereus, Bacillus circulans* and

paenibacillus polymyxa strains Table (4). Results of the standard curve and *Bacillus subtilis* group strains of template DNA primer and probe were shown in Table (5).

Table (4): Similarities of *Bacillus* strains closely related to our isolates in the comparative quantification RT-PCR.

Isolate symbol	Closely related strain	Strain No.	Amplification RT-PCR
SW2	*Bacillus atrophaeus*	ATCC 49337T*	+
SW3	*Bacillus pumilus*	ATCC 7061T	+
SW6	*Bacillus licheniformis*	ATCC 14580T	+
	Bacillus cereus	bc1	-
RS1	*Bacillus amyloliquefaciens*	ATCC 23350T	+
RS3	*Bacillus subtilis*	bs1	+
	Bacillus circulans	ATCC 4513T	-
	paenibacillus polymyxa	ATCC 842T	-
RS5	NA	-	-
SW9	NA	-	-
ECC2	NA	-	-

+: Sign indicates that the "*B. subtilis* group" amplification RT-PCR was positive.
-: Sign indicates that the "*B. subtilis* group" amplification RT-PCR was negative.
NA: Not amplified.

The technique of quantitative real-time PCR has emerged recently, owing to the advanced development of fluorogenic chemistry, as an effective means for the detection and quantification of microorganisms at very low concentrations. Comparing with the conventional hybridization and PCR based techniques; qRT-PCR not only has better sensitivity and reproducibility, but also is quicker to perform and has a minimum risk of amplicon carry-over contamination (Fang and, Zhang 2006).

To facilitate global quantification of *Bacillus* isolates and normalization to a single standard curve of total bacteria in this study, a pair of primers binding to highly conserved regions on the 16S rRNA gene were used to amplify the full-length 16S rRNA. Amplification of the whole 16S gene is necessary because the regions to identify and define the different bacterial species or groups are distributed over the full length of the 16S rRNA sequence. Contamination of TaqMan polymerase with bacterial genomic DNA that is not removed during the purification process is considered a serious problem with use of real-time PCR for bacterial quantification (Stephan *et al.*, 2004). Contamination usually occurs in last cycles of real-time PCR (after 40 cycles). To circumvent the problem, some authors

recommend treating Taq polymerase with DNase I to reduce contamination (Lyons *et al.,* 2000; and Nadkarni *et al.,* 2002).

Table (5): Standard curve results and "*B. subtilis* group" strains of template DNA primers and probe.

Parameter	Result
rRNA gene copies	3.1 x (10^3 ~ 10^5)
Slope average (±SD)	-3.421 ± 0.33
r^2	0.996
Intercept	43.244
"*B. subtilis* group" strains used	*B. atrophaeus* *B. subtilis* *B. pumilus* *B. amyloliquefaciens* *B. circulans* *B. cereus* *P. polymyxa*

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تعريف بعض عزلات باسيلس منتجة للإنزيمات الخارجية باستخدام تفاعل كمي للبلمرة المتسلسل

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الملخص العربي

الهدف الرئيسي من هذه الدراسة هو تحديد بعض عزلات من باسيلس معزولة من مصادر مختلفة لإنتاجها بعض المنتجات النشطة حيويا مثل إنزيمات سيلولاز وبكتيناز وتعريف هذه العزلات باستخدام تفاعل البلمرة المتسلسل كميا (qRT-PCR) اعتماداً على جينات 16S rRNA فى كل عزلة. تم عزل عدد 9,6,2 عزلات من جنس باسيلس من مياه الصرف الصحي وقش الأرز المتحلل فى صورة كومة وأدوية صيدلية منتهية الصلاحية (كابوزيد) على التوالي. تم اختيار أربع عزلات فقط من جنس باسيلس من مجموع تسع عزلات على أساس وجود البرايمر الخاص بمجموعة الباسيلس وهم عزلات SW2, SW3, SW6,SW9 لقدرتهم على إنتاج انزيمات سيلولاز وبكتيناز حيث أظهرت قدرة فائقة على إنتاج إنزيم بكتين لاياز وبولى جالاكتيرونيز 475.55 , 222.53 (وحدة /مجم) على التوالي بينما أظهرت ثلاث عزلات من مجموع ست عزلات أظهرت قدرة فائقة على إنتاج إنزيمات كربوكسى ميثيل سيلولاز وهم RS1,RS3,RS5 أظهرت العزلة RS3 قدرة على إنتاج 657.21 (وحده/ مجم) ومن جهة أخرى أظهرت عزلة واحدة فقط وهى ECC2 من عزلتان قدرة فائقة على إنتاج إنزيم أفيسيلاز 533.32 (وحده/مجم). تم استخدام طريقة تفاعل البلمرة المتسلسل باستخدام طريقة Taq Man RT-PCR لرصد وعد وتحديد جينات 16S rRNA فى أجناس باسيلس محل الدراسة. تم استخدام بادىء أمامي Bsub5F وبادىء عكسي Bsub3R لتعظيم جينات 16S rRNA مجموعة باسيلس ساتلس بالمقارنة بسلالات مرجعية حيث تم توضيح بنجاح الشبة الواضح أو التطابق بين عزلات الباسيلس المعزولة فى هذه الدراسة مع مجموعة باسيلس ساتلس المرجعية.