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ISOLATION AND IDENTIFICATION OF LOCAL ISOLATE OF *BACILLUS SUBTILIS* PRODUCED POLYGALACTURONASE FROM ROTTEN APPLE

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Abstract

This study was aimed to obtain a local isolate of *Bacillus* sp. from some of local sources which can produce polygalacturonase (PGase). There are 43 isolate were chosen from 170 isolate from different sources including samples from rotten apple (treatment A), samples from rotten tomato (treatment B), samples from soil of sunflower (treatment C) and samples from soil of corn (treatment D). These treatments were cultured on nutrient agar and incubated at 37°C and 55°C. Primary screening of these isolates were achieved on nutrient agar enriched with 1% pectin under same previous conditions. Fourteen isolate were revealed high efficiency for pectin hydrolysis because of their ability to produce polygalacturonase. So it gives high ratio for clear zone diameter to growth diameter (Z/G). The isolate A5 from rotten apple incubated at 37°C was revealed the highest ratio of (Z/G). So it attained to 3, while the isolate C3 from soil of sunflower which incubated at 37°C was revealed lowest ratio of (Z/ G) and attained to 0.2.

At secondary screening step the isolate A5 also revealed highest enzyme activity and attained to 265 unit / ml, while the isolate C1 was revealed lowest enzyme activity and attained to 159. 5 unit / ml. The isolate A5 was chosen as the best one for polygalacturonase production, so the morphological, biochemical and genetic identification by amplification of 16s rRNA gene for this isolate was achieved. The results of biochemical tests using Vitek 2 compact system were revealed that the isolate A5 was belong to *Bacillus subtilis* with compatibility of 95%. These results were identical to genetic analysis which revealed that the isolate A5 was belong to *Bacillus subtillus* also with compatibility of 100% in comparison with some of isolates in genetic bank of NCBI and the molecular size of 16S rRNA gene was attained to 1196 bp.

Key word: *Bacillus* subtilis; polygalaturonase; rotten apple; 16S rRNA; Vitek 2 system.

Introduction

Enzyme are proteins produced from most of biological cells. It consider one of an essential factor for activation many of bioreactions different in it's mechanism and characterizations. pectinases are one of these enzymes which produced from plants and microorganism like fungi and bacteria (Karine *et al*., 2011; Jayanie *et al*., 2005; Mehrnoush *et al*., 2011; Nakkeeran *et al*., 2010). These enzymes were used to provide carbon and energy sources for these organisms (Murad *et al*., 2011). The differential between pectinolysis enzymes due to the high complexity of pectins (Massa, 2006). Polygalcturonase (PGase) was considered one of pectinolysis enzyme which produced from bacteria, fungi and some of plant cells (Fons *et al*., 2003). There are many types of *Bacillus* sp. which have high ability to produce PGase. (Dharmik and Gomashe, 2013; Rehman *et al*., 2012). *Bacillus subtilis* and *Bacillus* licheniformis were responsiable for production of about 50% of industrial enzyme (Schallmey *et al*., 2004). The demand of PGase was increased day by day, So it must be necessary to find an inexpensive methods for industrial production of these enzymes either by soild stat fermentation depending upon fruit or rice wastes (Siumara *et al*., 2012) or by submerged fermentation (Kusuma and Reddy, 2014). Many of companies widly carried out production of PGase with good characterization by microorganisms like bacteria (Pedrolli *et al*., 2009; Jacob *et al*., 2008). There are many applications of pectinolytic enzyme such as oil extraction, juice calrification, tea and coffee fermentation and other applications (Pedrolli *et al*., 2009). The idea of this study is to obtain a local isolate have ability to produce one of pectinolytic enzyme (polygalcturonase) because of it's commercial importance and wide range uses in industrial applications.

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Material and Methods

All medium were sterilized in autoclave at 121°C, 15 psi for 15 min. Nutrient agar was prepared as instructions of provider company (Salucea) by dissolve 28 gm of media in one liter of sterilized distilled water and used to growth and preservation of isolates as slant. The supplemented medium was nutrient agar with pectin which used in primary screening (Chiliveri *et al*., 2012), containing the following components (gm / liter distilled water): yeast extract 10; pectin 10; $CaCl_2$ 0.5; Agar 20. Liquid medium (Nutrient broth) was prepared as instructions of provider company (Hemedia) by dissolving 13 gm of media in one liter of sterilized distilled water and the pH was calibrated to 7, then the medium was sterilized in autoclave at same previous conditions. This medium was used in preservation of isolates and in preparation of bacterial inoculum. The medium of polygalacturonase production was prepared according to Sanjay *et al*., (2016), by dissolving the following component in liter of distilled water : pectin 20 gm ; yeast extract 10 gm; K_2HPO_4 6 gm; KH_2PO_4 2gm; $(NH_4)_2$ SO_4 1.4 gm; $MgSO_4.7H_2O$ 0.1 gm. The medium was sterilized in autoclave at 121°C, 15 psi for 15 min. The pH was calibrated to 7 by sterilized sodium hydroxide (1 N). The medium was used in production of polygalacturonase at secondary screening.

Isolation of *Bacillus* sp. produced polygalacturonase

The sources of isolation were included four samples: the first one is from rotten apple (treatment A); The second from rotten tomato (treatment B) ; third from soil of sunflower (treatment C) and fourth from soil of corn (treatment D) belong to horticulture and field crops departments of college of agricultural engineering sciences / Baghdad university. Soil samples were taken after crops harvesting, then it cleaned from impurities and seived. Ten gram were suspended from each sample and dissolved in 90 ml of sterilized distilled water and heating at 80°C to remove vegetative cells and only spores will survived. Serial dilutions will done, then 0.1 ml of diluent was transfer to petridish with duplicate, then add nutrient agar with homogenous spreading. After solidification, medium was incubated in inverse direction at 37°C and 55°C for 48 hour.

Purification of isolates

The single isolates were purified by sub-culturing on nutrient agar and pick up those isolates that have similar characterstatic of *Bacillus* which have irregular edges, creamy color and somewhat sticky.

Isolates maintains

The Isolate that revealed high level of production of PGase at primary and secondary screening were maintained on slant of nutrient agar and in nutrient broth at refrigerator temperature. The pure isolate was used during the experiments and activation done whenever it takes.

Screening of Isolates

- Primary screening (semi - quantitative)

The supplemented medium of nutrient agar was used for primary screening of isolates. The selected bacteria was transfer by loop and spreading on this medium as spot. The petridishes were incubated at 37°C and 55°C for 48 h., the ability of isolates for producing PGase were detected by measuring the ratio of diameter of clear zone which represented pectin lysis (Z) to diameter of bacterial growth (G). This ratio was considered as primary indication of isolate efficiency for PGase production at experiment circumstances (Manasi *et al*., 2013).

- Secondary screening (Quantitative)
- McFarland Standard curve

The method of (3) was followed for getting of MacFrland standard curve and the linear equation was extracted and using in calculation of approximate number of CFU/ ml for activated cultures to determine the volume of inoculum which must be add to production medium of enzyme (Fig. 1).

Preparation of inoculum

Fresh **c**ultures were prepared after 48 h., of growth on nutrient broth at 37°C and 55°C from selected isolates of primary screening. The number of cell / ml were calculated by determination the absorbance on 600 nm and using linear equation of MacFarland standard curve. The dilutions were done to get the exact number of cell must be add to production medium.

Enzyme Production

Submerged culture was followed for enzyme

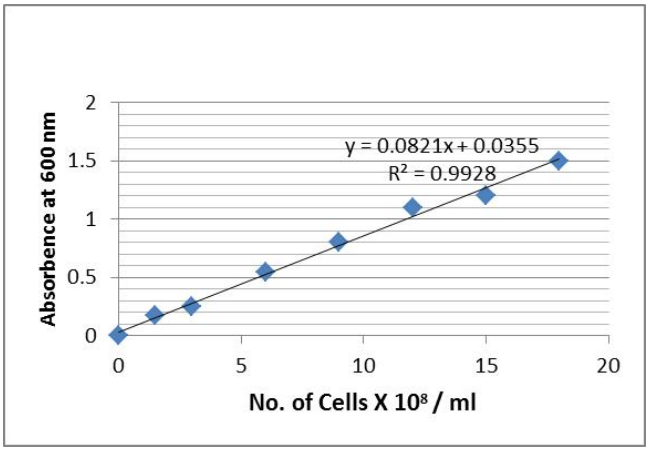

Fig. 1: Standard curve of MacFarland for calculation of cell / ml.

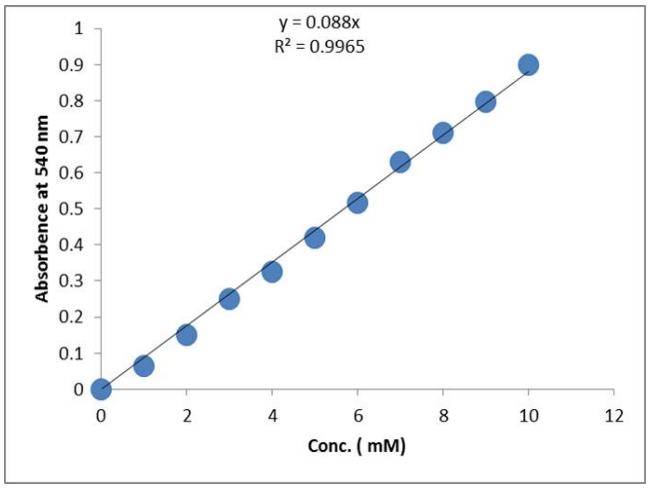

Fig. 2: Standard curve of galacturonic acid.

production (Sanjay *et al*., 2016), where 98 ml of production medium were transfer to Erlenmeyer flask (300 ml), 2ml of inoculum contain 1×10^7 CFU / ml and incubated in incubator shaker at 37°C for 48 h., with speed 1500 rpm.

The standard curve of galacturonic acid was carried out according to method mentioned by Padmavathi and Raghuram, 2016. Where gradient concentrations of galacturonic acid (1-10 mM) were prepared and stock solution of galacturonic acid was diluted by Sodium acetate buffer pH 5 (Fig. 2). one ml of diluents were transfer to cleaned tubes and 3ml of Dinitrosalcilic acid (DNSA) were add (Miller, 1959). Tubes were incubated in boiling water bath for 15 min. Volume were completed to 5 ml with distilled water. Absorbance of solutions were assayed at 540 nm.

Enzyme activity assay

The enzyme activity was assayed using method mentioned by (Kusuma and Reddy, 2014). 0.5 ml of enzyme extract was add to 1 ml of substrate solution (1% pectin), incubate in water bath at 40°C for 20 min, 3ml of DNSA were add to mixture and incubate in boiling

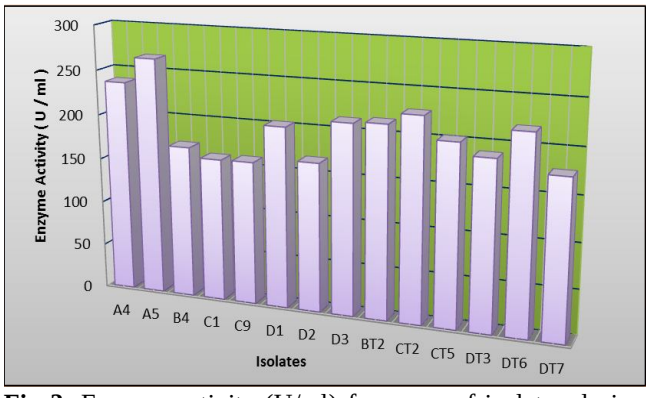

Fig. 3: Enzyme activity (U/ml) for some of isolates during secondary screening.

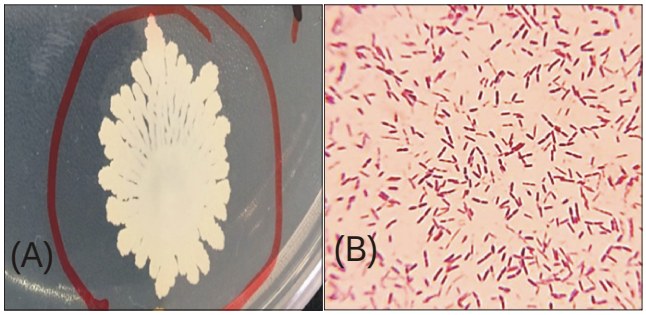

Fig. 4: A: Growth of isolate A 5 on Nutrient Agar; B: Microscopic characterization of isolate A5 after gram stain by simple compound microscope with power of magnification X 1000

water bath for 15 min. Complete volume to 5 ml with distilled water and the spectrophotometric absorbancey was determined at 540 nm. Blank was by prepared in same way exclude addition of enzyme was added after stopping of reaction to calibrate the spectrophotometric.

The unite of enzyme activity (U / ml) were defined as quantity of enzyme that liberate one Micromole of galacturonic acid in one minute under experiment conditions.

Identification assay

The identification tests of selected isolate from primary screening which included a microscopic and culturing characteristics. Gram stain, shap, aggregation, and spore position were assayed. Cultural. characteristics of colonie (size, appearance, color, edge and surface height) when growth on nutrient agar and supplemented nutrient agar at 37°C for 48 h., were studied. The isolate was also identified by Vitek 2 compact system after culturing on blood agar for 24 h., then 3 ml of physiological solution were inoculated with loopfull of pure isolate. Put the mixture in sterilized tube in Densi Chek apparatus and measure it's optical density at 600 nm. Identification kit of Bacillaceae which signed BLC was used. It contain 46 of biochemical test and the procedure of BioMerieux company was followed. The genetic analysis to identification of selected isolate was also carried out by amplification of 16S rRNA gene using PCR technique (Shriparna *et al*., 2013). The sequence of nucleotide were also studied and compared with those gene bank database in NCBI. The genetic analysis include extraction of selected isolate DNA using promega protocol and kit provided from this company. The purity of DNA extract (1 µl) was determined by Nanodrop spectrophotometer and the absorbance at 260 nm and 280 nm were measured and used the following equation : Purity of DNA extract = Absorbance at 260nm / Absorbance at 280 nm. The amplification of 16 S rRNA using forward primer (F27) 5'AGAGTTTGATCCTGGCTCAG'3 and Reverse primer

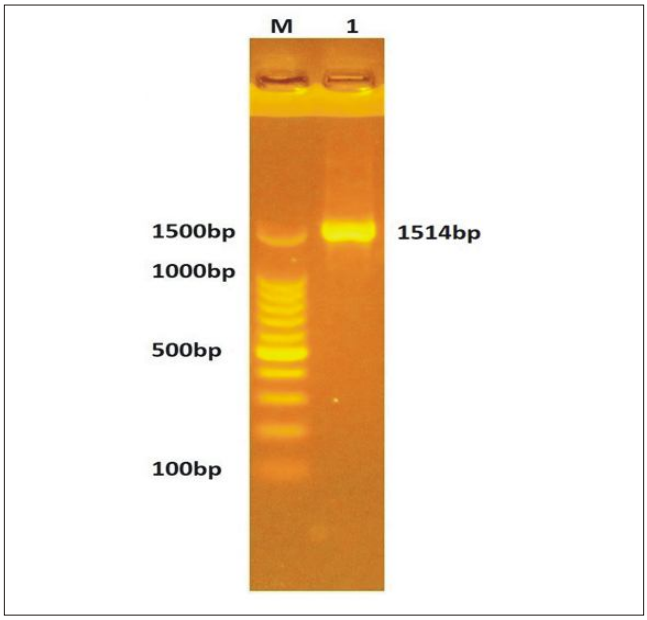

Fig. 5: Gel electrophoresis of gene 16S rRNA on 1% agrose for isolate A5, stained by Ethidium bromide, Lane M: ladder (100bp); lane 1 sample.

(R1492) 5'TACGGTTACCTTGTTACGACTT'3 according to (Shriparna *et al*., 2013). Adding 12.5 µl of Master mix and 1 µl of these primers and 8.5 µl of Nuclease free and 2 µl of DNA extract. The eppendruff tube was transfer to Thermocycler and the following program were used : First denaturation of the target DNA was carrid out at 94°C for 5 minutes followed by second denaturation for 30 sec., then primer annealing at 60°C for 45 sec. Extension at 72°C for 1min. the reaction was repeated for 30 cycles. Final extension were done at 72°C for 7 min. At the end of the cycling, the reaction mixture was held at cooling temperature (4°C) for 10 minutes. PCR amplification was detected by of agarose gel electrophoresis, So 5µl of product and ladder (100-1500 bp) provided from promega company were add to 1% of agrose prepared by dissolve 0.5 gm of agrose in 50 ml of 1 X TBE buffer. The mixture was heated in microwave oven for 2min. and 2 µl of ethidium bromid was added. The gel was poured at 50-55°C in electrophoresis mold. put the comb in gel to make samples slot and leave gel to solidification. Remove the comb and add 1 X TBE buffer to cover gel surface (16). The run of electrophoresis were carried out at 60 mA and 90 volt. Coming down of dye were noticed. At the end of run gel was transfer to UV-light transillminator to detect the separated bands. The amplicon and primers were sent to Macrogen company

Table 1: Results of primary screening for number of isolates grown on nutrient agar supplemented with pectin at 37°C and 55°C for 24-48 h.

Strain No.	*[1] Sample code	Incu. Tem. (°C)	*[2] Hydrolyis efficiency (Z/G)
1	A1	37	1.27
2	A2	37	0.5
3	A3	37	1
4	A4	37	**1.5**
5	A5	37	**3.0**
6	A6	37	0.8
7	A7	37	1.2
8	B1	37	1.07
9	B2	37	1.1
10	B3	37	0.6
11	B4	37	**1.3**
12	B5	37	1.0
13	C1	37	**1.42**
14	C2	37	1.17
15	C3	37	0.2
16	C4	37	1.0
17	C5	37	1.1
18	C6	37	1.2
19	C7	37	0.8
20	C8	37	0.9
21	C9	37	**1.4**
22	C10	37	1.3
23	D1	37	**1.5**
24	D2	37	**1.6**
25	D3	37	**1.5**
26	D4	37	1.3
27	D5	37	0.7
28	D6	37	1.0
29	D7	37	1.17
30	BT1	55	1.2
31	BT2	55	**1.7**
32	CT1	55	1.1
33	CT2	55	**1.4**
34	CT3	55	0.7
35	CT4	55	1.2
36	CT5	55	**1.5**
37	DT1	55	1.2
38	DT2	55	0.7
39	DT3	55	**1.4**
40	DT4	55	1.35
41	DT5	55	1.0
42	DT6	55	**2.4**
43	DT7	55	**1.4**

*[1] The treatments as follow : A (rotten apple, incubation temp. 37°C); B (rotten tomato, incubation temp. 37°C); C (sample of soil from sun flower field, incubation temp. 37°C); D (sample of soil from corn field, incubation temp. 37°C); BT (rotten tomato, incubation temp. 55°C); CT (sample of soil from sun flower field, incubation temp. 55°C); DT (sample of soil from corn field, incubation temp. 55°C).

*[2] Results represent the mean of two duplicate.

to determine the sequence of nucleotides. The sequence of nucleotide were compared with data base of gene bank belong to NCBI using Blast program to confirmed the identity of isolate.

Results and Discussion

Isolation and primary screening

Forty three isolate were chose from 170 isolate which obtained from many samples of rotten apple (treatment A), rotten tomato (treatment B). Samples from soil of sunflower (treatment C) and samples from soil of corn field (treatment D) belong to horticulture and field crops departments / College of Agricultural engineering and sciences / University of Baghdad. These isolates were distinguished by their ability to produce polygalacturonase (PGase). These isolates gives clear zone around growth area. The efficiency of pectin hydrolysis were determined by calculate the ratio of zone diameter (Z) to diameter of bacterial growth (G) at 37°C and 55°C on nutrient agar supplemented with pectin. Table 1, revealed the efficiency of pectin hydrolysis by isolates. 14 isolates were showed in this stage had high ratio of pectin hydrolysis compared with others isolates, so these isolates were chosen to submit to secondary screening. The differences between these isolates in pectin hydrolysis because to their genetic differences in polygalacturonase production. This agree with Chiliveri *et al*., (2012), which indicate that 12 isolates from 20 isolates of *Bacillus* sp. that be able to hydrolyze pectin. Another study revealed that 25 of fungi strains that be able to hydrolyze polygalacturonic acid as main carbon source and produce clear hydrolyze zone (Saeed *et al*., 2007).

Secondary screening

Fourteen isolates which revealed high efficiency to hydrolyize pectin during primary screening were subjected to secondary screening to evaluate their ability to produce polygalacturonase by determination enzyme activity. The results indicate that the isolate A5 from rotten apple which growth at 37°C give the highest production of PGase and the enzyme activity attained to 265.5 U/ ml, while isolate C1 from soil of sunflower field revealed a lowest enzyme activity and attained to 150 U/ ml (Fig. 3). Therefore the isolate A5 was chosen to complete this study. The results of primary and secondary screening were compatible in determination of the best isolate compared to others in achieving higher production of enzyme activity.

Identification of selected isolate

Identification of isolate A 5 was carried out by cultural and morphological assay moreover biochemical test by Vietik 2 system and genetic analysis of 16S rRNA gene. The isolate A5 revealed creamy colony with irregular edge when growth on nutrient agar at 37°C (Fig. 4a). The morphological test of isolate A5 revealed that this isolate was gram positive and it seem to be short rode shaped bacteria, endospore forming (central or subterminal position) and the colony were aggregate as chains (Fig. 4b). These results were agree with (Duguid, 1996; Logan and De Vos, 2009), where they pointed out the general characterizations of *Bacillus* genes, where it grow at surface of medium because it is aerobic and the colony with creamy color and have irregular edges and curly surface. The results of Vitek 2 (Table 2), revealed the isolate A5 was also belong to *Bacillus* subtilis with probability of 95 % when we compared with biochemical test of *Bacillus* genus.

The genetic analysis was achieved depending upon identification of 16S rRNA gene, Firstly the extraction of

Table 2: Biochemical test of isolate A5 by Vitek 2 system and using BCL kit for Bacillus genus.

Identification Information	Analysis Time:	13.82 hours	Status:	Final
Selected Organism	95% Probability	Bacillus subtilis		
	Bionumber:	1362170715447261		
ID Analysis Messages				

Biochemical Details																	
1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	(-)	10	PyrA	+	11	AGAL	+	12	AlaA	-	13	TyrA	+	14	BNAG	-
15	APPA	+	18	CDEX	-	19	dGAL	-	21	GLYG	+	22	INO	+	24	MdG	+
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	(-)	47	dTAG	-	48	dTRE	+
50	INU	+	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	-
60	OLD	-	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

Table 3: The sequence of nucleotides (FASTA) of 16S rRNA gene for isolate A5.

Total number of rules	16S rRNA gene sequences	Gene
1196	CCGAATGCGGCGTGCCTATTAATGCAGTCGAGCGGACCGACGGGAGCTT GCTTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGC TTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCA CTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCCCGTGAGTGATG AAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCG TTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCT AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC GGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATG TGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAAC TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATG CGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTG TAACTGACGCTAAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATA CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTT CCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGT ACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA GGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGG GCAGAATGACAGGGGGTGCAAGGGTTGTCCTCACCTCCGGTCCTGGAG AATTTGGGGTTAAATCCCCGCACCAAAGGCCACCCCTTGTTTTTTTTTT GCCGCCATTTACTTTGGGGGCCCCTTAGGGGAAGGCCGGGGACAA ACCGCAAGAAAGGGGGGGGTGAAGGTCAAATAAAAAAGGCGCC	1_27F16SrRNA

Table 4: Ratio of similarity of local isolate A5 with *Bacillus strains* in NCIB-blasted record.

Sequences producing significant alignments Download Manage Columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subtilis subsp. inaquosorum strain FJAT-46211 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MK859964.1
Bacillus subtilis subsp. inaquosorum strain FJAT-46184 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MK859944.1
Bacillus subtilis strain G1 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MK720397.1
Bacillus subtilis strain PK5-6 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MG988239.1
Bacillus subtilis strain JZ78 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KY194280.1
Bacillus subtilis strain BT5 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MK184985.1
Bacillus subtilis strain TT207 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MH819693.1
Bacillus subtilis strain cm45 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MF187639.1
Bacillus subtilis strain ban d 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KX530950.1
Bacillus subtilis strain p1 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	MF073326.1
Bacillus subtilis strain X502 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KU240496.1
Bacillus subtilis strain GQJK4 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KY952698.1
Bacillus subtilis strain G9 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KX343968.1
Bacillus subtilis strain Y5 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KU160383.1
Bacillus subtilis strain Kakrayal_1 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KT985358.1
Bacillus subtilis strain 1-2 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KT831431.1
Bacillus subtilis strain PWK36 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KJ620422.1
Bacillus subtilis strain GLMP6 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KF364495.1
Bacillus subtilis strain TUST018 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KC456632.1
Bacillus subtilis strain D31 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KC441769.1
Bacillus subtilis strain D29 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KC441767.1
Bacillus subtilis strain D23 16S ribosomal RNA gene, partial sequence	2608	2608	100%	0.0	100.00%	KC441763.1

DNA for selected isolate has been done and the purity was determined by measure the ration of absorbance at 260nm to absorbance at 280nm. Therefore the purity of DNA extract of isolate A 5 attained 1.8. This result conforms with many studies which pointed out that the purity of DNA extraction for prokaryota was considered highly purified if the ratio of absorbance at 260nm to absorbance at 280nm was equal ≥ 1.8 (Turner *et al*., 2005). Then amplification of 16 S rRNA gene by PCR technique was achieved. The results of electrophoresis were revealed that there is a single band of amplified gene (Fig. 5).

This indicate a successful engagement of primers with gene target (16S rRNA) without other parts of DNA. The molecular weight of amplicon was determined and attained to 1514 bp (Fig. 5). Many studies point out that using gene 16S rRNA with successfully for identification of many species of bacteria and it gives a decisive results (Aarti and Khusro, 2015; Rehman *et al*., 2012). Also Özdemir *et al*., (2011) indicate that the length of amplified target of *Bacillus subtilis* attained 1013 bp, While Salman *et al*., (2016) found that molecular weight of amplified gene of 16 S rRNA for *Bacillus* sp. were 1082 bp. The differences in molecular weight of amplified gene for 16S rRNA may be due to differences between sources of isolates, moreover the differences between procedure of DNA extraction and differences in program conditions which used in PCR technique (Yemisi *et al*., 2019). The products of PCR amplification and primers were sent to Macrogen Company to study the sequence of nucleotides for 16S rRNA gene. The results revealed that the sequence of 1_27 F 16s rRNA gene contain 1196 nucleotide (Table 3).

The sequence were evaluated and trimmed some of these sequences which had low quality especially irregular peaks. Formation of these parts may be due to primers coupling or it represent short results of amplification which disappeared during electrophoresis on agrose gel. After the analysis of these sequences using BLAST program to obtain the gene matching with gene bank in NCBI, the results revealed that sequences of 16S rRNA for isolate A5 were identical to those sequences of *Bacillus subtilis* with similarity 100 % when matched with NCBI-blasted record (Table 4). For this reason the local isolate A5 was considered belong to *Bacillus subtilis.*

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