CLASSICAL AND MOLECULAR APPROACHES FOR IDENTIFICATION OF *RHIZOBIUM LEGUMINOSARIUM, AZOTOBACTER CHROOCOCCUM* AND *BACILLUS MEGATERIUM*

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Abstract. The present study aimed to identify of *Rhizobium leguminosarium* from Broad bean nodules, *Azotobacter chrococcum* and *Bacillus megaterium* from the soil in Sulaimani city/Iraq. Cultural characteristics showed that the colonies of bacteria were circular, smooth convex, and white color in *Rhizobium leguminosarium* and *Bacillus megaterium*, while creamy colored for *Azotobacter chrococcum.* The isolated bacteria were motile, rod, and gram-negative in *Rhizobium leguminosarium* and *Azotobacter chrococcum,* and gram-positive in *Bacillus megaterium*. Biochemical tests found that *Rhizobium leguminosarium* was positive for Catalase and negative for Indole Production, Methyl Red, Vogas Proskauer, Citrate Utilization and Gelatinase, *Azotobacter chrococcum* was positive for Catalase and Citrate Utilization, while *Bacillus megaterium* was positive for Catalase, Citrate Utilization and Gelatinase. *Rhizobium leguminosarium* utilized Starch Glucose, Mannitol, Galactose, Raffinose, Trehalose, Mannose and Xylose, but *Azotobacter chrococcum* did not utilize Galactose and xylose, while *Bacillus megaterium* did not utilize Trehalose**.** The molecular method based on the detection of plasmid DNA, *nod*D2 and *nod*D3 in *Rhizobium leguminosarium*, chromosomal DNA, *nif*H2 and *nif*H3 in *Azotobacter chrococcum*, genomic DNA and two random primers in *Bacillus megaterium* on gel electrophoresis have been successfully applied. We conclude that cultural, morphological and biochemical tests used for identification of these isolates was equivalent to the molecular-based method.
Keywords: *biological nitrogen fixation, nod and nif genes, phosphate solubilization, PCR, gel electrophoresis*

Introduction

Rhizobia are soil bacteria able to form nodules and establish a symbiosis with the roots of leguminous plants. During the symbiotic process, rhizobia reduce atmospheric nitrogen into a form directly assimilated by plants (ammonium) (Berrada and Fikri-Benbrahim, 2014). *Azotobacter* is an aerobic and free-living bacterium that can fix atmospheric nitrogen into the soil. Plants are able to utilize the ammonia as a nutrient (Biomate India, 2008). *Bacillus megaterium* is a gram-positive bacteria; it has a very efficient protein secretion system, grow on several different and cheap carbon sources, lack endotoxins, and are nonpathogenic. Proteins produced from and with it are of great industrial importance (Bunk et al., 2010). *B. megaterium* has the ability to solubilize phosphorus in the soil (Patel et al., 2016).

Nitrogen (N) and Phosphorus (P) are mineral nutrients often limiting plant growth because they are required in large amounts in relation to their availability in the soil

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(Harpole et al., 2011). Biological Nitrogen Fixation (BNF) is known to be a key to sustain agriculture and to reduce soil fertility decline (Kahindi et al., 2009). (BNF) is an efficient source of fixed N2 that plays an important role in land remediation (Mohammadi et al., 2012). Nitrogen fixation in symbionts and free-living microbes is catalyzed by nitrogenase; an enzyme complex encoded *nif*DK and *nif*H genes. Nitrogenase itself consists of a molybdenum-iron protein (MoFe), subunit I and an iron-containing protein (Fe) subunit II. This biological process between *Rhizobium* strains and their legume partners can happen under low levels of available nitrogen with the help of many different genes such as a *nod, nif, fix,* production of polysaccharides, competition, infection process, and host specificity. *Nod*D is appositive transcription regulator from the Lysr family and present in all *rhizobia* (Shamseldin, 2013). The structural *nif* genes from taxonomically diverse microbes are nearly identical and function in a similar manner to encode nitrogenase (Ruvkin et al., 1980). *Azotobacter spp.* studies on the genetics in this genus should take into consideration their *nif* genes which are responsible for fixing nitrogen (Dashti, 2011; Khider, 2011; Abid, 2013; Mohamed, 2017). The *nif*H gene is the biomarker most widely used to study the ecology and evolution of nitrogen-fixing bacteria (Raymond et al., 2004). Phosphate solubilization ability of microorganisms is associated with the release of low molecular weight organic acids (Puenta *et a*l., 2004). The phosphate solubilization ability of microorganisms is regulated by several genes (Young and Lai, 2008), these genes are induced under phosphate starvation and constitute the Pho regulation (Bagyaraj et al., 2000). *Bacillus megaterium var. phosphaticum* was used to create a bio-preparation called Phosphobucterin with the purpose of enhancing mineral phosphorus solubilization (Sylvia et al., 1999).

The present study was carried out to compare two strategies for the isolation and identification of *Rhizobium leguminosarium* from bean nodules, *Azotobacter chroococcum* and *Bacillus megaterium* from soil depending on classical phenotypic approaches and through molecular methods by detection of *nod*D genes in *R. leguminosarium* and *nif*H genes in *A. chroococcum* and cheek the presence of *B. megaterium* that reported in different researches to do significant role in biological nitrogen fixation and phosphorus solubilization up to date. We used the molecular methods addition to the classical taxonomical process to support and to be sure that the bacteria which identified by the classical taxonomical process are same using molecular methods or not, because often the tests or characteristics of the classical taxonomical process of many bacterial species are similar which in some cases make the identification difficult. So, this classification should be confirmed and supported by molecular examination. This study consider the importance report and the first time identified of these bacteria depending on molecular approach in Bakrajow soil in Sulaimani city, Kurdistan region, Iraq.

Materials and methods

Isolation of bacteria

Rhizobium leguminosarium spp.

Nodulated Broad bean was selected from the organic farm of Bakrajow; the plant washed thoroughly with running tap water, then healthy, unbroken, firm and pink nodules were selected for the isolation. Isolation of *Rhizobium* was done on Yeast Extract Mannitol Agar (YEMA) (Handley et al., 1998; Castro et al., 2003; Kucuk et al., 2006).

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The selected nodules were repeatedly washed in sterile water for 3-4 min, then washed with 70% ethyl alcohol, next rewashed with sterile water, after that crushed with a sterile glass rod. The resulting suspension was streaked on YEMA medium and incubated at 28 ℃ for 2-3 days. The developed colonies were isolated and purified then identified (Agrawal et al., 2012).

Azotobacter chroococcum spp.

Modified Ashby's Medium (MAM) was used as the specific *A. chroococcum* medium (Astafyeva and Shalabayeva, 2016). Two grams of soil samples were added to 500 ml Erlenmeyer flasks containing 100 ml (MAM), then stirred on rotary shaker 180 rpm for 10 min, streaked out on (MAM) and incubated at 28 ± 2 °C for 2-5 days to be checked for purity (Marwa et al., 2010).

Bacillus megaterium

B. megaterium was isolated through suspending 10 g of soil in 100 ml distilled water in a conical flask. Aerobic spore formers pasteurized a diluted soil sample at 80 °C for 10-15 min and serial dilutions were made from soil suspension, then 100 µl of soil suspension at different dilutions powered onto the surface of Sperber's medium which is a selective medium for isolating *B. megaterium*. Incubated at 28-30 °C for 48 h. Developed colonies of *B. megaterium* appearing on the plates were observed (Shiva et al., 2010).

Purification of bacteria

A single colony of each isolated bacteria (48 h old surface film) was streaked on their selective media; the growth was observed depending on the type of bacteria, *R. leguminosarium* and *B. megaterium* after 24-48 h and *A. chroococcum* after 3-7 days at 28 °C of incubation. The well separated and apparently uncontamination colonies appearing on the plates were streaked on agar medium, plating and picking were repeated at least 4-5 times (Ausubel et al., 1987).

Identification of isolated bacteria by cultural and microscopical characteristics

Cultural characteristics for each isolated were achieved on their media including the shape, color and type of the colonies after 24-48 h for *R. leguminosarium* and *B. megaterium*, and after 3-7 days for *A. chroococcum* at 28 °C of incubation, while microscopical tests for each isolated were carried out for fixed smears using gram stain, shape, and motility followed the method described by Mahon and Manuselis (2000).

Identification of isolated bacteria by biochemical tests

The isolated bacteria were characterized biochemically such as Catalase test and Gelatinase test according to Forbes et al. (2002), methyl red, vogas proskauer and Indole production depending on Atlas et al. (1995) and Citrate utilization according to Mahon and Manuselis (2000).

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Identification of isolated bacteria by carbohydrate fermentation test

Carbohydrate fermentation profiles were conducted in Yeast manitol broth for *R. leguminosarium*, Modified Ashby's broth for *A. chroococcum* and Sperber's broth for B. *megaterium* as carbon sources amended with 0.004% of chlorophenol red and 1% of one of the investigated sugars namely: Starch, Glucose, Mannitol, Galactose, Raffinose, Trehalose, Mannose and Xylose, growth was observed after 5 and even up to 15 days at 28 °C of incubation. Acid and gas production were detected by observing the color change of chlorophenol red from red to yellow (acid) and accumulation of gas in Durham's tube (gas) (Forbes et al., 2002).

Maintenance and storage of bacterial culture

The cultures were maintained for a short time at slant medium, and for a long time without losing their activity in 20% glycerol and stored at - 70 °C (Ausubel et al., 1987).

Identification of bacteria by molecular basis protocol

Extraction of Chromosomal, Genome and Plasmid DNA from bacteria

Plasmid DNA was extracted from pure culture of *R. leguminosarium* using Genet bio, PrmePrep Plasmid DNA isolation kit, and Chromosomal DNA from pure culture of *A. chroococcum* and Genome DNA from pure culture of *B. megaterium* were extracted using the PrestoTM Mini gDNA Bacteria Kit Protocol.

PCR amplification conditions

The conditions of PCR amplification: for *nod*D2 and *nod*D3 were performed according to the modified method of Del Cerro et al. (2015a) at cycling conditions consisted of a single cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 2 min, 60 °C for 30 s and 72 °C for 30 s, and a final extension cycle at 72 °C for 6 min., while PCR reactions for *nif*H2 and *nif*H3 were done depending on Setubal et al. (2009), and for random primers 1 and 2 of *B. megaterium* carried out depending on Patil et al. (2013). The forward, reverse and random primers are shown in *Table 1*.

Gel electrophoresis

The PCR products were checked by electrophoresis by dissolve 0.5, 1, 1.5 and 2% (w/v) agarose gel depending on the size of amplified DNA fragments in 100 ml IX TBE buffer by heat in microwave oven for 3 min, then cooled solvent to 45 °C at room temperature, Gels were stained in ethidium bromide and then covered the gel tank by lid and electrophoresis was run at (80 V, 100 V) 10 V/cm. DNA fragments were visualized at 312 nm with a UV-transilluminator Image (Helmut et al., 2004).

Results

Isolation

R. leguminosairum was isolated from Broad bean plant nodules using yeast mannitol agar medium, and colonies observation were done after 2-3 days of incubation at 28 ± 2 °C (*Fig. 1*). The nodules were found positive for the presence of *R. leguminosarium*. *A. chroococcum* was isolated from the soil using Modified Ashby's

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medium, and the growth was observed after 3-7 days of incubation at 28 °C (*Fig. 2*). *B. megaterium* was isolated from soil using Sperber's medium, and the colonies were observed on the plates after 48 h of incubation at 28-30 °C (*Fig. 3*). Then the bacteria were characterized morphologically, biochemically and molecularly.

Table 1. *The forward, reverse and random primers used*

Primer	Sequence (5'-3')	Nucleotide	Reference
*nod*D2- F-	(GTA GGC CAT AAT GTC CAG A)	19	Del Cerro et al., 2015a
*nod*D2- R-	(GCG GCT TTA TAC TCA CCA)	18	
*nod*D3- F-	(GAG CTA CCT CGA CTG CTA)	18	Del Cerro et al., 2015b
*nod*D3- R-	(CTA CCG CCA TGA TCA CCA)	18	
*nif*H2- F-	(CGCCGGCGCAGTGTTTGCGG)	20	Setubal et al., 2009
*nif*H2 -R-	(CACTCGTTGCAGCTGTCGGC)	20	
*nif*H3- F-	(CGATGACTGAAGACTGAACGAG**)**	22	Setubal et al., 2009
*nif*H3 -R-	(AAGGTGCGGTCAGGAGAGAA)	20	
Random primer1	(GGT GCG GGA A)	10	Patil et al., 2013
Random primer2	(GTA GTC ATA T)	10	

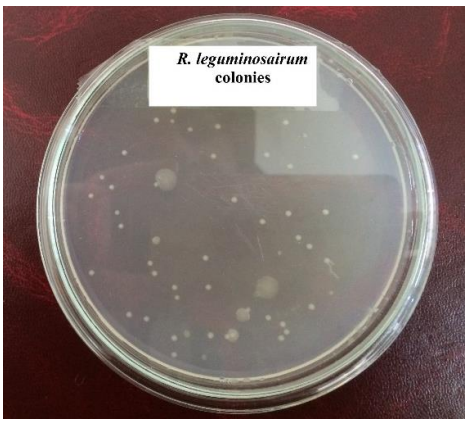

Figure 1. *Single colonies of R. leguminosarium growth on YEMA medium*

Figure 2. *Single colonies of A. chroococcum growth on modified Ashby's medium*

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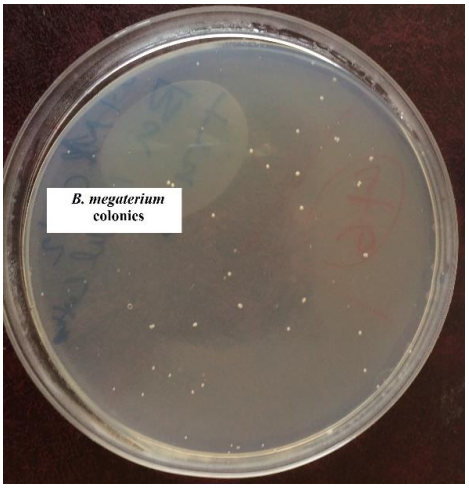

Figure 3. *Single colonies of B. megaterium growth on Sperber's medium*

Identification of isolated bacteria by the morphological and biochemical characteristics

To identify the species, the following were determined: the growth, morphological, biochemical properties, and carbohydrate fermentation tests according to Brenner et al. (2005).

Table 2 represents cultural and microscopical characteristics of *R. leguminosarium, A. chroococcum and B. megaterium*. The colonies of all the bacteria were circular in shape and smooth convex in type, but in color, *R. leguminosarium and B. megaterium* were white while *A. chroococcum* was creamy color. The all bacteria were rod in shape and motile, but in gram stain, *R. leguminosarium* and *A. chroococcum* were negative while *B. megaterium* was positive.

Table 2. *The cultural and microscopical tests of the isolated Rhizobium leguminosarium, Azotobacter chroococcum and Bacillus megaterium*

Test	**Bacteria**		
	Rhizobium leguminosarium	***Azotobacter chroococcum***	***Bacillus megaterium***
Colonies			
Shape	Circular	Circular	Circular
Color	White	Creamy	White
Type	Smooth convex	Smooth convex	Smooth convex
Bacterium			
Gram Stain	-	-	+
Shape	Rod	Rod	Rod
Motile	Flagella	Flagella	Flagella

Table 3 represents the biochemical characterizations of *R. leguminosarium, A. chroococcum* and *B. megaterium*. All the bacteria were found positive for Catalase test, and negative for Indole Production, Methyl Red, Vogas Proskauer tests, but in Citrate Utilization was positive in *A. chroococcum* and *B. megaterium*, and negative for *R.*

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leguminosarium, while Gelatinase test was negative for *R. leguminosarium* and *A. chroococcum* and positive for *B. megaterium*.

Table 3. *The biochemical characteristics of the isolated Rhizobium leguminosarium, Azotobacter chroococcum and Bacillus megaterium*

Characteristics	Bacteria		
	Rhizobium leguminosarium	***Azotobacter chroococcum***	***Bacillus megaterium***
Catalase	+	+	+
Indole Production	-	-	-
Methyl Red	-	-	-
Vogas Proskauer	-	-	-
Citrate Utilization	-	+	+
Gelatinase	-	-	+

R. leguminosarium, *A. chroococcum* and *B. Megaterium* were also examined for fermentation of the various sugars, *Table 4* shows that the *R. leguminosarium* found positive for assimilation of all sugars, *A. chroococcum* found negative for assimilation of Galactose and xylose while positive for other sugars, and *B. megaterium* found negative assimilation for Trehalose but positive for others.

Table 4. *The Carbohydrate fermentation tests of the isolated Rhizobium leguminosarium, Azotobacter chroococcum and Bacillus megaterium*

Carbon sources	Bacteria		
	Rhizobium leguminosarium	***Azotobacter chroococcum***	***Bacillus megaterium***
Starch	+	+	+
Glucose	+	+	+
Mannitol	+	+	+
Galactose	+	-	+
Raffinose	+	+	+
Trehalose	+	+	-
Mannose	+	+	+
Xylose	+	-	+

Identification of bacteria by the molecular basis

Moreover based on the molecular method, plasmid DNA and *nod*D genes of *R. leguminosarium*, chromosomal DNA and *nif*H genes of *A. chroococcum*, and genome DNA and random primers of *B. megaterium* were detected.

The chromosomal DNA from *A. chroococcum*, genome DNA from *B. megaterium* and plasmid DNA from *R. leguminosarium* were detected on the agarose gel electrophoresis, the result shows in *Figure 4*, lane 2, 4, 6 respectively.

The PCR was carried out for all tested isolates to check the presence of *nod*D2 and *nod*D3 genes in *R. leguminosarium*, *nif*H2 and *nif*H3 genes in *A. chroococcum*, and to know the presence of the *B. megaterium* in the region using two random primers. Their

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banding patterns of DNA on agarose gel electrophoresis were compared with their references' primers. The PCR products for *R. leguminosarium* were: *nod*D2 (100 bp) (*Fig. 5*) and *nod*D3 (150 bp) (*Fig. 6*), for *A. chroococcum* were: *nif*H2 (250 bp) (*Fig. 7*), and *nif*H3 (130 bp) (*Fig. 8*) lanes 2, while these bands did not appear in the negative control (lanes 3) in all figures.

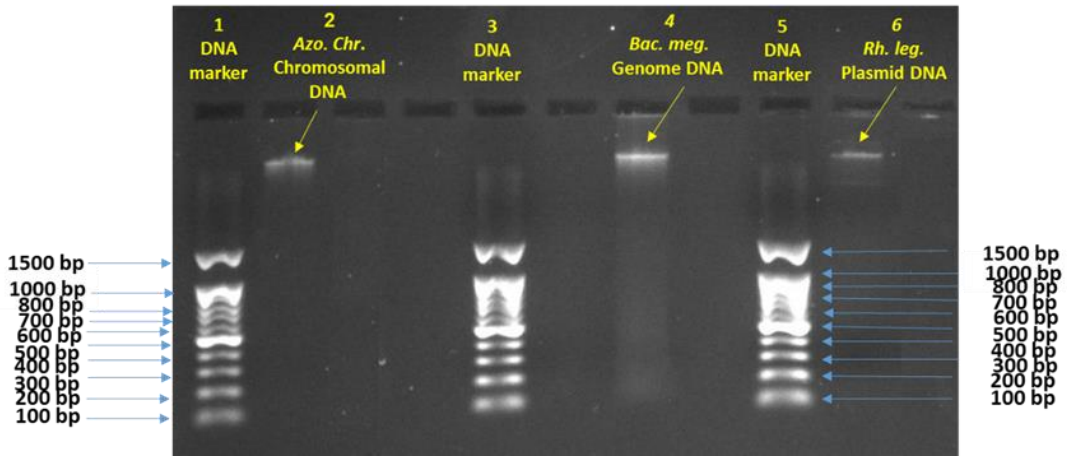

Figure 4. *Agarose gel electrophoresis shows DNA marker lanes (1, 3, and 5), extracted chromosomal DNA from A. chroococcum lane 2, genome DNA from B. megaterium lane 4, and plasmid DNA from R. leguminosarium lane 6*

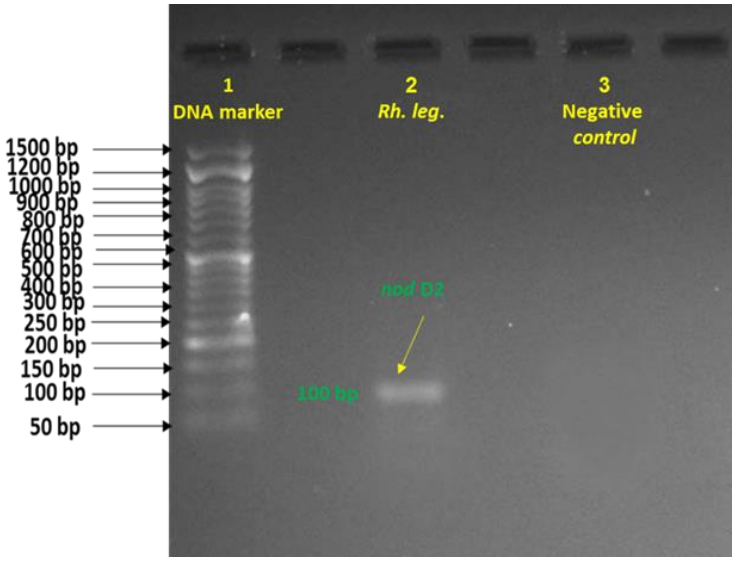

Figure 5. *Agarose gel electrophoresis shows the PCR amplified products of the nodD2 gene (100 bp). Lane 1: DNA marker, lane 2: R. leguminosarium (+ve PCR product), lane 3: negative control (-ve PCR product)*

For *B. megaterium* using random primers (1 and 2) were obtained high amplification rate and reproducible banding pattern which confirmed the existence of *B. megaterium* in the regions (*Fig. 9*) lanes 2 and 3. Therefore, all the bacteria that classified depending on the traditional approach were compared with that of molecular-based methods.

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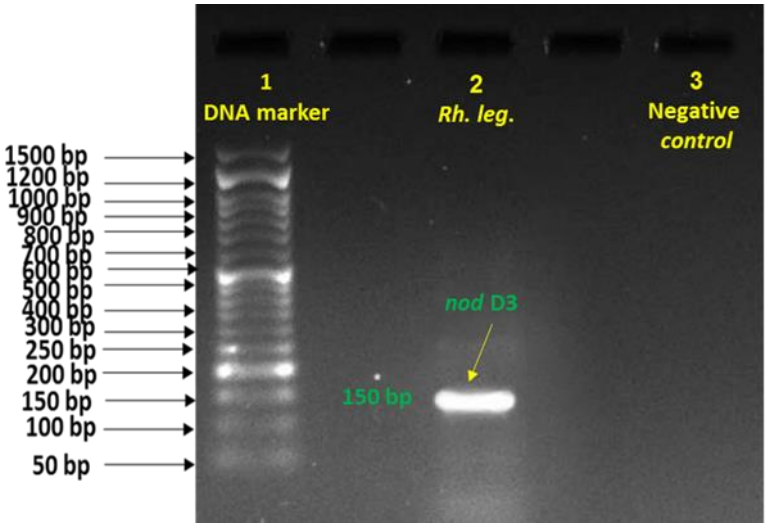

Figure 6. *Agarose gel electrophoresis shows the PCR amplified products of the nodD3 gene (150 bp). Lane 1: DNA marker, lane 2: R. leguminosarium (+ve PCR product), lane 3: negative control (-ve PCR product)*

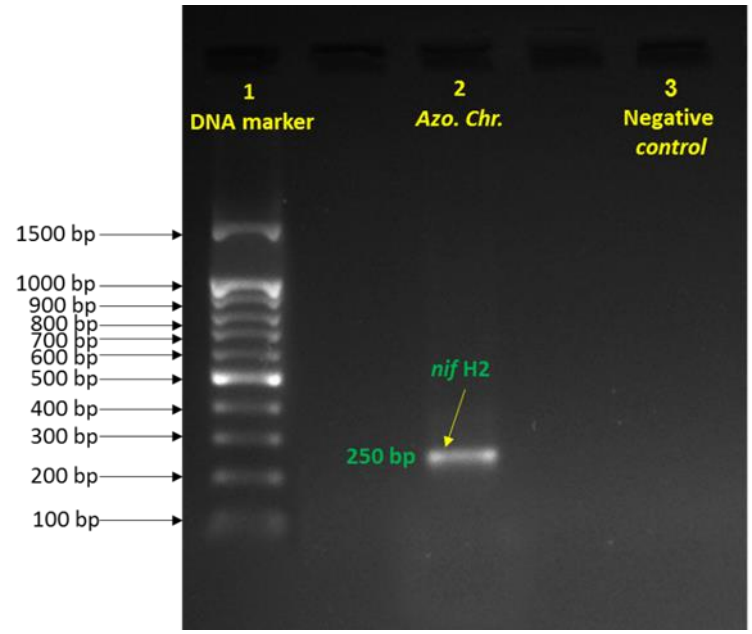

Figure 7. *Agarose gel electrophoresis shows the PCR amplified products of the nifH2 gene (250 bp). Lane 1: DNA marker, lane 2: A. chroococcum (+ve PCR product), lane 3: negative control (-ve PCR product)*

Discussion

This research work aimed to compare two different methods reported in the literature for the isolations of *R. leguminosarium* from Broad bean nodules, *A. chroococcum* and *B. megaterium* bacteria from Bakrajow soil in Sulaimani city, Iraq, in order to prove the presence and identify these bacteria. Different criteria have been presented by several researchers to delimit characteristic which can be used as a key for identification of all studied bacteria, generally included morphological, cultural, biochemical and molecular

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characteristics, highlighting the points which may be used as the diagnostic indications. All bacteria were processed through the characteristics using common procedures for isolation and identification of all isolated bacteria using selective media for each bacteria.

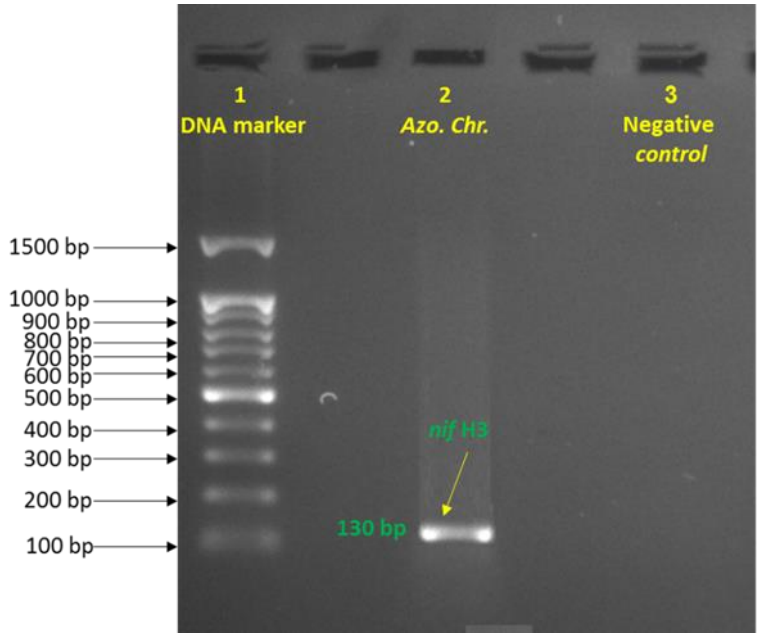

***Figure 8**. Agarose gel electrophoresis shows the PCR amplified products of the nifH3 gene (130 bp). Lane 1: DNA marker, lane 2: A. chroococcum (+ve PCR product), lane 3: negative control (-ve PCR product)*

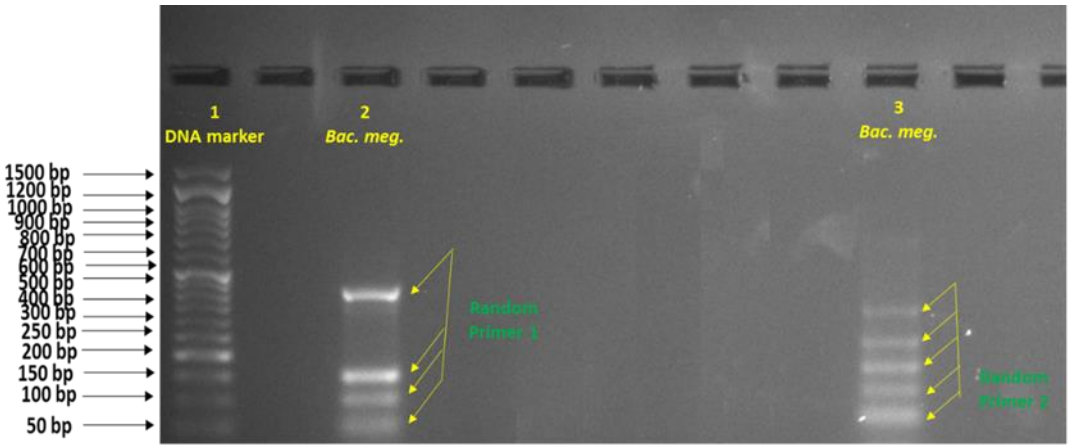

***Figure 9.** Agarose gel electrophoresis shows the PCR amplified products of the B. megaterium isolate generated using 10-mer random primers: Lane 1: DNA marker, lanes 2 and 3: random primers 1 and 2 respectively (+ve PCR products)*

The nodules of Broad bean showed presences of *R. leguminosarium* basing on the characteristics, the colony morphology on a selective medium is clearly recognizable (*Fig. 1*), so the isolated bacteria classified as *R. leguminosarium* when the colonies were circular in shape, white color, smooth convex in type, and the bacterium was motile, rod

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and gram-negative. The biochemical characterization found positive for Catalase test, and negative for other biochemical tests, when *R. leguminosarium* utilized starch and all other sugars and produced of gas and acid, considered the important observations to classify the isolated bacteria as *R. leguminosarium*, Depending on these characteristics the isolated *Rhizobium* was classified as *R. leguminosarium* according to Brenner et al. (2005). Same results have also reported by other researchers like Shoukry et al. (2013), Datta et al. (2015) and Tesema (2018).

Obviously molecular characterization of the isolated *Rhizobium* identified based on the extraction and purification of plasmid DNA from pure culture of *R. leguminosarium* to detect of *nod*D genes, because nodulation genes (*nod* genes) are located on the plasmid which is also called mega plasmid (Kumari and Sinha, 2011), which have been successfully applied and the banding pattern of plasmid DNA on agarose gel electrophoresis showed in *Figure 4* lane 6. The PCR was carried out for isolated bacteria by using complementary primers in order to amplify and cheek the presence of *nod*D2 and *nod*D3 for isolated *Rhizobium* which identified as *R. leguminosarium* by the traditional approach. The *nod*D2 and *nod*D3 genes were (100 bp) and (150 bp) in *Figures 5* and *6*, lanes 2, respectively. *Nod*D genes act as the family of regulatory (Kidaj and Wielbo, 2010). In legume nitrogen-fixing symbioses, rhizobial *nod* genes are obligatory for initiating infection thread formation and root nodule development (Fujishige et al., 2007). *Nod*D is the core signaling protein, reacting to plant flavonoids then binding to nod boxes, binding sites upstream of *nod* genes, typically *nod*A and/or *nod*B, triggering the expression of a *nod* gene cascade and thus the construction of the Nod Factor (Jones et al., 2007). The *nod* gene is controlled by *nod*D gene. *Nod*D proteins act as a transcriptional activator of inducible nod gene (Kumari and Sinha, 2011). So the importance of *nod*D in nitrogen fixation process gives importance to our choice of these genes (*nod*D2 and *nod*D3) which are located on the plasmid for this study. The common nodulation genes (*nod*DABC) are found in all bacteria that nodulate legumes (Giraud et al., 2007). Also Black et al. (2012) reported that all fourteen species of *Rhizobium* contained *nod*D, *nod*E, *nod*G, *nod*I, *nod*M, *nod*P, *nod*Q, *nod*V, and *nod*W. As well Rossen et al. (1985), Hu et al. (2000) and Zeze et al. (2001) showed and confirmed presence of *nod*D in *R. leguminosarium.*

The soil sample was processed through the colony characteristics, morphology, and biochemical tests, using common procedures for isolation and identification of *A. chroococcum* and *B. megaterium* species in the soil using selective media for each bacteria. The sample showed presences of the two species depending on their characteristics.

The colony morphology of *A. chroococcum* on Modified Ashby's medium clearly appeared (*Fig. 2*), and the medium that used is to be selective enough, so the isolated classified as *A. chroococcum* when the colonies were circular in shape, creamy color, smooth convex in type and the bacterium was motile, rod and gram-negative, in biochemical characterization found positive for Catalase test and Citrate utilization, but negative for other biochemical tests. The important observations that the isolates classified as *A. chroococcum* when utilized different carbon sources such as starch, glucose, mannitol, raffinose, trehalose and mannose. Marwa et al. (2010), Dashti (2011), Khider (2012), Abid (2013) and Mohamed (2017) obtained same results when they isolated different strains of *Azotobacter* from the soil of different regions in Kurdistan-Iraq. The isolated *Azotobacter* was classified as *A. chroococcum* according to Brenner et al. (2005) in Bergey's Manual of Determinative Bacteriology.

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Indeed molecular characterization of the isolated *Azotobacter* based on the extraction of chromosomal DNA from pure culture of *A. chroococcum* and the banding pattern of the chromosomal DNA on agarose gel electrophoresis showed in *Figure 4* lane 2. Then the *nif*H genes detected on chromosomal DNA of *A. chroococcum,* because *nif* genes in some free-living bacteria are located on the chromosome (Kumari and Sinha, 2011). Using of the complementary primers to well-conserved regions in the bacterial genome, led to amplify the *nif* genes (nitrogenase genes) *nif*H2 and *nif*H3 genes by PCR which identified as *A. chrococcum* by classical approach. The *nif*H2 and *nif*H3 genes were (250 bp) and (130 bp) in *Figures 7* and *8,* lanes 2, respectively. *Nif*H acts as dinitrogenase reductase, obligate electron donor to dinitrogenase during dinitrogenase turnover and is required for FeMo-Co biosynthesis and apodinitrogenase maturation (Shamseldin, 2013). The region of chromosome which contain *nif*K, *nif*D, *nif*M, *nif*A, *nif*N, *nif*B, *nif*Q, *nif*Z, *nif*P, *nif*F, *nif*W, *nif*B, *nif*L, and *nif*Y genes are located between the fragment of chromosome which contains *nif*H2 and *nif*H3 and the fragment contain *nif*V, *nif*S, and *nif*U (Hamilton et al., 2011). Importance of region of *nif*H2 and *nif*H3 genes on chromosome took into consideration to select these genes for this study. Previous studies by Kirshtein et al. (1991), Ueda et al. (1995), Zehr et al. (2003), Aquilantia et al. (2004), Mary Ann and Virginia (2007), Dashti (2011), Hamilton et al. (2011), Khider (2012), Abid (2013) and Mohamed (2017) indicated that N-fixing bacteria were investigated by the diversity of nitrogenase genes in different environments, through amplification of *nif* genes, i.e., *nif*H, *nif*D, *nif*V, *nif*K, *nif*U which encode nitrogenase complex from cultivated organisms.

Sperber's medium was used as a selective medium to identify the *B. megaterium* depending on the colonies (*Fig. 3*) and bacterium morphology, and other characteristics, the isolated classified as *B. megaterium* when the colonies were circular in shape, white color, smooth convex in type and the bacterium was motile, rod and gram-positive. In biochemical characterization found positive for Catalase, Citrate utilization and Gelatinase tests, but negative for Indole Production, Methyl Red and Vogas Proskauer. *B. megaterium* had the ability to utilize all carbon sources except trehalose. Other researchers have also reported same results such as Dunca et al. (2005), Tenzing et al. (2016), Patel et al. (2016) and Environment and Climate Change Canada (2018). So the isolated *Bacillus* was classified and identified as *B. megaterium* according to Brenner et al. (2005) in Bergey's Manual of Systematic Bacteriology.

Molecular characterization of the isolated *B. megaterium* was identified through extraction of the genome DNA from pure culture of *B. megaterium* culture, and the banding pattern of the genome DNA on agarose gel electrophoresis showed in *Figure 4* lane 4. The PCR was carried out for isolated bacteria by using two random primers 1 and 2 to detect and cheek the presence of *B. megaterium* in the region which identified as *B. megaterium* by classical approach. In the results found that the banding patterns were intense, clear and reproducible in *B. megaterium* (*Fig. 9*). Previous studies by Shiva et al. (2010) and Patil et al. (2013) indicated the presence of *B. megaterium* in the different area by these primers.

Conclusion

As the result of the morphological and growth characteristics, physiological and biochemical properties of all isolated bacteria according to Brenner et al. (2005) in Bergey's Manual of Systematic Bacteriology, and based on the molecular methods: the

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isolated *Rhizobium* was taxonomically classified as *Rhizobium leguminosarium*, isolated *Azotobacter* as *Azotobacter chroococcum* and isolated *Bacillus* as *Bacillus megaterium*, so the results of the classical approaches were parallel with molecular methods. Our recommendation is using molecular method with multiplex PCR for one step identification of each bacteria is faster, more accurate and low cost. Detection of other *nod* and *nif* genes required further work.

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