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ISOLATION AND CHARACTERIZATION OF RHIZOBIAL ISOLATES IN IRAQ: A FIRST RECORD OF SYMBIOSIS

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Abstract

Rhizobia can fix atmospheric nitrogen and they have a good potential of use as biological control agents against human pathogens and soil borne plant pathogens. This research aimed to isolate and characterize of local isolates of rhizobia using conventional methods. 42 rhizobial isolates were isolated from root nodules of different host plants from different cultural areas in different cities in Iraq. The rhizobial cultures were purified and their agronomic and biochemical characteristics were studied. Three rhizobial isolates, *viz*: OJ27, OJ29 and OJ31 were isolated from root nodules of *Bauhinia purpurea* L. and *Parkinsonia aculeata* L, respectively. Rhizobial genera and species of these isolates were still unknown. A first record of isolation of rhizobial bacteria from root nodules/swellings of *Catalpa speciose* L. All isolates were sensitive to Ciprofloxacin. A high tolerance to Pb $(CH3COO)_2$ were obtained with the exception of *R. grahamii* OJ26. A maximum mean value of inhibition zone was noticed 18.6 mm when filtrate culture of OJ31 were applied against *Candida albicans* AS43. A maximum effect of rhizobial filtrate culture of *R. leuguminisarm* bv. *phaseoli* OJ6 against a pathogenic plant *Aspergillus niger* BI1 was 15.6 mm inhibition zone diameter, whereas a minimum effect was 7.0 mm when *Ensifer fredii* bv. *fredii* OJ12 filtrate culture applied against *Fusarium solani* BI2. It was concluded that there was a potential with the isolates for detail research work for production of exopolysaccharides and antimicrobial activity.

Keyword: Rhizobia, Exopolysaccharide Production, Antimicrobial Activity.

1. Introduction

Rhizobia is a prokaryote microorganism that can fix atmospheric nitrogen when introduce a relationship with a leguminous plant [1]. Genera of rhizobia includes *Rhizobium*, *Ensifer*, *Shinella*, *Mesorhizobium*, *Phyllobacterium*, *Azorhizobium* and *Bradyrhizobium*. Rhizobia are of considerable scientific and economic interest not only for their ability to fix atmospheric nitrogen in leguminous plants, but also it has a good potential of use as biological control agents against human pathogens and soil borne plant pathogens [2].

Rhizobia produce types of surface molecules which contribute to various stages of symbiotic interaction like attachment to roots, host recognition infection thread formation and invasion of nodules. These molecules include extracellular polysaccharide (EPS), cyclic β-glucan and

cellulose fibrils. Rhizobial polysaccharides poorly connected with the bacterial surface and secreted in large amounts in to the environment are known exopolysaccharides (EPSs) [3]. Cyclic β-glucans (CGs) are sugar oligomers secreted by rhizobial cells into their periplasmic space. The lack of CGs decreases bacterial capability of infection thread formation and nodule occupation [4]. In addition, mutations in glucan synthesis genes affect bacterial sensitivity to detergents and motility [5]. Cellulose fibrils produced by rhizobia are involved in the second step of bacterial attachment to host root hairs and biofilm formation [6]. Antibiotic resistance is one of the biggest threats to global health and food security [7]. Bacteriocins are bactericidal substances produced by bacteria and have effective against other bacteria of the same or closely related species. Rhizobial bacteria produce bacteriocins previously have been characterized as phagelike, protease-sesitive or protease resistant [8]. This study aimed to isolate and characterize the rhizobia from root nodules of different plants. Also, to investigate the biochemical activity and antibiogram of the isolated rhizobia.

2. Results and Discussion

Isolation of rhizobia from root nodules of host plants

Different nitrogen fixing plants were collected from different areas of Nineveh, Al-Anbar and Baghdad Governorates and Duhok/Kurdistan/Iraq. Results revealed that a success of isolation of single pure colonies from squashed nodules after growing on TY plates. 42 different rhizobial isolates were isolated, named and the host plants were recorded as shown in Table 1. 27 rhizobial isolates were isolated from different leguminous herbs plants, whereas fifteen rhizobial isolates were isolated from different leguminous tree plants. The rhizobial strains OJ27 and OJ29 which isolated from root nodules of *Bauhinia purpurea* L., the genera and species are still unknown and not-mentioned in literatures till now. Also, in this study, we recorded for a first time in Iraq isolation of rhizobial bacteria from root nodules/swellings (Figure 1-A) of a non-legume tree plant of *Catalpa speciose* L. (Figure 1-B). The isolate was named OJ30, purified and included in cultural and biochemical assays. The isolated strain in this study OJ31 genus and species is still unknown and there is no information about rhizobia which infect this plant up to date (as we know). A success of isolation of different rhizobial isolates were also recorded by many researchers [2, 9].

Table 1. Rhizobial isolates in this study and their host plants.

Isolate No.	Rhizobial genera and species	Host plants
OJ1, OJ3, OJ7, OJ12, OJ13, OJ20, OJ21, OJ22, OJ23	*Ensifer fredii* bv. *fredii*	*Vigna unguiculate* L. (Cowpea)
OJ4, OJ9	*Rhizobium leguminosarum* bv. *viciae*	*Lens culinaris* L. (Lental)

OJ2	*Ensifer meliloti*	*Trigonella foenum-graecum* L. (Fenugreek)
OJ5, OJ33	*Mesorhizobium loti*	*Cicer arietinum* L. (Chickpea)
OJ6	*Rhizobium leguminosarum* bv. *phaseoli*	*Phaseolus vulgsris* L. (Green bean)
OJ8	*Rhizobium leguminosarum*	*Lathyrus sativus* L.(Grass pea)
OJ10, OJ38	*R. japonicum*	*Glycine max* L.
OJ11, OJ40	*R. vignae*	*Vigna radiata* L. (Mungbean)
OJ14, OJ15, OJ16, OJ17, OJ18, OJ19	*Ensifer meliloti*	*Medicago sativa* L. (Alfalfa)
OJ24, OJ26, OJ28, OJ35, OJ36	*R. grahamii*	*Leucaena leucocephala* L.
OJ25, OJ37, OJ39, OJ41, OJ42	*Neorhizobium huautlense*	*Sesbania herbacea* L.
OJ32, OJ34	*R. mesosinicum*	*Albizia odoratissima* L.
OJ27, OJ29	Not mentioned in literatures	*Bauhinia purpurea* L.
OJ30	A first record (in this study) of rhizobial symbiosis	*Catalpa speciose* L. *(Catawba tree)*
OJ31	Not mentioned in literatures	*Parkinsonia aculeata* L.

Figure 1*: Catalpa speciose L. plant.*
A: Root group showing root nodules/swellings.
B: Shoot group.

Reverse inoculation test

Results of reverse inoculation test revealed that the fourty-two isolated rhizobial strains were able to infect the root hairs of the host plants and induce root nodules of the specific host plants. These results confirming the success symbiotic relations with their specific host plants.

Cultural and microscopic characteristics

Cultural characteristics of the colonies of isolated rhizobia showed circular with entire margin, translucent appearance on TY agar medium. TY agar swarm plates showed ability of motility of all rhizobial isolates. Gram's staining of the isolates was confirmed by microscopic observations and the results was negative, i.e., pink, rod cells were noticed under microscope. Earlier studies revealed that rhizobial cells were negative for Gram stain [10].

Biochemical tests

Results of biochemical tests were shown in Table 2.

Table 2: Biochemical tests results of rhizobia isolates.

Rhizobial isolates	Growth on RMM	CU	GL	TSI	Catalase	Oxidase
OJ1	+	+	+	+	+	+
OJ2	+	+	+	+	+	+
OJ3	+	+	+	+	+	+
OJ4	+	+	+	-	+	+
OJ5	+	+	+	+	+	+
OJ6	+	-	-	+	+	+
OJ7	+	+	-	+	+	+
OJ8	+	+	+	+	+	+
OJ9	+	+	+	-	+	+
OJ10	+	+	+	+	+	+
OJ11	+	+	+	+	+	+
OJ12	+	+	+	+	+	+
OJ13	+	+	+	+	+	+
OJ14	+	+	+	+	+	+
OJ15	+	+	+	+	+	+
OJ16	+	+	-	+	+	+
OJ17	+	+	+	+	+	+
OJ18	+	+	+	+	+	+
OJ19	+	+	+	+	+	+
OJ20	+	+	+	+	+	+
OJ21	+	+	-	+	+	+
OJ22	+	+	-	+	+	+
OJ23	+	+	-	+	+	+
OJ24	+	+	+	+	+	+
OJ25	+	+	+	+	+	+
OJ26	-	-	-	+	+	+

OJ27	+	+	+	+	+	+
OJ28	+	+	+	+	+	+
OJ29	+	-	-	+	+	+
OJ30	+	+	+	+	+	+
OJ31	+	+	+	+	+	+
OJ32	+	+	+	+	+	+
OJ33	+	+	-	+	+	+
OJ34	+	+	+	+	+	+
OJ35	+	-	-	+	+	+
OJ36	+	+	+	+	+	+
OJ37	+	+	+	+	+	+
OJ38	+	+	+	+	+	+
OJ39	+	+	-	+	+	+
OJ40	+	+	+	-	+	+
OJ41	+	+	-	+	+	+
OJ42	+	+	-	+	+	+

RMM: Rhizobial Minimal Medium, CU: Citrate Utilization, GL: Gelatin Liquefaction, TSI: Triple Sugar Iron.

Results of study of rhizobial isolates on RMM revealed that ability of these isolates to grow on this medium indicating that intact flow biosynthetic pathways of the amino acids, nucleotides and vitamins [11]. Isolated rhizobial strains were able to utilize citrate as a sole carbon source with the exception of the strains *Rhizobium leguminosarum* bv. *phaseoli* OJ6, *R. grahamii* OJ26, OJ35 and OJ29 which showed negative results. A study showed that rhizobial isolates were varied to citrate utilization assay [12].

Only 13 out of 42 (30.95%) rhizobial isolates, were unable to liquefy gelatin medium. Kumari *et al.* [13] also reported different results with variant rhizobial isolates and mentioned that gelatinase enzyme plays an important role during nodule formation. All rhizobial isolates showed positive results for TSI test with the exception of three isolates *Rhizobium leguminosarum* bv. *viciae* OJ4, OJ9 and *R. vignae* OJ40 which were unable to utilize sucrose and lactose. Only one of the rhizobial isolate *Ensifer fredii* bv. *fredii* OJ13 showed the production of hydrogen sulphide in the medium which is indicated by the formation of a black precipitate that will blacken the medium in the butt of the tube, this indicates ability of this strain to produce cysteine desulfurase which in turn analysis cysteine and releasing hydrogen sulphide.

Utilization of different sugars

Results of utilization ability of rhizobial isolates of different carbon sources revealed that sugar alcohol (Mannitol) is preferred than other saccharides by all rhizobial isolates and supporting better growth than glucose. Variant utilization ability of hexoses monosaccharides, i.e. glucose, galactose, fructose and mannose were obtained by different rhizobial isolates. Five isolates were

unable to utilize hexoses monosaccharides, *viz*: *Ensifer meliloti* OJ16, OJ19 and *Ensifer fredii* bv. *fredii* OJ22 and *R. grahamii* OJ26 and OJ29. Utilization of disaccharides lactose and maltose were variant from very good growth to good and moderate.

Three isolates *viz*: *Rhizobium leguminosarum* OJ8, *Ensifer fredii* bv. *fredii* OJ21 and OJ29 were unable to utilize the disaccharides lactose and maltose. Five rhizobial isolates were unable to utilize the pentose sugars such as arabinose and xylose *viz*: *Rhizobium leguminosarum* OJ8 and *Ensifer fredii* bv. *fredii* OJ21, OJ22 and *R. grahamii* OJ26 and OJ29. The three isolates *Ensifer fredii* bv. *fredii* OJ22, *R. grahamii* OJ26 and OJ29 were couldn't utilize the hexoses monosaccharides. All isolated bacteria obtained from root nodules collected in this study were able to utilize mannitol and most of them were able to utilize the other different studied sugars. These carbon sources are generally utilized by rhizobial cells of the genus *Rhizobium*, the ability of metabolize a broad range of carbon substrates may be advantageous for survival in soil. Results of this study is compatible with the founds of Hossain *et al.* [14], whom recorded priority of mannitol on glucose as carbon source support growth of rhizobial cells.

Sodium chloride tolerance

Results of tolerance of rhizobial isolates to different concentrations of sodium chloride as follows: 2.0, 4.0, 6.0 and 8.0% (w/v) revealed that all the isolates were tolerate 2.0 and 4.0% concentration of NaCl, growth of rhizobial cells were variant from very good to moderate. Generally, there was tolerance to 6.0% NaCl with the exception to *Mesorhizobium loti* OJ33 isolate which were sensitive to this concentration. Three isolates, *viz*: *R. grahamii* OJ24, OJ35 and *Mesorhizobium loti* OJ33 were sensitive to 8.0% NaCl. The other isolates were tolerating this concentration. Gauri *et al*. [15], reported that some rhizobial isolates grown under 4.5% NaCl, therefore reported the isolation of strains highly tolerant to high salt concentrations. Khalid *et al*. [16] obtained eight rhizobial isolates from *Arachis hypogaea* nodules and found that the isolates were tolerate NaCl up to 3.0% but not 4.0%.

Intrinsic resistance to antibiotic

Results of antibiotic resistance assay of rhizobial isolates showed variable susceptibility toward the tested antibiotics. All isolates were sensitive to Ciprofloxacin (CIP) 10 µg/ml, whereas only one strain R. vigne OJ40 was sensitive for Erythromycin 10 µg/ml and the resistance percentage were 97.61%. The resistance percentage for the Tetracycline (TE) and Ceftriaxone (CRO) 10 µg/ml were 19.04 and 21.42%, respectively. The resistance to Rifampicin 78.57% was higher than Amikacin 14.28% at 10 µg/ml concentrations. Results of this study in contrast with results of Gauri *et al*. [15], which revealed that 33% of isolates were resistant to Tetracycline 10 µg/ml, whereas results of this study were compatible with founds of Khalid *et al*. [16].

Abdel-Hakim *et al*. [17]. founds that most isolates were sensitive to Tetracycline and susceptible to Erythromycin and Ceftriaxone. Also, studies revealed that rhizobial isolates were sensitive to Amikacin and majority of rhizobial strains were found to be resistance of a to Rifampicin. Active resistance involves three main mechanisms, namely modification of antibiotic target site, active

efflux of the antimicrobial agent from the bacterial cell and enzymatic modification/degradation of the antimicrobial agent [18].

Heavy metal tolerance test

Results showed in generally tolerance to the tested heavy metals and the higher tolerate were with lead acetate trihydrate Pb $(CH_3COO)_2$ with the exception of *R. grahamii* OJ26 which were sensitive. Also, this strain was sensitive to nickel chloride ($NiCl_2$), cadmium chloride ($CdCl_2$) and zinc sulfate ($ZnSo_4$). Variant tolerance indicated by very good growth to good and moderate growth were noticed. Some isolates were unable to tolerate one or two of studied heavy metals, whereas only two isolates, *viz*: *Ensifer fredii* bv. *fredii* OJ20 and OJ21 were unable to tolerate three of studied heavy metals. The isolated strains of this study were less sensitive to mercuric chloride ($HgCl_2$) (11.90%) in comparison with Wubie *et al*. [19], isolates (48.0%).

Rhizobial isolates were high sensitive to $HgCl_2$ 100 µg/ml concentration. The results founded in this study were similar to those obtained by Abdelnaby *et al*. [20], when isolated rhizobial strains from *Vigna unguiculata* whom founded that rhizobial strains resistant to $HgCl_2$ and Pb $(CH3COO)_2$ at 100 µg/ml concentration. Maatallah *et al*. [21], reported inhibition of some rhizobial isolates by $CoCl_2$ 25 µg/ml concentration, Pb $(CH3COO)_2$ 250 µg/ml concentration, but insensitive to $HgCl_2$ 25 µg/ml concentration. Makkar and Jangra [22], showed less effects of $ZnSO_4$ on rhizobial isolates. A gene encoding a DmeF ortholog has been identified in *R. leguminosarum* bv. *viciae* strain UPM791. DmeF proteins belong to the cation diffusion facilitator (CDF) protein family, which form metal/proton antiport systems to translocate heavy metals across the bacterial membrane [23].

Cell surface molecules production test of rhizobial isolates

Production of cellulose fibrils test

Results of production of cellulose fibrils by different rhizobial isolates revealed that the ability of the all isolates of production this molecules surface depending on uptake of the Congo red dye. Purwaningsih *et al*. [24], obtained 11 rhizobial isolates and Congo red test showed positive results on YEMA medium, whereas another results were negative for Congo red test [14].

Production of β-(1-3) and β-(1-2) glucans test glucans test

All the rhizobial isolates showed ability to produce β-(1-3) glucans indicated by up taking of rhizobial different cultures of Aniline blue dye. Determination of ability of rhizobial isolates to produce β-(1-2) glucans were done by making spots on TY swarm plates and the results revealed that the rhizobial isolates were motile according to expansion of growth circle after 24-48 hours. Clear swarming for each rhizobial isolate spotted plate, gave a good proof for production of β-(1-2) glucans. it was reported that absence of β-(1-2) glucans production has been linked to a defective flagellum and resulting absence of chemotactic response which in turn leads to formation of ineffective nodules [25].

Production of EPS test

Cultural characteristics of rhizobial isolates showed a gummy texture of growth of all isolates. The isolates *R. leguminosarum* bv. *viciae* OJ4 (Figure 2)**,** *Mesorhizobium loti* OJ5, *Ensifer fredii* bv. *fredii* OJ7, *R. leguminosarum* OJ8, *R. leguminosarum* bv. *viciae* OJ9, *R. japonicum* OJ10, *Ensifer meliloti* OJ16, *Ensifer fredii* bv. *fredii* OJ23 and *R. grahamii* OJ26 showed extreme production of EPS according to growth appearance. Janczarek *et al*. [3], were reported that EPS very necessary for establish active relationship between rhizobia and host plants.

Figure 2: *Rhizobium. leguminosarum bv. viciae strain OJ4 cultured on YEMA shows a gumy colonies.*

Determination of amounts of rhizobial EPS

Experiment determination of amount of EPS production by different rhizobial isolates revealed that a maximum EPS production were from *R. leguminosarum* bv. *viciae* OJ4 230 mg/l, and the minimum production was 70 mg/l by the isolate *Ensifer fredii* bv. *fredii* OJ23. A maximum cell dry weight was 280mg/l by the isolate *R. leguminosarum* bv. *viciae* OJ4 and the minimum cell dry weight was 110 mg/l by the isolate *Mesorhizobium loti* OJ5. Regarding to final pH a maximum decrease was pH 3.8 in *R. grahamii* OJ26 fermented cultures after two days' incubation. Mandal *et al*. [26], reported 346 mg/l production of EPS from *Rhizobium* sp. VMA301 and Zhou *et al*. [27], obtained 280 mg/l EPS from a rhizobial isolates EPS production. Earlier study revealed that *expR* active gene which gave a mucoid texture for rhizobial growth was responsible for productivity of EPS [28]. As well as a success symbiotic relationship between Rhizobia and host plants depends on EPS production by rhizobia, a rhizobial exopolysaccharide was a new agent enter in cancer treatment [29].

Antimicrobial activity of rhizobial filtrated cultures test

Human pathogens

The isolates showed a variable antimicrobial activity. A maximum inhibition zone 16.6 mm by *Ensifer fredii* bv. *fredii* OJ13 against *Klebsiella pneumoniae* AS63 (Figure 3, A), whereas a minimum inhibition zone was 8.0 mm by *R. mesosinicum* OJ32. A maximum inhibition zone against *Salmoneii typhi* AS39 were 14.3 mm by *Ensifer fredii* bv. *fredii* OJ13 (Figure 3, B) and a minimum inhibition zone was 7.6 mm by *Neorhizobium huautlense* OJ37. A maximum inhibition

zone was 15.6 mm by *Ensifer fredii* bv. *fredii* OJ13 against *Staphylococcus aureus*AS38 (Figure 3, C) and a minimum inhibition zone was 7.0 mm by *R. leuguminisarm* bv. *phaseoli* OJ6. A maximum effect was 16.3 by *R. leuguminisarm* OJ8 (Figure 3, D) and a minimum effect was 7.0 by *Ensifer fredii* bv. *fredii* OJ21 against *Pseudomonas aeruginosa* AS60.

A maximum inhibition zone was 16.3 mm by *R. vignae* OJ11 against *E. coli* AS37 (Figure 3, E), whereas a minimum inhibition zone was 7.6 mm by *Ensifer fredii* bv. *fredii* OJ1. A maximum effect of rhizobial cell free filtrate was 18.6 mm by OJ31 rhizobial isolated from *Parkinsonia aculeate* L. host plant, a minimum inhibition zone was 7.0 mm by *R. leuguminisarm* bv. *viciae* OJ9 against *Candida albicans* AS65 (Figure 3, F). Joseph *et al*. [8], reported antimicrobial activity of *Rhizobium leguminosarum* bv. *trifolii* against *Salmonella typhi* A and B, *Pseudomonas aeruginosa* and *Staphylococcus citrus*. The inhibition zones were 5.0, 6.0, 8.0 and 6.0 mm, respectively. Deora *et al*. [30], research results revealed that rhizobia isolated from *Vigna radiate* have antimicrobial activity against *E. coli* and *Staphylococcus aureus*. Inhibition zone were 13 and 15 mm, respectively.

Plant pathogens

Results of antimicrobial activity of rhizobial filtrate cultures test against plant pathogens showed a maximum effect of the filtrate culture of *R. leuguminisarm* bv. *phaseoli* OJ6 was 15.6 mm against *Aspergillus niger* BI1 (Figure 3, G), whereas aminimum effect were 7.0 mm when filtrate culture of *Ensifer meliloti* OJ15 applied against *Aspergillus niger* BI1. A maximum antimicrobial effect of the filtrate culture of *Ensifer fredii* bv. *fredii* OJ13 was 13.0 mm against *Fusarium solani* BI2 (Figure 3, H), whereas a minimum effect was 7.0 mm by the filtrate culture of OJ29 rhizobia which isolated from *Bauhinia purpurea* L. were applied. Most of rhizobial filtrate cultures showed an antimicrobial activity against *Penicillium* spp. BI3 isolate and a maximum effect 14.3 mm were found by the isolate OJ31 Rhizobia (Figure 3, I) which isolated from root nodules of *Parkinsonia aculeate* L., and the minimum inhibition diameter zone were 7.0 by the filtrate culture of *Ensifer meliloti* OJ19.

Antimicrobial resistance is an urgent global public health threat [31, 32]. Little studies were done about antimicrobial activity of rhizobial filtrate cultures against human and plant pathogens. The results of this study are very important and need a more detailed and in-depth study in order to separate the active substance. Al-Ani *et al*. [33], evaluated the activity of *Rhizobium japonicum* against the soil-borne pathogens *Fusarium solani* as causative agents of soybean root rot disease in broth culture medium and soil. Rhizobial culture filtrate caused an inhibition of the fungal radial growth of *Fusarium solani* on Potato Dextrose Agar medium amended with the filtrate compared with control. Results showed inhibition percentage of *Fusarium* 65.5%. Sowmya *et al*. [34], antifungal activity of *Rhizobium* spp. against *Aspergillus niger* and the inhibition zone diameter were 11.0 mm. Antimicrobial activity of *Rhizobium japonicum* and *Bradyrhizobium japonicum* on different plant pathogenic fungal strains were studied. The results showed 8.3 and 9.2 mm against *Aspergillus niger* by the filtrate cultures of *Rhizobium japonicum* and *Bradyrhizobium japonicum*, respectively. Filtrate cultures of *Rhizobium japonicum* were showed 7.4 mm inhibition zone when

applied against *Fusarium oxysporum*. *Bradyrhizobium japonicum* filtrate culture showed 7.6 mm inhibition zone when applied against *Fusarium oxysporum* [2].

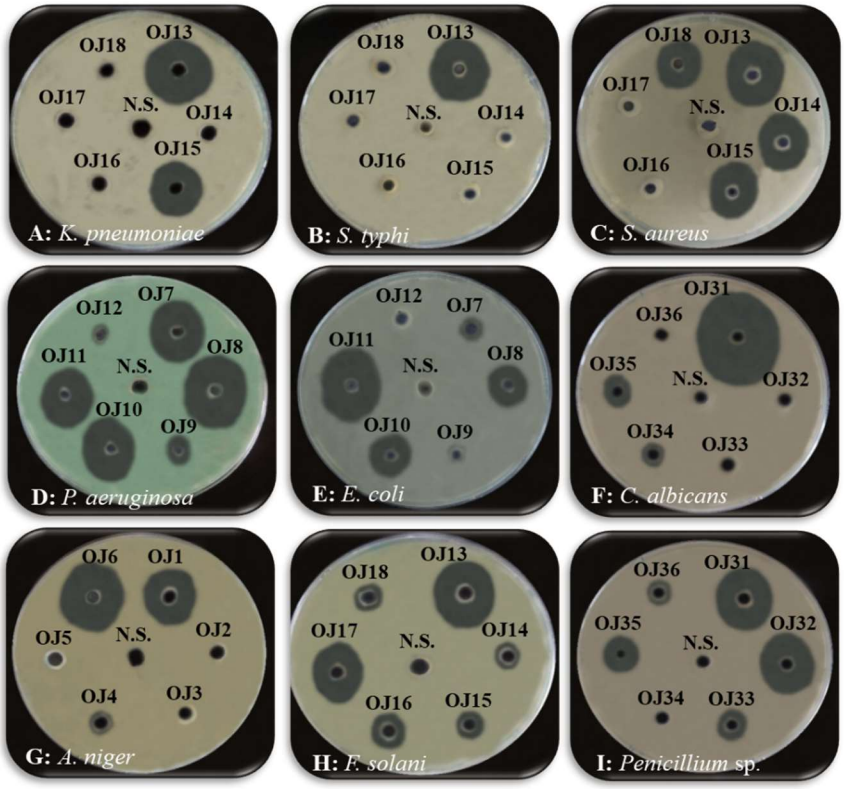

Figure 3: *Antimicrobial activity test of rhizobial filtrated cultures.*
A: E. fredii bv fredii OJ13 agaist K. pneumoniae AS63, B: E. fredii bv fredii OJ13 agaist S. typhi AS39, C: E. fredii bv fredii OJ13 agaist S. aureus AS38, D: R. leguminosarum OJ8 against P. aeruginosa AS60, E: R. vignae OJ11 against E. coli AS37, F: OJ31 against C. albicans AS65, G: R. leguminosarum bv. phaseoli OJ6 against A. niger BI1, H: E. fredii bv fredii OJ13 agaist F. solani BI2, I: OJ31 rhizobial strain against Penicillium sp. BI3, N.S: Normal saline as negative control.

3. Conclusions

Isolates of this research work considered as promising in different biotechnology fields as long as revealed variations in cultural, biochemical characteristics rather than some of them were high exopolysaccharide producer and have antimicrobial activity against some of human and plant pathogens. Research work on rhizobial antimicrobial activity is still needs more efforts to clarify the potential agents to serve as the next generation of antibiotics for use in human health and agricultural applications.

4. Material and Methods

Isolation of Rhizobia from root nodules of host plants

Nitrogen fixer plants were uprooted with an amount of soil and washed. Depends on pink color, the nodules were chosen and were cut with scissors with keeping a small part of the link that connects the nodule to the root plants. Then, immersion of root nodules in a normal saline for several minutes. Then, nodules immersed in ethanol 70 % for 3-4 minutes, and then sterilized with sodium hypochloride solution (3.0 % concentration) for 15 minutes [35]. After that, nodules were washed with physiological solution for three times and nodules were transferred, using sterile forceps, to a solid tryptone yeast extract medium (TY). After incubation for one day at 28±2 °C, to ensuring efficiency of sterilization, root nodules were destroyed under sterile conditions with sterile rod glass in 1 ml of normal saline. 0.1 ml of different solutions of bacterial suspensions were spread with glass spreader on tryptone yeast agar plates to obtain pure colonies. Finally, incubation was done at 28±2 °C for two days.

Reverse inoculation test

To confirm the isolated bacteria are a specific rhizobium, inoculation of 48 hours' seedlings of nitrogen fixer plants with certain isolate were done.

Cultural and microscopic characteristics

Growth and ability of motility of rhizobial isolates were noticed by necked eye. ability of staining with Gram stain and bacterial shape were examined under microscope.

Biochemical tests

Growth on Rhizobial minimal medium (RMM)

To examine the ability of rhizobial isolates to grow on minimal medium, plates of Rhizobial minimal medium (Solution A was prepared by adding the following (g/L): Na2HPO4.12H2O, 0.45; (NH4)2SO4, 2.0; FeCl3, 2.0; MgSO4.7H2O, 0.1; CaCl2.2H2O, 0.04 and distilled water to make volume 990 ml. The pH of the medium was adjusted to 7.0. Solution B was prepared by adding 20 gm of glucose to 100 ml of D.W., pH was adjusted to 7.0. After that solution B was added to solution A). streaked with different colonies of rhizobial isolates and incubated at 28±2 °C for 24-48 hours. Results recorded depends on the growth of the of rhizobial isolates [11].

Citrate utilization test

In order to determine the ability of isolated rhizobia to utilize citrate as sole carbon source. The isolates inoculated in simmon citrate agar medium then incubated at 28±2 °C for 24-48 hours, then color was observed [22].

Gelatin liquefaction test

Fresh rhizobial colonies were inoculated to gelatin medium by stabbing with inoculation needle and incubated was done at 28±2 °C for one day. Results were recorded after transfer the tubes into refrigerator for 30 minutes at 4 °C [9].

Triple sugar iron test

TSI test was achieved by inoculating the medium with a fresh of rhizobial isolates and then the tubes were incubated at 28±2 °C for three days. The positive result was recorded by changing the colour of the medium to dark brown color [36].

Catalase test

Catalase activity of rhizobial isolates was determined by placing one day colonies on a glass slide and adding one drop of 30 % H_2O. The appearance of gas bubbles indicated the activity of catalase enzyme.

Oxidase test

Oxidase reagent was prepared by dissolving 1 % *N, N, N, N*-tetramethyl-*p*-phenylene diamine in warm water and stored in a dark bottle at 25-30 °C. A strip of filter paper was dipped in this reagent then 24 hold rhizobial colonies were transferred to the filter paper strip with a sterile needle. Oxidase-positive colonies turned lavender, then dark purple to black within 5 minutes [14].

Utilization of different sugars

To determine the ability of rhizobial isolates to utilize several sugars as a carbon sources as follows: glucose, galactose, arabinose, fructose, maltose, xylose, mannose, mannitol and lactose, were added each alone to the Yeast extract mannitol medium with 1% (w/v) concentration. pH was adjusted at 6.8. Results were recorded after two days of incubation at 28±2 °C [3].

Sodium chloride tolerance

To determine the ability of rhizobial isolates to tolerate different concentrations of sodium chloride salt (2.0, 4.0, 6.0 and 8.0%). 0.1ml of fresh rhizobial liquid culture were spreaded on Tryptone yeast extract agar containing the mentioned concentration. Incubation was done at 28±2 °C for one day [22].

Intrinsic resistance to antibiotic

The susceptibility of rhizobial isolates against different antibiotics were assayed by Kirby-bauer disk diffusion susceptibility test. Studied antibiotics were as follows (10 mg/ml): Tetracycline, Erythromycin, Ceftriaxone, Ciprofloxacin, Amikacin and Rifampicin, on Mueller Hinton Agar plates. Plates containing the discs were incubated at 28±2 °C for 3-7 days [22].

Heavy metal tolerance test

To assess the ability of rhizobial isolates to grow on TY plates containing different concentrations of heavy metal salts, different heavy meatal were added to TY plates as follows: Pb $(CH_3COO)_2$, $COCl_2$, $NiCl_2$, $ZnSo_4$, $CdCl_2$ and $HgCl_2$ with concentration 100 µg/ml [37].

Cell surface molecules production test of rhizobial isolates

Production of cellulose fibrils test

Rhizobial isolates were streaked on Yeast extract mannitol agar (YEMA) medium containing 0.02% (w/v) Congo red. Observation of the growth were done after incubation at 28±2 °C for 72

hours. Absorption of Congo red indicating positive results, i.e. production of cellulose fibrils molecules [38].

Production of β-(1-3) glucans test

All rhizobial isolates were streaked on YEMA medium containing 0.02% (w/v) Aniline blue, incubation at 28±2 °C for 72 hours. Results were recorded and the presence or absence of blue growth depends on aniline blue dye uptake [39].

Production of β-(1-2) glucans test

Determination of ability of rhizobial isolates to produce β-(1-2) glucans were achieved by making spots on TY swarm plates followed by incubation at 28±2 °C for 72 hours. Results were recorded after every 24 hr. Swarming indicates the ability production of β-(1-2) glucans [25].

Estimation of cell dry weight

Cell dry weight as indicator for biomass, production was measured according to Castellane *et al.* [40]. Centrifugation 5000 rpm for 30 minutes was achieved, followed by drying to a constant weight in an oven at 60°C overnight.

Estimation rhizobial exopolysaccharide

Estimation of rhizobial EPS production were achieved by inoculation fresh rhizobial growth into YEM broth medium supplemented with 1.0% mannitol. Incubation was achieved at 28±2 °C at 150 rpm for 48 hours. Fermented culture broth was centrifuged 5000 rpm for 30 minutes, the supernatant was mixed with two volumes of acetone. Collection of rhizobial EPS were done by centrifugation 5000 rpm for 45 minutes. Washing of the EPS were done with D.W. and acetone. Then transferred onto a filter overnight at 105 °C [41].

Final pH assay

Final pH was recorded of rhizobial isolates fermented cultures after two days of incubation.

Antimicrobial activity of rhizobial filtrated cultures test

Each rhizobial isolates were grown in TY broth medium at 28±2 °C for 18-24 hours. After that centrifugation at 5000 rpm for 15 minutes was done. Filtration of a culture was done by passing the culture through 0.2 nm micro filter to exclude the rhizobial cells from the fermented medium. 0.1 ml of each of bacterial and fungal strains cultures were transferred into Mueller -Hinton Agar medium and spreaded using spreader. 200 µl of each rhizobial filtrated culture were transferred to the wells (6 mm). Replicates were done as well as a negative control with 100 µl normal saline [42]. Inhibition zone diameter (mm) was measured using calipers. The antimicrobial activity was determined according to diameter of clear zone around the wells.

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