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Improved selection medium and technique for isolation of *Azospirillum irakense*

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الخلاصة

تم تطوير تقنية ووسط انتخابي محسن لعزل بكتريا *Azospirillum irakense* وهذة التقنية تستند على استخدام وسط زرعي شبه صلب خالي من النتروجين يحوي السكر الثنائي Lactulose بدلا من الماليت كمصدر وحيد للكاربون والطاقة وجعل قيمة pH 7.4.

وجد أن هذا الوسط الانتخابي يشجع على نمو *A.irakense* لوحدها ويمنع بقية انواع *Azospirillum* من النمو, كما وجد ان هذا الوسط اكثرتفوقا من بقية الاوساط المتاحة.

هذه التقنية سهلة الاستخدام للكشف عن *A.irakense* حيث أظهرت زيادة عالية في اعداد العزلات لهذا النوع التي تم الحصول عليها من تربة الرايزوسفير والجذور وتقليل الخسارة في الوقت.

Abstract:

An improved selection medium and technique for Isolation of *Azospirillum irakense* was developed . This technique is based on the utilization of free-nitrogen semi-solid medium supplement with disaccharide, lactulose, as a sole source of carbon and energy instead of malate with a pH value of 7.4 .This selected medium was found to encourage the growth of *A. irakense* alone and to prevent the other species of *Azospirillum*.

The improved medium was superior than the other available media .The technique was simply applied to detect *A.irakense*, showed a high increase in numbers of *A.irakense* isolates obtaining from the soil rhizosphere and roots, and decreased the time cost.

Introduction:

Of the various rhizosphere-associated bacteria, *Azospirillum* species are probably the most studied and appears to have significant potential for commercial application [1], and their beneficial effect on plant yield [2].

The genus *Azospirillum* comprise free-living N2-fixing rhizosphere bacteria that have been isolated from different soil types and cultivated plants in tropical, subtropical,and temperate regions all over the world [3]. The identification of a

specific species of soil bacteria is usually difficult and cumbersome task [4], entailling either extensive immunological, biochemical, and DNA- hybridization test or the use of more advanced genetical studies [5].

Several proposal media for culturing N2-fixing bacteria and isolation procedure for *Azospirillum* were used [6].Selective media and techniques based on semi-solid nitrogen-free medium and malate as a source of carbon and energy was the key for the isolation of *Azospirillum* [7], but was not selective enough , enabling the development of many differential type bacteria [8]. The previous technique were concentrated on decreasing the quantity of malate [9], using Gongo red [10], or the utilization of malate with the addition of antibiotics [4].

In spite of all these techniques, they were insufficient to differentiate among the species within the genus *Azospirillum* [22].

At present, the genus *Azospirillum* comprises eleven species, including, in addition to *A. lipoferum, A. brasilense* [11], *A.amanzonense* [12],*A.halopreaferens* [13], *A.irakense* [14], *A.largimobile* [15], *A.doebereinera* [7], *A.oryzae* [16], *A.meli nis* [5], *A.canadense* [17] and *A.rugosum* [18].

However, all the above mentioned species are quite similar in morphology and biochemically, and still difficult to be differentiated enough, therefore the development of a selective medium and technique or reliable isolation procedure, or both, was urgently needed for the isolation and identification of *Azospirillum irakense.*

In this study we present efficient, quick and simple technique for the isolation of *A. irakense*.

Materials and methods:

Media: Two media were used:

1- Nitrgen fixing bactria medium (Nfb medium): Semi-solid free-nitrogen medium which used for routine isolation of *Azospirillum* [19]. The composition of the medium as following: g/L DL malic acid 5.o; K_2HPo4 0.5; $MgSo4.7H_2O$ 0.2; NaCl o.1;$CaCl_2$ O.2; trace element (Na_2Mo $04.2H_20$ 0.2 ,$MnSo_4.7H_2O$ O.2; H_3BO_3 0.28 ,$CuSO_4.5H_2O$ 0.008 , $ZnSO_4.7H_20$ 0.O24 , 1000 ml d.w) 2ml , bromothymol blue (o.5% aqueous solution dissolved in KOH 0.2M) 2ml , solution of FeEDTA (1.64%)4 ml , vitamin solution (biotin 0.01, pyridoxine o.o2 dissolved in 1000ml.d.w.); KOH 4.5 ; pH adjusted to 6.5 with 0. 1 N KOH. For semi-solid and solid media added 1.75 and 15 agar respectively.
When bacteria grow aerobically added 1g/l NH_4CL with 20 mg yeast extract.

2- Improved selective media named KLM in this study was applied for isolation of *Azopirillum irakense*. This medium contain the same composition of Nfb except that KOH 4.5 g/l was eliminated and DL malic acid was replaced by lactulose (syrup 67%) 7 ml . The pH of KLM was adjusted to 7.4 with 0.1N

KOH .For semi-solid and solid medium 1.75 and 15 g/l were added respectively.

Bacterial growth at NaCl concentration 3% was determined in Nfb and KLM media.

Strains used: All references strains used in this study are listed in (table-1). All the strains of *Azopirillum* were maintained in Nfb malate semi-solid media except Y1 strain (*A. amazonense*) was maintained in Nfb malate with pH 6.0.

Isolation: Rice root (Oryza sativa) and soil rhizosphere were collected from rice field in the Diwaniyah and Najaf district in Iraq. Rice root were washed extensively, then crushed in sterile mortar and suspended in sterile distilled water, the same steps were followed for soil rhizosphere.

Tubes (18x180 mm) of 10 ml Nfb and KLM semi-solid were inoculated separately with dilution of crushed root and soil and incubated for 48-72 h at 30^0C. Veil -like subsurface pellicle formation was observed indicate positive tubes for the growth of *Azopirillum* and were confirmed microscopically for the presence of very active curved rod or S-shaped cells spirillum [17]. A loopful of the growth (pellicle) from each of Nfb and KLM media were subcultured in the same fresh media for further purification. A loopful of pellicle was streaked on plate of Nfb and KLM solid media. After 5-7 days incubation at 30^0 C, single colonies were picked up and transferred to each of Nfb and KLM semi-solid media,and were assayed for nitrogenase activity by acetylene reduction as described by Mehnaz and Lazorovits[20]. Nitrogen fixing isolates were selected inclusion in this study.

Carbon-source utilization:

Carbon-source utilization was tested by using Nfb and KLM media .For this purpose, malate and lactulose at each media were replaced by sucrose,D-cellobiose, L-rhamnose, trehalose, and lactose.

The pH for Nfb and KLM were adjusted to 6.5 and 7.4 respectively [14, 17].

Results and discussion:

Twenty seven strains of the bacteria were isolated by KLM medium, sixteen from the crushed root and eleven from soil rhizosphere, while thirty one strains were isolated by Nfb medium, eleven from crushed root and twenty strains from soil rhizosphere.

All these strains could reduce acetylene, and showed veil-like subsurface pellicle, the cells were vibrio-shape, curved rod, or S-shaped. Poly-B-hydroxybutyrate (PHB) were observed in the cells examined microscopically. These properties corresponded and matched to the general characteristics of *Azospirillum* [14, 7, 17].

All the twenty seven stains isolated by KLM medium could grow and assimilate sucrose , D-cellobiose , L-rhamnose , trehalose and lactose sugars , while

all reference strains (table -1) failed to grow on these carbon sources in KLM medium except KBC1 which is a type strain of *A.irakense* . Eight strains only isolate by Nbf medium could utilize these carbon sources mentioned above either in Nfb or KLM media.

The reference strains couldn't use these sugars in Nbf medium except KBC1 (*A. irakense*) and Y1 (type strains of *A.amazonene*) .

From the above description and other studies it appeared that only *A irakense* and *A.amazonene* could use and assimilate these carbon sources and all of other species of *Azopirillum* couldn't [14, 7 ,17,18] .

From this study, it seems that the Y1 strain couldn't grow and assimilate these sugars mentioned in KLM medium but it could in Nfb medium because *A.amazonene* (Y1) couldn't tolerate the pH 7.4 of KLM medium and not support its growth [14].

The twenty seven and eight strains isolated by KLM and Nfb media respectively were shown to be able to grow with 3% NaCl concentration. Also KBC1 strain, but not Y1 had this ability because *A .irakense* can grow in 3% Nacl while the *A.amazonene* can not [14,5]. A summary of these results is presented in (table-2).

From all the descriptions mentioned above it appear that the 35 strains isolated by KLM and Nbf media (named KL strains in this study), correspond well with the given characteristics of *Azopirillum irakense* and suitable and could be used for differentiation from other *Azopirillum* species [14,8,17,18].

During isolation and purification of *Azospirillum* strains in KLM medium, we observed that the contamination of other N2-fixing bacteria was rare especially some enterobacteria species. This is because N2-fixing enterobacteria could not use lactulose as a sole carbon source [8] and from this point the purification of *Azopirillum* strains were easy, quick and took short time. In contrast , several isolates other than *Azopirillum* species capable to grow in Nbf medium because malate was as a good source of carbon for several species of N2-fixing enterobacteria , *Derxia* and *Clostridium* [21,8]. For this reason the purification of *Azopirillum* strains that grew in Nbf medium caused difficulties and time consuming by this study.

In this study the number of strains of *A.irakense* isolated by KLM medium were 27 strains while they were 8 strains only that were isolated by Nfb medium . Since KLM medium has more advantage than any other media for obtaining a high numbers of isolates by many studies [8,22] .

Conclusion:

We conclude from this study that KLM medium with pH value 7.4 was found to encourage the growth of *A.irakense* strains alone and prevent other species

of *Azopirillum* . The medium also decease the contamination of N2-fixing bacteria other than *Azopirillum*, quick and the isolation and purification time were short as well.

Species	Strain designation	Reference
A.irakense	KBC1,type (DSM 11586)	Khammas *et al* ,1989
A.lipoferum	SP 59b (ATCC 29707), type	Tarrand *et al* 1978
A.brasilense	SP7(ATCC 29145), type	Tarrand *et al* 1978
A.amazonense	Y1 (ATCC 35119), type	Magalhaes *et al* 1983
A.halopreaferens	Au4 (LMG 7108) ,type	Reinhold *et al* 1987

Table-1: Strains used

ACTT= American type culture collection, Rockville, Maryland.
DSM= Deutch Sammlung Von Mikroorganismen, Gottengen, Germany.
LMG= Microbiele Genetica, Ghent, Belgium.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12
Sucrose utilization	+	+	-	-	- (+)	-	-	-	-	-	-	-
D-Cellobiose utilization	+	+	-	-	- (+)	ND	-	-	-	-	-	-
L-Rhamnose utilization	+	+	-	-	- (+)	ND	-	-	-	-	-	-
Trehalose utilization	+	+	-	-	- (+)	-	-	-	-	-	-	-
Lactose utilization	+	+	-	-	- (+)	-	-	-	-	-	-	-
Growth in 3% Nacl	+	+	-	-	- (-)	+	-	-	-	-	-	-

Table-2: Physiological differences between *Azospirillum* species and strains of this study.

Strains 1,KL; 2, *A.irakense* (KBC1); 3, *A.lipoferum* (SP59b); 4, *A,brasilense* (SP7), *A.amazonense* (Y1) ; 6 ,*A.halopraeferens* (Au4) ; 7, *A.oryzae* ; 8, *A.doebereinerae* ; 9, *A.largimobile*; 10, *A.melinis*; 11, *A.canadense*,12 *A.rugosum*. Data for KL *A.irakense, A.lipoferum, A,brasilense, A.amazonense and A.halopraeferens* are from this study. The remaining data were taking from (17.18)

+ = Positive, - = Negative, ND = Not determined, ( ) =The results in Nfb medium

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