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PRODUCTION OF HYDROXAMATE SIDEROPHORES BY *AZOTOBACTER CHROOCOCCUM* BACTERIUM

Shimaa Dwedar[1], Marwa S. Abdel-Hamid[2], Doaa Keshk[3], A.A. Haroun[4] and A.F. El Baz[1]

[1] Industerial Biotechnology Dept., Genetic Engineering and Biotechnology Institute, Sadat city university, Egypt

[2] Microbial Biotechnology Dept.[2], Genetic Engineering and Biotechnology Institute, Sadat city university, Egypt

[3] Genetic Engineering and Biotechnology Institute , Agriculture Research Center, Giza- Egypt

[4] Division of Chemical Industries Research, National Research Center, Dokki, Egypt.

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ABSTRACT: *Siderophores are ferric ion specific chelating agents produced by bacteria and fungi growing under low iron stress. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is an essential metal, available to the microbial cells. Azotobacter sp. isolate Azo-4 isolated from Sadat City ,Egypt showed positive result for siderophores production by Chrome Azurol Sulfonate (CAS) assay. The organism was subjected to various biochemical tests and 16S rRNA analysis;* results indicated that the isolated fragment sequence (~1.3) Kbp is homologous to *Azotobacter chroococcum strain KCA1 16S ribosomal RNA gene. Deferrated medium concentration induced the siderophores production (45 µM). Hydroxymate siderphore was extracted and the antagonistic activity of partially purified siderphore was tested against bacterial pathogens Bacillus stbtlius and Salmonella sp.,in vitro. Result showed that the Azotobacter sp. under study is a good producer of siderophores, which can be beneficial for its antagonistic activity towards different pathogens as biocontrol agent.*

Key words: *Azotobacter chroococcum*, Hydroxamate Siderophores, 16SrRNA, SEM, biocontrol

INTRODUCTION

Azotobacter chroococcum is non-pathogenic, free-living bacterium that can fix atmospheric nitrogen with various crops without any symbiosis do not need a specific host plant thus increases germination in young plants and leading to improved crop yields (Zahir *et al.*, 2004) and (Marwa *et al.*, 2012). Most fungi and bacteria respond to Fe deprivation by producing ferric-specific Fe^{3+} transport system called siderophores (Nair *et al.*, 2007) and (Baakza *et al.*, 2004). Siderophores may be classed chemically as either hydroxamate or phenolate and catecholates (Neilands, 1980) and (Leong, 1986). Most aerobic microorganisms produce at least one siderophore, and in some cases, a single bacterial strain can produce two or more (Meyer *et al.*, 2002). The importance of these siderophores extends beyond their immediate role in microbial physiology and their applications in biotechnology (Messenger and Ratledge, 1985). Siderophores and their substituted derivatives have a lot of applications in the treatment of some human diseases as treatment of haemochromatosis (Nagoba & Vedpathak, 2011). New anti-parasitic siderophores and their substituted derivatives were obtained from *Klebsiella pneumonia*. The new siderophores act through a mechanism that is different from that of other antimalarial agents and is non-toxic (Gysin *et al.*, 1991). Siderophores conjugates were used as a diagnostic agent for prostate cancer (Ding & Helquist, 2007). Siderophores isolated from *Bacillus* species have good probiotic properties(Patel *et al.*, 2009). This work interested with the isolation of *Azotobacter* sp. from Sadat City soil; identification through biochemical tests and 16S rDNA sequencing and studying its ability to

produce hydroxamate and bioactivity of extracted siderphore.

MATERIALS AND METHODS

Isolation of *Azotobacter*

Azotobacter chroococcum (Azo-4) was isolated from Sadat City soil in Menoufyia Governorate. Five grams of soil samples were placed in 250 ml Erlenmeyer flasks containing 45ml of Atlas medium (Atlas,1997), then stirred on rotary shaker (150 rpm for 10 min) , streaked out on Atlas agar medium and incubated at 28± 2°C for five days then checked for purity.

Identification of *Azotobacter chroococcum*

The Physiological and biochemical characteristics of isolated bacterium was studied using the criteria of Bergey's Manual of Systematic Bacteriology (Brenner *et al.*, 2005) 16S rDNA gene sequencing was amplified on an applied Biosystem 380A DNA synthesizer. Sequences of the 16S rRNA specific primer for the amplification were 27F (5'–GAG AGT TTG ATC CTG GCT CAG–3)' and 16S rRNA(R) 1495R (5'–CTA CGG CTA CCT TGT TAC GA–3'). (Studholme ,1999) and (Sanger ,1977).

Detection of Siderophores Production

A. chroococcum isolate was grown in Atlas medium containing Glucose 20.0 g, $KHPO_4$ 0.8 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.2 g , $CaCl_2$ 0.05 g and NaMoO4.2H2O 0.05 g and pH was adjusted to 6.8. after 72 hours at 28 °C. The supernatant was collected by centrifugation at 6,000 rpm for 20 min. The Chrome Azurol Sulfonate (CAS) Assay solution was carried out according to (Schwyn and Neilands 1987). A 0.5 ml of supernatant was mixed with 0.5 ml CAS assay solution. A reference was prepared using exactly the same components except the supernatant which was replaced with the un- inoculated medium used for culture of the bacteria. After reaching equilibrium the absorbance was measured at 630 nm. (Amal *et al.*, 2014).

Effect of iron concentrations on siderophores production

The isolate was grown for 48 h at 25°C with shaking (200 rpm) in 500 ml Erlenmeyer flasks containing 125 ml medium, with pH adjusted to 7. To remove traces of iron, glassware was cleaned with 6 M HCl and with double distilled water. Atlas medium was supplemented with different $FeCl_3$ concentrations added in increasing amounts (0, 2, 4, 6, 8, 10, 15, 50, 100, 150 µg/ml) according to (Rachid and Bensoltane, 2005).

Extraction of hydroxamate siderophores

Bacterial culture filtrate was separated from cells by centrifugation at 6,000 rpm for 20 min. The supernatant was concentrated to one-ninth at 35°C. Five grams of $FeCl_3$/liter were added to the concentrated supernatant fluid. The solution was saturated with ammonium sulfate (50% saturation) (Hissen *et al*, 2004). The contents were then transferred into a separating funnel followed by an equal volume of phenol/chloroform (1:1 ratio) and the funnel was shaken with ample venting. The contents were allowed to separate in the dark for 24h. Following separation, the organic phase was collected and the aqueous phase was discarded. Twice the volume of ether/water (1:1 ratio) was added to the organic phase, followed by shaking, venting and separation in the dark. After separation, the organic phase was discarded and an equal volume of ether was added to the remaining aqueous phase. The aqueous phase was washed continuously with ether in the same way until separation occurred instantly. The aqueous phase was then lyophilized according to (Ams *et al*, 2002).

Bio control assay of partial purified siderphore

Sterile nutrient agar medium was prepared and solidified in petri dishes. The inoculum of pathogenic bacteria *Bacillus subtilus* and *Salmonella* sp. were swabbed over the surface of the plates. Disk size of

6mm diameter was sterilized thensaturated with partially purified siderphore added to the plates and incubated at 37C° for 48 hours. The zone of inhibition of growth was an indicator of positive effect.

Scanning electron microscopes

Scanning electron microscope was used to clarify the shape and sizes of hydroxamate siderophores crystals. The sample was coated with gold/palladium and imaged using a JEOL JSM 5400 Scanning Electron Microscope operated at 20 kV.

RESULTS AND DISCUSSION

Isolation and identification of the *Azotobacter* local isolates:

The isolate was Gram negative short oval shape rod in nature and motile. The biochemical test showed that the isolate could be considered as *Azotobacter* sp. Results in Fig (1) showed that the isolated *Azotobacter* colonies on nitrogen free medium (Atlas, 1997) . colonies were slightly viscous, semi-transparent during the early growth and changed into dark brown on aging. (Mishustin and Shilnikova, 1969), (Marwa *et al.,* 2012) mentioned that *A. chroococcum* produces a black pigment "melanin" especially in the older cultures and this pigmentation is due to the oxidation of tyrosine by tyrosinase enzyme (Fig.1). The cells are motile; ovoid to rod shaped, occurs in pairs and Gram negative, aerobic and catalase positive. The isolate can hydrolyze starch, utilize citrate, produce acetone and can utilize different carbon source such as glucose, mannitol, insoitol, rhamnose, arabinose, ethanol, sorbitol, butanol 0.2 %, trehalose and glutrate. The isolate was sensitive to Erythromycin (2 µg / ml), phenol, isopropanol and methanol. The isolate was classified as *Azotobacter chroococcum* according to the above criteria of Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005). For confirmation of identification, molecular identification was carried out using 16S ribosomal RNA gene identity. The amplified gene product showed around 1.3 Kbp fragment in agarose gel electrophoresis (Fig.2). The Blast result revealed that the obtained nucleotide sequence showed 99% homology with *Azotobacter chroococcum* (Accession KM043465.1) 16S ribosomal genes sequence (Fig 3).

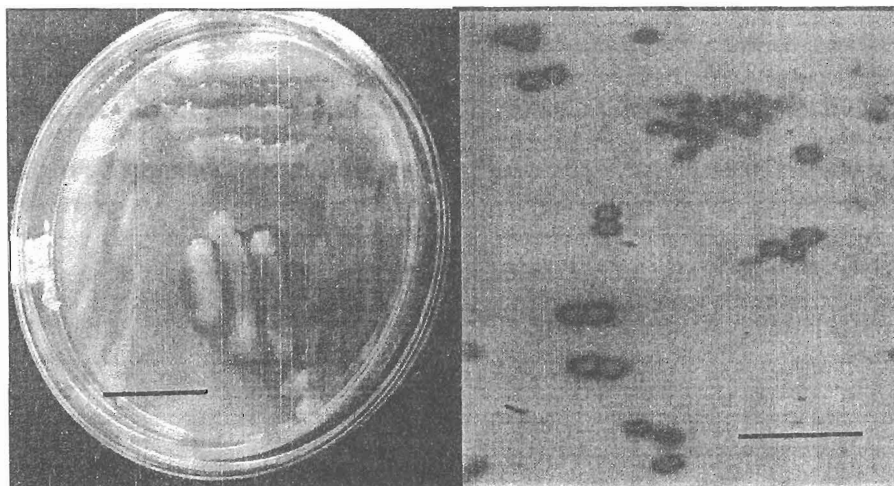

Fig (1). ***Azotobacter chroococcum*** **colonies and cell shape by light microscope x 100**

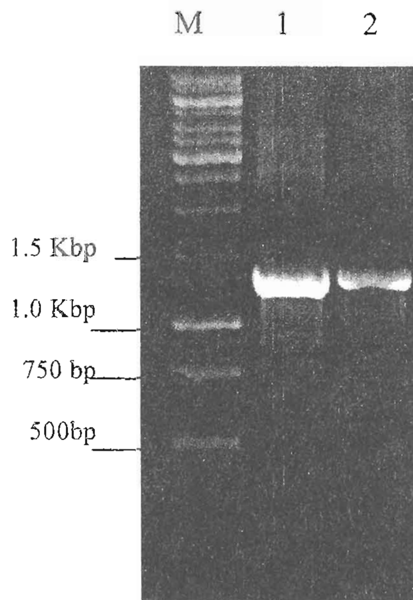

Figure (2): Ethidium bromide stained agarose gel resolving the PCR amplification fragment of 16S rRNA gene ~1.3Kb from local isolates *Azotobacter* sp. where (1 and 2): 16S rRNA gene as PCR product. Lane M: DNA marker (1Kbp).

Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Azotobacter chroococcum strain KCA1 16S ribosomal RNA gene, partial sequence	737	737	99%	0.0	94%	KM043465.1
Azotobacter chroococcum strain RK3 16S ribosomal RNA gene, partial sequence	737	737	99%	0.0	94%	KJ511860.1
Azotobacter chroococcum strain CL13 16S ribosomal RNA gene, partial sequence	737	737	99%	0.0	94%	KJ001770.1
Azotobacter chroococcum strain CD5 16S ribosomal RNA gene, partial sequence	737	737	99%	0.0	94%	JX913866.1
Azotobacter chroococcum strain 59A 16S ribosomal RNA gene, partial sequence	737	737	99%	0.0	94%	JX036396.1
Azotobacter chroococcum strain YCYS 16S ribosomal RNA gene, partial sequence	737	737	99%	0.0	94%	JQ692178.1

Figure (3): Blast search results of 16SrRNA sequence of the isolated bacteria aligned with those deposited in the gene bank.

Siderophore Detection

Azotobacter chroococcum Azo-4 strain was grown in Atlas medium for 48h then the culture was centrifuged and 1 ml of supernatant was added to 1 ml of CAS assay shuttle solution. Un-inoculated Atlas medium with no added iron was used as a control. The ability of hydroxamate siderophores production was confirmed by the color change from blue to reddish yellow (Fig.4) as mentioned by (Rachid and Bensoltane, 2005). The siderophore production is quiet common phenomenon exhibited by various organisms like *Pseudomonas* spp., *Bacillus* spp., clinical isolates like *E. coli, Klebsiella* etc. (Syed and Vidhale, 2011) reported for the acquisition of iron complex from the soil. The production of siderophores and nitrogen fixation by this isolate can provide dual benefit for the plants (Amal *et al.*, 2014).

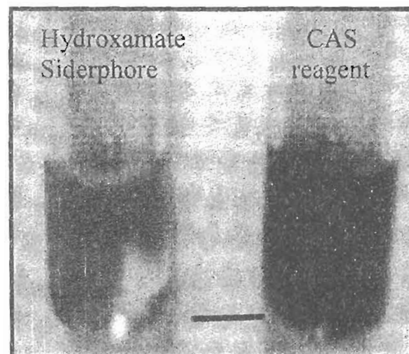

Figure (4): Detection of hydroxamate siderphore

Results in Fig (5) showed that iron-stressed conditions lead to production of strong iron-chelating agents such as siderophores and this results were in agreement with those obtained by (Diaz de Villegas *et al,* 2002).Maximum siderophores production was obtained at deferrated and 2 μM/ml concentration (Tailor and Joshi, 2012) which was a vital factor affecting the siderophores production. In this regards (Vellore 2001) reported that only under iron-restricted conditions for 48 hours that siderophores production could be induced. High iron media conditions repress the siderophores-mediated iron uptake system, and, therefore, low concentration of siderophores is produced (Duhme *et al.*, 1998).

Extraction of hydroxamate

The supernatant fluids of cultures grown for 72 h were easily extractable into chloroform-phenol solvents (1:1 v/v). Aqueous samples of the iron complexes, derived from the chloroform-phenol extracts of culture supernatants were positive for reaction in the universal CAS assay but were nonreactive in the Arnow assay for catecholates. SEM photomicrographs of chemically fixed, dehydrated and critical point-dried showing a rod crystal Fig (6). *Azotobacter* sp. had drawn a worldwide attention because of production of secondary metabolites such as siderphore, enzymes and phyto-hormones and involved in nitrogen fixation. Iron has numerous, diverse functions in bacterial cells. It influences cell composition, intermediary metabolism, secondary metabolism, enzyme activity and host cell interactions which would include pathogenicity (Messenger and Barclay 1983). The alternative strategies for disease management include the use of *Azotobacter* sp. that show beneficial effects on plants and are known as Plant Growth Promoting Rhizobacteria (PGPR). The positive effects of PGPR are normally divided into two categories: growth promotion and biological control (Kleopper, 1997). Also, certain root colonizing bacteria can protect plants from soil-borne pathogens (Slininger *et al.*, 1996). Hydroxamate-based chelators of iron are potent inhibitors of *in vitro* growth of *Plasmodium falciparum* causes malaria (Golenser *et al.,* 1995). *Azotobacter* sp. has utility as an extremely economical and eco-friendly bio-pesticide (Rachid and Ahmed, 2005).

The production of microbial metabolites and their applications in various fields were gaining attention that it could be more control and less risk.

Antagonistic activity

Azotobacter sp. was evaluated for its ability to control some gram negative and positive bacterium such as *B. subtlius* and *Salmonella* sp. by Minimum Inhibitory Concentration technique (MIC). It was found that the extracted siderophores inhibite the tested pathogens. The percentage of

inhibition was increased from 40% to 50.5% in partially purified siderophore (Fig. 7). This proved the ability of siderophore as biocontrol agent in our study. *Azotobacter* sp. produces azotobactin type siderophore under iron starving conditions with high stability and affinity for iron that restricts growth of microorganism with low iron competition ability such as phytopathogenic fungi (Kloepper, 1977). Purified siderophore of *A. calcoaceticus* at 500 μg/mL concentrations inhibited the growth of phyto-pathogens up to 30.00%, suggested that both siderophore rich supernatant as well as pure siderophore has the inhibitory potential against phyto-pathogenic fungi (Prashant *et al.*, 2009). The isolates of *Azotobacter* spp. were found to effectively inhibit the mycelia growth of both fungal pathogen in dual cultures with rhizospheric bacteria and soil borne pathogens (Sapna *et al.*, 2012).

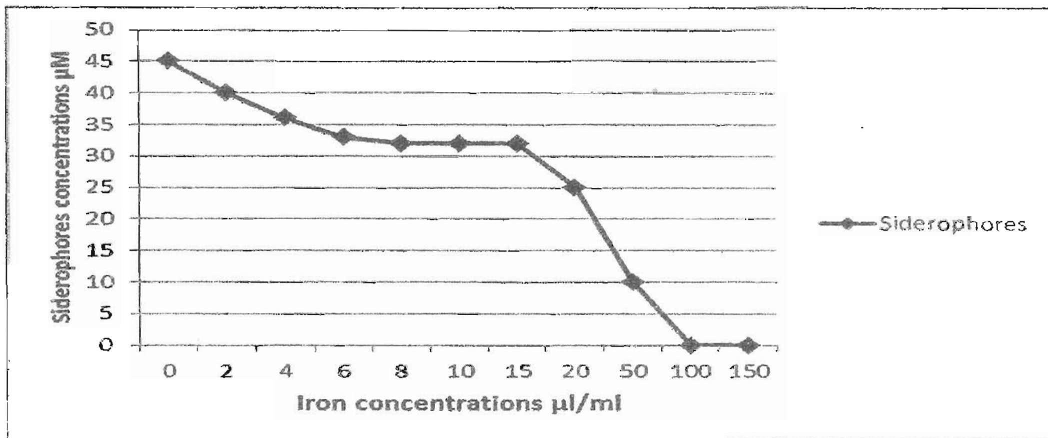

Fig (5): Effect of different concentration of iron on Siderophore production by *Azotobacter chroococcum*

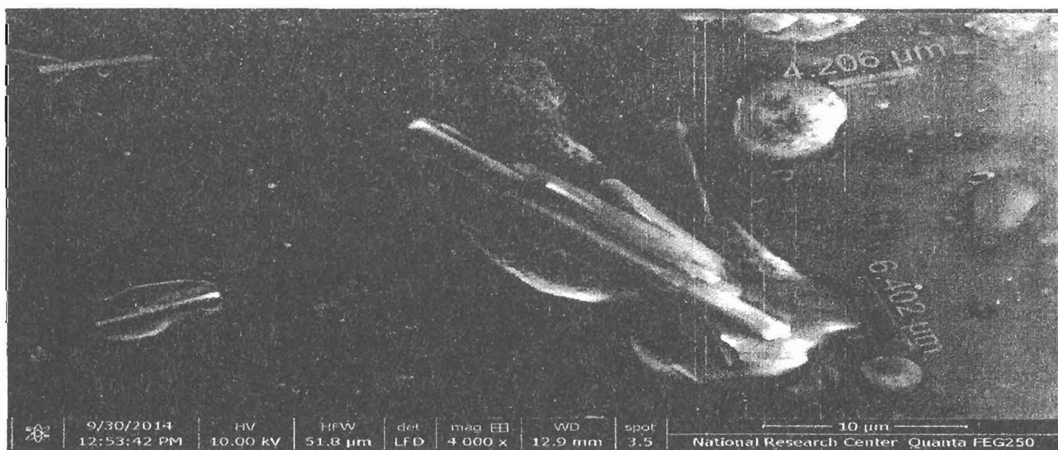

Fig (6): Scanning electron microscope photograph showing crystal hydroxamate siderphore

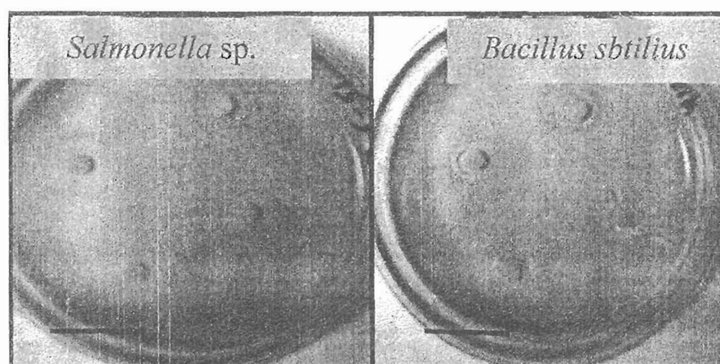

Fig (7): Biological activity of partial purified siderphore

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انتاج مخلبيات الحديد الفينولية بواسطة بكتريا ازوتوبكتر كروكوكم

شيماء دويدار[١] ، مروا صلاح[٢] ، دعاء كشك[٣] ، احمد هارون[٤] ، اشرف الباز [١]

[١] قسم البيوتكنولوجيا الصناعية-معهد الهندسة الوراثية والتكنولوجيا الحيوية-جامعة مدينة السادات-مصر.

[٢] قسم البيوتكنولوجيا الميكروبية-معهد الهندسة الوراثية والتكنولوجيا الحيوية-جامعة مدينة السادات-مصر.

[٣] معهد الهندسة الوراثية والتكنولوجيا الحيوية-مركز البحوث الزراعية- جيزة- مصر.

[٤] قسم الصناعات الكيماوية-المركز القومي للبحوث-الدقي -جيزة -مصر

الملخص العربى

مادة السيدروفور او مخلبيات الحديد من المواد التي تفرز من البكتريا او الفطريات التي تنمو تحت تأثير انخفاض تركيز الحديد. ان هذه المركبات لها دور هام في جعل العناصر متاحة في البيئة الميكروبية. تم عزل ميكروب الازتوبكتر من مدينة السادات بمحافظة المنوفية بمصر تستطيع انتاج مخلبيات الحديد الفينولية وتم التحقق من ذلك باختبار Chrome Azurol Sulfonate .وتم تعريف الميكروب تحت الدراسة باستخدام الاختبارات الفسيولوجية وايضا على مستوى تحليل التتابع الجيني واكدت النتائج ان العزلة قد تتشابه مع الازتوبكتر كروكوكم بنسبة ٩٤ في المائة .واوضحت النتائج ان البيئة الميكروبية للعزلة المستخدمة الخالية من الحديد تنتج ٤٥ ميكرومولر سيدروفور . تم استخلاص هذه المادة واختبار مدى قدرتها على مقاومة بعض اجناس البكتيرية الممرضة مثل *Bacillus stbtlius*, *Salmonella* sp التي اعطت نتائج موجبة على مستوي المعمل.وبذلك يمكن القول بان بكتريا الازوتوبكتر هي منتج جيد لمادة السيدروفور التي لها اهمية تطبيقية.