In vitro study the Characterization of *Azotobacter spp*. isolates from Sudanese soils

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Abstract

An attempt was made to look for indigenous , ecofriendly N_2 fixing microorganisms inhabitant to Sudanese soils. Isolates were made from a clay soil in central Sudan, where most of the soils are classified as nitrogen deficient, and where most of the economical crops are grown including sorghum , wheat, and sugarcane. The isolates were identified using standard methods as *Azotobacter sp.*

Characterization of the isolates was made including morphological and physiological tests. The obtained results revealed similar results to what had been reported earlier in the literature.

Tests were made for the isolates tolerance to different temperatures, different pHs, different salt concentrations, and to antibiotic resistance in a Mannitol N-free Agar Medium. The data collected indicated that *Azotobacter* isolates from Sudanese soils grow best in media with neutral pH (7) , tolerate temperatures in the mesophylic range (30^0C) , and can withstand salt concentrations from 0.2-0.4% NaCl.

Further studies were stressed to look into their efficiency in fixing atmospheric nitrogen when exposed to the harsh environmental conditions prevailing in Sudan.

Key words: Azotobacter, Indigenous, Isolates, Nitrogen Fixation, Salt concentration ,Antibiotic resistance.

Introduction

Reducing the use of chemical fertilizers by adoption of bio-fertilizer technology is recently advocated, (Zarabi *et al.*, 2010). Extensive studies have therefore been conducted in this aspect due to the negative impact of chemical fertilizers on the plant, soil, animal and human health, (Venuturupalli, 2010). Therefore, attention is focused on bio-fertilizers sources and use, since they are classified as eco-friendly and safe to human and animal health. The world, therefore started to find living organism and detect them to act as bio-fertilizers to promote plant growth. Many bacteria were found to promote plant growth and were called plant promoting rhizobacteria, (Joseph *et al.*, 2007). They received a great attention due to their role in nitrogen fixation.

Biological nitrogen fixation is the most important process that promotes plant growth and increases its productivity, (Kizilkaya, 2008). It could be symbiotic or non-symbiotic according to the relationship between plant and bacteria.

One of the major groups of bacteria that can fix nitrogen non-symbiotically is Azotobacter spp., which is a free living bacterium, indigenous to the soil (plant rhizosphere). Extensive studies were therefore performed in isolation, identification, characterization, growth and ability of these bacteria to fix nitrogen, (Breed *et al.*, 1957). However, scanty information is available regarding their isolation, characterization, and identification of the strains dominating Sudanese soils.

Their characterization regarding their tolerance to different temperatures, pH, and salinity is of utmost importance in Sudan due to the harsh environmental conditions prevail in the country.

This study was therefore conducted with the following objectives:

1- To isolate Azotobacter sp. from Sudanese soil.

2- To physiologically characterize the obtained isolates in an attempt to identify them.

3-To study the effect of pH, temperature and different salt concentrations on the growth of the isolates.

4-To test the tolerance of the isolates to different antibiotics concentrations.

Materials and Methods

Soil samples collection

This study was conducted at the College of Agricultural Studies –Sudan University of Science and Technology demonstration farm. Soil samples were collected using sterile tools from the rhizosphere of sorghum (0-15 cm), and transferred directly to the laboratory and kept refrigerated (4C°) until used.

Isolation of bacteria

The bacteria was isolated using the *Azotobacter* Mannitol Agar as described by (Pelczar, 1957), and (HiMedia Laboratories, 2010).For counting, the Sodium Benzoate Medium described by (aleem, 1953) was used. Standard Plate count and gram staining was made according to the method described by (Gerhardt, 1985).

Biochemical tests

The biochemical tests were performed according to (Gerhardt, 1985). The following tests were made: catalase test, citrate test, indole test, urease test, and methyl red test.

pH Tolerance Test

Six pH ranges of *Azotobacter* media were prepared by adjusting the pH, either by adding Hcl (0.1 N) to acidify the medium, or by adding NaOH (1N) to increase the alkalinity. A pH-Meter was used for these adjustments as described by (Gerhardt, 1985).Two pH levels were used; 5.0 and 10.0. The inoculated plates were incubated for seven days at 30C°.

NaCl Tolerance Test

Five different NaCl concentrations were used in this test ; 0.2%, 0.4%, 0.6%, 0.8% and 1%. The inoculated plates were incubated at 30 C° for seven days.

Temperature Tolerance Test

The temperatures used were 10 C°, 20 C°, 30 C°, 40 C°, and 50 C°. Inoculated plates on *Azotobacter* Mannitol Agar medium were incubated for seven days.

Antibiotic Resistance Test

This test followed the method described by (Gerhardt, 1985) using streptomycin antibiotic in a gradient plate technique.

This test was made by adding streptomycin sulfate (100 mg/ ml) to the *Azotobacter* Mannitol Agar medium in petridishes and incubated at 30 C° for seven days.

Results and Discussions

The data obtained are presented in tables 1 through 6.

The isolated *Azotobacter spp.* were cultured on *Azotobacter* Agar (Mannitol) medium. This medium is selective to growth, and suitable for identification and characterization of Azotobacter spp, according to Pelczar, (1957), and HiMedia Laboratories, (2010). The colony morphology of the isolated *Azotobacter spp* (table1), showed that the size of colonis ranged from 1-5 mm diameter. The shape is circular. Pigmentation is creamy beige white, with similar color and hue as the agar. Elevation is raised, with smooth edge and surface,

glossy, viscous and musky in odor. This agrees with the findings of (Jensen, 1965 Saribay *et al*, 2003, and Aquilantia *et al*, 2004).

The isolated bacteria were gram stained and the results showed a gram negative reaction as visuallized by the red color of cells, (table2). This matched with the findings of Breed *et al.*, 1957, Mali, and Bodhankar,(2009).

The cells of isolated bacteria are oval rods and big in size. There are vegetative cells and there is evidence of cysts formation in the mature cells when observed under the microscope after a long incubation period. These observations noted with the isolates obtained agreed with the findings of Breed *et al.*, 1957; and Jensen, 1965.

The results of biochemical tests that have been made on the isolated bacteria gave a positive reaction with mannitol, indole, methyl red, citrate utilization and catalase test. All of these obtained results gave consistent positive reactions with the isolated bacteria (table 3).

These results, therefore confirmed the identity of the isolated bacteria which could be classified as *Azotobacter spp* as described by (Jensen, 1965).

Response of isolates to salt concentrations:

The growth of *Azotobacter* is negatively influenced by salt concentrations (Kaushik and Sethi, 2005). Table 4 presents thegrowth rate of the isolated at different NaCl concentrations. The rate of *Azotobacter* growth varied according to salt concentrations. The maximum rate of growth occurred at 0.2% and 0.4% NaCl, which revealed that both concentration have a same impact on the growth of *Azotobacter spp*. Medium growth occurred at 0.6% and 0.8% NaCl concentration. Also, no significant differences were observed between 0.6% and 0.8% concentrations. Despite the minimum growth observed at 1% NaCl concentration this finding indicated clearly the presence of some tolerant strains to salts ,which agreed with the findings of Islam *et al*.,(2008). Such isolates needs further screening to confirm their tolerance to

different salt concentrations, which might be of use in soils affected by high salinity. However, their efficiency in fixing atmospheric nitrogen at such high salt concentrations needs further evaluation.

Effect of pH on *Azotobacter* growth:

The effect of pH on *Azotobacter* varies according to different pH values (Jensen, 1965). The obtained results presented in table 5 showed no growth of *Azotobacter* at pH 5 . There was minimum growth at pH 6. Growth at pH 7, however, was optimum. This clearly indicated the suitability of neutral pH to the growth of the *Azotobacter* isolates. This agrees with the findings of Burk *et al*.,(1933) , and Aquilantia *et al*.,(2004). (Aquilantia *et al*, 2004) reported similar results. Increasing the pH to 10 showed no growth of the isolates (Saribay *et al.*, 2003), which clearly confirmed the results of Aquilantia *et al.*, (2004) that both alkalinity and acidity are not favorable for *Azotobacter* growth, and the best growth occurs at the neutral pH value (7).Wide range of soil pHs prevail in Sudanese soils, although alkalinity dominates most of the soils. Further testing of the isolated bacteria needs to be conducted to find pH tolerant *Azotobacter* strains to add to the different variable soils in Sudan.

Effect of different incubation temperatures on *Azotobcter spp*:

The results explained that the isolated *Azotobacter* did not tolerate a temperature of 10 C° (table 6) . But, growth was observed upon raising the incubation temperature to 20 C°, although growth was generally weak to moderate. The high growth occurred when the incubation temperature was adjusted at 30 C°. A decrease in growth was observed when raising the incubation temperature to 40 C°. No growth was reported at 50 C° incubation temperature. The results were consistent with the findings of (Joseph *et al.*, 1964, Jensen, 1965).

Antibiotic resistance test for the isolated *Azotobacter spp*:

Antibiotic resistant of *Azotobacter*:

The *Azotobacter* isolates obtained from Sudanese soils showed resistance to the added antibiotic; streptomycine in the range (0- 5 µ/ml).This agrees with the findings of Garrity (2005) who reported that his *Azotobacter* isolates were susceptible to 0.2 µ/ml concentration of streptomycine. Further research regarding the efficiency of *Azotobacter* isolates in Sudan to fix atomosheric nitrogen under field conditions will be possible by using such resistant isolates. This will ease their identification in the field in future studies.

Table (1). Colony Morphology of *Azotobacter spp* isolated in Mannitol N-free Agar Medium.

Size	1-5 mm diameter
Shape	Circular
Pigmentation	creamy beige white, similar color and hue as the agar
Elevation	Raised
Edge	Smooth
Surface	Smooth
Optical Characteristics	
Under reflected light	Glossy
Consistency	Viscous
Odor	Musky
Other Colonies	There were no other colony types visible.

Table (2). Cell Morphology of *Azotobacter spp* Cells isolated in Mannitol N-free Agar Medium.

Shape	rectangular rods, and mostly oval rods, many were peanut shaped
Axis	Straight
Ends	Rounded

Grouping	single, most were paired end to end
Gram Stain	Negative

Table (3) *Azotobacter spp* biochemical characterization according to Bergey's Manual(1981)

Biochemical characterization	
Mannitol	+
Indole	+
Methyl red Vogues Proskauer	+
Citrate utilization	+
Urease test	+
Catalase test	+

Table (4) Growth rating* of the isolated *Azotobacter* in Mannitol N-free Agar Medium at different salt (NaCl) concentrations

Rep.	**0.2 % NaCl**	**0.4 % NaCl**	**0.6 % NaCl**	**0.8 % NaCl**	**1.0 % NaCl**
1	3	3	2	2	1
2	3	3	2	2	1
3	3	3	2	2	1
Mean	2.67	2.33	2.00	2.00	2.00

*Growth Rating: **3**=high growth, **2**=moderate growth, **1**=weak growth

Table (5) Growth rating* of the isolated *Azotobacter* in Mannitol N-free Agar Medium adjusted to different pH Values .

Rep.	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
1	1	3	4	3	2	1
2	1	3	4	3	2	1
3	1	3	4	3	2	1
Mean	2.3	2.3	2.3	2.3	2.3	2.3

*Growth Rating: 4=high growth, 3=moderate growth, 2=weak growth, 1=No growth.

Table (6) Growth rating* of the isolated *Azotobacter* in Mannitol N-free Agar Medium adjusted to different temperatures

Rep.	10 C°	20 C°	30 C°	40 C°	50 C°
1	1	2	3	2	1
2	1	2	3	2	1
3	1	2	3	2	1
Mean	1.67	2.00	1.67	2.00	1.67

* Growth Rating: 3=high growth, 2=moderate growth, 1=no growth

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