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SCREENING, ISOLATION AND CHARACTERIZATION OF *AZOTOBACTER VINELANDII* IN SOILS OF VARIOUS FIELDS AND ORCHARDS

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ABSTRACT: *Azotobacter vinelandii* is a free-living and heterotrophic bacteria. It also has the ability to produce a yellow-green pigment which gives fluorescence under ultraviolet light. They are generally found in alkaline soils, marine sediments, marsh water etc. Present work was done to isolate strains of *Azotobacter vinelandii* from soil samples collected from different locations as its polysaccharide alginate has widespread applications. Derx medium was used for enrichment and nitrogen free and Burk's medium for isolation. Relevant biochemical and antimicrobial sensitivity tests were done to identify the isolates. In all 81 soil samples were collected and 39 cultures were isolated and identified to be as *Azotobacter vinelandii*. Cultures were found to be resistant to the antimicrobial agents at the recommended concentrations. Higher concentration of streptomycin showed inhibition.

KEYWORDS: *Azotobacter vinelandii*, Derx medium, Burk's medium, antimicrobial agents, biochemical tests.

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1.INTRODUCTION

Azotobacteraceae are a group of aerobic, free-living, heterotrophic bacteria whose main characteristic is the ability to fix atmospheric nitrogen [1,2]. They are hence associated with higher plants as a non-symbiotic nitrogen fixing bacteria. They influence growth yield in plants [3,4] and hence used in biofertilizer formulations [5,6].They are large cells, Gram negative (although some may be Gram variable), and often motile. Non-endospore formers, but some species may form cysts. It includes four genera : *Azomonas, Beijerinckia, Derxia* and *Azotobacter.* The key taxonomic characters are cyst formation , G+C ratio ,cell form, motility,the formation of characteristic lipoid bodies (usually poly-β-hydroxybutyrate), and catalase reaction. Members of this genera are regular inhabitants of soil, including aerially transported dust, aqueous habitats, plant surfaces such as the surface of roots (rhizospheres) and leaves (phyllosphere). Since these organisms are aerobic , heterotrophic , they can grow on any medium having suitable pH that contains an organic carbon source, minerals (especially phosphate), some trace elements (in particular, molybdenum), and no combined nitrogen [7]. *Azotobacter vinelandii* was found to be mostly present in the alkaline soils, such as calcareous soils and soil derived from marine sediments, sea sludge, or sea muds that had been pumped up for leveling land. This organism can be easily enriched in the media designed by Derx [8].The underlying principles of this method were the addition of sodium benzoate (1.0%) to the enrichment medium in order to suppress the development of *Azotobacter chroococcum*, and the use of special carbon source, such as mannitol or ethanol, which is very readily assimilated by *A. vinelandii*. Jensen (1961) mentioned that L-rhamnose (1.0%) could be used as carbon source for selective accumulation of *A vinelandii* , since only a fraction of other strains of *Azotobacter* and *Azomonas* could utilize this compound. When grown in pure culture on L-rhamnose, *A vinelandii* develops profusely within 3-5 days, while the other species develop after 1-2 weeks. Claus and Hemperl (1970) observed that resorcine, ethylene glycol, or glutarate, all in 0.1 or 0.2 % (wt/vol.) concentration , are very selective source of carbon in enrichment media. The presence of *A vinelandii* can be detected by a colour change of the enrichment medium, which turns yellow, green , or violet. On nitrogen-free mineral agar plates, a water-soluble yellow, green, or violet fluorescent pigment is excreted into the medium. Pigment production is stimulated by low iron concentration of the medium [7]. Researchers are more interested in *Azotobacter* because of its versatility in being applicative in many spheres. The exopolysaccharide i.e. alginate produced by these organisms have widespread applications of being used as a thickener, stabilizer, cosmetics, gelling agent in textile, pharmaceutical, paper, printing, beverage and food industries,viscofiers, emulsifiers, manufacturing of ceramics and production of welding rods, [9] drug delivery system, waste water treatment as a flocculant, metal adsorption etc. [10,11].Moreover, *Azotobacter vinelandii* is the preferred organism being non-pathognic [12,13] as compared to other species of *Pseudomonas* which are pathogenic or

opportunistic pathogens [14,15].

2. MATERIALS AND METHODS

2.1 **Collection of soil samples:** Soil samples were collected from various fields and orchards in sterile zipped plastic bags from the depth of 10-15 cm.

2.2 **Treatment of soil:** To support the growth of desired organism i.e. Azotobacter vinelandii, soil was treated with extract of grapes, 1.0% sodium benzoate and kept at 37^0C for a week.

2.3 **Enrichment in selective medium:** Enrichment was done in Derx media at 37^0C (1 ml Ethanol, 0.5 g K_2HPO_4,1.0 g Sodium benzoate,0.1% phenol,100 ml distilled water,7.2 pH) by using treated (treatment of soil with 1% sodium benzoate and crushed grapes) soil samples from different fields and orchards. It was incubated for a week. Gram staining was performed before proceeding for isolation.

2.4 **Isolation of microorganisms :** Isolation was done on Nitrogen free medium (1.0g K_2HPO_4,0.1 g $CaCl_2.2H_2O$,0.2g $MgSO_4.7H_2O$,0.05 g $FeSO_4.7H_2O$,0.005 g $Na_2MoO_4.2H_2O$, Sucrose 10.0g 1000 ml distilled water) and Burk's medium (0.08g K_2HPO_4, 0.02g KH_2PO_4, 0.02g NaCl, 0.02g $MgSO_4.7H_2O$, 0.01 g $CaSO_4$, Fe-Mo mixture 0.01 ml, 2.0g Sucrose, 10µg H_3BO_3, 10µg $ZnSO_4.7H_2O$, 1 µg $MnSO_4.7H_2O$, 0.30 µg $CuSO_4.5H_2O$, 0.1 µg KI , 100ml distilled water) (Fe-Mo mixture:1.45g $FeCl_3.6H_2O$, 0.253 g $Na_2MoO_4.2H_2O$) at 37^0C. Growth of colonies was observed for 3-5 days. Small, glistening colonies were observed. They were further transferred on medium which enhances the pigment production maintaining the same cultural conditions [8,9,16].

2.5 **Pigment production:** Basal nitrogen free medium was used with omission of $FeSO_4.7H_2O$ and reduction of $Na_2MoO_4.2H_2O$ to 1 µg/ml and adding Glucose 10g/lit as 'C' source. Plates were incubated at 37^0C for about a week [8,9,17].

2.6 **Morphological, Cultural and Biochemical characterization:** Relevant biochemical tests were performed along with motility and Gram-staining to identify the bacteria. (Cyst forming ability, oxidase, catalase, amylase, nitrate reductase, urease, proteolytic activity, H_2S production, growth at 9^0C, 14^0C, 18^0C, 32^0C, 37^0C, growth in presence of 1% NaCl, utilization of sugars - rhamnose, fructose, glucose, sucrose, ethanol, dulcitol, maltose, raffinose, lactose, mannitol, mannose, galactose, m-inositol, sorbitol, propan-1-ol, butan-1-ol, glycerol) [8,16].

2.7 **Determination of sensitivity to antimicrobial agents:** Antibiotic sensitivity test (streptomycin-0.2µg/ml,25µg/ml,Chloramphenicol-5µg/ml,oxytetracycline-0.2µg/ml, Pen-G5U/ml, Erythromycin-2µg/ml) along with other antimicrobial agents (Phenol-0.05%, Sodium benzoate-0.05%, Mercuric Chloride – 10 g/ml) was done which is helpful in identification of the isolated strains [8].

2.8 **Preservation of the cultures:** Cultures were preserved at -20^{0}C in 20% of glycerol solution for further studies.

3. RESULTS AND DISCUSSION

Table 3.1: Collection of soil samples from fields and orchards of various regions and number of cultures preliminary identified as *Azotobacter vinelandii*

S.No	District	Place	Fields/ Orchards	No. of samples collected	No. of identified isolates	Culture Code
1.	Nasik (Mah)	Nasik road	Grapes	6	1	1
2.	Aurangabad (Mah)	Himayat Bagh	Guava	3	2	2,3
			Mango	3	3	4,5,6
			Sweet lemon	3	2	7,8
		Phulambri	Sugarcane	6	4	9,10,11,12
			Figs	6	2	13,14
3.	Parbhani(Mah)		Sugarcane	6	6	15,16,17, 18,19,20
			Jowar	6	2	21,22
			Cotton	6	2	23,24
			Chickoo	6	3	25,26,27
			Wheat	6	2	28,29
			Corn	6	3	30,31,32
			Gram	6	2	33,34
			Tur	6	1	35
4.	Kurnool(A.P.)		Mango	6	4	36,37,38, 39

Table 3.2a: Morphological, cultural and biochemical characterization of isolates.

Culture Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gram's nature	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	d	+	+	+	+	+	d	+	+	+	+	+
Cyst formation	+	+	+	+	+	+	+	+	+	+	d	+	+	+	+	+	+	+	+	+
Pigmentation	+	+	+	+	+	+	+	+	+	+	d	+	+	+	+	+	d	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nit. red.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Prot. acty.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 9^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14^0C	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
18^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37^0C	+	+	+	+	d	d	+	+	+	+	+	+	d	+	+	+	+	+	+	+
Gro.in 1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Utilization of sugars																				
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	d	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	d	+	+	+	+	+	+	+	+	d	+	+	d	+	+
m-inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propane-1-ol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Butane-1-ol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Legend: (+) Positive results, (-) Negative results, (d) Partially positive results, (Nit.red.) nitrate reductase, (Prot. Acty) proteolytic activity, (Gro.in 1% NaCl) Growth in 1% NaCl.

Table 3.2b: Morphological, cultural and biochemical characterization of isolates.

Culture Characteristics	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Gram's nature	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cyst form	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pigmentation	d	+	+	+	+	d	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nit. red.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Prot. acty.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 9^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14^0C	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+
18^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37^0C	+	+	+	+	d	d	+	+	+	+	+	+	d	+	+	+	+	+	+
Gro. in 1% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on sugars																			
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	d	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	d	d	+	+	+	+	+	+	+	+	d	+	+	+	+	+	d
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	d	+	+	+	+	+	+	+	+	+	d	d	+	+	+	+

m-inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propane-1-ol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Butane-1-ol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Legend: (+) Positive results, (-) Negative results, (d) Partially positive results, (Nit.red.) nitrate reductase, (Prot. Acty) proteolytic activity, (Gro.in 1% NaCl) Growth in 1% NaCl.

Table 3.3: Determination of sensitivity to various antimicrobial agents.

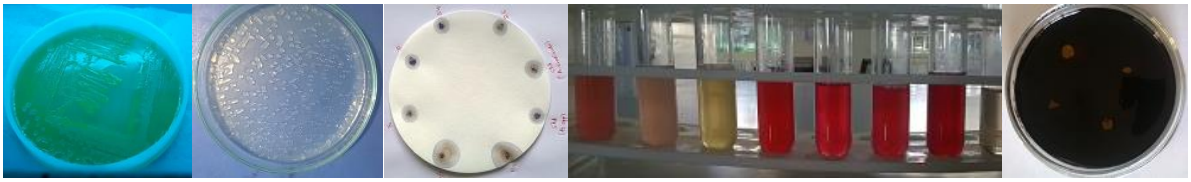
Clts.	Streptomycin		Chl	O-Tet	Pen-G	Phenol	Na-Ben	Ery	$HgCl_2$
	0.2µg/ml	25µg/ml	5µg/ml	0.2µg/ml	5 U/ml	0.05 %	0.5 %	2µg/ml	10µg/ml
1	R	S	R	R	R	R	R	R	R
2	R	S	R	R	R	R	R	R	R
3	R	S	R	R	R	R	R	R	R
4	R	S	R	R	R	R	R	R	R
5	R	S	R	R	R	R	R	R	R
6	R	S	R	R	R	R	R	R	R
7	R	S	R	R	R	R	R	R	R
8	R	S	R	R	R	R	R	R	R
9	R	S	R	R	R	R	R	R	R
10	R	S	R	R	R	R	R	R	R
11	R	S	R	R	R	R	R	R	R
12	R	S	R	R	R	R	R	R	R
13	R	S	R	R	R	R	R	R	R
14	R	S	R	R	R	R	R	R	R
15	R	S	R	R	R	R	R	R	R
16	R	S	R	R	R	R	R	R	R
17	R	S	R	R	R	R	R	R	R
18	R	S	R	R	R	R	R	R	R
19	R	S	R	R	R	R	R	R	R
20	R	S	R	R	R	R	R	R	R
21	R	S	R	R	R	R	R	R	R
22	R	S	R	R	R	R	R	R	R
23	R	S	R	R	R	R	R	R	R
24	R	S	R	R	R	R	R	R	R

25	R	S	R	R	R	R	R	R	R
26	R	S	R	R	R	R	R	R	R
27	R	S	R	R	R	R	R	R	R
28	R	S	R	R	R	R	R	R	R
29	R	S	R	R	R	R	R	R	R
30	R	S	R	R	R	R	R	R	R
31	R	S	R	R	R	R	R	R	R
32	R	S	R	R	R	R	R	R	R
33	R	S	R	R	R	R	R	R	R
34	R	S	R	R	R	R	R	R	R
35	R	S	R	R	R	R	R	R	R
36	R	S	R	R	R	R	R	R	R
37	R	S	R	R	R	R	R	R	R
38	R	S	R	R	R	R	R	R	R
39	R	S	R	R	R	R	R	R	R

Legend: (Clts) Cultures, (R) Resistant, (S) Sensitive, (Chl) Chloramphenicol, (O-Tet) Oxytetracycline, (Pen-G) Penicillin G,(Na-Ben) Sodium benzoate, (Ery) Erythromycin, ($HgCl_2$) Mercuric Chloride.

3.4 Images

3.4a 3.4b 3.4c 3.4d 3.4e



3.4a-Fluorescence under U.V, 3.4b-Growth on Burk's medium, 3.4c-Oxidase test, 3.4d-Nitrate reductase test, 3.4e-Amylase plate assay.

Derx medium is a selective medium for enrichment of *Azotobacter vinelandii* from soil or water. Presence of sodium benzoate suppresses the growth of *Azotobacter chroococcum* and other species. Ethanol/mannitol is easily assimilated by *A vinelandii*. Treatment of soil with benzoate and crushed grapes displaces the other normal population with *A vinelandii*. It produces yellow-green pigment on nitrogen free glucose agar (Fig.3.4a). But pigment production depends on various factors. Hence proper pigmentation was observed only by using medium which induces pigment production [18,19]. As shown in (Table 3.1) out of 81 soil samples only 39 cultures were isolated which resembled the characteristics of *A vinelandii*. Cultures were found to be showing resistance to streptomycin at 0.2 µg/ml concentration as shown in (Table 3.3) which may be due to adaptation and almost all the

results of biochemical tests were found to be similar as observed in (Table 3.1,Table 3.2 and Fig. 3.4c, 3.4d, 3.4e). Selectively enriching soil with grape extracts, sodium benzoate and incubation at 37^0C are very much helpful in proliferation of the desired organism. Pigment production and the other relevant biochemical methods are helpful in identifying the bacteria at preliminary level. Such selected organisms can further be confirmed by any of the advanced sophisticated technique like FAME, MALDI-TOF MS, 16S rDNA sequencing etc. or by polyphasic identification system.

4. CONCLUSION

The isolated organisms are further intended to be exploited for its polysaccharide alginate having commercial applications. Hence it has a lot of scope for further studies which includes developing the cultures for maximum yield of product under optimized conditions or maintaining conditions to produce polysaccharides of varying M/G ratio, molecular weight and viscosity. As alginates used for various purposes are being extracted from sea weeds. It has its own disadvantages of non-reproducibility of results and also some environmental concerns as the consumption level is increasing day by day many folds. Hence its synthesis using cheap substrates and a totally non-pathogenic bacteria would be advantageous to fulfill the demands.

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6. CONFLICT OF INTEREST

The authors declare that there is no financial interest or any conflict of interest.

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