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Antimicrobial Activities of Actinomycetes Against Urease Producing Bacterial Pathogens

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ABSTRACT
Ureolytic bacterial pathogens are clinically important group of organisms in which urease acts as an important biomarker and virulence factor during onset of infection. Multidrug resistant ureolytic bacteria are posing additional threat due to their involvement in potentially fatal conditions such as gastric and duodenal cancers. This induced the need to search novel bioactive molecules acting against ureolytic bacteria. In the present study48actinomycetes strains isolated from soil are screened against four ureolytic bacteria including Proteus mirabilis, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa. The ethyl acetate extracts of five actinomycetes inhibited growth of Proteus mirabilis, 13 extracts inhibited growth of Staphylococcus aureus, six inhibited Pseudomonas aeruginosa growth and significant inhibition of Klebsiella pneumoniae growth was found in presence of 16extracts. Based on the results theCLA1strain was selected as potent ureolytic bacterial inhibitor and primarily identified at morphological level as Streptomyces species. The study highlights the inhibitory activity of different actinomycetes strain and can be useful to direct the development of novel bioactive molecules against ureolytic bacterial pathogens.
Keywords: *Urease, bioactive, actinomycetes, ureolytic.*

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INTRODUCTION

Urease is a nickel ion dependent hydrolytic enzyme accelerating the rate of urea breakdown to ammonia and carbon dioxide (1). Ureases have been isolated from a range of organisms including bacteria, fungi and plants. Bacterial urinary tract infections (BUTI's) are most common infections affecting more than 150 million patients globally. Multidrug resistant uropathogens are gaining worldwide attention due to interference in treatment and increase in hospital stay. The complications also arise due to catheterization procedure and pregnancy (2). Urease dependent disease process is connected with BUTI's caused *Proteus* and *Klebsiella* species. The infections due to these urease positive bacteria may result in infection stone formation that offers protection to pathogens (4). In addition to this urease mediated pH changes causing host epithelial cells damage are considered important in promoting some bacterial infections. Ureolytic bacteria are also involved in formation of infectious kidney stones, gastroduodenal inflammation and pyelonephritis (5-7). Emergence of rapid drug resistance in ureolytic bacteria has been proved to make available line of treatment ineffective (8). The search for novel bioactive molecules from natural sources for modulating growth and activity of ureolytic bacteria can be helpful in overcoming the drawbacks associate with it. Actinomycetes are most important and dominant group of Gram-positive bacteria with a proven track record of producing a wide variety of bioactive metabolites (9, 10). Among the all known antimicrobial agents used in medicines and agriculture, 70 to 80% products have been isolated from actinomycetes. The present study aimed to screen some actinomycetes strains isolated from rhizosphere soil of *Ficus religiosa*,*Curcuma longa* and *Azadirachta indica* for antibacterial activity against ureolytic bacterial pathogens.

MATERIAL AND METHODS

Isolation of actinomycetes:

Rhizospheric soil samples associated with different medicinal plants viz,*Ficus religiosa*,*Curcuma longa* and*Azadirachta indica*cultivated in Vishnupuri region of Nanded, MS, India were collected from 15cm depth in sterile polythene bags, transferred to laboratory. The soil samples were air dried at room

temperature (35°C) for 48 hrs and after removing recognizable stone and debris, crushed and sieved to get fine powder. The serial dilutions of soil sample were prepared till 10^{-8}dilution. 0.1 ml of dilutions 10^{-4} to 10^{-7} was spread plated onto surface of sterile starch casein agar (HiMedia) supplemented with Streptomycin (50 µg/ml) and nystatin (50 µg/ml) reduce the growth of other bacteria and fungi. The plates were incubated at 30°C till fifteen days and well developed morphologically distinct colonies of actinomycetes were selected and maintained on starch casein agar slant at 4°C till further use.

Test organisms:

Three clinical isolates of *Proteus mirabilis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* and one strain of *Pseudomonas aeruginosa* isolated from urine sample of urinary tract infected patient were used in the present study.

Screening of bacteria for urease production:

The selected test bacteria were tested for urease activity on Christensen's urea agar (CUA) (HiMedia) plates as suggested by Hammad et al. The active cultures of test bacteria were spot inoculated onto surface of sterile CAU plates. The plates were incubated for 24-48 hrs at 37°C. The appearance of bright pink color surrounding the growth of bacteria indicated urease production (9).

Screening of actinomycetes for antibacterial activity against urease producing bacteria:

Antibacterial activity of ethyl acetate extracts of 48 actinomycetes isolates against urease producing bacteria was tested by agar well diffusion method. The actinomycetes isolates were cultivated in 100 ml of sterile soybean casein digest broth (HiMedia) supplemented with 1% dextrose in a 250 ml capacity Erlenmeyer flask for seven days at 30°C in an orbital shaking incubator at 120 rpm rotation. After incubation the culture broth was centrifuged at 10,000 rpm and 4°Cfor 30 min to separate cell debris and secondary metabolites. The supernatant was mixed to ethyl acetate in 1:1 proportion and shaken vigorously in a separating funnel for 20 min. The organic phase was collected and evaporated to dryness in a desiccator. The dried residues were dissolved in dimethyl sulfoxide and used for next studies (10).

Antimicrobial activity of extracts:

The partially purified ethyl acetate extract of 48 actinomycetes isolated were tested for antibacterial activity using agar well diffusion method. Active cultures of test bacteria were prepared in sterile nutrient broth till approaching the OD 600 nm similar to 0.5 McFarland turbidity standards. The best bacterial cultures were spreaded on Muller Hinton agar plates with the help of alcohol sterilized glass spreaders. Well of 5mm area were made with the help of flame sterilized cork borer and 0.05 ml of each extract was added into wells. The plates were kept for diffusion in refrigerator for 20 min and later incubated at 37°C for 24 hrs (11). The antibacterial efficacy of actinomycetes was determined based on sizes of zone of inhibition obtained. The isolate CLA1 showing strong antibacterial activity was selected for further study.

Characterization of CLA1 isolate:

The isolate CLA1was characterized further by coverslip culture technique to observe the number and arrangement of spores, shape of spores and presence or absence of submerged mycelium. The ability to form diffusible pigments was tested by observing colony front and reverse in starch casein agar plate. The color of spore'smass and aerial mycelium was identified using National Bureau of Standards(NBS)color chart as indicated previously (12).

RESULT AND DISCUSSION:

Urease dependent bacterial pathogens including *Helicobacter pylori*, *Proteus* species and *Klebsiella* species are posing serious threat clinically especially due to emergence of drug resistance among them. Urease production is particularly helpful in these bacteria to cope with highly acidic conditions at infection site like stomach (13) as in *Helicobacter pylori*. It is also beneficial for those bacteria who infects urinary tract where they form infection stones around them to protect themselves from host defences. In fungi like *Cryptococcus neoformans* their virulence is totally dependent on urease production as the products of urease action are toxic to human cells and thus helps to develop a systemic infection (14). The ureolytic bacteria are also involved in Pneumonia, kidney stone formation, dental plaque formation, UTI and calculus deposition (15, 16). Urease is an important virulence marker and ten of twelve antibiotic resistant pathogens listed by WHO as "priority pathogens" are ureolytic (17). In order to cope up with challenges associated with these bacteria new strategies focusing on novel bioactive molecules are required to control ureolytic activity. In view of this, in present study 48 actinomycetes strains isolated from rhizosphere soils of medicinal plants were screened for their antibacterial activity against four urease producing test bacteria. The urease activity of test bacteria was identified based on pink color formation on CUA plates with 24-48 hrs of incubation. Appearance of pink color was noticed faster in *Proteus mirabilis* inoculated plate (12 hrs) followed by *Pseudomonas aeruginosa* (16 hrs),*Klebsiella pneumoniae* (24 hrs) and *Staphylococcus aureus* (24 hrs).

a. b. c. d.

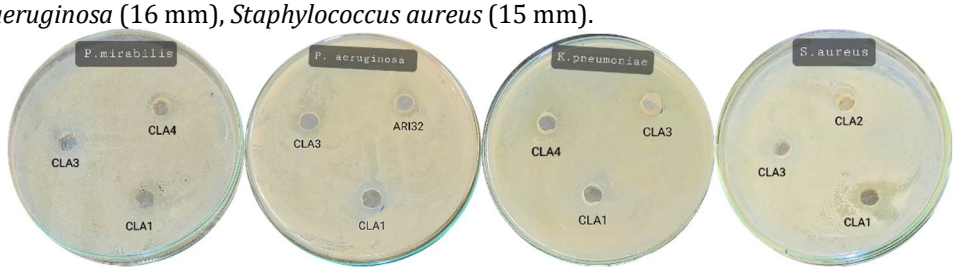
Fig. 1:Urease Activity of bacterial isolates on CUA after 24 hrs incubation. a.*Proteusmirabilis*b. *Pseudomonas aeruginosa* c.*Klebsiella pneumoniae* and d.*Staphylococcus aureus*.

Table 1: Antibacterial activity of actinomycetes isolates against ureolytic bacteria

Sr. No.	Actinomycetes Isolates	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
		Zone of Inhibition in mm			
1.	ARI1	10	-	-	-
2.	ARI2	-	-	12	-
3.	ARI3	-	-	13	-
4.	ARI4	-	-	10	-
5.	ARI5	-	-	11	-
6.	ARI6	-	-	-	10
7.	ARI7	-	-	-	-
8.	ARI8	-	-	12	12
9.	ARI9	-	-	-	-
10.	ARI10	-	-	-	-
11.	ARI11	-	-	-	-
12.	ARI12	-	-	11	10
13.	ARI13	-	-	-	-
14.	ARI14	-	-	-	12
15.	ARI15	-	-	-	11
16.	ARI16	-	-	-	10
17.	ARI17	-	10	10	-
18.	ARI18	-	12	-	10
19.	ARI19	-	-	-	-
20.	ARI20	-	10	10	-
21.	ARI21	-	-	11	-
22.	ARI22	-	-	12	-
23.	ARI23	-	-	10	-
24.	ARI24	-	-	-	10
25.	ARI25	-	-	-	10
26.	ARI26	10	-	11	-
27.	ARI27	-	-	-	-
28.	ARI28	-	-	10	-
29.	ARI29	-	-	-	-
30.	ARI30	-	-	-	-
31.	ARI31	-	-	-	-
32.	ARI32	-	10	-	11
33.	CLA1	12	16	14	15
34.	CLA2	-	-	-	-
35.	CLA3	10	11	10	11
36.	CLA4	11	-	13	-
37.	CLA5	-	-	-	12
38.	CLA6	-	-	-	-
39.	CLA7	-	-	-	-

40.	CLA8	-	-	-	-
41.	CLA9	-	-	-	-
42.	CLA10	-	-	-	-
43.	CLA11	-	-	-	-
44.	FRA1	-	-	-	-
45.	FRA2	-	-	-	-
46.	FRA3	-	-	-	-
47.	FRA4	-	-	-	-
48.	FRA5	-	-	-	-

The antibacterial activity CLA1 of ethyl acetate extracts of actinomycetes against all four test ureolytic bacteria is shown in table 1 and fig. 2. The extracts from five isolates were highly active against *Proteus mirabilis*, 16 extracts strongly inhibited *Klebsiella pneumonia* whereas six and 13 extracts inhibited *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively. Two extracts were able to inhibit the growth of all four test pathogens. The extract from isolate CLA1 showed highest inhibition activity against *Pseudomonas aeruginosa* (16 mm), *Staphylococcus aureus* (15 mm).



a **b** **c** **d**

fig.2 Antibacterial activity exhibited by crude extract of streptomyces CLA1 against a. *Proteus mirabilis* b. *Pseudomonas aeruginosa* c. *Klebsiella pneumoniae* and d. *Staphylococcus aureus*.

The isolate CLA1 produced greyish colonies with light brown diffusible pigment when grown starch casein agar plate for seven days. Microscopic observation of isolate indicated the presence of branched substrate mycelia, aerial hyphae and long spore chain with more than 10 spores in each chain. Based on morphological and microscopic features, the isolate was primarily identified to belong Streptomyces group.

a **b** **c**

fig.3 Streptomyces CLA1 on starch casein agar a. greyish colonies with mycelium b. light brown colony reverse and micrograph showing spore chain of CLA1 (c).

Streptomycetes are prominent producers of bioactive molecules and a predominant group of actinomycetes widely available in different ecological niches such as soil, sediment or water, more than since last 50 years, streptomycetes are raising the academic, industrial and research interests due to their probiotic nature. However, the antibacterial property of streptomyces against ureolytic bacteria has not revealed and reported much earlier. The present study highlighted the bioactive potential of CLA1 in controlling the infections caused by urease active bacteria and we also strongly that rhizosphere soil of *Curcuma longa* can serve as potent source of novel antibacterial compounds.

CONCLUSION:

The ureolytic bacterial pathogens are clinically important group of microbes and needs utmost attention due to emergence of multidrug resistance among them. The actinomycetes isolates from rhizosphere region of studied medicinal plants offers a new hope for development of novel bioactive compounds against these bacteria. The present study identified the potential of CLA1 strain isolated from *Curcuma longa* rhizosphere soil against priority pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Staphylococcus aureus*. However further studies regarding the characterization of

CLA1 and its bioactive secondary metabolite are needed to understand its nature and mode of action against ureolytic bacteria.

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