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Antagonistic activity of *Pseudomonas aeruginosa* ATCC 27853 against pathogenic bacteria

Sara Tayeb, Zouaouia Chama, Abdelkarim Tifrit, Fatima Zohra Imène Reffas and Bouziane Abbouni

Laboratory for Molecular Microbiology Proteomics and Health, Department of Biology, Faculty of Natural Science and life, University of Djillali Liabès of Sidi Bel Abbés, Algeria

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ABSTRACT

Different biological control agents are used to fight various pathogens or even harmful organisms in different environments. Among these agents, a wide range of bacteria belonging to the genus Pseudomonas spp are capable to produce a large number of secondary metabolites with antibacterial activity. The main aim of the present work is the evaluation of the antagonistic activity of Ps. aeruginosa ATCC 27853 against pathogenic bacteria strains such as E. coli ATCC 25921, P. mirabilis ATCC 12453, B. subtilis ATCC 6633 and B. cereus ATCC 11778 by the using agar well diffusion method and by the study of the bacterial growth in the absence and in the presence of the supernatant of Ps. aeruginosa ATCC 27853. In order to characterize the molecules responsible for the antagonistic activity of Ps. aeruginosa ATCC 27853, various parameters such as temperature, pH, and incubation time has been investigated. The obtained results showed that the tested antagonistic activity of Ps. aeruginosa ATCC 27853 against, S. aureus, Enterobacter, S. typhi, P. vulgaris and B. subtilus ATCC6633 has manifested an excellent antibacterial activity with a maximal diameters of inhibition zone 32, 15, 14.14, 13 mm respectively. Furthermore, the study of bacterial growth of B. subtilus ATCC 6633 in the absence and in the presence of the supernatant of Ps. aeruginosa ATCC 27853 indicated a considerable biomass reduction accompanied with unbalanced growth after adding of the supernatant.

Keywords: *Pseudomonas aeruginosa*, antagonistic activity, optimization, pathogenic strains.

INTRODUCTION

Bacterial resistance to antibiotics poses a serious challenge to the prospect of chemotherapy, because the traditional antibiotics and its derivatives are becoming nonfunctional**.** The whole world is thus confronted with a looming drug crisis which has motivated the pursuit of new antibiotic compounds with novel mechanisms of action [1]**.** The bacterial products have served the development of new pharmaceutical drugs that are widely used to fight bacterial infections [2]**.**

Particularly, the genus *Ps. aeruginosa* has received much attention because of their suggested involvement in natural disease suppressiveness of certain soils [3]**.** Iron is essential for the growth of almost all organisms but, in many environments [4, 5, 6, 7, 8]**.**

The antibacterial properties of *Ps. aeruginosa* have therefore led to the characterization of various antimicrobial substances as organics acids (lactic acid and formic acids), diacetyl, and hydrogen peroxide alone or in combination [9]. Therefore, the objective of this work were the investigation of the antagonistic activity of *Ps. aeruginosa* ATCC27853 against such pathogenic strains *E. coli* ATCC 25921, *P. mirabilis* ATCC 12453, *B. subtilis* ATCC 6633 and *B. cereus* ATCC 11778 and the characterization of their inhibitory substances.

EXPERIMENTAL SECTION

Microbial strains

The used antagonistic bacterial strain in this investigation *Ps. aeruginosa* ATCC 27853 was provided by institute of Pasteur Algiers, whereas the used pathogenic bacterial strains such as *P. mirabilis* ATCC 12453, *B. subtilis* ATCC 6633, *B. cereus* ATCC 11778, *E. coli* ATCC 25921 were furnished by the Microbiological laboratory of the veterinary institute of Tiaret (Algeria). Furthermore, the microorganisms were maintained by routine culture on agar slants, stored at 4°C between transfers. At least two additional subcultures (24 h, 30 °C) were made in fresh medium before use in the experiment.

Detection of antagonistic activity

The antagonistic activity of *Ps. aeruginosa* ATCC 27853 towards the growth of pathogenic bacteria was carried out by the using of the streak according both methods described by Fokkema and Adetuyi [10, 11]. For the conventional streak method 40 mm streak was made from 24 hours old culture of bacteria, 23 mm away from the centre of petridish. The using of 7 mm diameter sterile cork borer, the growing edge of a 24 hours old bacteria culture was aseptically placed at the centre of the plate already inoculated with the tested antagonistic bacterium.

Plates were incubated at 30°C and monitored for 2 days. Measurement of the percentage of inhibition and intercolony distance were taken daily. For the Novel ring method of Adetuyi and Cartwright [11], bacteria were cultured on nutrient agar plates into confluent growth. Prepared Malt Extract Agar plates were inoculated separately with concentric ring culture of the organism. The suspension of the pathogenic bacteria strains (7 mm) was transferred aseptically into the centre of the plate already inoculated with the test antagonistic. Plates were incubated at 30 °C and monitored for 2 days. The measurement of the percentage of inhibition and intercolony distance were taken daily.

The well diffusion assay

The described well diffusion assay by Schillinger and Lucke [12] was used for the in-vitro test of antagonistic activities of *Ps. aeruginosa* ATCC 27853 against other bacteria. Plates containing solidified Nutrient Agar (20 ml) were overlaid with 10 ml of soft Nutrient agar and inoculated with 0.05 ml of an overnight culture of pathogenic bacteria. Four wells, three of the periphery and one at the centre, each 7 mm in diameter were made in the agar using cork borer and 0.1 ml of culture supernatant of test bacterium were transferred into each well. The plates were incubated for 24 h at 30°C, examined for clear inhibition zone around the well. The assay was carried out in duplicate for all the test organisms.

Inoculum preparation

The used isolates were inoculated in nutrient broth, incubated with agitation at 150 rpm on a rotary shaker, at 30°C for 48 h and maintained as stock cultures in starch agar slants, grown at room temperature for 2 days and stored at 4 °C for regular subculturing. The used culture media had the following composition (in g/L): 10 g starch, 10 g of nutrient broth, 15g agar, served as inoculation media for all the experiments. The pure culture was inoculated into sterile broth medium and incubated at 30 °C on a rotary shaker at 75 rpm for 72h. The fresh over night culture was used as inoculum for the growth study [13, 14]**.**

Preparation of culture supernatant

The used antagonistic strain *Ps.aeruginosa* ATCC 27853 was inoculated in a nutrient broth, incubated at temperature 30°C on rotary shaker at 120 rpm for 24 h. The bacterial cells were removed by centrifugation at 6000 g for 10 min and the supernatant was used immediately for further investigation.

Antagonistic activity of the bacterial biomass

In order to explore the antagonistic activity of the bacterial biomass of *Ps. aeruginosa* ATCC 27853, inoculated in a nutrient broth, incubated at temperature 30°C on rotary shaker at 120 rpm for 24 h, the bacterial cells were harvested by centrifugation at 6000 g for 10 min and the sediment was washed twice with sterile distilled water. The deposed disk on the agar surface was inoculated with a volume of 5µl of the bacterial biomass of *Ps. aeruginosa* ATCC 27853**.**

Effect of various parameters on the antagonistic activity

Determination of the thermo-resistance of antagonistic activity

In order to explore the thermo-resistance of the molecule responsible for antagonistic activity of *Ps. aeruginosa* ATCC 27853, 3 sterile tubes containing a volume of 1.5 ml of supernatant of broth of the inoculated antagonistic strain, were treated at the following temperatures (37, - 80, 120°C) for 30 min. After that, a volume of 30 µl of each treated tube spotted on the disk and placed on the surface plate agar. The antagonistic activity was estimated by measuring of the diameter of zone of inhibition formed around the disks, after incubation at 30°C for 18 hours [15].

Effect of incubation time on the antagonistic activity

In order to optimize the incubation time for antagonistic activity of *Ps. aeruginosa* ATCC 27853, an inoculated culture media with the tested bacterial strain were incubated at 30°C on a rotary shaker at 120 rpm for regular time intervals (24, 48, 72h). After that, a volume of 30 µl of each treated tube spotted on the disk and placed on the surface plate agar. The antagonistic activity was estimated by measuring of the diameter of zone of inhibition formed around the disks, after incubation at 30°C for 18 hours [15].

Effect of the pH on the antagonistic activity

In order to investigate the effect of the pH-value on the antagonistic activity of *Ps. aeruginosa* ATCC 27853 against the tested pathogenic bacteria, the pH-value of the supernatant of inoculated broth of the antagonistic strain, was adjusted to the pH-value in the range (3-8) by the using 1N HCl and 1N NaOH. The antibacterial activity was then measured by the agar spot test, where the plates were seeded with 0.1 ml of 18 hours inoculated broth of the tested pathogenic bacteria (approximately $5x10^5$ CFU/ml). The agar plate was dried, and then 5µl of the supernatant on the antibacterial substance of *Ps. aeruginosa* ATCC 27853 adjusted at different pH-value were spotted on the disks placed on the surface plate. The antagonistic activity was estimated by measuring of the diameter of zone of inhibition formed around the disks, after incubation at 30°C for 18 hours [15].

RESULTS

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants and microbes [16]**.** In the present study, the antagonistic activity of *Ps. aeruginosa* ATCC 27853 against various Gram positive (*B. subtilis* ATCC 6633 *and B. cereus* ATCC 11778 and Gram negative bacteria such *E.coli* ATCC 25921, *P. mirabilis* ATCC 12453 has been investigated. For this purpose, the bacterial growth of various Gram positive and Gram negative bacteria in the absence and presence *Ps. aeruginosa* ATCC 27853 has been investigated by the using the disk and the wells diffusion methods.

The illustrated results in (Figure 1, 2, 3) on the antagonistic activity of *Ps. aeruginosa* ATCC27853 highlighted against the pathogenic strains such as *E. coli* ATCC 25921, *P. mirabilis* ATCC 12453, *B. subtilis* ATCC 6633 *and B. cereus* ATCC 11778 showed a excellent antibacterial activity for all tested strains, with a clear zones inhibition at least 7 mm. Furthermore, the obtained diameters of zones inhibitions by the methods of disks and well diffusion assay were 18, 25, 30, 22, 35, 30 mm respectively.

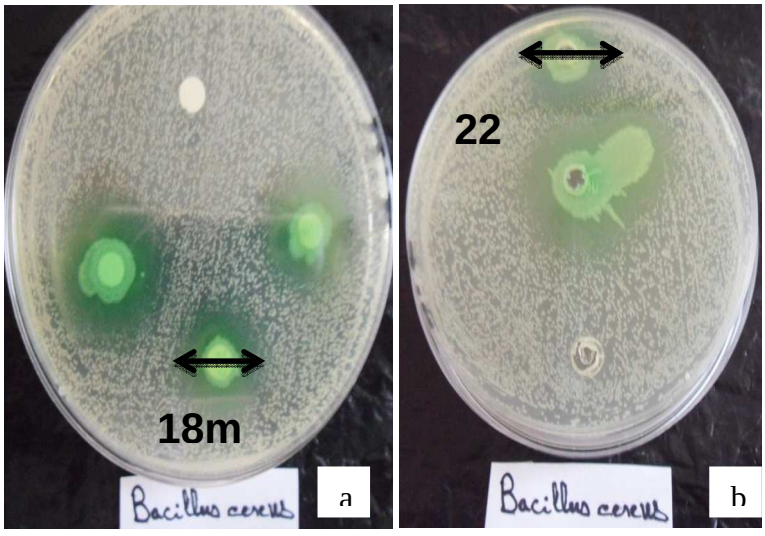

Figure 1: Illustration of the antagonistic activity of *Ps. aeruginosa* ATCC 27853 against *B. cereus* ATCC 11778 by the method of disks (a) and wells diffusion assay (b)

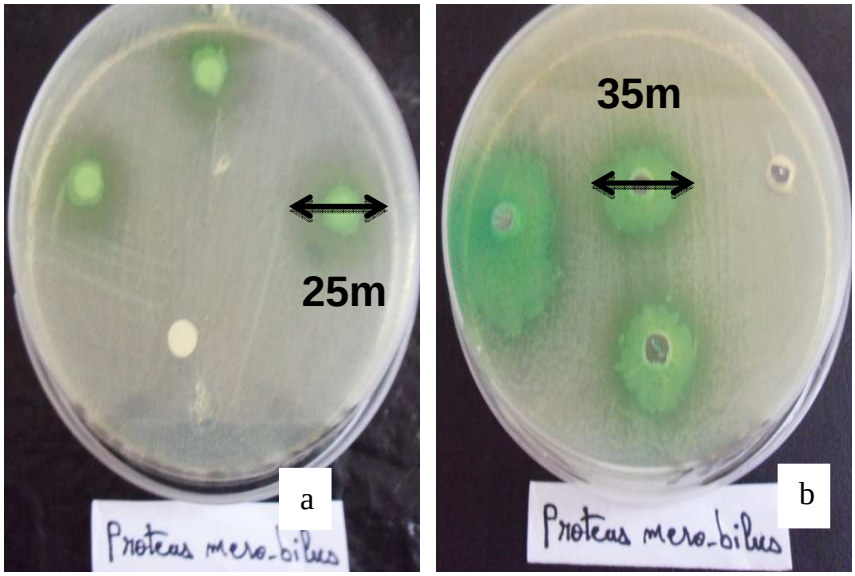

Figure 2: Illustration of the antagonistic activity of *Ps. aeruginosa* ATCC 27853 against *P. mirabilis* ATCC 12453 by the method of disks (a) and wells diffusion assay (b)

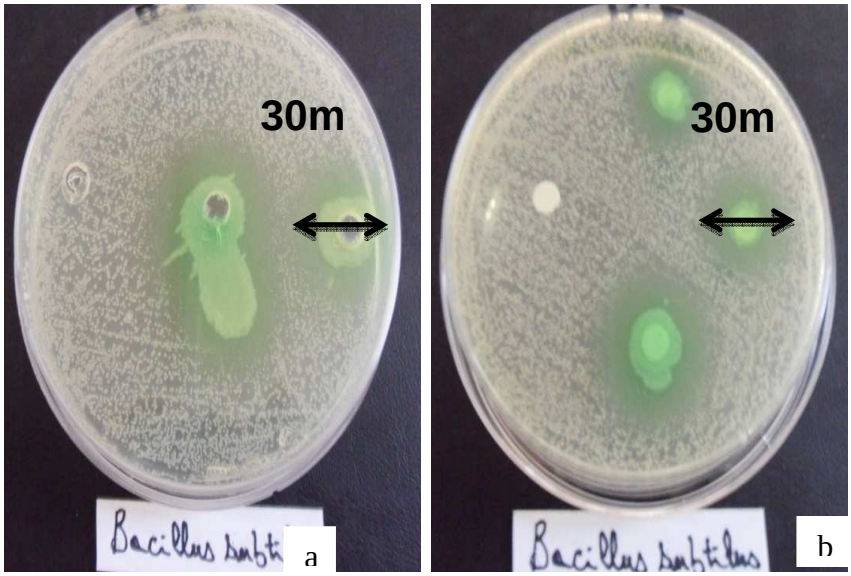

Figure 3: Illustration of the antagonistic activity of *Ps. aeruginosa* ATCC27853 against *B. subtilis* ATCC 6633 by the disk method (a) and wells diffusion assay (b)

The effect of pH-value on the antagonistic activity

In order to explore the effect of the pH-value on the antagonistic activity of the supernatant of *Ps. aeruginosa* ATCC 27853 against *B. subtilis* ATCC 6633, *P. mirabilis* ATCC 12453, the disk diffusion method was used, where the pH-value of the used culture media was adjusted in the range between 3-8.3. The antagonistic activity was evaluated by the measurement of the diameter of the inhibition zone around discs impregnated with supernatant of *Ps. aeruginosa* ATCC 27853. The obtained results indicated the absence of the zone of inhibition in the range of pH-value between 3-7 and the appearance of clear zone of inhibition around the discs impregnated with the supernatant by pH-value 8.3.

Table 2: Diameters of the inhibition zones of *P. mirabilis* ATCC 12453 and *B. subtilis* ATCC 6633 in the presence of the supernatant of *Ps. aeruginosa* ATCC 27853 adjusted at different pH-value

The tested strains	pH-value		
	3	**7**	**8,3**
P. mirabilis	0 mm	0 mm	1,3 mm
B. subtilis	0 mm	0 mm	1,1 mm

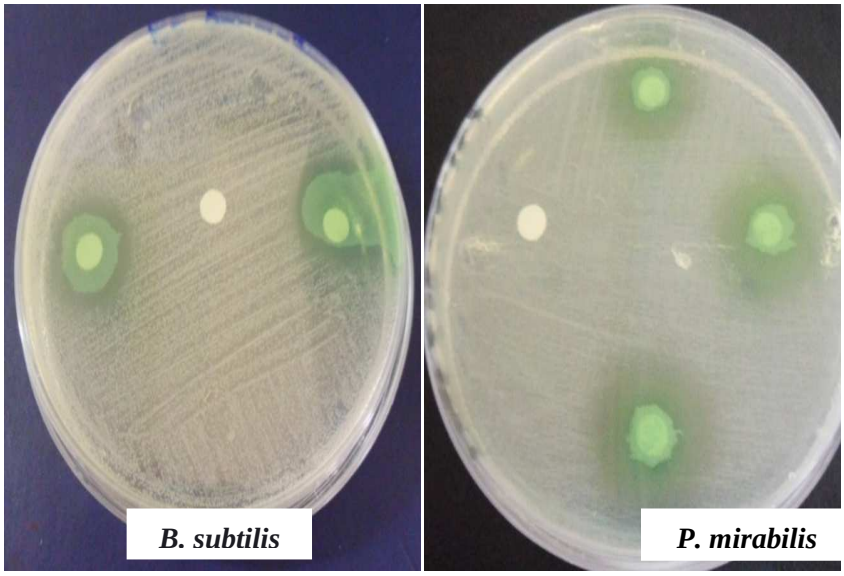

Figure 4: The antagonistic activity of supernatant of *Ps. aeruginosa* ATCC 27853 against *B. subtilis* ATCC 6633 and *P. mirabilis* ATCC 12453 adjusted at pH 8, 3

Effect of the incubation time on the antagonistic activity

Furthermore, the effect of the incubation time on the antagonistic activity of the supernatant of *Ps. aeruginosa* ATCC 27853 against *B. subtilis* ATCC 6633, *P. mirabilis* ATCC 12453 has been studied. The evaluation of the antagonistic activity was estimated by the measurement of the diameter of the inhibition zone around discs impregnated with supernatant of *Ps. aeruginosa* ATCC 27853. The results indicated that an excellent antagonistic activity was obtained after incubation time 72 hours, with the diameters of the zones of inhibition of 35 mm by *P. mirabilis* ATCC12453 and 30 mm by *B. subtilis* ATCC 6633.

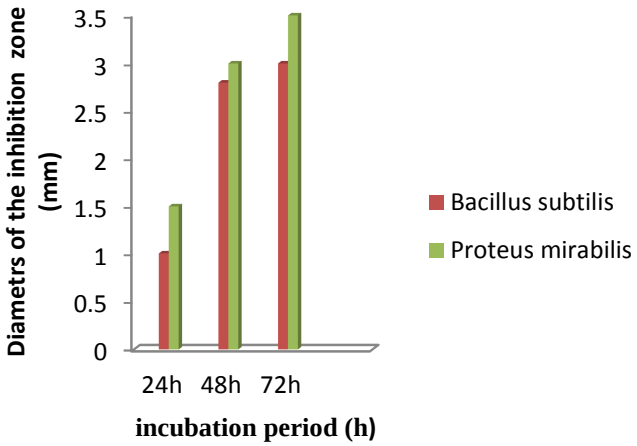

Figure 5: The diameters of the zone of inhibition *of B.subtilis* ATCC 6633, *P. mirabilis* ATCC 12453 in the presence of the supernatant of *Ps. aeruginosa* ATCC 27853, harvested from culture medium after 24, 48, 72 hours of incubation time

Study of the pathogen bacterial growth in the presence of the supernatant of antagonistic strain

In order to explore the effect of the used compounds present in the supernatant of *Ps. aeruginosa* ATCC 27853 against tested pathogenic bacteria, the bacterial growth of *P. vulgaris* in the absence (control) and in the presence of the supernatant of *Ps. aeruginosa* ATCC 27853 has been investigated. For this purpose, *P. vulgaris* was inoculated in seed media with initial optical density of 0.5 at 578 nm according the protocol described by Abbouni and coworkers [17, 18]**.** A volume of 1 ml of the recovered supernatant of *Ps. aeruginosa* ATCC 27853 was added 6 hours after the onset of the bacterial growth. The obtained results (Figure 8) showed a considerable inhibition of the growth of *P. vulgaris*, after the adding a volume 1 ml of the supernatant of *Ps. aeruginosa* ATCC 27853 in the culture of *P. vulgaris* during early exponential growth phase. The growth curve of the test strain is plotted as a function of time. In conclusion, the molecules present in the supernatant of *Ps. aeruginosa* ATCC 27853 has

induced unbalanced growth and furthermore the arrest of the cell cycle of *P. mirabilis* ATCC 12453 in comparison with the untreated biomass (balanced growth).

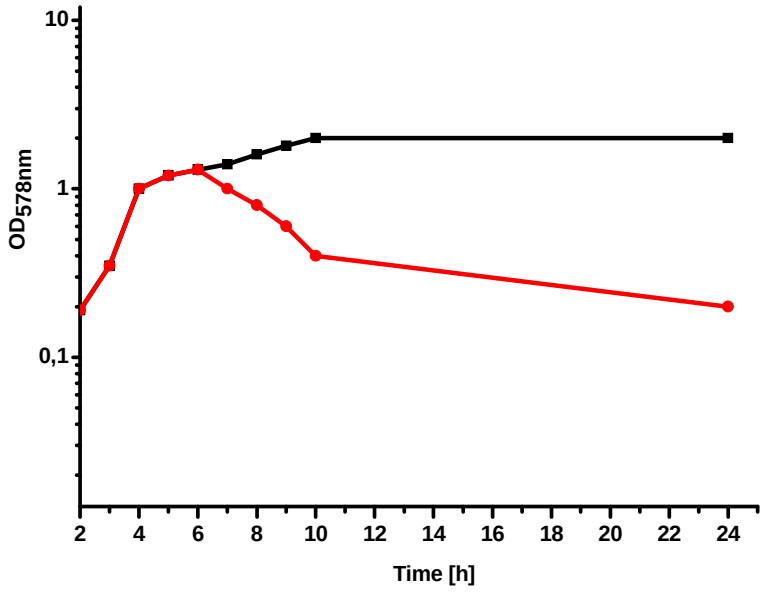

Figure 6: The study of *P. mirabilis* ATCC 12453 growth in the absence and the presence of the supernatant of *Ps. aeruginosa* ATCC 27853

DISCUSSION

Many studies suggested the ability of members belonging to the genus *Pseudomonas* to produce a various secondary metabolites, with antibacterial activity [19].

The tested antagonistic strain *Ps. aeruginosa* ATCC 27853 against *B. subtilis* ATCC 6633, *P. mirabilis* ATCC 12453 and *B. cereus* ATCC 11778 has indicated a considerable inhibition of bacterial growth with the following diameters of zone inhibition (30, 35, 22 mm) respectively. Hays and their co-workers [20] has reported that that *Ps. aeruginosa* manifested a feeble antagonistic activity against gram-negative bacteria. Whereas, Young [21] showed that *Ps. aeruginosa* reduced the growth of *E. coli*.

In order to explore the molecules responsible for antibacterial activity, the selected antagonistic strains *Ps. aeruginosa* ATCC 27853 was inoculated in the culture medium and incubated at temperature of 30°C for 24 hours and the supernatant was harvested by using the centrifugation. Furthermore, the supernatant was investigated for antibacterial activity against *B. subtilis* ATCC 6633, *P. mirabilis* ATCC 12453 and *B. cereus* ATCC 11778. The obtained results indicated that the tested supernatant has considerably induced the inhibition of bacterial growth of *P. mirabilis* ATCC 12453 and *B. subtilis* ATCC 6633 and *B. cereus* ATCC 11778.

The optimization of several parameters implicated in the antagonistic activity has been in details investigated. For this purpose, the selected the antagonistic activity of *Ps. aeruginosa* ATCC 27853 has been incubated at different period time The obtained results indicated that the formed inhibition zone diameter remains constant but decreases its transparency. this slight variation over time may be due to the dynamics of change in the extra cellular concentrations of metabolites produced by *Ps. aeruginosa* strain.

Machan and colleagues [22] has reported that the 18 hours old culture of *Ps.aeruginosa* P 3940 culture filtrate decreased considerably the bacterial biomass of *St. aureus* after 18 h of incubation. However the 4 hours culture filtrate manifested any antagonistic activity against *Staphylococcus aureus*. Whereas, James and Gutterson [23] reported that the constituents of the environment influence widely antibiotic production.

Shanahan and their co-workers [24] has reported that the molecules responsible for antagonistic activity, may be antibiotics such as bacteriocins (including pyocin), antibiotics phenaziniques or non-nitrogenous compounds such as 2.4 diacetyl phloroglucinol.

To order to study the influence of the variation of pH-value on the antagonistic activity, the harvested supernatant of the pre-culture of *Ps. Aeruginosa* was adjusted with solution tampon of phosphate. The obtained results manifested the absence of the zones of inhibition around the impregnated discs with the acid and the neutral supernatant. Whereas, the appearance of a zone of inhibition around the impregnated discs with alkaline supernatant by *B. subtilis* ATCC 6633 and *P. mirabilis* ATCC 12453. Avril and co-workers [25] has reported that the production of the pigments (pyocyanin, pyoverdin) is associated with the alkalization of the culture medium.

To study the nature of the inhibitory agent, the supernatant of pre-culture of strain of *Ps. aeruginosa* ATCC 27853 was submitted at different heat treatments. The different thermal treatments carried out showed that secondary metabolites responsible for antagonist activity are not thermo-resistant. These results do not go with the earlier work carried out by Jayaswal [26], they showed that the agent responsible for the antagonistic activity produced by *Ps. spp*. strains appeared very resistant to heat treatment. Furthermore, the study of the bacterial growth of the *P. mirabilis* ATCC 12453 has been achieved on nutrient broth (BN) in the presence and absence of supernatant of pre-culture of antagonistic strain *Ps. aeruginosa* ATCC 27853. The obtained results indicated that the kinetics of growth of *P. mirabilis* ATCC 12453 has revealed a important sensitivity towards the supernatant of pre-culture of antagonistic strain *Ps. aeruginosa* ATCC 27853. These substances allow inhibition and the potentially pathogenic micro-organisms elimination by deprivation of nutrient resources (carbon substrates, trace element essential) necessary for their development or by production of antibiotic or lytic enzymes [27].

CONCLUSION

From our results we could demonstrate the potential value of antagonistic activity of *Pseudomonas aeruginosa* ATCC 27853 against pathogenic bacteria. It would be of interest to find out which functional group is responsible for the antagonistic activity and also whether any of them is a novel compound with antimicrobial activity which would make it a promising candidate for the production of new antimicrobials.

Further studies involving the purification of the chemical compounds of the secondary metabolite produced by *Pseudomonas aeruginosa* ATCC 27853 by the using a modern technique such HPLC, IRM, will require for determination of this active molecules.

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