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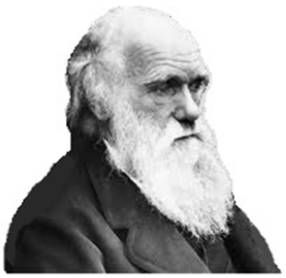

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# Isolation of bacteria from soil sample of Tamil Nadu and their *in vitro* interaction

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**ABSTRACT**

The present study was investigated to determine the competitive interaction between Gram positive (*B. subtilis*) and Gram negative (*P. aeruginosa* and *E. coli*) bacteria *in vitro*. Bacteria of interest were isolated from soil sample and were identified through morphological and biochemical tests. Bacterial cultures were grown alone and as co- culture in test tubes containing sterile broth. Bacterial growth and their growth inhibition in co- culture tubes were analyzed till 96 h through spectrophotometric assay and colony forming unit (CFU) assay. Spectrophotometric analysis and CFU/mL assay showed that the effect of *B. subtilis* on *P. aeruginosa* and *E. coli* in co- culture tubes was growth inhibitory. This is accompanied by the reduction in absorbance value and CFU/mL of the co-culture tubes. The bactericidal activity of *B. subtilis* was measured on *P. aeruginosa* and *E. coli* using agar well diffusion method. *B. subtilis* showed zone of inhibition of 8 mm and 6 mm against *P. aeruginosa* and *E. coli*, respectively. The antagonistic activity test of *B. subtilis* favoured the spectrophotometric and colony forming assay results. These results suggest that *B. subtilis* develop adaptational pathways by extracellular signaling molecules and antibacterial factors in order to compete with bacterial neighbors.

***Keywords*:** Antagonism, *B. subtilis*, CFU, *E. coli*, Interaction, *P. aeruginosa*

## 1. INTRODUCTION

Interaction among microorganisms is the diverse mechanism which is proving to be as important as their habitats. When exposed to adverse environmental conditions, bacteria can

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develop unique strategies for their adaptation. Many bacteria undergo non-growing state because of drastic changes in their natural habitats. In order to compete with other microorganisms or stress response, bacteria develop adaptational changes in their physiology and/or morphology [1]. In general, all bacteria are equipped with a set of survival mechanisms and their corresponding regulatory cascades [2].

Bacteria can secrete bacteriocins when competing with other bacteria for the same resource. The antibacterial compound of specific bacteria inhibits the growth of other microorganisms growing as dual culture for the same resource. *Escherichia coli* is a gram negative pathogenic bacteria and the production of enterotoxins is one of the pivotal characteristics of virulent *E. coli* [3]. *E. coli* is a major pathogen of poultry causing colibacillosis with manifestations such as airsacculitis, pericarditis and septicemia [4]. *E. coli* is one of the causative agents for severe illness in human beings. *Pseudomonas aeruginosa* is an obligate aerobic, saprophytic, and gram negative pathogenic bacteria. Pseudomonades are commonly found in aquatic and soil habitats.

These bacteria display extraordinary versatility for surviving their natural habitats, which involves unusual primary metabolisms such as aromatic hydrocarbon degradation [5], production of antibiotic secondary metabolites [6], and biofilm formation [7]. These features enable the bacterium to survive in the presence of competing organisms in their natural habitats. *Bacillus* genus is a group of gram positive, aerobic, and endospore forming rod-shaped bacteria. *Bacillus* bacteria are among the most widespread microorganisms in nature. One of the most important species of genus *Bacillus* is *Bacillus subtilis* that can survive in extreme conditions because of the production of endospores.

Some strains of *Bacillus* synthesize bacteriocins, which are only effective against bacteria of the same species, others produce antibiotics against gram negative bacteria and still other strains have a wide spectrum of antibiotic activity [8]. The ability of *Bacillus subtilis* to secrete grams per liter of proteins directly into the growth medium has also made them prime producer of heterologous proteins [9, 10]. In the present context, we determined the *in vitro* interaction between the bacteria isolated from soil samples of Tamil Nadu. Growth inhibition of bacteria in co-culture (dual culture) was quantified by spectrophotometric and colony forming unit assay.

## 2. MATERIALS AND METHODS

### 2. 1. Sample collection

Soil samples were collected from places in and around Chennai district (Tamil Nadu). Soil samples were stored at room temperature for further experimental analysis.

### 2. 2. Isolation of bacteria

Nutrient agar, King's B agar and EMB (Eosin Methylene Blue) agar medium were used for the isolation of *B. subtilis*, *P. aeruginosa*, and *E. coli*, respectively. Serial dilution (upto $10^{-6}$ dilution) of soil samples was performed aseptically. Aliquot of 0.1 ml suspension from $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions was poured on the surface of respective sterile agar plates and were streaked using L-rod. The plates were incubated overnight at 37 °C in inverted position. Isolates were named as 'Isolate 1', 'Isolate 2', and 'Isolate 3'. Isolated bacterial colonies were sub-cultured by quadrant streaking and pure bacterial cultures were preserved for further analysis.

### 2. 3. Morphological and Biochemical tests of isolates

Purified isolates were characterized by biochemical analysis using indole test, methyl red test, voges-proskauer test, citrate utilization test, catalase test, and urease test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining, endospore staining, and motility test were performed under morphological tests.

### 2. 4. Bacterial growth as dual culture

Sixty milliliters of Nutrient broth were prepared and transferred to 6 tubes (each tube contains 10 ml of broth). The tubes containing broth were autoclaved and allowed to cool. Bacterial isolates were inoculated in respective tubes aseptically. Tube 1 was inoculated with *P. aeruginosa*, Tube 2 was inoculated with *B. subtilis*, Tube 3 was inoculated with *P. aeruginosa* + *B. subtilis*, Tube 4 was inoculated with *E. coli*, Tube 5 was inoculated with *B. subtilis* and Tube 6 was inoculated with *E. coli* + *B. subtilis*. Tubes were incubated in rotatory shaker at 37 °C for 96 h.

### 2. 5. Spectrophotometric analysis

The growth of bacteria growing alone and growth inhibition of bacteria in dual culture or co-culture was determined spectrophotometrically by determining absorbance at 420 nm. Absorbance was read every 24 h interval.

### 2. 6. Colony forming unit (CFU) assay

Colony forming unit was determined for quantitative analysis. Luria Bertani (LB) agar plates were prepared for counting the bacterial colonies. One ml of 24 h, 48 h, 72 h, and 96 h grown bacterial cultures from respective tubes was taken and spread on LB plates using L-rod. The plates were incubated overnight at 37 °C. Number of bacterial colonies grown on agar plates was enumerated and CFU was determined after 24 h [11].

### 2. 7. Antagonistic activity of *B. subtilis*

*B. subtilis* grown after 48 h of incubation was centrifuged at 8000 rpm for 10 min at 4ºC. The supernatant was collected and filter sterilized using 0.2 µm syringe filter. The antagonistic activity of *B. subtilis* against *E. coli* and *P. aeruginosa* was determined through agar well diffusion method. The broth culture of each tested bacteria was spread over the Nutrient agar plates using sterile cotton swabs. Wells were created on agar plates with 5 mm cork borer. Hundred microliters of supernatant from *B. subtilis* were poured into the wells. The plates were incubated at 37 ºC for 24 h and the zone of inhibition was measured.

## 3. RESULTS AND DISCUSSION

### 3. 1. Isolation and identification of bacteria

The isolated bacterial strain ('Isolate 1', 'Isolate 2', and 'Isolate 3') was identified as *B. subtilis, P. aeruginosa*, and *E. coli* based on morphological and biochemical characteristic (Table 1). The characteristic morphology (circular, smooth, creamish, and opaque) of colonies of 'Isolate 1' on Nutrient agar indicates the presence of *Bacillus* genus. The green pigmented

colonies of 'Isolate 2' on King's B agar medium indicate the isolation of bacteria that belongs to *Pseudomonas* genus. The 'Isolate 3' was identified as *E. coli* based on the characteristic green metallic sheen colonies on the EMB agar plates.

**Table 1.** Morphological and biochemical test reports of isolates.

| Tests | Isolate 1 | Isolate 2 | Isolate 3 |
|---|---|---|---|
| Gram staining | Gram (+) | Gram (-) | Gram (-) |
| Motility | Positive | Positive | Positive |
| Endospore | Spore | Non-spore | Non-spore |
| Indole | Negative | Negative | Positive |
| Methyl Red | Positive | Negative | Positive |
| Voges-Proskauer | Positive | Negative | Negative |
| Citrate utilization | Positive | Positive | Negative |
| Urease | Negative | Negative | Negative |
| Catalase | Positive | Negative | Positive |

### 3. 2. Spectrophotometric analysis of bacterial growth

Optical density of bacterial culture grown alone and as co-culture was read at 420 nm (Fig. 1 and 2). Optical density of *P. aeruginosa* and *B. subtilis* grown as dual culture (Tube 3) was showing less absorbance compared to bacterial culture growing alone (Tube 1 and 2). The absorbance value of *E. coli* and *B. subtilis* growing as dual culture (Tube 6) was also less compared to the cultures grown alone (Tube 4 and 5).

On the other hand, the growth inhibition in Tube 3 was increased with respect to to Tube 6. It clarifies that the competitive interaction between *B. subtilis* and *P. aeruginosa* for the same resource is more than that of *B. subtilis* and *E. coli*. The growth of bacteria in tubes was increased after 48 h and again it decreased after 72 h.

The growth in dual culture tubes were always found to be less when compared to the cultures grown alone till 96$^{th}$ h. Results indicate that the bacteriocin production from *B. subtilis* was showing more inhibitory effect on *P. aeruginosa* when compared to *E. coli*, which was later confirmed by the antagonistic report. In general, cells in biofilms are surrounded by a self-produced matrix, and their growth is slow because of insufficient nutrients and space [12, 13].

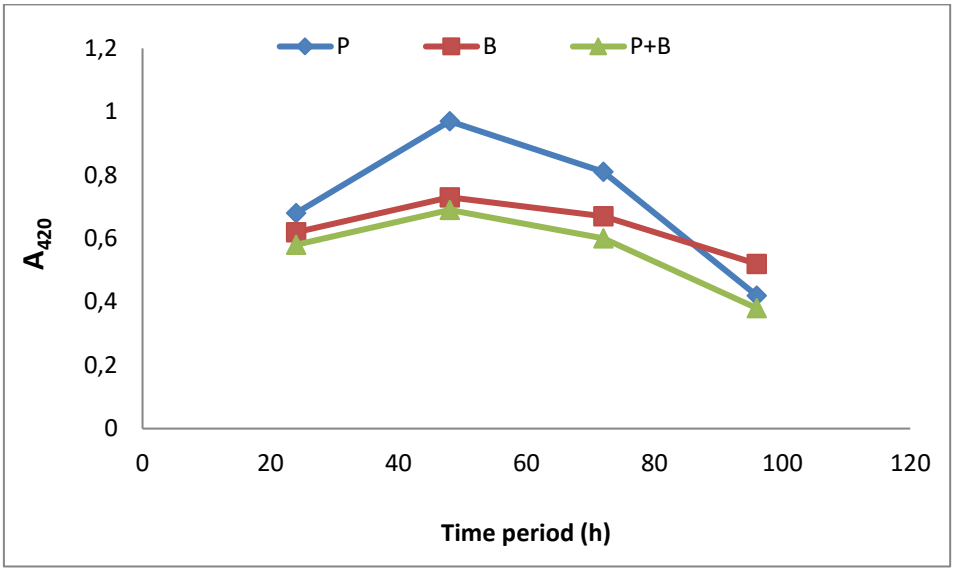


**Fig. 1.** Growth determination of bacteria in Tube 1 (P = *P. aeruginosa*), Tube 2 (B = *B. subtilis*) and Tube 3 (P+B = *P. aeruginosa* + *B. subtilis*).

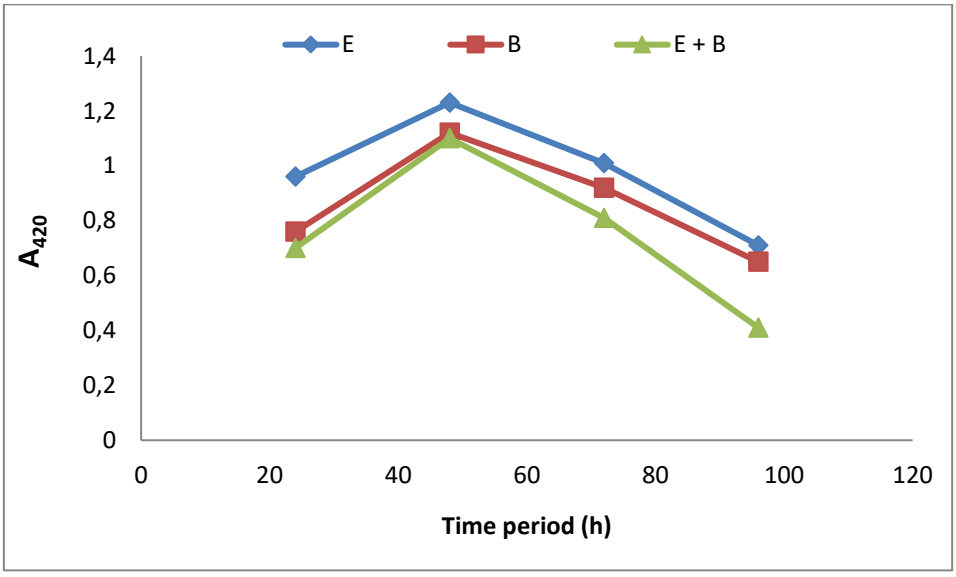


**Fig. 2.** Growth determination of bacteria in Tube 4 (E = *E. coli)*, Tube 5 (B = *B. subtilis*) and Tube 6 (E+B = *E. coli* + *B. subtilis*).

### 3. 3. Colony forming unit assay

Colony forming unit assay was performed in order to check the bactericidal effect of bacteria in dual culture. Both the spectrophotometric and CFU assays provided similar results. After 24 h, growth of bacteria in Tube 3 was inhibited when compared to Tube 1 and 2 (P = $13.7 \times 10^8$ CFU/mL; B = $12.8 \times 10^8$ CFU/mL; P+B =. $9 \times 10^8$ CFU/mL). Growth of bacteria in

Tube 6 was also reduced after 24 h when compared to Tube 4 and 5 (E = 14.5 × $10^8$ CFU/mL; B = 12.5 × $10^8$ CFU/mL; E+B = 11× $10^8$ CFU/mL). After 48 h, CFU/mL was increased as per the spectrophotometric analysis. As the growth of bacteria was inhibited in dual culture so the number of bacterial colonies in dual culture was also reduced at successive time interval. When the growth of the cultures reached at exponential phase then it showed increment in CFU/mL (Fig. 3 and 4).

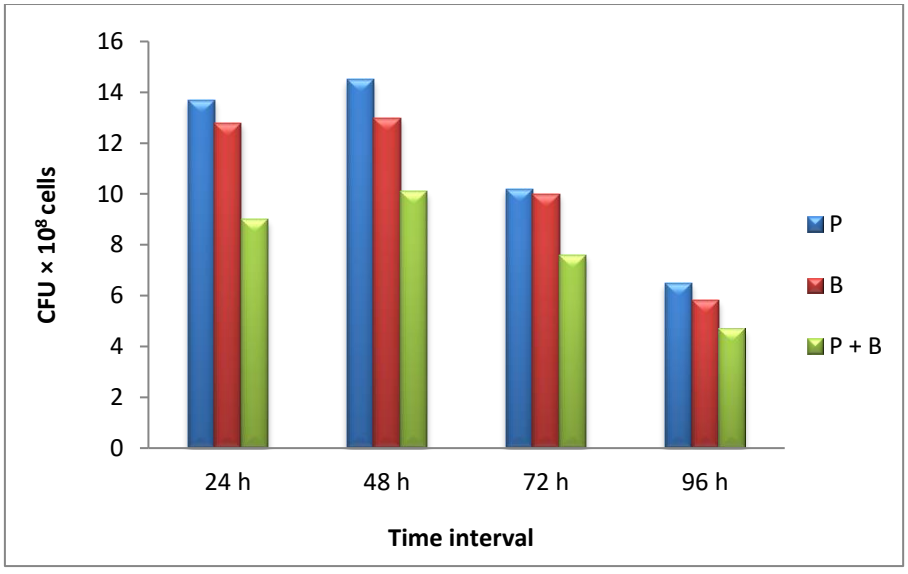


**Fig. 3.** CFU/mL of bacteria in Tube 1 (P = *P. aeruginosa*), Tube 2 (B = *B. subtilis*) and Tube 3 (P+B = *P. aeruginosa* + *B. subtilis*)

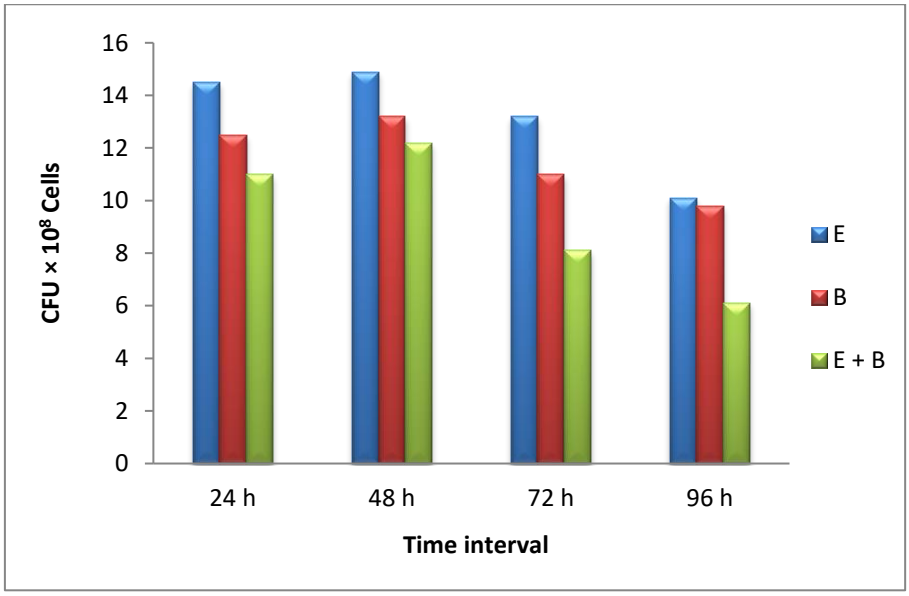


**Fig. 4:** CFU/mL of bacteria in Tube 4 (E = *E. coli*), Tube 5 (B = *B. subtilis*) and Tube 6 (E+B = *E. coli* + *B. subtilis*).

### 3. 4. Antagonistic activity

As the growth of bacteria was inhibited in co-culture tubes, the antagonistic activity of bacteria was performed using agar well diffusion method. Antagonistic activity of *B. subtilis* was determined against *E. coli* and *P. aeruginosa*. *B. subtilis* inhibited the growth of *P. aeruginosa* with maximum zone of inhibition of 8 mm. On the other hand, *E. coli* was found to be less sensitive to the bacteriocin of *B. subtilis* with 6 mm of zone of inhibition. From the spectrophotometric analysis and CFU/mL it was clear that growth inhibition in dual culture of *B. subtilis* and *P. aeruginosa* was more when compared to *B. subtilis* and *E. coli*. The antagonistic results of *B. subtilis* against *P. aeruginosa* and *E. coli* indicate that the bacteriocin produced by *B. subtilis* has potential inhibitory effect against *P. aeruginosa*. There is more competition between *B. subtilis* and *P. aeruginosa* for the limited source. This nutritional stress results in production of antibiotic peptides by *B. subtilis* which showed more inhibitory effect on *P. aeruginosa*. Our results strongly favor the finding of Pinchuk *et al*. [14] who reported that *Bacillus* strains exhibited promising activity against *E. coli*. But in the present investigation, *B. subtilis* showed less antagonistic activity against *E. coli*. The antagonistic nature of the bacteria depends upon the type of strain, source of isolation, and their genetic organization [15]. Bacteria can secrete a wide array of antibacterial compounds such as bacteriocins when competing with other bacteria for the same resources. Bacteriocins can affect bacteria of similar or closely related strains. These secretions have been found to affect sibling cells that belong to the same colony. From the current investigation it is clear that the virulence mechanism of *B. subtilis* is crucial for competition with *P. aeruginosa* and *E. coli*. Bacteriocins production from *B. subtilis* caused reduction in viability of *P. aeruginosa* and *E. coli*. The apparent competitive interrelation between *B. subtilis* and gram negative bacteria may be because of conserved antagonistic mechanism of *Bacillus* sp. [16]. Further computational strategy is essential to understand the mechanism of action of metabolites against indicator bacteria [17].

## 4. CONCLUSION

Our results suggest competitive interaction between *B. subtilis*, *P. aeruginosa*, and *E. coli*. Bacteriocins production from *B. subtilis* has potential growth inhibitory effect on *P. aeruginosa* and *B. subtilis* are capable of producing antibacterial factor against *P. aeruginosa.* Further investigation on the interaction between these bacteria and released extracellular signaling molecules of *B. subtilis* may help to elucidate the molecular mechanism of antagonistic nature.

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