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DISTRIBUTION OF HAEMOLYTIC THIOBACILLI IN SEWAGE SAMPLES

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ABSTRACT

Sulphur is one of the essential plant nutrients and it contributes to yield and quality of crops. *Thiobacillus* play an important role in sulphur oxidation in soil and domestic wastewater.The present study is sought to understand the frequency of *Thiobacillus* in sewage water samples collected from hospital and domestic area.Distribution of sulphur oxidizing bacteria were analysed by using modified Thiobacilli agar. Five isolates were selected and analysed their selected putative virulence properties phenotypically.Also they were showed biocompatible within the group of the *Thiobacilli*.The extracellular products (culture supernatant) of *Thiobacilli* showed a substantial level of haemolytic activity and antagonistic activity. Among the isolates from sewage sources, TB-D1 and TB-D2 produced higher level of bacteriocin against pathogenic bacteria due to the formation of maximum inhibition zone. It reveals that the bacteriocin from *Thiobacillus* showed the elevated antagonistic activity against most of the pathogenic bacteria, as we tested.The strains TB-D4 and TB-D5 showed beta and alpha haemolysis, it might have virulent properties and cause infection in human and animals.The strain TB-D5 showed the maximum inhibitions while compared with others. It is visibly showing presence of virulence factors in the extracellular metabolites of *Thiobacilli.*

Key words: Extracellular products, Biocompatibility, Antagonistic activity, Haemolytic activity

1. INTRODUCTION

Sulphur is now considered the fourth major plant nutrient after N, P and K and is one of the sixteen elements which are essential for the growth and development of plants. The role of chemolithotrophic bacteria of the genus *Thiobacillus* in this process is essential.Organisms belonging to the group of colorlesssulfur bacteria oxidize sulfide to elemental sulfur under oxygen limiting conditions. Based on this feature many researchers worked for biological oxidation using various types of microorganisms (Gadre, 1989). The advantage of this biological sulfide oxidation system is that, no chemicals are required except oxygen (Buisman*et al*., 1990).

*Thiobacilli*can use reduced inorganic sulphur compounds as an energy source (Kelly and Harrison, 1989) and are therefore used for removing sulphide from industrial outlets, domestic outlets and also from wastewater (Kim *et al*., 2002; Kleerebezem and Mendez, 2002 and Martin *et al*., 2002; Cha *et al* 1999) have also studied the removal of organic sulphur compounds.Various workers have proposed the assignment of some species to other genera or the creation ofnew genera for most of the former species of *Thiobacillus* (Katayama *et al*., 1995; Moreira andAmils,1997; Hiraishi*et al*., 1998; Kelly and Wood, 2000).

Considerable interest has been shown in *Tferrooxidans* because of its use in industrial mineral processing and its unusual physiology. The major contribution of *T. ferrooxidans* to metal extraction is its ability to attack sulphide containing minerals and convert the insoluble sulphides of metals such as copper, lead, zinc or nickel to their soluble metal sulphates.An alternative way to replace chemical methods in removing heavy metals is microbial leaching with *Acidithiobacillus*sp. (Tyagi and Couillard, 1989; Tyagi *et al*., 1993; Blasis *et al*., 1993; Couillard and Mercier, 1993; Sreekrishnan *et al*., 1996). This method shows several advantages over chemical methods extraction, lower acids and alkali consumption and minimum reduction in sludge nutrients such as N and P (Tyagi*et al*., 1988; Couillard and Mercier, 1993).

2. MATERIALS AND METHODS

2.1. Collection of samples

Sewage samples were collected from in and around Kongunadu College campus. Sewage discharge received from the hospitals, house, hotels, bakery and college hostel premises. Sterile bottles were taken to collect the samples from the

respective places and transported to laboratory for further analysis.

2.2. Isolation and enumeration of total heterotrophic bacteria

All the sewage samples were subjected to enumerate the bacterial population in respective samples by total plate count method. The dilutions 10^{-4} and 10^{-5} of each sample were selected and 1 mL of suspension was transferred to *Thiobacillus* agar medium and incubated at 37°C for 24 h. After incubation colonies were counted and tabulated. Different colonies were selected and inoculated into *Thiobacillus*agar slants, and maintained it by frequent sub culturing and stored in refrigerator at 4°C.

2.3. Presumptive and Biochemical Identification of Thiobacilli

Identification of *Thiobacillus*was performed based on morphological, cultural and biochemical identification methods. The isolates were selected from *Thiobacillus* agar based on colony morphology with whitish blue colour.The presumptive colonies were subjected to Gram's stain;spore stain; motility test and biochemical test such as catalase test and oxidase test are followed.

2.4. Biocompatibility assay

Symbiotic relationship between the *Thiobacilli* were assessed by inhibiting within groups. *Thiobacillus* agar plates were prepared, the loop full of *Thiobacillus*isolates (n=5) were streaked on the agar plates. The plates were incubated at 37°C for 24 hrs. The growth and inhibitory activity of each organism were noticed.

2.5. Antagonistic activity

Thiobacilli isolates (n=5) were qualitatively tested for the production of antimicrobial compounds like bacteriocin at 37°C for 24hrs. Overnight culture of indicator bacterium *Aeromonashydrophila, Pseudomonas aeruginosa, Acinetobactorbaumanii, Serratiamarcescens* and *Bacillus subtilis)* approximately 5mm×10^{-7}cells were swabbed over nutrient agar plate and 0.5mm×0.5 mm sized wells were made using well cutter and kept it for incubation for 30 min. About 500µL filter sterilized (Sartorius) bacterial supernatant (culture supernatant) loaded on to the wells of plates seeded with indicator bacterium (pathogenic bacterium) obtained from Kongunadu College Culture, Kongunadu Arts and Science College, Coimbatore. After incubation of 24hrs at 37°C the plates were checked for the zone of inhibition. The inhibition as positive if the width of clear zone around the colonies of producer strains is 0.5 mm or larger. Inhibition zone could be around the wells, thus demonstrating bacteriocins-mediated inhibition of the sensitive microorganism.

2.6. Hemolytic activity

Haemolytic activity was determined as a zone of haemolysis around the colonies on blood agar plates containing 5 % (v/v) human blood, after 24 hrs incubation at 37°C (Brenden and Janda, 1987).Blood agar plates were prepared and the loop full of *Thiobacillus*isolates [n=5] were streaked on the blood agar plates and the growth and inhibition zone was noticed in all the plates tested.

2.7. Determination of haemolysin production

Extracellular products: Brain heart infusion broth (BHIB) (15mL) was prepared and loop full of *Thiobacillus*isolates [n=5] were inoculated. The tubes were incubated at 37°Cat 24 hrs. After 24 hrs, broth was taken and centrifuged at 12,000 rpm for 15 min. After centrifugation, collect the supernatant in fresh tubes and ammonium sulphate (5mL) was added to it and kept the content for overnight in cold room. The content was then centrifuged at 15,000rpm for 30 minutes and discards the supernatant immediately. Phosphate buffer saline [1X concentration] (1mL) was addedto the pellet and stored at cold room.

*Haemolysin assay: Thiobacillus*was subjected to haemolytic efficiency by well diffusion method. About 10μl of each extracellular protein was loaded on plates and incubated at 37°C for 24 hrs. After 24 hours incubation the zone was observed.

3. RESULTS AND DISCUSSION

3.1. Total heterotrophic population in sewage samples

Collected samples were serially diluted and the total heterotrophic bacterial population were noticed on the respective dilutions. Sewage samples and Hospital sewage/discharges were labelled as TB-D1 to D5 and TB-H1 to TB-H2 respectively. The population count was maximum (68X10^{-7} cfu mL^{-1}) in sewage samples collected from Saibaba colony, Coimbatore. Hospital samples showed too numerous countable numbered colonies which indicates the existence of bacteria is more in hospital sewage.

Table 1. Estimation of *Thiobacillus*

S.No.	Source	Sample label	*Thiobacillus* Population (cfu/ml)
1.	Kavundamplayam	TB-D1	43 X 10^3
2.	Cheran Nagar	TB-D2	71 X 10^3
3.	G. N. mills	TB-D3	19 X 10^3
4.	Thudialur	TB-D4	26 X 10^3
5.	Saibaba Colony	TB-D5	49 X 10^4

*TB – *Thiobacillus; D* – sewage samples / isolates

3.2. Preliminary and biochemical identification of Thiobacillus

All the five isolates showed Gram negative, motile and endospore production positive by performing Gram's staining; motility assay and Malachite green staining method respectively. The biochemical tests such as catalase, hydrogen suphide production ferrous ion oxidation and nitrate respiration were studied and noticed that results were favour for *Thiobacillus*.

Table 2. Biochemical properties of *Thiobacillus*

Colony on MW-agar plate	Whitish-yellow with sulfur Deposited 1-1.5 mm in diameter
Morphology	Short rod, 0.5 × 1-1.5
Motility	Positive
Gram-staining	Negative
Intracellular sulfur	Negative
Autotrophic growth with	
Hydrogen sulfide	Positive
Elemental sulfur	Positive
Thiosulfate	Positive
Tetrathionate	Positive
Hetrotrophic growth	Negative
Ferrous iron oxidation	Negative
Nitrate respiration	Negative

3.3. Biocompatibility of the thiobacillus

The results of biocompatible assay showed the significant level of symbiotic relationship with in the group of sewage isolates by showing the non-inhibitory growth on *Thiobacillus* agar after 24 and 48 hrs growth. It exposes the biocompatibility of the isolates and provides an evidence for supporting the growth of each other.

3.4. Antagonistic Activity

Influence of environmental factor on the production of extracellular products that could account for the differences found in virulence for trout and mile of the strains studied, a comparative study was made of the enzymatic and toxic activities contained in culture supernatant fluids growth at 28^{o}C and 37^{o}C after incubation for 24 to 48 hrs (Mateos*et al*., 1993).

Bacteriocin as extracellular metabolites, which has a potential inhibitory activity against pathogenic microorganisms. In the present study, we qualitatively measured the efficiency of bacteriolytic activity of sulphur oxidizing bacteria, *Thiobacillus*, which produces bacteriocin, were used to analyse bacteriolytic activity by the method of antagonistic activity with pathogenic microorganisms (*Aeramonashydrophila, Pseudomonas aeruginosa, Acinetobactorbaumanii, Serratiamarcescens*and *Bacillus subtilis*).

Table 3. Antagonistic activity of *Thiobacillus.*

Pathogens	TB-D1	TB-D2	TB-D3	TB-D4	TB-D5
Aeromonas hydrophila	++	+++	+	+	+
Pseudomonas aeruginosa	+++	++	+++	+	++
Acinetobactorbaumanii	+++	+++	+	+	++
Serratiamarcescens	++	+++	+	-	++
Bacillus subtilis	+++	+	++	+	+

*TB – *Thiobacillus; D* – sewage samples / isolates; +++ - High; ++ Medium; + Low

3.5. Haemolytic Activity

*Thiobacillus*is known to produce a variety of virulence factors. Among them, haemolysin is the important one, also considered as the primarytoxin, produced by most of the pathogenic strains of *Thiobacillus*. On blood agar plate, all the *Thiobacillus* isolates were showed growth after 24 hrs incubation. *Thiobacillus* were showed inhibition zone around the colonies due to the RBC's lysis effectively. The strains TB-D4 and TB-D5 were showed beta and alpha haemolysis; it might have virulent properties and cause infection in human and animals.

In a study, Wong *et al*. (1996) reported bacterial haemolysin – positive genotype was virulent in the suckling mouse model assay. They also observed that after 24 hours at 37° C, the production of hemolysin was found high, whereas Wretlind*et al*. (1973) and Riddle *et al*., (1981) observed the haemolytic activity during the exponential growth phase, reaching a maximum before maximal growth, and then falling on prolonged incubation.

Table 4.Haemolytic activity of *Thiobacillus* on blood agar.

Isolates	α	β	γ	Inhibition zone of Extracellular metabolites
TB-D1	-	-	+	1.2 cm
TB-D2	-	-	+	1.1 cm
TB-D3	-	-	+	1.0 cm
TB-D4	-	+	-	1.1 cm
TB-D5	+	-	-	1.4 cm

*TB – *Thiobacillus; D* – sewage samples / isolates

3.6. Hemolytic activity in extracellular protein

Allen and Stevenson (1981) reported that haemolytic activity appeared extracellularly during the early stages of growth, reading a peak just before an increase in the haemolytic activity. The cell free culture supernatant was showed significant level of haemolytic activity on blood agar. The extracts were showed inhibition zone around the well with clear inhibition zone and it was revealed the RBC lytic

exists in culture supernatant. The strain TB-D5 showed the maximum inhibitions while compared with others. It is visibly showing presence of virulence factors in the extracellular metabolites of *Thiobacilli.*

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