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ASSESSMENT OF ANTIBIOTIC SUSCEPTIBILITY AND HEAVY METAL RESISTANCE OF PIGMENTED BACTERIA ISOLATED FROM SOIL

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ABSTRACT

Aim of this study was to isolate and characterize soil pigmented bacteria for their industrial and environmental use. In this investigation, soil pigmented bacteria were subjected to antibiotic susceptibility screening and heavy metal tolerance assays. As a result, it was noted that 85-95% pigmented isolates showed sensitivity to Gentamicin, Trimethoprim and Tetracycline; 40 and 35% isolates were resistant to Penicillin and Ampicillin, respectively. Heavy metal tolerance tests indicated that soil pigmented microflora pose effectual tolerance even at higher concentrations of heavy metals (3mM and 5mM). Strain S13 grew well in 5.0mM concentration of copper sulfate while S17 tolerated all concentrations (0.5mM to 5.0mM) of lead acetate. This strain flourished more as the molar amount of lead acetate increased (up to 5.0mM). Isolate S3 was capable to thrive under 5.0mM concentration of mercuric sulfate.
These findings can be helpful to select those pigmented bacterial strains that are susceptible to antibiotics with the ability of heavy metal tolerance, thus they can be used for large scale production of pigments for food and pharmaceutical applications and detoxification of heavy metals in various ecological niches.

Keywords: Pigmented bacteria, heavy metal, antibiotic susceptibility, resistance, biopigment, detoxification.

INTRODUCTION

Color is very important element of food that is linked with the quality and sensory features of food (Joshi *et al*., 2003). The aim of enrichment of color in food is to make food product appealing and recognizable along with its acceptance by consumers (Samyuktha and Naphade, 2016). World Health Organization (WHO), U.S. Food and Drug Administration (FDA) and the European Food Standard Authority (EFSA) have suggested the secured dosage of synthetic colors in food, drugs and cosmetic products (Clydesdale, 1993; Wissgot and Bortlik, 1996; Wodieka, 1996). Nevertheless the consumption of many artificial colorants has been banned due to their connectivity with hyperallergic reactions, carcinogenic properties and other toxicological issues. Such unpleasant effects of artificial colors have led scientific community to explore natural pigments from biological sources (Reyes *et al*., 1996). Biocolor is any pigment derived from biological origin such as plants, animals or microorganisms. Natural colors possess fascinating characteristics including stability to heat, pH, and light (Joshi *et al*., 2003; Sharma, 2014). Most common biological sources of biocolor range from fruits, vegetables, roots, seeds, leaves, insects to microorganisms (Arulselvi *et al*., 2014). Microbes are most versatile microfactories to obtain broad spectrum of molecules for instance enzymes, antimicrobial agents, vitamins, organic acids, texturizing agents and pigments. Additionally, use of microorganisms for natural color production is beneficial and convenient over plants.

Current researches highlight that microorganism are encouraging source for natural pigments. A large number of work have been documented regarding the extraction and purification of colors from bacteria, fungi, yeast, algae and protozoa for their utilization in industrial and medical sectors (Tuli *et al*., 2014).

Variety of microbial genera has been identified for bicolor production with proposed bioactivities. These include *Monascus* (Hsu *et al*., 2011; Duffose, 2009; Blanc *et al*., 1994; Zheng *et al*., 2010), *Rhodotorula* (Sakaki *et al*., 2000; Ungureanu and Ferdes, 2012), *Phaffia* (Florencino *et al*., 1998; Ramirez *et al*., 2000; Flores- Cotera and Sanchez, 2001), *Bradyrhizobium* (Lorquin *et al*., 1997; Chew *et al*., 1998), *Pseudomonas* (Baron and Rowe, 1981), *Cyanobacteria* (Stevenson *et al*., 2002), *Xanthomonas* (Rajagopal *et al*., 1997), *Bacillus, Yarrowia, Achromobacter* etc. (Joshi *et al*., 2003). A diverse range of biopigments have been isolated from these sources, namely chlorophyll, carotenoids, flavins, anthoquinone, melanins, violancein, monascino (Duffose, 2006). Pigmented microbes are found everywhere in nature. They can be isolated and purified from several ecological slots such as fresh and marine water, soil, plants and animals (Rao *et al*., 2017; Tuli *et al*., 2014). Advances in fermentation procedures have allowed easy production and extraction of pigments. Techniques of solid state fermentation or submerged fermentation can be employed for the production of microbial pigments (Araujo *et al*., 2010; Grossart *et al*., 2009). Beside these, scientists also exploited the effects of various physiochemical factors like temperature, pH, carbon sources, nitrogen sources, minerals, aggitation rate on pigment production (Vasanthabharathi *et al*., 2011). But the

large scale production cost of microbial pigments using synthetic medium is high which can be reduced by finding inexpensive substrates. Cost of production can be lower down through the bioconversion of agroindustrial wastes like sugarcane bagasse, molasses, pine apple wastes, whey, apple pomade, crushed pasta etc. in to industrially important biocolors. Such type of waste usage activities not only reduce the production expenditure but also appear as an efficient waste management approach (Lampila *et al*., 1985).

Natural pigments of microbes have commercial importance. They function as coloring agents in food and cosmetic markets. They are also associated with biopharmacological properties. Microbial biopigments have anticancer, antimicrobials, anti inflammatory (Venil and Lakshmanaperumalsamy, 2009) and antioxidant potential (Duran *et al*., 2012; Lampila *et al*., 1985; Patel *et al*., 2007). Consequently, they are ideal tools in different pharmaceutical formulations. Moreover, these coloring agents have huge demand in textile, paper, printing, plastic and paint industries (Tuli *et al*., 2014).

One of the main health problems around the globe is the emergence of antibiotic resistance in bacteria towards numerous antimicrobial agents. Furthermore, the problem has been more provoked by elevated use and misuse of accessible antibiotics in veterinary medicines, in humans and in agriculture (Ibrahim *et al*., 2010). Therefore, industrially potent bacteria must be sensitive to antibiotics and the interest of applications of pigmented bacteria in medical and industrial processes must be cautiously considered for the presence of resistance to anitmicrobials (Nageswaran *et al*., 2012). With this respect, screening of their antibiotic susceptibility becomes necessary, so that they can be safely used commercially.

The pollution with heavy metals is a significant ecological issue. Environmental biotechnology provides solution by offering various methods of bioremediation. Pigmented bacteria in soil have remarkable ability to detoxify heavy metals, present in soil. Thus pigmented bacteria of soil are also involved in bioremediation process.

The present study emphasizes the isolation of pigmented bacteria from soil and their evaluation of antibiotic sensitivity and heavy metal tolerance.

MATERIALS AND METHODS

Sample collection

Eleven soil samples were collected aseptically from compost and different botanical gardens in Karachi and stored at 4˚C in laboratory.

Isolation and identification of pigment producing bacteria

Pigment producing bacteria were isolated by serially diluting soil samples in sterile saline and least dilutions were plated on nutrient agar plates. The plates were incubated at 37˚C for 24 h. Isolated colonies of pigmented bacteria were purified by quadrant streak plate method and stored as master plates.

For the characterization of isolates, Gram's staining, study of cultural morphology and series of biochemical assays were carried out by using Bergey's Manual of Determinative Bacteriology.

Antibiotic susceptibility analysis

Antibiotic susceptibility test for each pigmented strain was conducted based on Kirby-Bauer disc diffusion method as explained by Bauer *et al.* (1966). Antibiotics used were Penicillin G (10 units), Ampicillin (10mcg), Gentamicin (10mcg), Tetracycline (30mcg), Streptomycin (10mcg) and Sulfamethoxazole/Trimethoprim (5mcg).

The isolated cultures were inoculated in nutrient broth and incubated at 37˚C for 24 h. The inoculated cultures were spread on nutrient agar plates with the help of sterile glass spreader. Antibiotics were then placed on plates. Plates were incubated at 37˚C for 24 h. An interpretation of inhibition zones of the isolates as resistant (R), intermediate (I) and sensitive (S) was made according to the CLSI standards. Data were recorded as duplicates.

Evaluation of heavy metal tolerance

All the isolated pigmented bacteria were screened for their potential to tolerate different heavy metals. For this assay, the salts of heavy metals selected were copper sulfate, lead acetate and mercuric sulfate as these metals are abundant in most of ecological sites. Isolated cultures (1% v/v) were grown in nutrient broth containing different concentrations of heavy metal salts (0.5mM, 1.0mM, 3.0mM and 5.0mM) and incubated at 37˚C for 48 h. The determination of bacterial growth was done by estimating optical density at 600nm. Optical densities were then compared with control tubes without heavy metal salts (Chanda *et al*., 2014). All of the experiments were conducted in duplicates.

RESULTS AND DISCUSSION

Isolation and characterization of soil pigmented bacteria

Total twenty pigmented bacteria were isolated and purified from soil samples. Afterwards, cellular, cultural and biochemical features of all isolated strains were studied. According to Gram's staining, nine cultures were Gram positive while eleven were Gram negative. Most of them were cocci. Isolated bacterial cultures hold wide variety shades of pigments range from orange, yellow, pale yellow to pink (Table 1).

Table 1. Characterization of isolated pigmented bacteria according to Bergey's Manual of Determinative Bacteriology.

Culture Code	Gram's Reaction	Biochemical Characterization						Cultural Characteristics
		Catalase Test	Indole Test	Citrate Test	Methyl Red Test	Vogues-Proskauer Test	Urease Test	(Shape, Margin, Elevation, Size, Texture, Appearance, Opacity, Pigmentation)
S1	Gram +ve, cocci	+	-	+	-	+	-	Circular, entire, convex, small, smooth, shiny, opaque, **orange**
S2	Gram +ve, cocci	+	-	+	-	+	+	Circular, entire, flat, small, smooth, shiny, opaque, pale **yellow**
S3	Gram +ve, cocci	+	+	+	-	-	-	Irregular, undulate, flat, moderate, rough, dull, opaque, **yellow**
S4	Gram +ve, rods	+	+	+	+	-	-	Irregular, undulate, flat, moderate, rough, shiny, opaque, **pink**
S5	Gram –ve, cocci	+	+	+	-	-	+	Spindle, undulate, flat, moderate, smooth, shiny, opaque, **yellow**
S6	Gram –ve, cocci	+	+	+	+	-	+	Spindle, undulate, flat, moderate, rough, dull, opaque, **yellow**
S7	Gram –ve, short rods	+	+	+	-	+	-	Circular , undulate, flat, small, smooth, shiny, opaque, **orange**
S8	Gram –ve, cocci	+	+	+	-	-	+	Irregular , lobate, flat, small, rough, dull, opaque, **yellow**
S9	Gram +ve, cocci (in chain form)	+	-	+	-	+	-	Circular , entire, flat, small, smooth, shiny, opaque, **orange**
S10	Gram –ve, cocci	+	-	-	-	+	-	Irregular , lobate, flat, moderate, rough, dull, opaque, **orange**
S11	Gram –ve, cocci	+	-	+	+	-	+	Irregular , undulate, flat, small, rough, dull, opaque, **orangish yellow**
S12	Gram +ve, cocci	+	-	+	-	+	-	Spindle , undulate, flat, small, rough, dull, opaque, **yellow**
S13	Gram +ve, rods	-	-	+	+	-	+	Irregular , undulate, flat, small, rough, dull, opaque, **white**
S14	Gram –ve, cocci	+	-	+	+	-	-	Spindle, undulate, flat, small, rough, dull, opaque, **yellow**
S15	Gram +ve, cocci	+	+	-	-	+	-	Circular, entire, flat, moderate, smooth, shiny, opaque, **orange**
S16	Gram +ve, cocci	+	+	+	+	-	-	Circular, entire, punctiform, rough, dull, opaque, **yellow**
S17	Gram –ve, rods	-	+	-	-	+	-	Irregular , undulate, flat, moderate, smooth, shiny, opaque, **orange**
S20	Gram –ve, rods	+	+	-	-	-	-	Irregular , undulate, flat, moderate, smooth, shiny, opaque, **orange**
S21	Gram –ve, rods, (cocobacillus)	+	+	+	-	+	-	Irregular , undulate, flat, moderate, rough, dull, opaque, **pink**
S22	Gram –ve, rods	-	+	+	-	-	-	Irregular , undulate, flat, moderate, rough, dull, opaque, **orange**
Note: + Positive, - Negative								

Antibiotic sensitivity of isolates

All the pigmented bacterial isolates were screened for their antibiotic sensitivity against six commonly used antibiotics by Kirby-Bauer disc diffusion protocol (Table 2).

It was observed that all the bacterial strains (85-95%) were highly susceptible to Gentamicin, Sulfamethoxazole/Trimethoprim and Tetracycline. While 25% isolates showed intermediate effects to Streptomycin. High degree of resistance was analyzed for Penicillin (40%) and Ampicillin (35%) as compared to other antibiotics (Fig. 1).

Similar outcomes were recorded by Jafarzade *et al.* (2012) that bacterial isolates from marine environment were extremely resistant to Penicillin and Ampicillin. According to Rashid *et al.* (2014), 73.3% bacterial strains possessed resistance to Ampicillin, 60% were resistant to Penicillin while 53.3% soil microflora tolerated Streptomycin.

In the same way, Nageswaran *et al.* (2012) reported that all pigmented bacteria from soil and water were susceptible to different antibiotics such as Gentamicin, Tetracycline, Erythromycin and Vancomycin.

With respect to the results of current work, pigmented bacterial isolates S1, S2,S3,S4, S5, S15, S17, S20 and S21(Fig. 2) can be recommended for industrial use in biopigment production, as none of the strain showed resistance to any of tested antibiotics. However, advance studies are needed to buildup understanding towards the molecular mechanisms of development of antibiotic resistance of isolated pigmented bacteria. In present era, variety of new bacteria are emerging with resistance to nearly all commonly used antibiotics, which are unfit for commercial consumption (Begum *et al*., 2017).

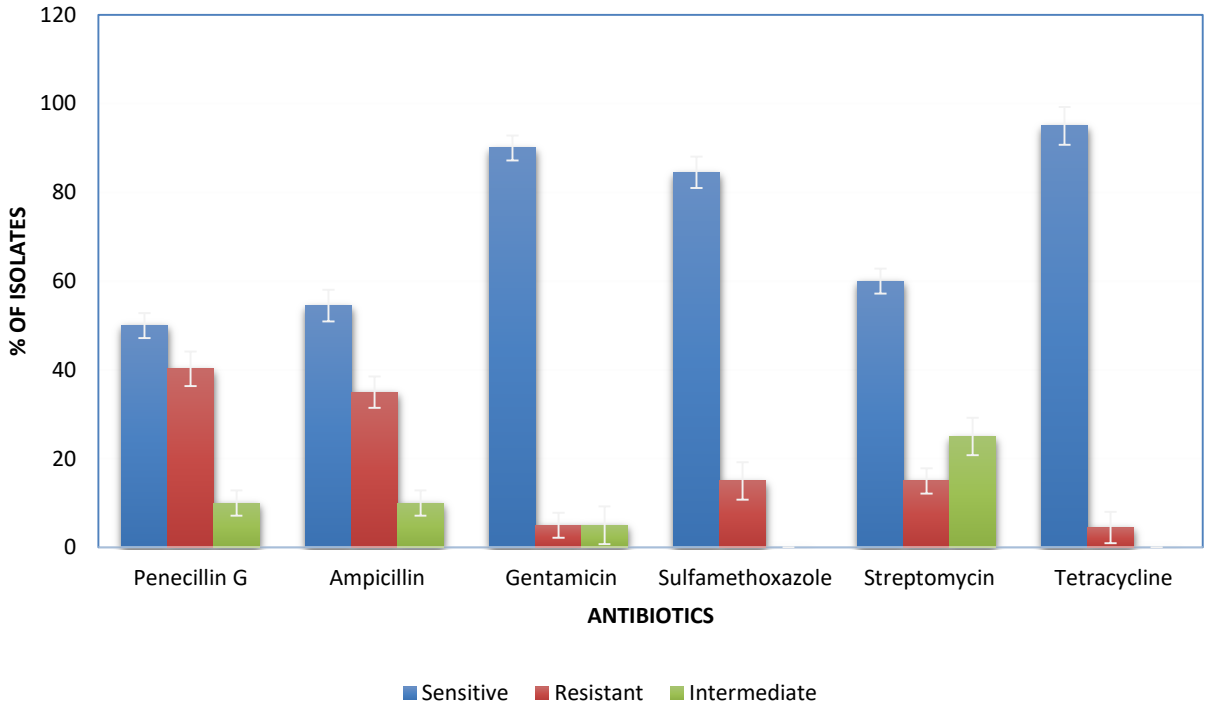

Fig.1. Antibiotic susceptibility of soil pigmented bacteria towards six commonly used antibiotics.

Heavy metal tolerance of isolated pigmented bacterial cultures

Microorganisms may be able to survive under certain concentration of heavy metals in their polluted microenvironment. Such microbial species can be ideal candidate for the bioremediation of heavy metal contaminated environments. Pigmented bacteria of soil have been reported to be involved in heavy metal detoxification. Based on current investigation, it is revealed that pigment producing bacteria isolated from soil presented high metal tolerance even at higher concentration of 3mM and 5mM. Strain S13 showed highest tolerance at 5.0mM concentration to copper sulfate. While strain S2 exhibited least metal tolerance at all concentrations (0.5 to 5.0mM) of copper (Fig. 3).

Table 2. Antibiotic Susceptibility of Pigment Producing Bacteria.

Isolates	**Interpretation of Zones**					
	Penicillin	**Ampicillin**	**Gentamycin**	**Sulphamthoxazole/ Trimethoprim**	**Streptomycin**	**Tetracycline**
S1	S	S	S	S	S	S
S2	I	S	S	S	S	S
S3	S	S	S	S	S	S
S4	S	S	S	S	I	S
S5	S	S	S	S	S	S
S6	R	I	S	S	R	S
S7	S	R	S	S	I	S
S8	R	R	S	S	S	S
S9	S	S	S	R	S	S
S10	R	R	S	S	I	S
S11	R	R	S	R	S	S
S12	R	R	S	S	I	S
S13	R	R	S	S	S	S
S14	R	S	I	R	R	R
S15	S	S	S	S	I	S
S16	S	S	S	S	R	S
S17	I	I	S	S	S	S
S20	S	S	S	S	S	S
S21	S	S	S	S	S	S
S22	R	R	R	S	S	S
Note: S = Sensitive, I = Intermediate, R = Resistant						

All isolated pigmented strains were also tested for their resistant to lead. Strains S3, S7, S10, S11, S15, S16, S17, S21 and S22 displayed remarkable tolerance in all concentrations of lead (up to 5.0mM) and among these S17 gave significant and interesting consequences, the optical density of bacterial growth was increased as the concentration of lead acetate increased (Fig. 4) while growth of isolates S1, S6, S8 and S14 was declined with raised concentration of lead acetate (5.0mM). Nageswaran *et al.* (2012) documented that the most of bacterial isolates from soil and water grew well in the presence of lead, copper, chromium, nickel and cobalt, which supports the findings of present analysis, so it is elucidated that heavy metal tolerant pigmented bacteria can be utilized for bioremediation of soil and water.

For mercury tolerance, pigmented isolate S1 & S3 expressed highest tolerance at 5.0mM concentration of mercuric sulfate, though growth of S6 was crucially inhibited at 5.0mM of mercuric sulfate (Fig. 5).Same work was presumed by Lima e Silva *et al.* (2012). According to them, higher metal tolerance to mercury was detected by bacteria isolated from sewage.

Some pigmented bacterial isolates expressed co resistance to heavy metals and antibiotics. It has been suggested that presence of higher level of metal contamination in soil, makes soil microflora tolerant to these metal contaminants. Although low concentration of some metals like zinc, copper and cobalt etc. are important for microbes, as these metal act as cofactors for microbial enzymes and metalloprotein. Heavy metal tolerance in soil pigmented bacteria may be due to intrinsic or induced mechanisms. One of the mechanism states that these metals impose selective pressure on microbes, thus enhancing their resistivity to metals (Ahmed and Malik, 2014). Heavy metal tolerance of bacteria is associated with antibiotic resistance. It is found that genes of metal and antibiotic resistance are present on same plasmid/ or transposons, hence producing co resistance (Onuoha *et al*., 2016).

However, in current work, co resistance to heavy metals and antibiotics was also found in few isolated strains. Bacterial isolates S10, S11, S13 and S22 showed tolerance to antibiotics and heavy metals, tested in this study. Nakahara *et al.* (1997) proposed that combined expression of antibiotic and heavy metal tolerance do not occur randomly or by chance, but they are due to selection pressure on microbes, exert by metal contaminants present in soil.

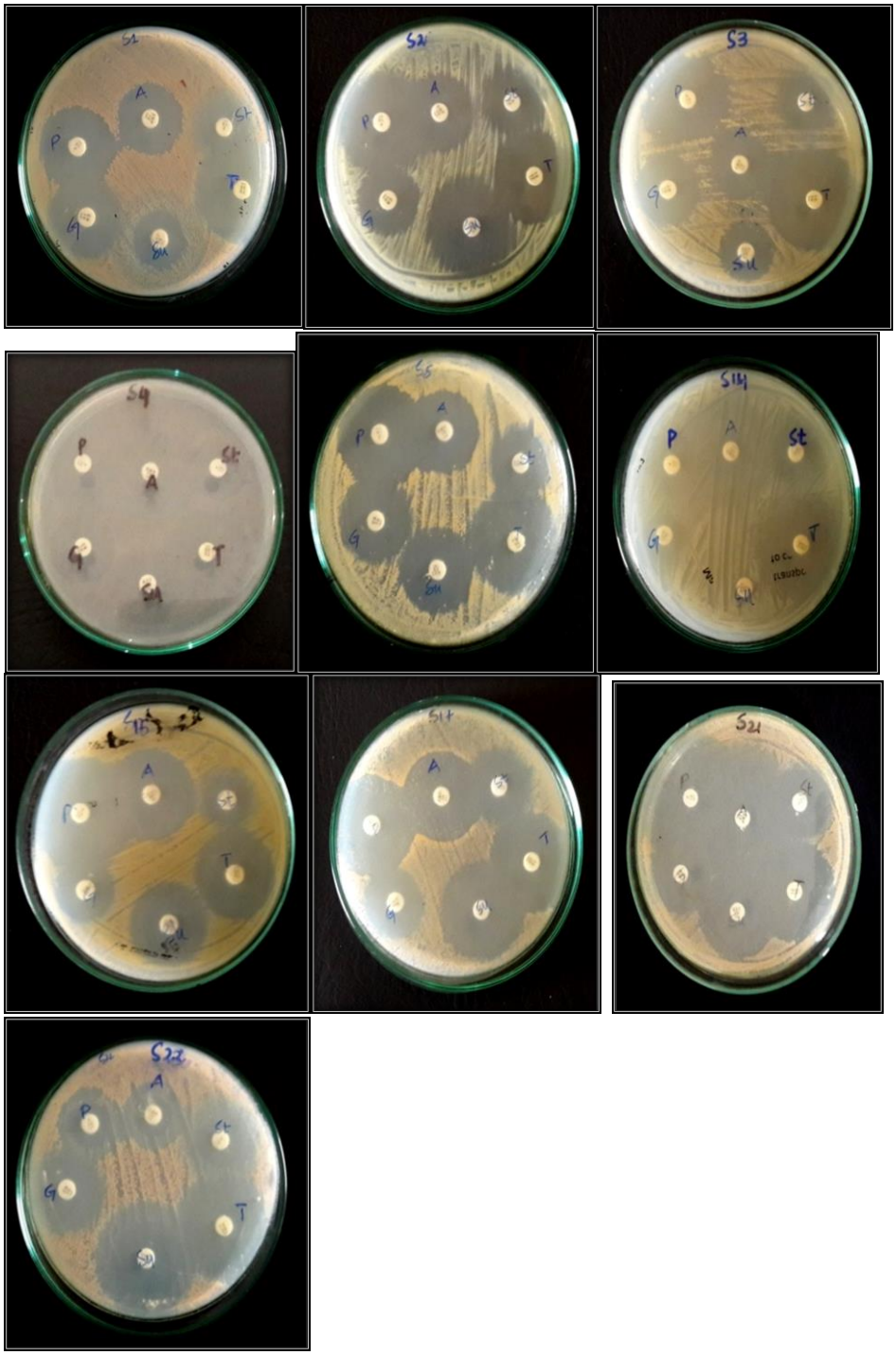

Fig 2. Antibiotics Sensitivity testing: Strain S1, S2, S3, S4, S5, S15, S17, S20 and S21 showed considerable susceptibility to various antibiotics while Strains S14 and S22 showed resistance to antibiotics.

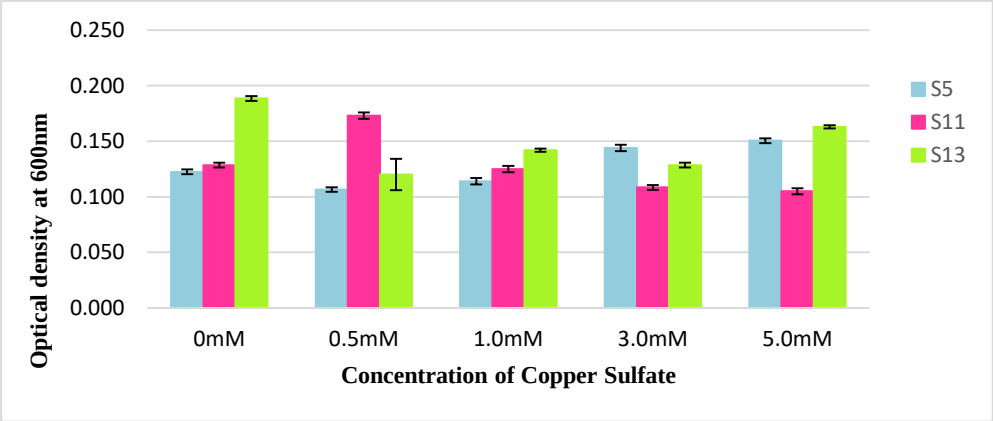

Fig. 3. Tolerance of pigmented isolates S5, S11 and S13 to copper sulfate.

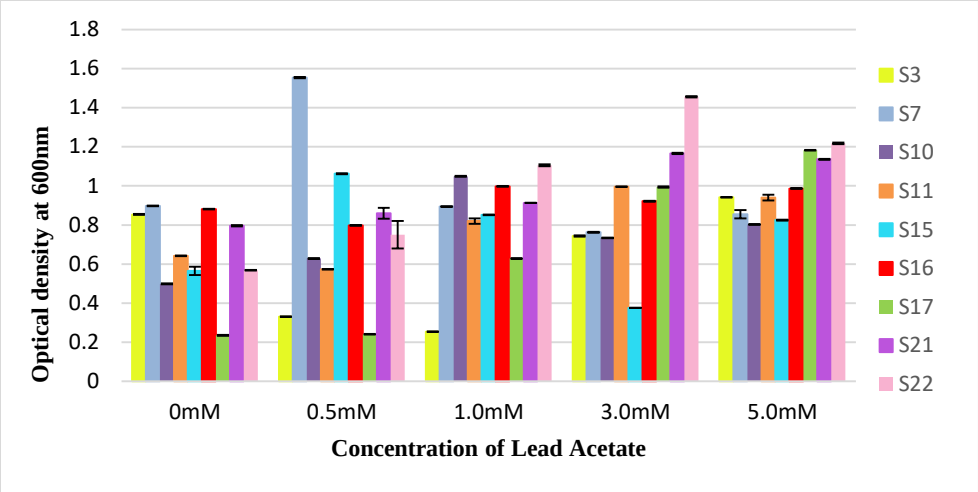

Fig. 4. Tolerance of pigmented isolates S3, S7, S10, S11, S15, S16, S17, S21, and S22 to lead acetate.

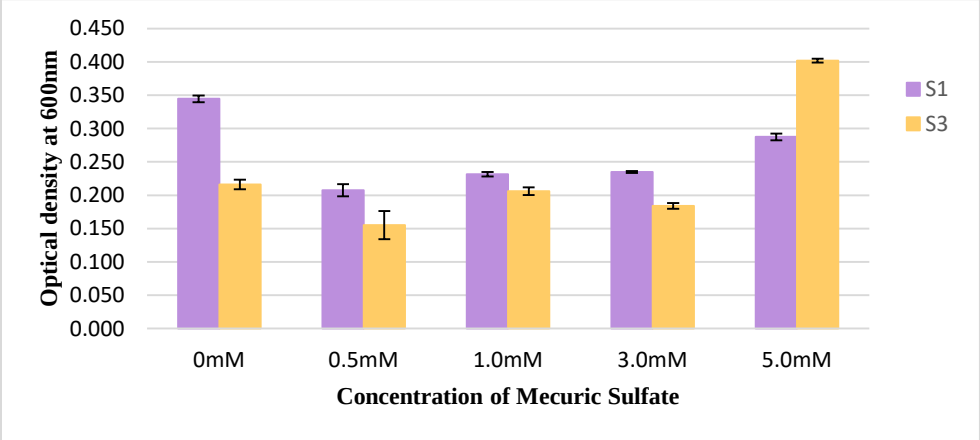

Fig. 5. Tolerance of pigmented isolates S1 and S3 to mercuric sulphate.

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