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CORRELATION STUDIES OF INDIGENOUS BIOFILM FORMING BACTERIA FOR RESISTANCE AGAINST SELECTED METALS, ANTIBIOTICS AND DYES DEGRADATION

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ABSTRACT

This study determines correlation of biofilm-forming bacteria for selected metals, antibiotics and dyes. Total 12 strains having biofilm production potential were targeted in this study. Majority of them were Gram negative excluding MB377 and MB378, and facultative anaerobes. Isolates were mesophilic and exhibited growth capacity over a wide variety of pHs. Almost all isolates conferred resistance capacity against a wide range of multiple antibiotics having 0.0063 MAR for bacitracin, ampicillin, clindamycin, amoxicillin and sulphamethoxazole. Bacterial isolates displayed tolerance to diverse variety of metals i.e. cadmium, lead and iron 1000 mg L^{-1}, copper 600-1000 mg L^{-1}, chromium 700-1000 mg L^{-1} and nickel 200-600 mg L^{-1}. Maximum heavy metal tolerance of 0.033 was observed for all metals excluding nickel. Correlation among metal tolerance and antibiotic resistance was extremely significant for chromium and bacitracin 0.862. Furthermore, moderate relationship (r= 0.682) between copper resistance and Basic Green 4 dye degradation was observed. Correlation coefficient for cadmium and Acid Red 2 dye was recorded as 0.512, while that for bacitracin and Basic Green 4 was noted as 0.665. These correlations proposed that resistance conferring genes might be present on same locus, genetic elements, chromosome or on a plasmid. 16S rRNA sequenced isolates were identified to be *Klebsiella pneumoniae* MB375, *Staphylococcus* sp. MB377, *Klebsiella oxytoca* MB381, *Klebsiella pneumoniae* MB394 and *Klebsiella pneumoniae* MB398. Potential of these biofilm forming strains to thrive under stressed environmental conditions proves them good entrants for being used in bioremoval of polluted sites after further studies on their metabolic pathways.

Keywords: Biofilms; metal/antibiotic resistance; dye decolorization; correlation coefficient; phylogenetic identification

INTRODUCTION

Microbial communities have the capability to thrive in all the ecological niches. Among them bacterial species exist in various habitats including soil, water and other living organisms. Bacteria depend on other microbes for their nourishment and existence. Therefore, bacterial communities establish an extracellular polymeric matrix (EPS) that acts as a micro-ecosystem in which different bacteria communicate and interact with each other forming a biofilm. Bacterial biofilm is a complex and highly organized structure consisting of microbial communities living in a self-produced matrix or slime adhered to a biotic or abiotic substratum **(Asri *et al.*, 2018; Johanna and Priyanka, 2014; Turki *et al.*, 2017; Irankhah *et al.* 2019).** The bacterial species in slime contain structural compounds including proteins, lipids, hydrated biopolymers, carbohydrates and DNA in huge amounts. These microbial communities are diverse in terms of genetic phylogeny **(Gbejuade *et al.*, 2015; Mohammed *et al.*, 2013; Sehar and Naz, 2016; Irankhah *et al.*, 2019)**. The bacterial cells have the ability to adhere to various exteriors e.g. solid substrates where there is availability of liquid and nutrients **(Garrett *et al.*, 2008; Sehar and Naz, 2016)**. Different substrates including various plant cells, natural fresh or aquatic organizations, tooth coating and living tissues/surfaces are analyzed for their biofilm formation. Moreover, bacterial biofilms are also reported on hospital implanted health devices, piping system of water and different surfaces of ships/hulls **(Khatoon *et al.*, 2018; Chan *et al.*, 2019; Oliveira *et al.*, 2019)**.

Bacterial biofilm formation is a survival mechanism for the bacterial cells; it offers protection against various factors including different toxins and antibiotics. The extracellular matrix helps in the maintenance and regulation of various enzymatic reactions and gene expression. Furthermore, it also offers the residing bacteria to adapt to harsh environmental conditions including lack of desired nutrients, temperature and pH fluctuations etc. Biofilm formation is linked to especially horizontal gene transfer as rate of conjugation increases during this process **(Fröls *et al.*, 2012; Irankhah *et al.*, 2019; Nisha and Thangavel, 2014; Sehar and Naz, 2016)**. Now-a-days bacterial biofilms gained a lot of attention because these microbes played a dynamic part in waste water treatment and remediation. These bacteria also degrade environmental pollutants and several organic compounds including petroleum oil, hydrocarbons as well as sewage water by forming Bio-Barriers (BBs) as reported by **Irankhah *et al.* (2019), Nancharaiah and Venugopalan (2019), Sehar and Naz (2016),** and **Sujana *et al.* (2013)**. Since these microorganisms are equipped with natural diversification in terms of adaptation to extreme/stressed conditions within the biofilm mode, thus, such microbial communities play an important role in degradation and detoxification of hazardous compounds. Moreover, microbial species possess the natural tendency to propagate (i.e. abundantly available in nature and renewable resources). Due to inexpensive propagation with little nutrients input and abundant supply, these microbial species have gained more attention in the treatment of wastes and/or detoxification of contaminants, especially in developing countries where pollution producers are unable to afford the expensive high performance treatments (comprising of chemical and physical ones). With these facts in mind, current research was planned for phenotypic and biochemical identification of indigenous biofilm forming bacteria obtained from different ecological sources, their aptitude towards metal and antibiotic tolerance/resistance, potential to metabolize the provided dyes (sole carbon source) and identify correlation among dyes, antibiotics and metals.

MATERIALS AND METHODS

Site selection, sample collection, isolation and purification of bacteria from biofilm samples

Different samples in the form of biofilms (i.e. slimy layers) to obtain biofilm forming bacteria were collected from multiple sites of Pakistan [including Flour Mill (Hassan Ibdaal), Ayub Park Lake (Rawalpindi), Plywood Industry (Golrah Mor), Tops Food Industry (Hattar) and Nullah at the back side of EPA (Environmental Protection Agency) dumping site for waste from flour mills, marble factories and laboratory waste (Rawalpindi)]. These layers were scrapped from the particular sites by disinfected spatula, placed in disinfected plastic bags and taken to laboratory for bacterial isolation. Slimy layers were resuspended in autoclaved distilled water by vortexing, and then spread (via spread plate method) on to the nutrient agar plates maintaining the temperature at 37 °C for 24 and 48 hours. Colonies with distinct phenotypic features were then selected and further purified by streaking single colony.

Screening of isolates for biofilm formation

Congo red agar (CRA) method

Screening CRA method was used for evaluating the capability of bacteria to form biofilm, and appearance of black bacterial colonies on plate after incubation (24-48 hrs) at 37°C showed the production of slime **(Freeman *et al.*, 1989)**.

Ring test/Tube test assay

This screening system was carried out to evaluate the amount of slime produced in broth [tryptic soy broth (TSB)] containing carbon source as 1% glucose. In autoclaved medium, 0.04% Congo red dye was added along with inoculum of overnight grown bacterial culture. Incubation was performed at temperature 37°C (100 rpm). The appearance of black color with the formation of rings was observed after 24 and 48 hours **(Atshan and Shamsudin, 2011)**.

Microtiter plate assay

The assay was used to assess the extent of biofilm formation by the bacterial isolates. It was performed via methodology of ***Cerca et al.* (2007)** and **Stepanovic *et al.* (2007)** using few amendments. Firstly, a sterile loop was used to inoculate the bacterial culture in a separate glass tube which contained growth medium (nutrient broth) and incubation for 24 hours was performed at 37°C to get primary/mother culture. Optical density was adjusted to 0.2 OD at 600 nm in order to get appropriate colony forming units i.e. 10^5-10^8 CFU.ml^{-1}. In this assay, 96 well plates were used to evaluate biofilm production. For this purpose, bacterial cultures (20 µl) were vortexed and added into 80 µl of autoclaved nutrient broth. The broth (devoid of bacteria) was represented as a control/blank in the assay. Cultures were incubated for 24 and 48 hrs at 37 °C and then the bacterial suspension was discarded from respective wells by moderate tapping. Washing was done to remove non-adherent bacterial cells from microtiter plates by using 200 µl volumes of saline solution (0.85% NaCl). After washing, the plates were dried for about 10 minutes and remaining bacterial cells in the microtiter plates were fixed with fixative solution [methanol (200 µl)] for 15-20 minutes. In the last step, attached bacterial cells were stained using 200 µl of 0.1% aqueous crystal violet (CV) stain for 10 minutes. To remove unnecessary CV stain, each well was washed three times with saline solution and 200 µl glacial acetic acid (35%) was dispensed in individual well for quantifying bound bacterial cells using the microtiter plate reader system (Microplate Reader BioRad$_{680XR}$) at 578 nm. All experimentations were done twice.

Phenotypic and biochemical characterization

Isolates were studied for distinct phenotypic and biochemical characteristics through standard procedures (Gerhardt *et al.*, 1994). Effects of different pHs and temperature ranges on bacterial growth was analyzed using UV-VIS spectroscopy. Overnight grown bacterial inoculum was dispensed in test tubes containing nutrient broth and incubated at a range of temperatures (30, 37 and 45°C). Similar experiments were performed with pH (5, 6, 7, 8, 9, 10 and 11) at 37°C under shaking conditions. After 24hrs, UV/VIS spectrophotometer at OD_{600} was used to observe the effects on physiological activity of isolates.

Bacterial Resistance to various metal salts

Resistance ability of biofilm forming bacteria against heavy metals was analyzed via variable amounts (50-1000 mg.L^{-1}) of different metallic salts including cadmium ($CdSO_4$), copper ($CuSO_4$), chromium (K_2CrO_4), lead [$Pb(NO_3)_2$], iron [$Fe_2(SO_4)_3$] and nickel ($NiCl_2$) using minimal M9 medium (Tahir and Yasmin, 2019). In the autoclaved medium, above mentioned concentrations of metal salts were added and growth patterns of isolates were observed following incubation (at 37 ˚C, 24 hrs).

Bacterial Antibiotic resistance profile

Bacterial isolates were also assessed for resistance potential against different antibiotics including Imepenem (IMP10), Ampicillin (AMP10), Amoxicillin (AML10), Streptomycin (S10), Erythromycin (E15), Gentamicin (CN10), Rifampicin (RD5), Bacitracin (B10), Sulphamethaxozole (SXT25), Neomycin (N30), Clindamycin (DA2), Tetracycline (TE30), Nalidixic acid (NA30), Kanamycin (K30), Chloramphenicol (C30) and Ofloxacin (OFX5). This resistance profiling was performed via disc diffusion method.

Evaluation on the basis of HMT and MAR index

According to source of isolation, bacteria were grouped into five categories for the analysis of resistance and tolerance against various metal salts and antibiotics. Following formula was employed for calculation of HMT (heavy metal tolerance) index of bacterial cells against each metal salt.

$$\text{Heavy metal tolerance (HMT) index for a metal} = \frac{\text{No. of metal tolerant bacteria}}{\text{No. of metals tested X No. of isolates}}$$

Whereas, MAR (multiple antibiotics resistance) index of bacterial groups against various antibiotics was quantified using formula

$$\text{Multiple antibiotics resistance (MAR) index for an antibiotic} = \frac{\text{No. of antibiotic resistant bacteria}}{\text{No. of antibiotics tested X No. of isolates}}$$

(Kawane, 2012)

Correlation among metals tolerance and antibiotics resistance

The correlation between metals and different antibiotics was determined through Pearson's correlation coefficient using SPSS statistics software (version 24). Perfect positive correlation was interpreted as +1, strong positive +0.70, moderate positive +0.50, weak positive +0.30, no linear correlation 0, weak negative correlation -0.30, moderate negative -0.50, strong negative -0.70 and perfectly negative was inferred as -1.

Assessment of similarity between isolates

Similarities between the isolates on basis of staining, physiological and biochemical features, motility patterns, metal and antibiotic resistances were assessed via Jaccard coefficient and un-weighted average clustering (UPGMA) algorithm. Positive results and negative results were assigned binary codes as 1 and 0. Paleontological statistics software (PAST$_{3.08}$, **Hammer *et al.*, 2001**) was employed for estimating similarities dendogram.

Evaluation of color removal proficiency of isolates for dyes

Different dyes including C.I. Acid Red 2, C.I. Solvent Yellow 2, C.I. Acid Orange 52, C.I. Mordant Black 11, C.I. Basic Green 4, C.I. Basic violet 3 and C.I. Direct Red 28 were used to assess decolorization potential in the current study by employing streak plate procedure **(Saranraj and Sivasakthivelan, 2014)**. M9 medium **(Tahir and Yasmin, 2019)** augmented with variable concentrations (50-1000 mg.L^{-1}) of dye stocks was used without any carbon source (glucose) for detection of dye decolorization activity of bacteria. Clear zones around biofilm forming bacterial colonies showed the decolorization ability of bacteria. This method was performed three times.

Association of antibiotics resistance with dye degradation capability

The association of antibiotics resistance with dye degradation ability was evaluated by Pearson's correlation coefficient method through SPSS statistics software (24 version). The results for correlation were analyzed as described above.

Correlation among metal tolerance and dye degradation capability

For evaluating the association between metal tolerance and ability of bacteria to degrade dyes, Pearson's correlation coefficient method (SPSS statistics software24 version) was used. Results were inferred as described above.

Molecular characterization of selected bacterial isolates

Pure bacterial cultures for further identification at molecular level were submitted to Macrogen (UK). 16S rRNA gene sequences obtained from Macrogen was submitted to NCBI database in GenBank. Accessions numbers of these sequences

were given by GenBank and investigated using BLAST search tool and MEGA6 software **(Tamura *et al.*, 2013)**.

RESULTS AND DISCUSSION

In the current study, bacterial isolates were evaluated for their biofilm formation capability isolated from various polluted sites. These microbes were collected on the basis of phenotypic variations among them and then purified as a single colony by using nutrient agar medium.

Screening for biofilm formation

Different screening methods like Congo red agar method, ring test and microtiter plate assay were used to assess the biofilm forming capacity of isolates.

CRA assay

Around 75%, 9/12 of isolates showed black colonies on plate while two bacterial isolates including MB386 and MB387 showed colonies in reddish black color.

Ring test/tube test assay

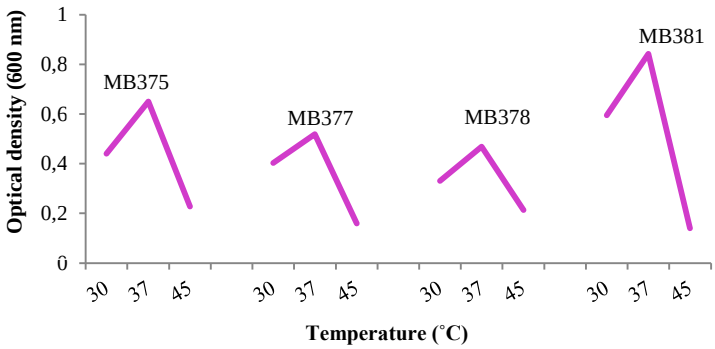
All of the bacterial isolates produced black colored pigmentation in addition to formation of rings around the walls of flasks, thus conferred positive potential for biofilm formation, expect MB386 and MB387 as they appeared non-biofilm formers.

Microtiter plate assay

The bacterial isolates displayed variations in biofilm forming ability over a period of 24-48 hrs. Despite of this variability all isolates showed an escalation towards biofilm formation potential with lapse of time (Figure S1 Supplementary Data). MB394, MB396 and MB398 displayed highest extent of biofilm formation with OD values 1.241, 2.035 and 1.094, respectively, indicating their ability to survive by forming biofilms in diverse environments. Pui et al. (2017) reported *Leptospira* biofilm producers P38 obtained from rats, soil and water samples displaying biofilm formation at 16.700±0.265 OD_{600} on 8th day, P18 with OD value 21.760±0.332 on 7th day and P22 with OD 19.793±0.144 on 7th day of incubation.

Phenotypic and biochemical characterization of bacteria

Except MB377 and MB378 all other isolates were gram negative in nature. Capsule was present in 75% of isolates and only two isolates (MB386 & MB387) formed spores. Bacterial isolates also displayed the capability of producing enzymes urease (6%), gelatinase (12%), cytochrome oxidases (42%), dihydroxylases (100%), catalases (100%), and decarboxylases (50%). The isolates targeted in this study were all facultative anaerobes. At 37 ˚C, all strains showed an increase in growth pattern indicating mesophilic nature. But isolate MB386 also exhibited good growth pattern at 45 ˚C (Figure 1). **Habibi *et al.* (2011)** and **Liaqat and Sabri (2010)** documented almost similar findings about the role of optimum temperature on growth of bacteria. Though variable range of pHs favored the growth of bacterial isolates, but it declined at extreme pHs (pH 5 and 11). Around seven isolates (MB375, MB378, MB381, MB387, MB394, MB396 and MB398) were acidophilic in nature as they displayed optimal growth towards acidic pH (5 and 6), along with good potential to grow at alkaline pH up to 10. At pH 7 and 8, MB391 and MB392 revealed maximum growth, while MB377 showed optimal growth towards neutral pH (neutrophile), MB393 grew at pH 8, and MB386 towards pH 9, thus categorized as moderate alkaliphiles (Figure 2).



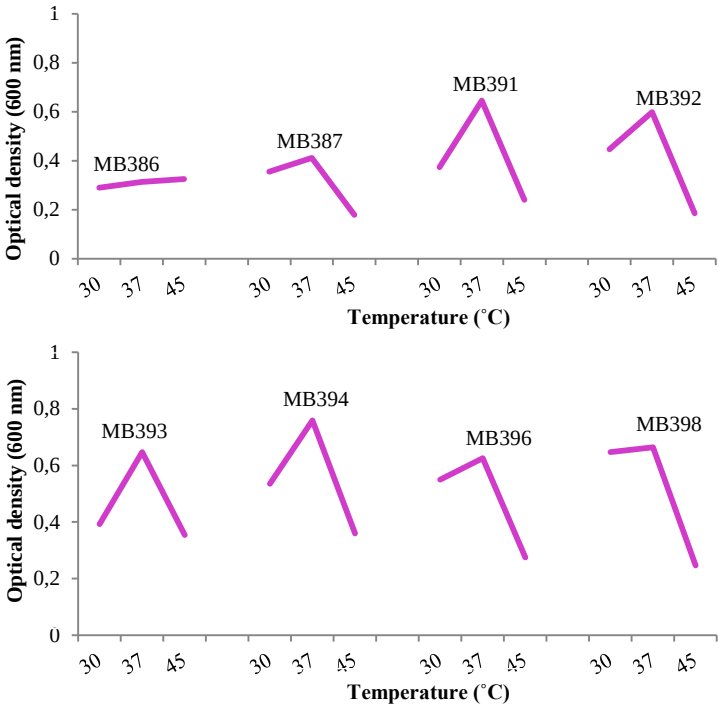


Figure 1 Growth patterns of bacteria at different temperatures

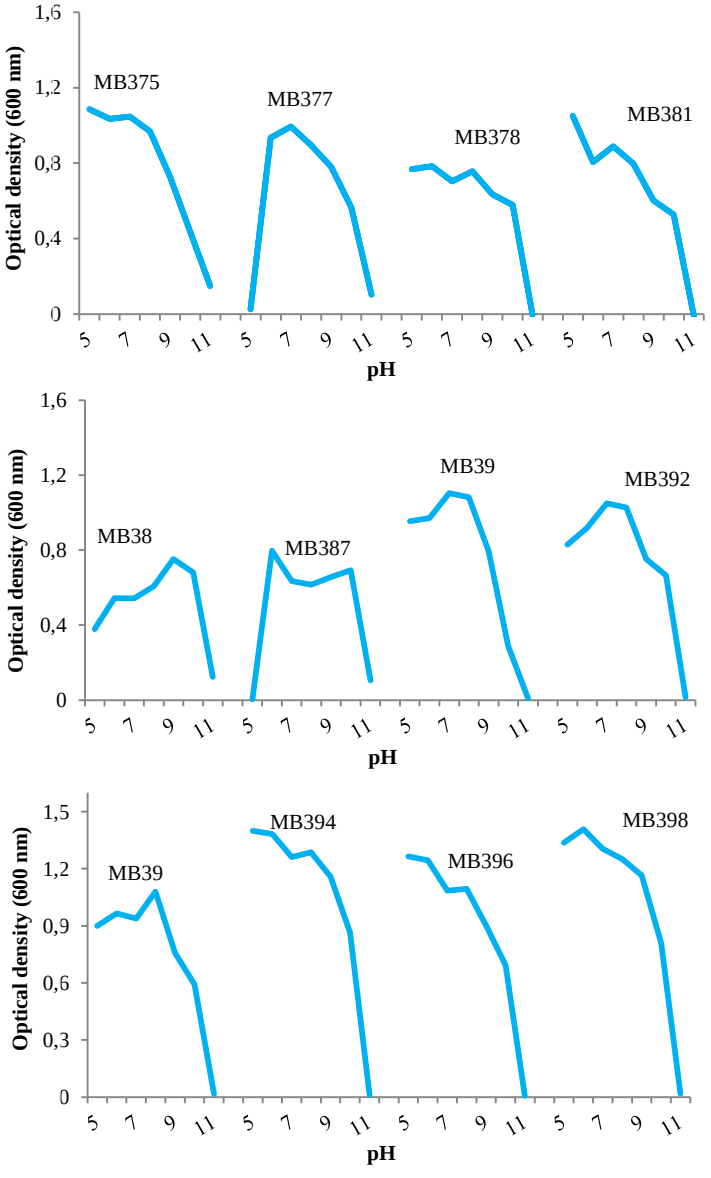


Figure 2 Growth patterns of bacteria at different pHs

Metal resistance

A large proportion of bacteria (MB377, MB378, MB386, MB387 and MB392) possessed the ability to grow and resist o chromium metal (1000 mg.L^{-1}). MB381 exhibited strong growth up to 600 mg.L^{-1}, however very weak growth patterns were observed at 700 mg.L^{-1} of chromium. Isolates MB391 and MB396 exhibited almost the same pattern for chromium metal at 800 and 900 mg.L^{-1}. MB381 exhibited a color change of growth medium to pale yellow. All isolates exhibited good resistance capability in presence of iron up to 1000 mg.L^{-1}. Likewise, all presented good resistance potential against lead metal up to 1000 mg.L^{-1} (Table 1).

All isolates grew optimally in medium containing cadmium (up to 1000 mg.L^{-1}), whereas, MB377 and MB378 displayed good resistance at lower concentrations (200 and 100 mg.L^{-1}). Most of them showed strong resistance potentials towards copper 1000 mg.L^{-1} excluding four isolates (MB377, MB378, MB386 and MB387) they showed fluctuations in their resistance potentials. MB386 and MB387 appeared more sensitive towards higher concentrations of copper (tolerated up to 600 mg.L^{-1}). MB378 displayed strong growth at lower concentration of copper (500 mg.L^{-1}). Variability in growth patterns of bacterial isolates was noted in presence of nickel metal (up to 600 mg.L^{-1}). Four isolates including MB386, MB387, MB394 and MB398 showed good resistance at 400 mg.L^{-1} of nickel, whereas, MB375, MB377, MB378 and MB396 grew strongly at 300 mg.L^{-1}. Only one isolate MB391 didn't show any growth in medium containing nickel. These results indicated competence of biofilm forming bacteria to survive and resist different metals. Bacteria have the ability to tolerate or detoxify metals by different mechanisms including segregation, attachment at protoplast and to different surfaces present on living cell surfaces. It also involves the formation of biofilms and mechanism like bioaccumulation process and precipitation. Bacteria has the ability to synthesize proteins that can trap heavy metals e.g. metallothionein and then convert them into volatile and unstable compounds. Furthermore, the negative charges on bacterial surfaces and presence of different anionic structures lead to the complexation of metals ions (iron, copper, manganese) by providing active sites for metal adsorption **(Selenska-pobell and Merroun, 2010; Tripathi *et al.*, 2011)**.

Bacterial antibiotic resistance profile

Isolates were assessed for their capability to withstand multiple antibiotics. Substantial changes had been detected among these biofilm forming bacteria. MB378 displayed good resistance against a broad range of antibiotics including ampicillin, rifampicin, erythromycin, bacitracin, clindamycin, sulphamethoxazole, and chloramphenicol. Similar resistance patterns were observed in MB398 against 6 antibiotics, whereas, MB392 and MB394 were resistant to 5 antibiotics (Table 1). A large number of isolates were sensitive to antibiotics including streptomycin, imipenem, gentamycin, chloramphenicol, kanamycin, erythromycin, nalidixic acid, neomycin and ofloxacin. *Aeromonas* sp. isolated from drinking water exhibited variable resistance patterns against chloramphenicol, ofloxacin, ampicillin, amoxicillin, sulphamethoxazole, ciprofloxacin, amikacin, tetracycline and ceftazidime whereas it was sensitive towards cefotaxime, ertapenem, imipenem, cefoxitin, meropenem and ceftriaxone **(Ifeanyichukwu *et al.*, 2015)**. *Proteus mirabilis* having biofilm formation potential exhibited tolerance against nystatin, cephotaxine penicillin, whereas, showed sensitivity against amikacin, ciproflaxin, rifampicin and tetracycline **(Rajivgandhi *et al.*, 2014)**.

Index values of MAR and HMT

Higher HMT indices of around 0.333 against all the metals were calculated for Group 1 isolates (MB375, MB377, MB378) and Group 2 isolates (MB394, MB396, MB398) from 2 separate locations. Similarly, highest indices (0.333) for all the tested metals were recorded for MB391, MB392 and MB393, with lowest (0.111) for nickel (Table I supplementary data). These higher indices represented greater exposure of isolates towards tested metals. Maximum MAR value for bacitracin and sulphamethoxazole was observed at 0.063 for MB381, and MB394, MB396 and MB398 (2 groups). Highest MAR values for MB381 were recorded against amoxicillin, sulphamethoxazole, bacitracin and ampicillin in contrast to others. Likewise, MB391, MB392 and MB393 exhibited higher MAR values for bacitracin, amoxicillin and sulphamethoxazole. MB394, MB396 and MB398 displayed 0.063 index against four antibiotics (ampicillin, clindamycin, bacitracin and amoxicillin). Streptomycin, gentamycin, imipenem, neomycin, nalidixic acid, ofloxacin and kanamycin exhibited no imperative differences in MAR indices (Table II supplementary data). Heavy metal and antibiotic indices revealed the capability of isolates to resist antibiotics (one or more) and tolerate various heavy metals; as reported by **Saidi *et al.* (2013)**.

Table 1 Multiple metal and antibiotic resistance profile, and assessment of color removal proficiency of bacteria

Bacterial Isolates	Metal resistance (mg.L^{-1})		Antibiotic resistance (µg)		Dye decolorization (mg.L^{-1})
MB375	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(600)}$	3/16	$Bacitracin_{(10)}$, $Ampicillin_{(10)}$, $Sulphamethoxazole_{(25)}$	7/7	C.I. Acid Red $2_{(100)}$, C.I. Solvent Yellow $2_{(100)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(400)}$, C.I. Basic violet $3_{(100)}$, C.I. Direct Red $28_{(1000)}$
MB377	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(200)}$, $Cu_{(600)}$, $Ni_{(600)}$	1/16	$Tetracycline_{(30)}$	7/7	C.I. Acid Red 2_{100}, C.I. Solvent Yellow 2_{100} , C.I. Acid Orange 52_{1000}, C.I. Mordant Black 11_{1000}, C.I. Basic Green 4_{400}, C.I. Basic violet 3_{100}, C.I. Direct Red 28_{1000}
MB378	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(100)}$, $Cu_{(700)}$, $Ni_{(600)}$	7/16	Erythromycin (15), Ampicillin (10), Bacitracin (10), Rifampicin (50), Chloramphenicol (30), Clindamycin (10), Sulphamethoxazole (25),	7/7	C.I. Acid Red $2_{(100)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(400)}$, C.I. Basic violet $3_{(100)}$, C.I. Direct Red $28_{(1000)}$
MB381	6/6	$Cr_{(700)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(300)}$	4/16	Sulphamethoxazole (25), Ampicillin (10), Bacitracin (10), Amoxycillin (10)	7/7	Acid Red $2_{(100)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(400)}$, C.I. Basic violet $3_{(100)}$, C.I. Direct Red $28_{(1000)}$
MB386	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(700)}$, $Ni_{(600)}$	1/16	Amoxycillin (10)	5/7	C.I. Acid Red $2_{(100)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(0)}$, C.I. Basic violet $3_{(0)}$, C.I. Direct Red $28_{(1000)}$
MB387	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(600)}$, $Ni_{(600)}$	1/16	Ampicillin (10)	5/7	C.I. Acid Red $2_{(50)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(0)}$, C.I. Basic violet $3_{(0)}$, C.I. Direct Red $28_{(1000)}$
MB391	5/6	$Cr_{(800)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(0)}$	3/16	Sulphamethoxazole (25), Bacitracin (10), Amoxycillin (10)	7/7	C.I. Acid Red $2_{(50)}$, C.I. Solvent Yellow $2_{(100)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(1000)}$, C.I. Basic violet $3_{(400)}$, C.I. Direct Red $28_{(1000)}$
MB392	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(400)}$	5/16	Sulphamethoxazole (25), Bacitracin (10), Rifampicin (50),	7/7	C.I. Acid Red $2_{(100)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I.

				Amoxycillin (10), Tetracycline (30)		Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(1000)}$, C.I. Basic violet $3_{(300)}$, C.I. Direct Red $28_{(1000)}$
MB393	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(200)}$	4/16	Sulphamethoxazole (25), Ampicillin (10), Bacitracin (10), Amoxycillin (10)	7/7	C.I. Acid Red $2_{(500)}$, C.I. Solvent Yellow $2_{(100)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(1000)}$, C.I. Basic violet $3_{(300)}$, C.I. Direct Red $28_{(1000)}$
MB394	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(600)}$	5/16	Sulphamethoxazole (25), Ampicillin (10), Clindamycin (10), Bacitracin (10), Amoxycillin (10)	7/7	C.I. Acid Red $2_{(500)}$, C.I. Solvent Yellow $2_{(500)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(300)}$, C.I. Basic violet $3_{(400)}$, C.I. Direct Red $28_{(1000)}$
MB396	6//6	$Cr_{(900)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(500)}$	4/16	Ampicillin (10), Clindamycin (10), Bacitracin (10), Amoxycillin (10)	7/7	C.I. Acid Red $2_{(200)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(1000)}$, C.I. Basic violet $3_{(200)}$, C.I. Direct Red $28_{(1000)}$
MB398	6/6	$Cr_{(1000)}$, $Pb_{(1000)}$, $Fe_{(1000)}$, $Cd_{(1000)}$, $Cu_{(1000)}$, $Ni_{(500)}$	6/16	Erythromycin (15), Ampicillin (10), Clindamycin (10), Bacitracin (10), Rifampicin (50), Amoxycillin (10)	7/7	C.I. Acid Red $2_{(100)}$, C.I. Solvent Yellow $2_{(200)}$, C.I. Acid Orange $52_{(1000)}$, C.I. Mordant Black $11_{(1000)}$, C.I. Basic Green $4_{(1000)}$, C.I. Basic violet $3_{(400)}$, C.I. Direct Red $28_{(1000)}$

Pearson's relation between metal tolerance and multiple antibiotics resistance

According to analysis by Pearson's correlation coefficient, the relation between copper and antibiotic bacitracin tolerance was very significant (r = 0.862). A moderate relation was found amongst copper metal tolerance and resistance against antibiotic amoxicillin (r = 0.668), while, the relationship among copper tolerance and sulphamethoxazole was detected to be moderate (r = 0.521). Similarly, cadmium resistance showed moderate correlation with that of amoxicillin (r = 0.631). The correlation coefficient was lowest at (r = 0.343) in case of nickel metal tolerance with clindamycin resistance. Whereas, in case of chromium metal and rifampicin resistance, the correlation was detected to be minimum (r = 0.302) and the antibiotic chloramphenicol conferred strongly negative correlation with cadmium i.e. r = -0.720. The resistance against other antibiotics was not related to metals tolerance. All these findings proposed that resistance conferring genes for both metals and antibiotics might be present on same locus, genetic elements, chromosome or on a plasmid. Moreover, strongly positive correlations were detected among various metallic salts and targeted antibiotics (Table 2), indicating the probability of stimulation/induction of same resistance mechanisms for different metal salts and antibiotics due to their co-existence within the environmental compartments.

Table 2 Pearson's correlation between metals and antibiotics resistance

	E15	SXT25	AMP10	C30	DA2	B10	RD5	AML10	TE30	Cr	Fe	Pb	Cd	Cu	Ni
E15	1	-.076	.316	.674*	.632*	.258	.775*	-.158	-.200	.234	0	0	-.434	-.089	.217
SXT25	-.076	1	.120	.255	-.120	.683*	.098	.120	-.076	-.265	0	0	.049	.521	-.454
AMP10	.316	.120	1	.213	.500	.408	0	-.125	-.632*	0	0	0	.130	.246	.218
C30	.674*	.255	.213	1	.426	.174	.522	-.426	-.135	.157	0	0	-.720*	-.330	.226
DA2	.632*	-.120	.500	.426	1	.408	.408	.125	-.316	.185	0	0	-.186	.176	.343
B10	.258	.683*	.408	.174	.408	1	.333	.408	-.258	-.302	0	0	.227	.862*	-.432
RD5	.775*	.098	0	.522	.408	.333	1	0	.258	.302	0	0	-.288	.057	.127
AML 10	-.158	.120	-.125	-.426	.125	.408	0	1	-.158	-.369	0	0	.631*	.668*	-.530
TE30	-.200	-.076	-.632*	-.135	-.316	-.258	.258	-.158	1	.234	0	0	-.364	-.222	.099
Cr	.234	-.265	0	.157	.185	-.302	.302	-.369	.234	1	0	0	-.233	-.364	.621*
Fe	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pb	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cd	-.434	.049	.130	-.720*	-.186	.227	-.288	.631*	-.364	-.233	0	0	1	.614*	-.334
Cu	-.089	.521	.246	-.330	.176	.862*	.057	.668*	-.222	-.364	0	0	.614*	1	-.522
Ni	.217	-.454	.218	.226	.343	-.432	.127	-.530	.099	.621*	0	0	-.334	-.522	1

Legends: Correlation between metals tolerance, Correlation between antibiotics resistance, Positive association between metals and antibiotics resistance, Negative association between metals and antibiotics resistance, * = Significant correlation, + = Positive correlation, - = Negative correlation

Similarity assessment among isolates

Bacterial isolates were grouped into three clusters based on phenotypic, functional (physiological) and biochemical features (Figure 3). First group (Cluster A) contained isolates which had the ability to grow in acidic environments. Bacterial isolates in cluster A varied from second group (Cluster B) by the presence of capsule structure and formation of resistant spores, diverse patterns in motility, enzymatic activities (decarboxylase, deaminase and gelatinase), production of chemicals (indole, acetoin), utilization of citrate molecules, capability to ferment various sugar derivatives e.g. melibiose rhamnose, amygdalin and sorbitol, and growth on brilliant green bile and MacConkey's agar media, and resistance potential against various antibiotics (clindamycin, erythromycin, bacitracin, ampicillin, sulphamethoxazole, rifampicin, amoxicillin, and chloramphenicol). The third group (Cluster C) was further divided into two sub-groups, C1 comprised of neutrophilic isolates while C2 involved acidophilic bacterial isolates.

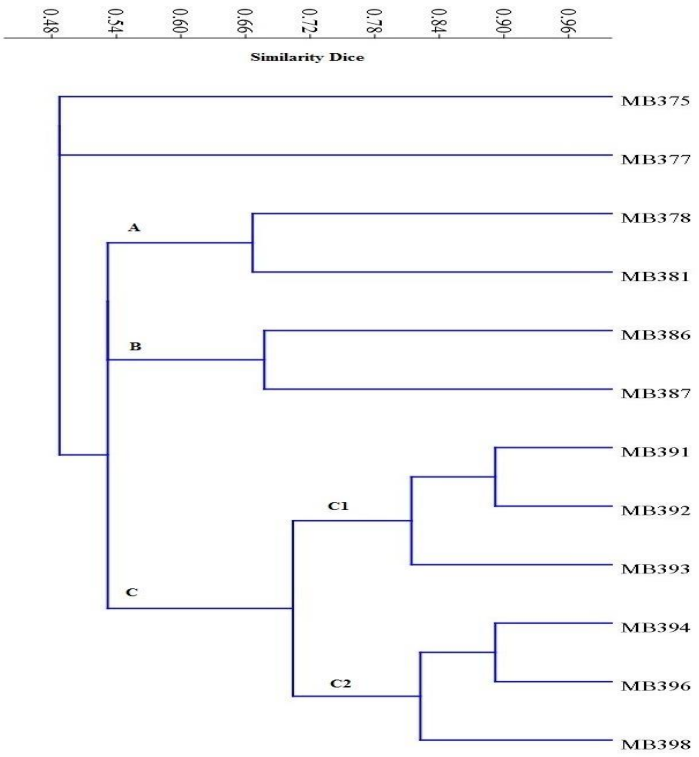


Figure 3 Similarity indices of biofilm forming bacteria via clustering through Jaccard's similarity coefficient

Evaluation of decolorization potential by biofilm forming bacteria

To evaluate the bacterial aptitude towards various dyes decolorization, C.I. Acid Red 2 dye concentration was slowly increased from 25 mg.L^{-1} to 500 mg.L^{-1}. Information regarding isolates' growth and percentage of decolorization capacity was mentioned in Table 1. Only one isolate MB394 had the ability to decolorize C.I. Solvent Yellow 2 dye up to 500 mg.L^{-1} concentration whereas all other isolates presented decolorization potential around 200 mg.L^{-1}. MB381 decolorized the dye supplemented in solid medium in addition to yellow pigment production. All isolates decolorized C.I. Acid Orange 52 dye in the range of 100-1000 mg.L^{-1}. Almost all the isolates excluding MB377, MB381 and MB396 decolorized up to 1000 mg.L^{-1} of C.I. Mordant Black 11.

All the isolates showed the capability of growing and decolorizing C.I. Direct Red 28 dye at various concentrations 100-1000 mg.L^{-1}. At low concentrations of dye, bacterial isolates revealed different trends of decolorization for dye molecules, whereas, upon increasing the concentrations, bacterial growth/cells transformed in to dark red color indicating the elimination of respective dye via adsorption. Decolorization of C.I. Basic violet 3 dye was also observed at 400 mg.L^{-1} concentration by four isolates (MB377, MB391, MB394 and MB398), and C.I. Basic Green 4 dye was decolorized by all isolates (except MB386) at 300-1000 mg.L^{-1} concentration.

Association of multiple antibiotics resistance with dye degradation capability

Moderately significant correlation was noticed between bacitracin and C.I. Basic Green 4 dye degradation ability. Results of correlation coefficient analysis are given in Table 3. All these findings indicated the possible reasons for coexistence of resistance conferring genes as mentioned above regarding the correlation for both metals and antibiotics.

Table 3 Pearson's correlation between antibiotic resistance and dye degradation capability

	E15	SXT25	AMP10	C30	DA2	B10	RD5	AML10	TE30	E15	SY	AO	DR	BG	BV	MB
E15	1	-.076	.316	.674*	.632*	.258	.775*	-.158	-.200	-.253	0	0	0	.146	.073	0
SXT25	-.076	1	.120	.255	-.120	.683*	.098	.120	-.076	.048	0	0	0	.210	.138	0
AMP10	.316	.120	1	.213	.500	.408	0	-.125	-.632*	.050	.177	0	0	-.046	-.230	0
C30	.674*	.255	.213	1	.426	.174	.522	-.426	-.135	-.171	0	0	0	-.138	-.246	0
DA5	.632*	-.120	.500	.426	1	.408	.408	.125	-.316	.100	.530	0	0	.185	.230	0
B10	.258	.683*	.408	.174	.408	1	.333	.408	-.258	-.054	0	0	0	.665*	.345	0
RD5	.775*	.098	0	.522	.408	.333	1	0	.258	-.327	0	0	0	.339	.157	0
AML10	-.158	.120	-.125	-.426	.125	.408	0	1	-.158	.050	.177	0	0	.507	.346	0
TE30	-.200	-.076	-.632*	-.135	-.316	-.258	.258	-.158	1	.253	0	0	0	.146	.364	0
AR	-.253	.048	.050	-.171	.100	-.054	-.327	.050	.253	1	.401	0	0	.049	.522	0
SY	0	0	.177	0	.530	0	0	.177	0	.401	1	0	0	-.326	.217	0
AO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BG	.146	.210	-.046	-.138	.185	.665*	.339	.507	.146	.049	-.326	0	0	1	.634*	0
BV	.073	.138	-.230	-.246	.230	.345	.157	.346	.364	.522	.217	0	0	.634*	1	0
MB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Legends: Correlation between antibiotics resistance, Correlation between dyes decolorization, Positive correlation between antibiotics resistance and dyes decolorization, Negative correlation between antibiotics and dyes, * = Significant correlation, + = Positive correlation, - = Negative correlation
AR= C.I. Acid Red 2, SY= C.I. Solvent Yellow 2, AO= C.I. Acid Orange 52, MB= C.I. Mordant Black 11, BG= C.I. Basic Green 4, BV= C.I. Basic violet 3, DR= C.I. Direct Red 28

Association of metal tolerance with dye degradation capability

Interpreted data of correlation coefficient analysis is presented in Table 4. Results revealed moderate relationship (r= 0.682) between copper resistance and C.I. Basic Green 4 dye degradation capability. Correlation coefficient for cadmium and C.I. Acid Red 2 dye was recorded as 0.512. All these findings indicated the possible reasons for coexistence of resistance conferring genes as mentioned above regarding the correlation for both metals and antibiotics.

Table 4 Pearson's correlation between metal resistance and dye degradation capability

	Cr	Fe	Pb	Cd	Cu	Ni	AR	SY	AO	DR	BG	BV	MB
Cr	1	0	0	-.173	.399	-.551	-.254	-.191	0	0	.379	0.049	0
Fe	0	0	0	0	0	0	0	0	0	0	0	0	0
Pb	0	0	0	0	0	0	0	0	0	0	0	0	0
Cd	-.173	0	0	1	-.510	.226	.512	0	0	0	-.138	0.344	0
Cu	.399	0	0	-.510	1	-.522	0	0	0	0	.682*	.405	0
Ni	-.551	0	0	.226	-.522	1	.075	.441	0	0	-.624*	-.366	0
AR	-.254	0	0	.512	0	.075	1	.401	0	0	.049	.522	0
SY	-.191	0	0	0	0	.441	.401	1	0	0	-.326	0.217	0
AO	0	0	0	0	0	0	0	0	0	0	0	0	0
DR	0	0	0	0	0	0	0	0	0	0	0	0	0
BG	.379	0	0	-.138	.682*	-.624*	.049	-.326	0	0	1	.634*	0
BV	.049	0	0	.344	.405	-.366	.522	.217	0	0	.634*	1	0
MB	0	0	0	0	0	0	0	0	0	0	0	0	0

Legends: Correlation between metals tolerance, Correlation between dyes decolorization, Positive correlation between metals tolerance and dyes decolorization, Negative correlation between metals tolerance and dyes decolorization, * = Significant correlation**,** + = Positive correlation, - = Negative correlation, AR= C.I. Acid Red 2, SY= C.I. Solvent Yellow 2, AO= C.I. Acid Orange 52, MB= C.I. Mordant Black 11, BG= C.I. Basic Green 4, BV= C.I. Basic violet 3, DR= C.I. Direct Red 28

Molecular characterization of selected strains by phylogenetic analysis

Molecular characterization was done through sequencing universal gene 16S rRNA. After sequencing specific bacterial strains universal gene, the analysis was performed by using phylogenetic tools exhibited highest similarity of particular strains with 2 genera including *Staphylococcus* (MB377) and *Klebsiella* (MB375, MB381, MB394 and MB398). As a result, these bacteria were recognized as *Klebsiella pneumoniae* (MB375), *Staphylococcus* sp. (MB377) and *Klebsiella oxytoca* (MB381). In addition, the isolates MB394 and MB398 were identified as *Klebsiella pneumoniae* respectively (Figure 4).

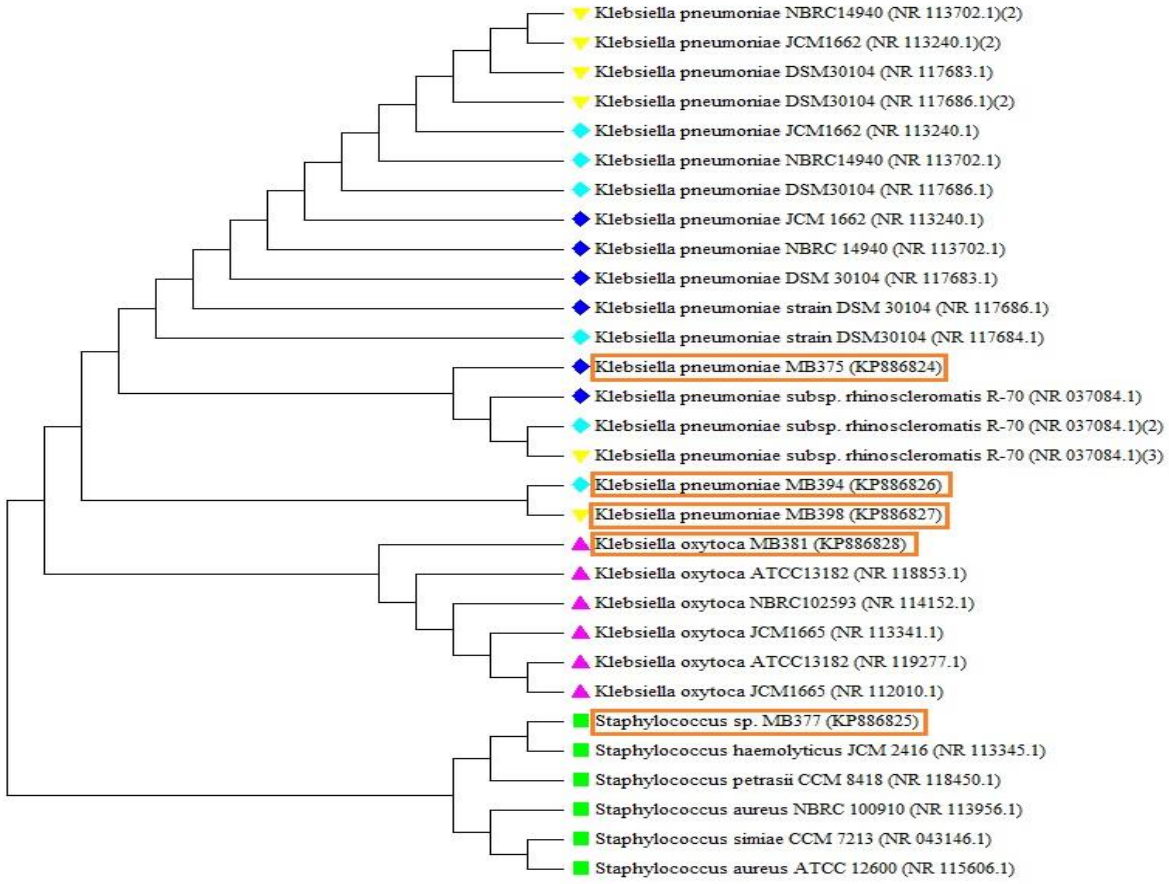


Figure 4 Molecular phylogenetic relationships among selected bacteria and other closely related representatives based on 16S rRNA genes

CONCLUSION

Current findings showed greater diversity among the bacterial strains based on the phenotypic, functional, biochemical and physiological characteristics. Furthermore, these biofilm producing bacteria possessed the potential to survive and thrive in stressed environments by showing the ability to resist various groups of antibiotics as well as heavy metal salts. In addition to toxic metals metabolism these biofilm forming strains revealed the potential to remove and degrade diverse dyes through different methods involving adsorption as well as enzymatic pathways. These diversified features specified the capabilities of bacteria of naturally adapting to withstand and grow in the existence of hazardous compounds and exploit these compounds by transforming them into different metabolite. Moreover, detailed studies about bacterial genetics and metabolic pathways will be greatly helpful in determining their potential roles in pollution abatement.

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REFERENCES

Asri M., Elabed S., Ibnsouda Koraichi S. & El Ghachtouli N. (2018). Biofilm-Based Systems for Industrial Wastewater Treatment. In: Hussain C. (eds) Handbook of Environmental Materials Management. Springer, Cham. https://doi.org/10.1007/978-3-319-58538-3_137-1.

Atshan. S.S. & Shamsudin, M.N. (2011). Evaluation of phenotypic and genotypic detection methods for biofilm-forming methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* clinical isolates. *Annals of Microbiology*, 61, 825-831.

Cerca, N., Jefferson, K. K., Maria-Litran, T., Pier, D. B., Kelly-Quintos, C., Goldmann, D. A., Azeredo, J. & Pier, G. B. (2007). Molecular basis for preferential protective efficacy of antibodies directed to the poorly acetylated form of *Staphylococcal* poly-N-acetyl-β-91,6)-glucosamine. *Infection and Immunity*, 75(7), 3406-3413.

Chan, S., Pullerits, K., Keucken, A., Persson, K. M., Paul, C. J. & Rådström P. (2019). Bacterial release from pipe biofilm in a full-scale drinking water distribution system. *NPJ Biofilms Microbiomes*, 5, 9. https://doi.org/10.1038/s41522-019-0082-9

Freeman, D. J., Falkiner, F.R. & Keane, C. T. (1989). New method for detecting slime production by coagulase negative *Staphylococci*. *Journal of Clinical Pathology*, 42, 872-874.

Fröls, S., Dyall-Smith, M. & Pfeifer, F. (2012). Biofilm formation by haloarchaea. *Environmental Microbiology*, 14(12), 3159-3174.

Garrett, T. R., Bhakoo, M. & Zhang, Z. (2008). Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science*, 18, 1049-1056.

Gbejuade, H. O., Lovering, A. M. & Webb, J. C. (2015). The role of microbial biofilms in prosthetic joint infections: A review. *Acta Orthopaedica*, 86(2), 147-158.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N.R. (1994). Methods for general and molecular bacteriology. Washington, D.C. American Society for Microbiology.

Habibi, N., Soleimanian-Zad, S. & Zeinoddin, M. S. (2011). Exopolysaccharides produced by pure culture of *Lactobacillus*, *Lactococcus* and Yeast isolated from Kefir Grian by microtiter plate assay: optimization and comparison. *World Applied Sciences Journal*, 12(6), 742-750.

Hammer, Ø., Harper, D. A. T. & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. Available from: http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Ifeanyichukwu, I., Nicodemus, M., Chika, E., Emmanuel, N. & Emmanuel, U. (2015). Detection of biofilmproducing isolates of *Aeromonas* species from drinking water sources in Ezza South local Government area of Ebonyi State, Nigeria. *Journal of Pharmaceutical, Chemical and Biological Sciences*, 3(2), 316-323.

Irankhah, S., Ali, A. A., Mallavarapu, M., Soudi, M. R., Subashchandrabose, S., Gharavi, S. & Ayati, B. (2019). Ecological role of Acinetobacter calcoaceticus GSN3 in natural biofilm formation and its advantages in bioremediation, *Biofouling*, 35(4), 377-391. http://10.1080/08927014.2019.1597061

Johanna, S. K. S. & Priyanka, R. M. (2014). Isolation and identification of biofilm producers from the implant infected individuals. *Journal of Global Biosciences*, 3(3), 633-638.

Kawane, R. S. (2012). Studies on antibiotics and heavy metal resistance profiling of *Escherichia coli* from drinking water and clinical specimens. *Bioscience Discovery*, 3(3), 292-295.

Khatoon, Z., McTiernan, C. D., Suuronen, E. J., Mah, T-F. & Alarcon, E. I. (2018). Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon*, 4, e01067. https://doi.org/10.1016/j.heliyon.2018.e01067

Liaqat, I. & Sabri, A. N. (2010). Characterization of biocides resistant isolates from dental unit water line biofilms by culture dependent approach. *African Journal of Clinical and Experimental Microbiology*, 11(1), 129-136.

Mahmood, R., Sharif, F., Ali, S. & Hayyat, M. U. (2013). Bioremediation of textile effluent by indigenous bacterial consortia and its effects on *Zea mays* LCV C1415. *Journal of Animal and Plant Sciences*, 23(4),1193-1199.

Nancharaiah, Y.V. & Venugopalan, V. P. (2019). Microbial Biofilms in Bioremediation and Wastewater Treatment1st Edition, Boca Raton,CRC Press. https://doi.org/10.1201/b22046

Nisha, P. & Thangavel, M. (2014). Isolation and characterization of biofilm producing bacteria from Arabian Sea. *Research Journal of Recent Sciences*, 3, 132-136.

Oliveira, D. R., Larsson, L. & Granhag, L. (2019). Towards an absolute scale for adhesion strength of ship hull microfouling, *Biofouling*, 35(2), 244-258. https://doi.org/10.1080/08927014.2019.1595602

Rajivgandhi, G., Vijayarani, J., Kannan, M., Murugan, A., Vijayan, R. & Manoharan, N. (2014). Isolation and identification of biofilm forming uropathogens from urinary tract infection and its antimicrobial susceptibility pattern. *International Journal of Advanced Life Sciences*, 7(2), 352-363.

Saidi, N., Lagha, R. & Abdalla, F. B. (2013). Slime producing, heavy metals and antibiotics resistance in *Aeromonas hydrophila* isolated in Tunisia. *African Journal of Microbiology Research*, 7(50), 5697-5708.

Saranraj, P. & Sivasakthivelan, P. (2014). Prevalence of bacterial isolates in textile dye effluent and analysis of its dye degrading efficiency. *Middle-East Journal of Scientific Research*, 21(5), 721-725.

Sehar, S. & Naz, I. (2016). Role of the Biofilms in Wastewater Treatment in: Microbial Biofilms - Importance and Applications (Dharumadurai Dhanasekaran and Nooruddin Thajuddin (eds). IntechOpen, doi: 10.5772/63499. Available from: https://www.intechopen.com/books/microbial-biofilms-importance-and-applications/role-of-the-biofilms-in-wastewater-treatment

Selenska-Pobell, S. & Merroun, M. (2010). Accumulation of heavy metals by microorganisms: biomineralization and nanocluster formation. *In:* König, H., Claus, H. & Varma, A. (eds) *Prokaryotic Cell Wall Compounds*. Springer Berlin Heidelberg. pp. 483-500.

Stepanovic, S., Vukovic, D., Hola, V., Di-Bonaventura, G., Djukic, S., Cirkovic, I. & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *Staphylococci*. *APMIS*, 115, 891-899.

Sujana, K., Abraham, K. P., Mikkili, I. & Prabhakar, K. (2013). Biochemical and molecular characterization of biofilm producing bacteria. *International Journal of Pharma and Bio Sciences*, 4(1), 702-712.

Tahir U, Yasmin A (2019) Role of bacterial extracellular polymeric substances (EPS) in uptake and accumulation of co-contaminants. *International Journal of Environmental Science and Technology*. https://doi.org/10.1007/s13762-019-02360-0

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725-2729.

Tripathi, M., Vikram, S., Jain, R. K. & Garg, S. K. (2011). Isolation and growth characteristics of chromium (VI) and pentachlorophenol tolerant bacterial isolate from treated tannery effluent for its possible use in simultaneous bioremediation. *Indian Journal of Microbiology*, 51(1), 61-69.

Turki, Y., Mehri, I., Lajnef, R., Rejab, A. B., Khessairi, A., Cherif, H., Ouzari, H. & Hassen, A. (2017). Biofilms in bioremediation and wastewater treatment: characterization of bacterial community structure and diversity during seasons in municipal wastewater treatment process, *Environmental Science and Pollution Research*, 24, 3519-3530.

Pui, C.F., Apun, K., Jalan, J., Bilung, L.M., Su`ut, L. & Hashim, H. F. (2017). Microtitre plate assay for the quantification of biofilm formation by pathogenic leptospira. *Research Journal of Microbiology*, 12, 146-153. https://dx.doi.org/10.3923/jm.2017.146.153

Supplementary data

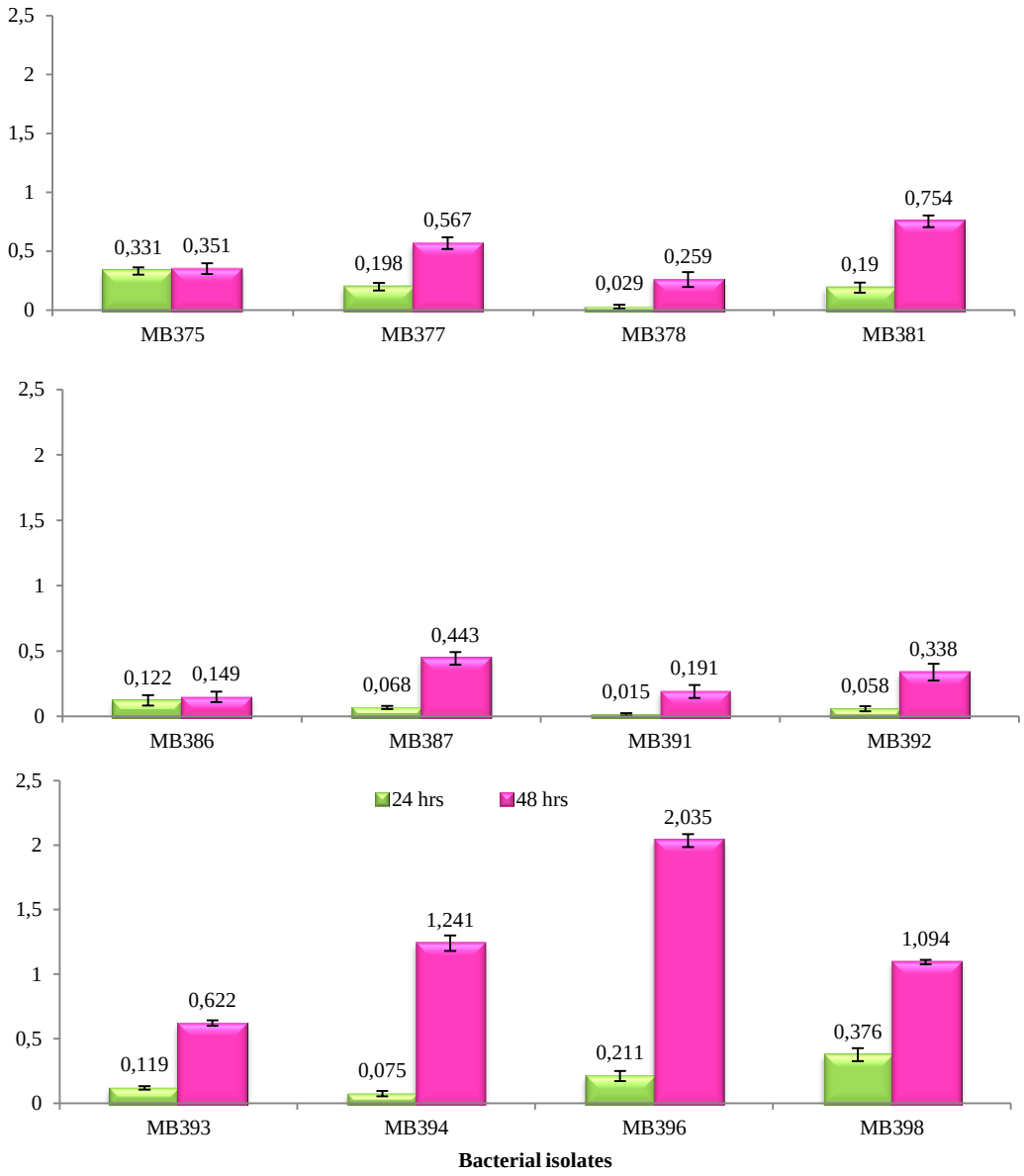

Figure S1 Biofilm formation proficiency of bacteria over time (24-48 hours)

Table I Heavy metal tolerance (HMT) indices for biofilm forming bacteria

Bacterial isolates	Lead	Chromium	Nickel	Iron	Copper	Cadmium
MB375, MB377, MB378	.333	.333	.333	.333	.333	.333
MB381	.167	.167	.167	.167	.167	.167
MB386, MB387	.167	.167	.167	.167	.167	.167
MB391, MB392, MB393	.333	.333	.111	.333	.333	.333
MB394, MB396, MB398	.333	.333	.333	.333	.333	.333

Table III Multiple antibiotic resistance (MAR) indices for biofilm forming bacteria

Bacterial isolates	IPM10	TE30	S10	E15	AMP10	B10	C30	SXT25	K30	DA10	CN10	RD50	OFX5	N30	AML10	NA30
MB375, MB377, MB378	0	.021	0	.021	.042	.042	.021	.042	0	.021	0	.021	0	0	0	0
MB381	0	0	0	0	.063	.063	0	.063	0	0	0	0	0	0	.063	0
MB386, MB387	0	0	0	0	.031	.000	0	0	0	0	0	0	0	0	.031	0
MB391, MB392, MB393	0	.021	0	0	.021	.063	0	.063	0	0	0	.021	0	0	.063	0
MB394, MB396, MB398	0	0	0	.021	.063	.063	0	.021	0	.063	0	.021	0	0	.063	0