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PHENOTYPIC CHARACTERIZATION AND CORRELATION ANALYSIS OF HEAVY METAL TOLERANT BACTERIA

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ABSTRACT

High concentrations of metals strongly influence the composition of soil microflora. Tanneries being one of major sources of metal pollution in Pakistan pose serious threat to the soil bacterial communities. Present study aimed to investigate the bacterial diversity from tannery effluents affected soil. Total Sixteen bacterial isolates were purified and characterized morphologically and biochemically. All selected isolates were facultative anaerobes and able to grow in wide pH ranges from 5-11. Only TS3, TS4, TS8, TS9, TS13 were resistant to Erythromycin. On the basis of phenotypic characters, cluster analysis was done to determine the similarities among selected bacterial isolates using Jaccord's (SJ) coefficients by UPGMA clustering algorithm. Two major clusters while eight subgroups of selected isolates were obtained on the basis of similarity indices. Bacterial isolates in present research were tolerant to the higher concentrations of lead, chromium and cadmium up to 1000µg/ml. Variable level of resistance was observed against nickel, manganese, copper and iron, while mercury, zinc and cobalt was toxic to these bacteria at higher concentrations. Pearson correlation analysis revealed a strong correlation among some heavy metals as well as antibiotics. Moreover, strong negative correlation was also observed between few antibiotics and heavy metals. The heavy metal profiling indicated the potential of these bacterial isolates to be used in bioremediation of heavy metal polluted soils after further research on their genetic mechanisms.

Keywords: Tanneries, facultative anaerobes, heavy metals, bioremediation

INTRODUCTION

Soil is a three dimensional, dynamic, natural entity on the surface of earth and it is a basic medium for plant growth. It plays a fundamental role in the regulation of pollutants in the ecosystem **(Ranjan, 2005)**. Pollution due to industrial activities has created a serious problem for the safe and rational use of soils **(Ashraf *et al.*, 2007)**. Emissions of liquid effluents due to industrial activities are capable of causing soil and groundwater pollution **(Surita *et al.*, 2007)**. When the effluent comes in contact with land it affects the soil and leads to its degradation in varying degrees depending upon its physicochemical characteristics **(Dwivedi *et al.*, 2006)**.Environmental contamination with heavy metals due to anthropogenic sources is raising new pollution concerns. Heavy metals like Pb, Hg, Zn, and Cu are most deleterious contaminants of soil. Application of metal containing sewage sludge, effluents and fertilizers to agricultural land is a common practice all around the world (**Seiler and Berendonk, 2012**).

Tanning is one of the oldest industries in Pakistan and is the major foreign exchange earners for the country. Tanning industrial wastes are of serious consequences from the pollution point of view. The wastes from this industry rank among the most polluting of all industrial wastes. Non degradable persistent trace metals present in tanneries are the most pressing problem these days. Soils act as the sinks for these metals. Tanneries discharge effluents containing toxic heavy metals like copper, nickel, zinc, lead, arsenic, cadmium, and chromium **(Sharma *et al.*, 2009)**. Heavy metal pollution of soil deteriorates environmental quality by influencing the yield and quality of crops, atmosphere and quality of water environment. Human health is also affected via food chains **(Huamain *et al.*, 1999)**.

Soil contains wide variety of microbial populations and these microorganisms significantly contribute to the maintenance of the matter and energy turnover in land environment **(Paul, 2007)**. Microbes such as fungi and bacteria play essential role in nutrient transformations **(Critter *et al.*, 2002)**. They affect the soil erosion and metal remediation efforts and also play important role in plant interaction with soil environment. These microbes may be essential for the revegetation efforts by aiding in removal of excessive soil metals **(Khan, 2003)**. Microorganisms mineralize, oxidize, reduce and immobilize inorganic and organic materials in soils. These compounds may alter the number or activity of the microorganisms and can affect soil biochemical process and ultimately influence the soil fertility and plant growth **(Araujo *et al.*, 2006)**. As tanneries are major source of heavy metal pollution, they may have damaging effects on the decomposition and nutrient mineralization in soil near pollution sources. This can be the result of harmful effect of metals on microbes **(Komulainen, 1992)**. However, some times exposure to metals may lead to the evolution of resistant microbial populations often characterized as genus *Bacillus*, *Corynebacterium*, *Arthrobacter* and *Alcaligenes* etc. (**Moghannem *et al.*, 2015**).

The objectives of this study were to isolate and characterize bacteria from the soil affected with tannery effluents and also to check their heavy metal resistance, which may have a potential role in biodegradation/ bioremediation of these metals from the polluted environment.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Tannery effluents affected soil samples were collected in airtight sterilized plastic bags from the vicinity of Leather Field Industry, Sambarial, where these effluents were thrown. Bacterial population was determined by serial dilution and plating of soil suspension on nutrient agar medium. After incubation at 37°C for 24-48 hrs plates were observed for the presence of bacterial colonies. Pure cultures of morphologically distinct colonies were obtained by single colony streaking. After many rounds of streaking purified bacterial cultures were obtained. Morphological and biochemical characterization of pure cultures was done following standard methods.

Antibiotic resistance of bacterial isolates

To test antibiotic resistance of selected isolates, various antibiotics like Vancomycin (VA30), Erythromycin (E15), Chloramphenicol (C30), Streptomycin (S10), Ampicillin (AMP25), Ciprofloxacin (CIP5), Kanamycin (K30), Rifampicin (RD5), and Tetracycline (TE30) were used. Disk diffusion method was employed. Zones of inhibition were recorded after 24 hrs of

incubation to determine the resistance spectra of selected isolates against different antibiotics.

Heavy metal resistance of bacterial isolates

To check heavy metals resistance bacterial strains were grown in the presence of salts of different heavy metals. Sterilized autoclaved molten nutrient agar was supplemented with known concentrations of salts of various heavy metals **(Yasmin and Hasnain, 1998)**. Bacterial strains were grown in the presence of salts of heavy metals of K_2CrO_4 (Cr), $ZnSO_4$ (Zn), $CdCl_2$ (Cd), $CoCl_2$ (Co), $Fe_2(SO_4)_3$ (Fe), $HgCl_2$ (Hg), $NiSO_4$ (Ni), $CuSO_4$ (Cu), $(CH3COO)_2Pb$ (Pb), $MnSO_4$ (Mn). Growth of bacterial strains was examined after incubation at 37°C for 24 to 48 hrs.

Effect of pH on growth of bacterial isolates

Effect of pH on growth of bacterial strains was observed through spectrophotometric analysis. Nutrient broth was prepared and adjusted at different pH from 5-11.Test tubes containing 5ml of nutrient broth of each pH was autoclaved and inoculated using micropippeter and incubated at 37°C for 24hrs. A spectrophotometer was used to measure the optical density at 600nm to determine the bacterial biomass.

Data analysis

Twenty-one different morphological, biochemical and antibiotic susceptibility tests were performed for phenotypic characterization of selected bacterial isolates. Positive and negative results were assigned 1 and 0 codes. Similarities among different strains were estimated with Jaccard coefficient and clustering was attained by un-weighted average linkage using PAST software. Cophenetic correlation was also achieved to determine the goodness of clustering **(Hammer *et al.*, 2001)**. Pearson's correlation coefficient (r) was used to determine correlation between antibiotics and metal resistance. Strong positive and negative relationships were interpreted as (r > +0. 5 to +1.0) and (r > -0.5 to -1.0) respectively, while weak relationship was inferred as (+0.5 > r < -0.5).

RESULTS AND DISCUSSION

The effects of sludge and liquid wastes produced from leather tanneries on soil biodiversity can be amplified through the food chain and also threaten sustainability of natural ecosystems **(Iram *et al.*, 2009)**. Survival of microbial communities in metal contaminated soils depend on inherent morphological and biochemical characters as well as physiological and genetic adaptation of individual cells. Beside this environmental alterations of metal speciation also plays a crucial role in their existence. Microbes employ different resistance mechanisms in response to elevated concentrations of heavy metals which may result in development of drug resistance among them (El-Sayed, 2016).

In present study sixteen bacterial strains were isolated and their morphological and biochemical characterization was done from tannery effluent affected soil. Majority of the total isolates under study were gram-negative (69%), while remaining were gram- positive and majority of them were rods. All the isolates were facultative anaerobes and showed negative nitrate reduction but exhibited positive catalase test. **Srinath *et al.* (2001)** reported five facultative anaerobes from tannery effluents in Kanpur, India which showed negative nitrate reduction and catalase test.

Presently 6% bacterial isolates showed positive methyl red, 69% bacterial isolates showed positive Voges Proskuer, 19% showed positive urease test, 38% were motile and all were unable to hydrolyze starch. Whereas, **Pal *et al.* (2004)** reported an isolate AND303 from serpentine polluted soil in India that exhibited negative methyl red, VP, urease and starch hydrolysis test. **Yazdi *et al.* (1999)** reported 20 bacterial isolates from industrial wastewater treatment unit. These bacterial strains gave positive catalase test and negative Voges Proskuer test. Twelve of them showed positive nitrate reduction test, 4 strains showed positive urease test and nine were motile.

Haq *et al.* (1999) reported three bacterial isolates from industrial effluents of chemical and textile mill industries. These isolates exhibited pink growth on MacConkey agar. Four strains documented by **Faisal *et al.* (2004)** from tannery effluent showed positive growth on MacConkey agar and no growth on Brilliant Green Bile agar while three of them exhibited growth on EMB agar as well. However, in present study only TS11 isolate showed pink growth on EMB agar. Two isolates TS2 and TS11 exhibited growth on Simmons Citrate agar. While TS7 and TS11 isolates were able to grow on brilliant green bile agar and MacConkey's agar.

Cluster analysis revealed that out of total sixteen isolates, TS3 was similar to TS12 isolate on the basis of phenotypic characters and it also showed about 80% similarity with TS4, TS12, TS13 and TS15 (Figure 1). Isolate TS5 exhibited 75% similarity with TS16 while, TS8 showed similarity of 80% with TS10. Isolate TS12 explicated 80% similarity with TS13 and TS15 whereas TS13 was similar to TS15 and showed 75% similarity with TS16. Though maximum similarities were observed among the bacterial isolates on the basis of selected characters yet they displayed variability in colony morphology (Table 1) which indicates diversity in species. The value of cophenetic correlation obtained showing the fitness cluster analysis was 0.90.

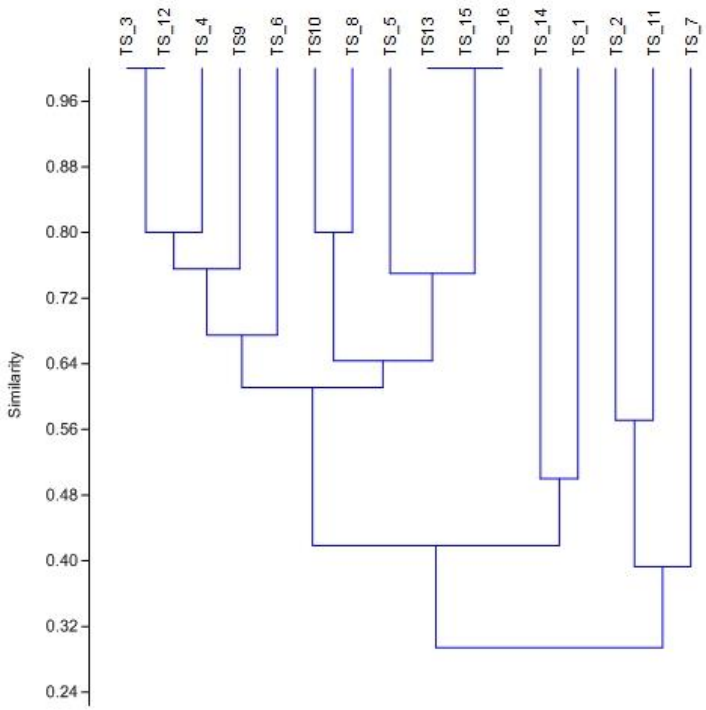

Figure 1 Similarity indices among bacterial strains using Jaccord's coefficient

Shafiani *et al.* (2003) documented 35% chloramphenicol and 20% tetracycline antibiotic resistant strains from wastewater irrigated soil. **Altaf *et al.* (2008)** reported 15 bacterial isolates from agricultural soil as well as from *Trifolium alexandrinum* plant nodule. They were 26.6% resistant against ampicillin and 20% against kanamycin. **Verma *et al.* (2001)** documented streptomycin resistant but chloramphenicol sensitive bacteria from tannery effluents. In the present study all the bacterial isolates were sensitive to tetracycline and 13% of bacterial isolates were resistant to chloramphenicol. Only one bacterial isolate TS4 was resistant to ampicillin, one isolate TS14 was resistant to streptomycin and no resistance was observed by any bacterial isolate to kanamycin.

About 75% of bacterial isolates in current study were resistant to cadmium up to 1000µgml^{-1}, 50% were resistant to chromium up to 1000µgml^{-1} and isolate TS11 was sensitive to chromium and exhibited no growth at any concentration. Majority of the isolates were resistant to lead at maximum concentration of 1000µgml^{-1}. Six strains showed growth against zinc at 100µgml^{-1} and two isolates TS2 and TS5 exhibited growth at 200µgml^{-1} as well, while remaining were sensitive to zinc and no growth was documented. 31% bacterial isolates were resistant against copper up to 500µgml^{-1}, 44% were resistant up to 400µgml^{-1} and 13% exhibited no growth. Majority of bacterial isolates (75%) were resistant to nickel (up to 500µgml^{-1}). Only three isolates TS3, TS5, and TS6 were resistant to mercury and could tolerate 200µgml^{-1} of mercury in medium, while all others were sensitive (Table 1). **Ashraf *et al.* (2007)** isolated stress tolerant bacteria from the soil amended with varying concentrations of heavy metal salts including silver (Ag), zinc (Zn) and lead (Pb). Many researchers reported multiple heavy metal resistant bacteria from different industrially polluted environments including the tannery effluents **(Haq *et al.*, 1999; Afrasyaab *et al.*, 2002; Zahoor *et al.*, 2009)**.

Table 1 Tolerance of Bacterial Isolates against Different Heavy Metals

Bacterial Strains	Metal Tolerance (µgml^{-1})									
	Cr^{+3}	Mn^{+2}	Ni^{+2}	Zn^{+2}	Cu^{+2}	Pb^{+2}	Co^{+2}	Fe^{+3}	Cd^{+2}	Hg^{+2}
TS 1	800	600	200	200^{w}	500	1000	-	900^{br}	1000	100^{g}
TS 2	1000^{w}	1000	500	300^{wp}	400	1000	-	900	1000	100
TS 3	900	900	600^{w}	100^{w}	400	1000	200^{w}	900^{br}	1000	200^{w}
TS 4	900	900	500^{w}	200^{w}	400	1000	200^{w}	900	1000	100
TS 5	1000	700	500^{w}	100^{p}	400	1000^{w}	200^{w}	800^{w}	800^{w}	200^{w}
TS 6	900	-	500^{w}	-	-	900^{w}	200^{w}	300^{w}	400^{w}	200^{w}
TS 7	900	700	500	100^{wy}	400^{w}	1000^{y}	200^{w}	800	1000	-
TS 8	1000^{w}	900^{w}	600^{w}	-	500	1000	200^{w}	800^{br}	1000	100^{w}
TS9	1000	700	500	-	600^{w}	1000	200^{w}	900^{w}	1000	100^{w}
TS10	500^{w}	1000	-	100^{wp}	-	1000^{w}	200^{w}	500	500w	-
TS 11	-	800^{w}	500	-	400	1000 ^{w}	200^{w}	900	1000	100^{w}
TS 12	1000^{w}	800	700^{w}	-	500	1000	200^{w}	800^{br}	1000	100
TS13	900	800	700^{w}	100^{w}	500^{w}	1000	200^{w}	900	1000	100
TS 14	1000	400	600^{w}	-	400^{w}	1000	200^{w}	900	1000	100^{w}
TS 15	1000^{w}	400	600^{w}	-	500	1000	200^{w}	900	1000	50
TS 16	1000	400^{w}	600^{w}	-	200	1000^{w}	200^{w}	500	500	100^{w}

Legend: w = weak growth, wp = weak growth with pale color, wy = weak growth with yellow color, y = growth of yellow color, br = growth of brick red color, g = growth of gray color

Data analysis by Pearson's correlation coefficient revealed a strong correlation among different antibiotics i.e. S(10), RD(5) and TE(30) which confirms the presence of same antibiotic resistance mechanism for different antibiotics in bacteria however, significant negative correlation was also found between few metals and antibiotics (Table 2). This showed that antibiotic resistance is negatively affected by metal resistance. Moreover, strong correlation among some metals (Table 2) is attributed to the presence of same metal resistance mechanism in bacteria for different metals. However in few investigations positive correlation among antibiotic and heavy metal resistance revealed that tolerance in bacteria may be induced due to heavy metals and antibiotics co-existence in the environment. Beside this some studies also demonstrated that metal resistant bacteria may induce antibiotic tolerance in sensitive bacteria (**Chen *et al.*, 2015**).

Table 2 Pearson's correlation among various antibiotics and heavy metal resistance

	S(10)	**RD(5)**	**TE(30)**	**C(30)**	**Cr^{+3}**	**Mn^{+2}**	**Ni^{+2}**	**Zn^{+2}**	**Cu^{+2}**	**Pb^{+2}**	**Co^{+2}**	**Fe^{+3}**	**Cd^{+2}**
S(10)	1	.683	-.067	-.098	.139	-.283	.141	-.215	.029	.067	.098	.162	.139
RD(5)	.683	1	.683	-.143	.056	-.271	-.234	.105	.156	.098	-.429	.236	.204
TE(30)	-.067	.683	1	-.098	-.063	-.086	-.462	.358	.184	.067	-.683	.162	.139
C(30)	-.098	-.143	-.098	1	-.538	.307	-.014	.314	.043	.098	-.429	.236	.204
Cr^{+3}	.139	.056	-.063	-.538	1	-.223	.421	.068	.249	-.038	-.056	.031	.073
Mn^{+2}	-.283	-.271	-.086	.307	-.223	1	-.179	.490	.310	.678	-.162	.488	.454
Zn^{+2}	-.215	.105	.358	.314	.068	.490	-.354	1	.052	.215	-.734	.289	.216
Cu^{+2}	.029	.156	.184	.043	.249	.310	.464	.052	1	.591	-.156	.889	.909
Pb^{+2}	.067	.098	.067	.098	-.038	.678	.009	.215	.591	1	-.098	.700	.603
Co^{+2}	.098	-.429	-.683	-.429	-.056	-.162	.345	-.734	-.156	-.098	1	-.236	-.204
Fe^{+3}	.162	.236	.162	.236	.031	.488	.287	.289	.889	.700	-.236	1	.961
Cd^{+2}	.139	.204	.139	.204	.073	.454	.369	.216	.909	.603	-.204	.961	1

Legend: Chloramphenicol (C30), Streptomycin (S10), Rifampicin (RD5), Tetracycline (TE30), chromium (Cr^{+3}), Manganese (Mn^{+2}), Nickel (Ni^{+2}), Zinc (Zn^{+2}), Copper (Cu^{+2}), Lead (Pb^{+2}), Cobalt (Co^{+2}), Iron (Fe^{+3}), Cadmium (Cd^{+2})

All the bacterial isolates in present study were able to grow at different pH values ranged from 5 to 11. Variable growth pattern was observed by bacteria at different pHs. Isolates TS7 and TS12 showed approximately same growth at 5 and 6 pH. TS11 isolate was neutrophile however, optimum growth of TS15 isolate was observed at 6 and 7 pH. Most of the isolates were slightly acidophilic or acidophilic in nature (Figure 2a-b). **Haq *et al.* (1999)** reported optimal growth of bacterial isolates at different pH values from 5 to 9. **Zahoor *et al.* (2009)** also documented bacterial strains explicating maximum growth at pH 6 and 7.

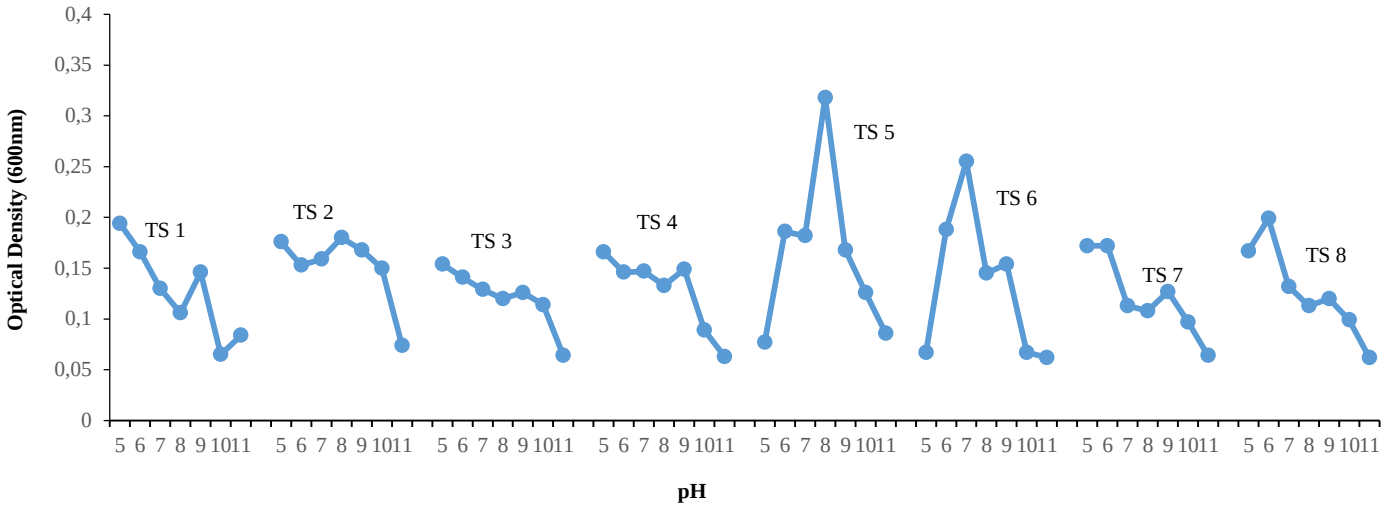

Figure 2a Growth of bacterial isolates at different pHs

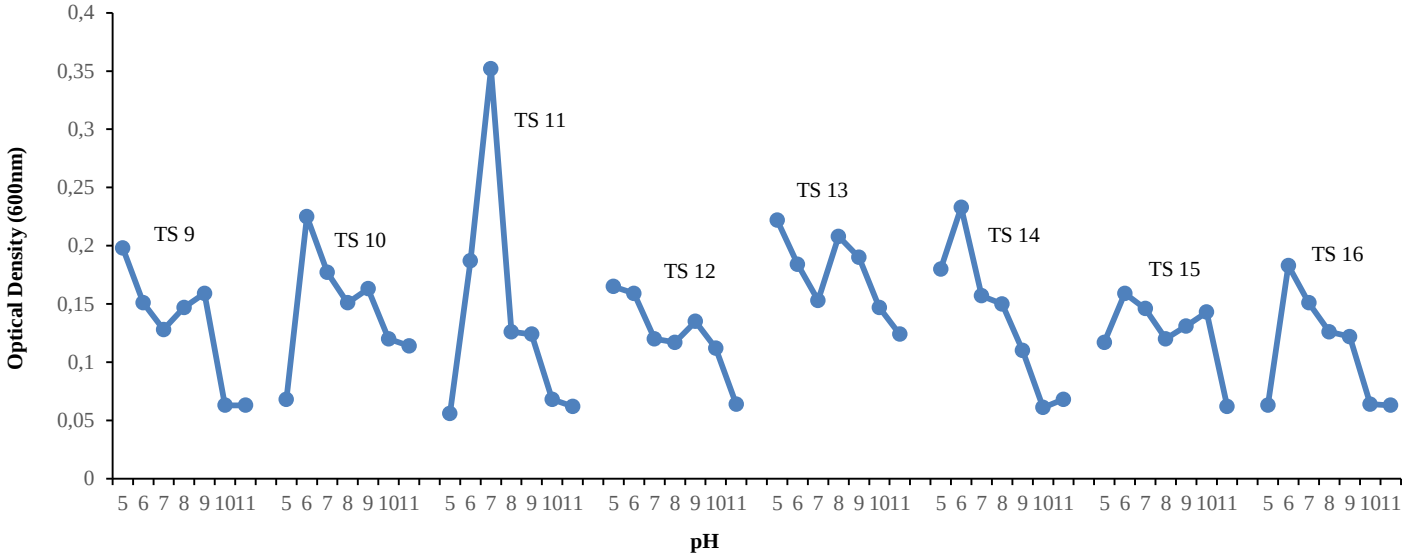

Figure 2b Growth of bacterial isolates at different pHs

CONCLUSION

This study demonstrates the preliminary screening of heavy metal tolerant bacteria from tannery effluent affected soil. The soil affected with tannery effluent was inhabited by a number of bacteria having diverse characters. Although they shared few characteristic like all of them were facultative anaerobes and they were highly tolerant to different heavy metals but the resistance pattern was different for different isolates. Majority of them were sensitive to various antibiotics. Further studies on their genetics and mechanism of heavy metal resistance will provide insight to their role in bioremediation.

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