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Multiple metal tolerance of *Paenibacillus dentritiformis* isolated from metal contaminated soils west Godavari district (Andhra Pradesh)

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Abstract
Sugar industrial effluents possess high amounts of toxic pollutants and contaminate the receiving sites. Treatment of contaminated sites by using microorganisms provides an alternate to conventional methods hence demands in the identification of metal tolerant microorganisms has been increasing day by day. Therefore in this study soil samples collected from Tanuku sugar factory residual effluent point (bank of Gosthani river), west Godavari district A.P were analyzed for the bacterial tolerance to Copper (Cu), Zinc (Zn) and Lead (Pb) in their chloride forms. Additionally, the study was carried out to identify the metal tolerant bacteria by morphological, biochemical and 16S rRNA gene sequencing studies. Four potential bacterial isolates were selected to analyze metal tolerance against $CuCl_2$, $ZnCl_2$, and $PbCl_2$. The sequences were compared with those in NCBI and submitted in gene bank with accession numbers MK100333 (*Paenibacillus* cookie), MK100334 (Bacillus cereus), MK100335 (Aneurini bacillus *sp*) and MK100387 (*Paenibacillus sp.*). A Phylogenetic tree was constructed to *Paenibacillus sp*. the highly efficient bacterial strain among the four isolates using MEGA 7 soft ware. The results of this study showed that *P. dentritiformis* had multiple metal tolerances (Cu, Zn and Pb) up to 500mg/L after 72 hrs. The identified bacterial strain proved to be the strong heavy metal tolerant bacterial strain. Hence, its usage will be helpful in the treatment of heavy metals *sp*ecifically Cu, Zn and Pb contaminated soils and further optimization of these cultures is required to improve its metal resistant capacity.

Keywords: Copper, Lead removal, Metal tolerance, *Paenibacillus species*, Zinc removal, 16s rRNA gene sequencing

INTRODUCTION

Heavy metals have relatively high atomic weight and density compared to water and occurring naturally in environment. Now a day's heavy metal contamination is an important environmental concern globally. These heavy metals can become mobile in soils depending on soil pH and can leach to nearby aquatic forms or to living organisms (Hookoom and Puchooa, 2013). The concentration of heavy metals is being increased due to rapid industrialization day by day. The industrial wastewater mainly contains the heavy metals like Cadmium, chromium, mercury, lead, nickel, cobalt, Zinc and copper (Smrithi and Usha, 2012). Removal of heavy metals by using microbes has attained a great attention from past few years. Many literatures have been reported so far on metal tolerant microbes and removal of heavy metals from aqueous solutions by using microorganisms such as bacteria (Shuttleworth and Unz, 1993), yeast (Salinas and Melorza, 2000), fungi (Anoop and Viraraghvan, 1999) and algae (Ahuja and Gupta, 1999). The rapid industrialization and urbanization creates environmental pollution. This situation highlights the need to find out more environmental friendly and cost effective treatment methods (Yasar *et al*., 2013). The industrial effluents rich in metal contaminants leach into the nearby water bodies and agricultural field changes the ecosystem (Sridevi *et al*., 2018). The sugar industry is playing an important role in the economic development of the Indian sub-continent, but the effluents create organic pollution (Jeyanthi Rebecca *et al*., 2014). Sugar industries play a major role in polluting the water bodies than other industrial effluents and re*sp*onsible to change the physicochemical characteristics of the water bodies and affect aquatic flora (Suresh *et al*., 2015).

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In addition, the use of stream and river water that has contaminated with sugar factory effluent causes a serious health hazards in humans. Untreated effluent from sugar factory creates an unpleasant odor when released into the environment (Usha Damodhar and Vikram Reddy, 2012).

Therefore, it is need of the time to analyze these wastes for some indigenous strains of heavy metal tolerant bacteria and to explore their potential in bioremediation of common heavy metals founds in such effluents. Keeping in view the above, present study was conducted for the isolation, identification and molecular characterization of heavy metal tolerant bacteria against Cu, Zn and Pb which are common in Tanuku sugar factory industrial effluents west Godavari district A.P

MATERIALS AND METHODS

Study area: Samples were collected from metal contaminated site i.e Gosthani River bank one of the residual effluent point near by Tanuku sugar factory, Tanuku, West Godavari district Andhra Pradesh.

Sample collection: Soil samples were collected from the bank of Gosthani River at a depth of 1cm, 5cm and 10 cm into sterile zip lock bags with a sterile *sp*atula and transferred to the laboratory. Samples were stored at room temperature.

Metal analysis: Collected soil samples were analyzed for the presence of 9 heavy metals *sp*ecifically Ni, Cd, Hg, Mn, Mg, Ar, Pb, Cu and Zn by Atomic absorption *sp*ectrophotometer (Model number: OPTIMA 8000, Category ICP-OES) following the Elemental analysis technique method.

Standard preparation: Mixed elemental standard was prepared using 100 ppm is mentioned below. Transferred 0.25ml, 05ml and 0.75ml of 100 ppm mixed standard in to three separate 50 ml volumetric flasks and diluted up to mark with milli Q water to obtained 0.5ppm, 1ppm and 1.5wg/L re*sp*ectively.

Sample preparation: Five ml of concentrated HNO3 **a**nd 20 ml of water was added to five grams of soil sample in a beaker. The sample was digested at 80^0C by using a hot plate for about 30 minutes; followed by cooling and diluted in 100 ml flask with water (Filtered the solution and a*sp*irated it).

Isolation of metal resistant bacteria: Soil samples were immediately processed after bringing to the laboratory by following 10 fold serial dilutions up to 10^{-7}in sterile test tubes. 0.1 ml from10^{-5}, 10^{-6} and 10^{-7} of each soil sample was *sp*read uniformly on to a sterile nutrient agar plates individually with a bent glass rod. Plates were incubated at 37°C for 24 hours. Nearly 256 bacterial colonies were screened for metal tolerance out of which four bacterial strains were selected randomly with different morphological appearance from that culture plates (Table: 1). Purification is done by streaking repeatedly on sterile nutrient agar plates and until further analysis pure cultures are maintained on agar slants at refrigerator temperature (Ansari.,2016).

Determination of metal tolerance: The maximum metal tolerable efficiency of selected strains were performed (Seralathan *et al.,* 2008; Shameer Syed and Paramageetham Chinthala, 2015; and Murthy *et al*., 2013) by inoculating bacterial strains into serial concentrations of $Cucl_2$, $Zncl_2$ and $Pbcl_2$ (100mg/L, 200mg/L, 300mg/L, 400mg/L, 500mg/L and 600mg/L) in nutrient broth at their re*sp*ective pH and maintained each tube at 37°C in triplicates and O.D values were taken at 72 hours.

Stock solution preparation: Stock solutions of the heavy metals were prepared by using copper chloride, Zinc chloride, and lead chloride. The stock solutions with 1000ppm concentration of re*sp*ective metal were prepared in de-ionized water by dissolving 2.68gm, 2.08gm and 1.3gm of $Cucl_2$, $Zncl_2$ and $Pbcl_2$ re*sp*ectively per 1000 ml and stored at refrigerator temperature (Seralathan *et al*., 2008; Shameer and Paramageetham, 2015).

Metal solution preparation: The metal solutions of three salts ($Cucl_2$, $Zncl_2$ and $Pbcl_2$) were prepared from stock solution in different concentrations 100mg/L , 200mg/L, 300mg/L, 400mg/L, 500mg/L and 600mg/L (Seralathan *et al*., 2008; Shameer and Paramageetham, 2015).

Inoculation of culture into different metal concentration (Yılmaz IE., 2003):

The metal tolerance of each isolate was assayed by tube dilution method in sterile conical flasks of 250ml capacity. 100ml of metal solutions of each metal inoculated with 10 ml overnight culture of isolates into properly labeled conical flasks. The pH of the metal microbe su*sp*ension was determined from the previously reported data (Murthy *et al*., 2012; Areco *et al*., 2012; Chang *et al*., 1997; Govarthanan *et al*., 2016) and was adjusted to their re*sp*ective pH to examine the metal tolerance capacity at their optimum pH. The conical flasks were incubated at their optimum temperature (37°C) for three days. A control without bacterial culture was also maintained. Each maintained in triplicates. The metal tolerance was analyzed calorimetrically by taking O.D values at 620n.m after 72 hours (Figs. 3, 4 and 5).

Morphological characterization: A standard procedure namely Gram Staining technique was performed as described by (Reddy *et al*., 2007 and Bergey's *et al*., 1974). The slides are observed under 45X objective lenses for examining Gram reaction and its morphological appearances such as color and the shape of bacterial colony are tabulated in Table 1.

Biochemical characterization: A few biochemical tests were performed for selected bacterial colonies, such as Indole, Methyl red-Voges proskauer, Citrate utilization, Catalase, Starch hydroly-

sis, multiple sugar analysis as described by Reddy *et al*. (2007) and Bergey's *et al*. (1974) (Table 2).

Molecular characterization

DNA extraction: The Insta GeneTM Matrix Genomic DNA isolation kit was used to extract genomic DNA from bacteria. As per the instructions *sp*ecified on the kit, genomic DNA was extracted from the individual isolates.

PCR protocol: Gene fragment was amplified in MJ Research Peltier Thermal Cycler by utilizing 16SrRNA universal primerswhich are mentioned below.

PCR amplification: The primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' are used for the PCR of 16S rRNA.

Purification of PCR products: Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore).

The PCR product was sequenced using the 785F/907R primers.

Sequencing primers: The purified PCR were sequenced by using 2 primers those are:

785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' are used for the Sequencing of 16S rRNA.

The sequences obtained were BLAST in NCBI to attain homologous sequences followed by phylogenetic analysis of the individual isolates using MEGA 7soft ware (Kumar *et al*., 2007) (fig: 4) by Neighbour Joining method (Saitou and Nei., 1987). The evolutionary distances were computed using the maximum composite likelihood joining method (Tamura *et al*., 2004).

RESULTS AND DISCUSSION

Metal analysis: The analysis of Heavy metals concentration present in the selected soil sample showed that heavy metals *sp*ecifically Cu, Zn and Pb are present in high concentration in compliance with WHO (2006) standards and these heavy metals have also been recognized as metals of immediate concern by WHO (2010) and (Hamid *et al* ., 2017).

Isolation and identification of metal tolerant bacterial strains: The range of metal tolerance in selected strains against Cu, Zn and Pb was reported previously. The metal tolerance against three heavy metals ranging from 60ppm to 100ppm were selected for further analysis of metal concentration above 500mg/L and identification studies (Sridevi *et al*., 2018). The strains were designated as VSR201, VSR202, VSR203 and VSR204. Four bacterial strains showed different cell shapes, colony characters and gram staining reactions (Table 1). The characterization of bacterial isolates (VSR201, VSR202, VSR203 and VSR204) based on their cultural, morphological and biochemical studies are *sp*ecified in Table 1 and 2 re*sp*ectively.

The colony morphological characters were distinct among isolates. VSR201 was small circular off white, translucent, creamy and yellowish to creamy in color. VSR 202 was off white raised, rough colony with irregular margin, VSR203 was creamy opaque, and VSR204 was like white branched but with medium size.

The morphological characterization studies revealed that among four bacterial strains, two are gram positive rods and two are gram negative rods. Further identification was done by biochemical (Table 2) and molecular studies. Isolates

Table 1. Morphological characterization of the selected isolates.

Strain	Gram's staining	Colony color	Colony elevation	Surface	Shape and size
P. cookie	G^-ve-rod	Yellowish to creamy	Raised	Smooth	Circular-small
B. cereus	G^+ve -rod	Off white	Raised	Rough	Irregular edge- medium
Aneurini bacillus sp.	G^+ve -rod	Creamy	Opaque	Smooth	Slightly crenate edge-medium
P. dentritiformis	G^-ve-rod	White	Flat	Smooth	Branched-medium

Table 2. Biochemical characterizations of the selected isolates.

Test	VSR 201	VSR 202	VSR 203	VSR 204
Indole	-	-	-	+
Methyl red	+	-	-	+
Voges proskauer	-	-	-	-
Citrate utilization	-	-	-	-
Catalase	-	+	+	+
Starch hydrolysis	-	-	-	+
Multiple sugar analysis:				
Glucose	+	-	-	+
Sucrose	+	-	+	-
Mannitol	+	-	-	-

+ = Positive; - = Negative; +/- = Variable

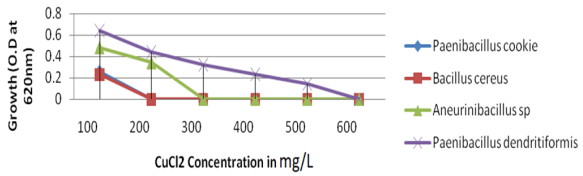

Fig.1. *Growth of selected isolates (O.D at 620n.m) at different concentrations of CuCl2 after 72 hours of incubation.*

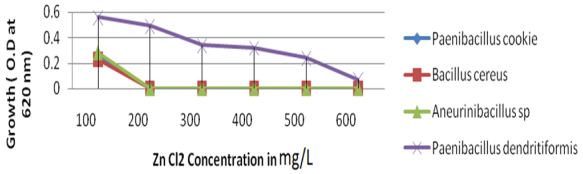

Fig. 2. *Growth of selected isolates (O.D at 620n.m) at different concentrations of ZnCl2 after 72 hours of incubation.*

VSR202 and VSR203 showed negative result for almost all biochemical tests except for catalase and sugar fermentation test. Only one isolate VSR201 ferment the mannitol.

The isolates finally identified up to *sp*ecies level were as *Paenibacillus cookie* (VSR201), Bacillus cereus (VSR202), *Aneurini bacillus* (VSR203), *Paenibacillus dendritiformis* (VSR204) by 16s rRNA characterization studies and based on partial sequence comparison by NCBI BLAST. 16S rRNA studies were performed for nine bacterial strains using universal primers with a length of 1492 bp. Amplified products were run in 1.0 % agarose gel electrophoresis and observed' in UV light. The Amplified DNAs were sequenced and isolates were identified by Gene Bank database by comparing the sequences with those in NCBI and identified as strains which show more than 99% similarity using BLAST search programme and sequences were submitted to NCBI. The assigned accession numbers for the submitted sequences are MK100333 (*Paenibacillus* cookie), MK100334 (Bacillus cereus), MK100335 (Aneurini bacillus *sp*) and MK100387 (*Paenibacillus Sp.*). The phylogenetic tree is shown as Fig. 4 based on 16S rRNA gene sequences. With the bootstrap value of 100%, strain *Paenibacillus Sp*. VSR204 was grouped with *P. dentritiformis* strain CS2a4 (KU601317) and *Paenibacillus sp*.BAB-3430 (KF917150), and had a closer genetic relationship with *P. dentritiformis*. Thus, strains VSR204 belonged to *P. dentritiformis*. Though there was a much less generic diversity among bacteria, strains showed biochemical diversity within them.

Determination of metal tolerance: Four bacterial isolates exhibited metal tolerance against selected metals up to 100ppm. Hence further study was carried out by subjecting bacterial isolates to high metal concentration (100mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L, and 600 mg/L) at their optimum pH and temperature.

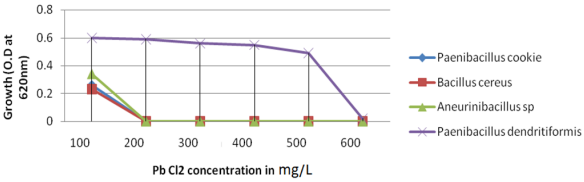

Fig. 3. *Growth of selected isolates (O.D at 620n.m) at different concentrations of PbCl2 after 72 hours of incubation.*

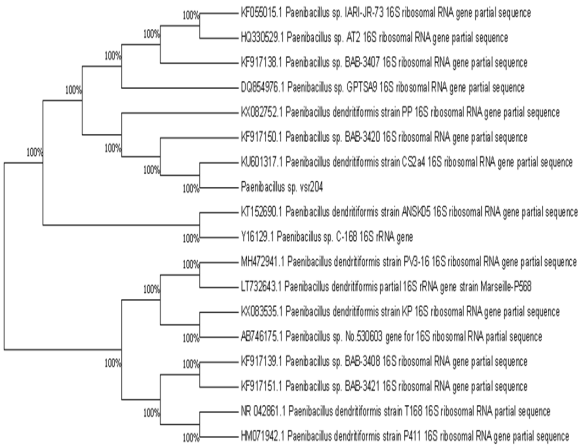

Fig. 4. *Phylogenetic Analysis of 16s rRNA gene sequence of P. dentritiformis by Neighbour Joiing Method.*

Out of four strains particularly one strain that is *P. dentritiformis* exhibited high metal tolerance against all three metals (Pb>Zn>Cu) at its optimum pH, this is a unique feature of *P. dentritiformis* to survive in different environmental metal pollutants. Rest of three strains *Paenibacillus* cookie, Bacillus cereus and *Aneurini bacillus sp*ecies showed their maximum growth at 100ppm. The growth was effected totally from 200 mg/L and completely absent after 300 mg/L against lead and zinc even at their optimum pH after 72 hours of incubation (Figs. 1, 2 and 3).

Unlike that of *Paenibacillus* cookie and *Bacillus cereus, A. bacillus sp*ecies showed its growth at least up to 200 mg/L concentration of copper (Fig.1) but after that the growth was affected by increasing metal concentration. *P. dentritiformis* is the only strain among all strains tested exhibited highest lead tolerance up to 500 mg/L at acidic pH after 72 hours. There was a steady growth rate observed until 400mg/L concentration of lead and then the growth was reduced suddenly by increasing lead concentration from 500 mg/L even at its optimum pH (Fig. 3). Against to copper and zinc the growth was reduced gradually by increasing metal concentration (Figs. 2 and 3) and it was observed to absent after 500 mg/L. The results of *P. dentritiformis* to zinc and copper tolerance was similar with the earlier studies reported by Shruti Murthy *et al*. (2013). In accordance with those studies it was clearly evident that the growth rate

was reduced gradually by increasing metal concentration.

However, there are some studies reported that the microorganisms have the ability to grow even at high concentration of metals without affecting their growth this is due to the results of intrinsic factors or due to induced mechanisms as well as other environmental factors (Xiao *et al*., 2010; Zouboulis *et al*., 2004; Leedjarv *et al* ., 2008). The similar scenario was observed in case of *P. dentritiformis* which showed unique pattern of growth rate in lead solution. Surprisingly the growth rate was constant until 400mg/L and latter it became slower at 500mg/L concentration followed by absences of growth after 72 hours at 600mg/L concentration of lead, this is may be due to the presence of its favorable temperature and pH.

From the literature, it is evident that Zn will get precipitate at pH 6-7 (Areco *et al*., 2012) and pH beyond 5 will decreases the lead solubility (Chang *et al* 1997) may be this is the reason why *P. dentritiformis* showed zinc and lead tolerance up to 500mg/L at pH 5. The lead tolerance of *P. dendritiformis* reported in this study similar to *Bacillus cereus* showed lead tolerance up to 500mg/L at temperature 30^{o}C and pH 5 reported by Shruti Murthy *et al*., (2012). And it is relatively high than the recently reported *Paenibacillus sp*. RM showed lead resistant up to 450mg/L after 48 hours (Govarthanan *et al*., 2016).

Hao *et al*. (1994) reported that zinc concentration ranging from 25 to 40 ppm can cause death of microorganisms. In contrast to this Govarthanan *et al*., (2016) reported that the Paenibacilius *sp*.RM exhibited highest metal resistant to zinc up to 500mg/L and similarly in this study the ability of strain *P. dendritiformis* to grow at their optimum pH and temperature in presence of a wide range of 100mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L and 600 mg/L) $Zncl_2$ have suggested that they have the zinc tolerance up to 500 mg/L. Mohammed Umar and Normala (2015) reported that bacterial isolate (MH4) showed high degree of resistance to copper up to 200 mg/l. However, the previous study done by Govarthanan *et al*., (2016) stated that Paenibacilius *sp*.RM exhibited copper resistant up to 750 mg/L. In this study *P. dendritiformis* showed copper tolerance up to 500 mg/L at temperature 37°c, pH 7.2 for 72 hours and taking the result of this study in to consideration we can assume that the strains at their optimum pH and temperature can able to exhibit a high metal tolerance.

Conclusion

In this study, *P. dentritiformis* MK100387 showed tolerance against three selected metals. The metal presence not effected much the growth of selected isolate during analysis. The results presented in this study proved that the isolated strain i.e *P. dentritiformis* is best suitable for the treatment of metal contaminated soils, industrial effluents that contains copper, zinc and lead as metal contaminants. And further genomic studies are required for the isolation and characterization of metal tolerant genes.

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