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Characterization of a yellow pigmented, alkali and heavy metal tolerant *Glutamicibacter* sp. isolated from red mud

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ABSTRACT

Red mud ponds are waste dumping sites of bauxite mines where highly alkaline and polluted red mud orbauxite residues are deposited. They harbour many microbial strains of environmental importance. In the present study, a yellow coloured bacterial colony was isolated which showed tolerance to high pH, and metals like lead, chromium and arsenic. Genomic DNA extraction followed by partial 16S rDNA amplification and sequencing study revealed the isolate to be *Glutamicibacter* sp. The bacterial strain showed luxuriant growth in pH ranging from pH5 to pH 11, and salt concentration up to 8% NaCl. The pigment was isolated by methanol extraction and identified to be beta-carotene through FTIR spectroscopy. The pigment showed absorption peaks at 436 nm and 466 nm. The pigment could not show antibacterial activity significantly. The pigments showed a good free radical scavenging activity which increased significantly under stress conditions. No such studies were done from the red mud ponds of Utkal Alumina earlier. Tolerance of the bacterial strain to various heavy metals indicates presence of heavy metals in the red mud. Identification of molecular mechanisms underlying the heavy metal resistance and use of the pigments as cosmetics can be carried out in future.

Key words *: Red mud, 16S rDNA, Glutamicibacter, Heavy metal tolerant, Alkali tolerant, FTIR spectroscopy.*

Introduction

Utkal Alumina International Limited-Aditya Birla Group is one of the important alumina refineries in India located near Rayagada district of Odisha state. During the process of alumina extraction through Bayer's process, this industry produces a huge quantity of red mud (bauxite residues) as waste. The red mud is transported to impervious ponds through pipelines, and the water is recycled to reuse in the refinery. These ponds acquire a huge area and are found to be highly alkaline, thus no flora or fauna exists on these ponds (Zampieri *et al*., 2019). Microbial diversity is also scarce due to high-temperature treatment for the extraction of aluminium in Bayer's process (Babu and Reddy, 2011). However, Microbes invade the region through air current and grow in the high alkaline condition by producing different acids to neutralize their milieu (Nogueira *et al*., 2017). Red mud is also contaminated with various heavy metals (Gräfe, 2010). Adaptability to these conditions leads development

of microbial communities. These communities also help in recycling of inorganic elements (Harris, 2009). Survivability of microbes in these harsh conditions leads to develop many enduring mechanisms. Pigment production may also be such an adaptive response by some bacteria. They produce various pigments as secondary metabolites via metabolic pathways. These pigments protect the cell from harmful oxidants, ultraviolet rays, desiccants and extreme conditions (Wada *et al*., 2013). The pigments also act as antimicrobial compounds against other bacteria (Suresh *et al*., 2015).

The use of bio-pigments is gaining importance in global market due to their safety values and antioxidant nature. Consumers are now highly aware of the damage caused by the use of artificial colours and shift towards the use of natural colours in every household item such as food and cosmetics. Pigments from bacteria can be an alternative source asthey are easily available, extractable, and compatible with the environment (Venil *et al*., 2013). Pigment producing bacteria are abundantly found in extreme conditions like marine environments, deserts and hyper-saline regions (Azman *et al*., 2018).

In the present study, isolation of a yellow pigmented bacterial strainis reported. The isolate, identified as *Glutamicibacter* sp. through 16S rDNA study, was characterized for its ability to tolerate stressful conditions like salinity, alkalinity and heavy metal contamination. The pigment was identified and its production efficiency under stress conditions was also studied.

Materials and Methods

Sample collection and characterization

The Red mud samples were collected from 15 cm depth of red mud pond of Utkal Alumina, Rayagada, Odisha, India (19.1831^0 N, 83.0269^0 E), They were immediately kept in ice packs to bring to the laboratory and stored in the refrigerator maintained at 4 ^{0}C until use.

Isolation of Bacterial strain

One gram of red mud was suspended in 100 ml of PBS and serially diluted down to 10^{-6} dilutions. One ml from the last dilution was spread on Nutrient Agar (NA) medium (Tryptone 0.5 gm; yeast extract 0.3 gm, NaCl 0.5 gm; agar agar 1.6%, pH 7) and incubated at 32 ^{0}C. Colonies were observed every 24 hrs for 3 days.

Biochemical and Molecular characterization

Biochemical tests were done using Hi-Biochemical test kit (HiMedia, India) according to the instructions provided by the supplier. The tests included carbon source utilization, nitrogen source utilization, catalase, urease, oxidase, H_2S production, Methyl red test and motility test.

Genomic DNA was extracted following Cooper (2007) and quantified spectrophotometrically by recording absorbency at 260 nm (Sambrook and Russel, 2001). The 16S rDNA sequence was amplified by polymerase chain reaction using primer sets 27F(5′AGAGTTTGATCMTGGCTCAG) and 1492R (5′TACGGYTACCTTGTTACGACTT). PCR reaction mixture (50 µl) consisted of 25 µl of 2 x PCR Master mix (Aura Biotech, Chennai, India), 0.4µM primers (forward and reverse each), 50 ng template DNA, and water (remaining amount). The PCR reaction was performed in a thermocycler (HIMEDIA, India) with the following programme: Initial denaturation at 95^{0}C for 2 min., 30 cycles of denaturation at 95 ^{0}C for 45 sec., annealing at 48 ^{0}C for 90 sec., elongation at 72 ^{0}C for 60 sec., and a final extension at 72 ^{0}C for 7 min. The PCR product was separated by electrophoresis using 2% agarose gel containing 1:10000 SYBR safe stain (HiMedia, Mumbai) for visualization. The desired band was cut and purified using gel extraction kit (Genei, India). The purified DNA sample was sent for Sanger's dideoxy sequencing, service provided by BioServe Biotechnologies (India) Pvt. Ltd. The sequences (in.ab1 files) thus obtained, were edited to remove low-quality sequence stretch from the ends using Bio Edit version 7.2.5 (Hall, 1999). The consensus sequence was subjected to similarity search from GenBank using BLASTN programme (Altschul *et al*., 1990). The Phylogenetic analysis of 16S rDNA sequence was done using MEGA X (Kumar *et al*., 2018). The evolutionary distance matrix was calculated using the distance model of Jukes and Cantor (1969). The evolutionary tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with bootstrap analysis based on 1000 resamplings.

Growth Characteristics under various stress conditions

The isolate was grown on various salt concentrations, pH conditions and heavy metal stress condi-

tions such as arsenate (as sodium arsenate), lead (as lead tetra chloride), and chromium (Potassium dichromate). To observe growth on salt, the Nutrient Broth (NB) was amended with 2,5, and 8 % NaCl, individually. Effect of pH on growth was observed by growing the isolate in NB medium maintained at pH 5,7,9 and 11. Citrate Buffer (50mM) was used to maintain pH 5, Phosphate buffer (50mM) was used to maintain pH7 and carbonate buffer (50mM) was used to maintain pH9 and 11. Growth characteristics under metallic stress condition was observed by adding arsenate, lead, and chromium separately into NB medium to get desired final concentrations for each metal. The conical flasks inoculated with the isolate were kept at 32 ^{0}C for 24 h on a rotary shaker (Remi, India) operated at 120 rpm. Growth of the isolate was measured by recording optical density at 620 nm. The LD_{50} value, defined as the metal concentration that inhibits 50 percent bacterial growth in comparison to control, was calculated from the linear graph plotted between per centages of growth against metal concentration as per El-Deeb and Al-Sheri (2005).

Pigment extraction and characterization

Bacterial pigment was isolated and degree of pigment production was determined following the method of Sasidharan *et al*., 2013. The isolate was grown in NB medium and kept on a rotary shaker for 24 hours. Cells were collected by centrifuging the cultures at 4280xg for 5 minutes followed by washing with sterile normal saline for three times. The pellet was then resuspended in 5 ml of 90% methanol and incubated in water bath maintained at 55^0 C for 15 minutes. The extract was again centrifuged at 4280xg for 5 minutes to collect the methanolic extract of pigment.

The extracted pigment was subjected to UV-visible scanning in a UV-Vis spectrophotometer (Systronics 110, India) to observe the absorption maxima of the pigments. The scanning range was selected from 300 nm to 800 nm.

The methanol extract of yellow pigment from the isolate was directly subjected to FTIR (Fourier Transformation Infrared Spectroscopy). A volume of 10 µl was placed in the diamond window of the spectroscope and scanned with a resolution of 4 cm^{-1} ranging from 400 to 4000 cm^{-1}.

Effect of abiotic stress on pigment production

The effect of pH, NaCl and metal stress conditions on the production of pigment was studied by growing the strains in NB medium under different conditions. The pigment production under these conditions was determined by measuring the degree of pigment production in the absence of molar coefficient value for the particular pigment. This was calculated as Degree of pigment production = A_{466} value/A_{620} value.

Free radical scavenging activity and antimicrobial activity

The free radical scavenging activity of the pigment was measured by using DPPH (1-Diphenyl-2-picrylhydrazil) assay following the method used by Pan *et al*. (2018). Pigment was extracted using 90% methanol and degree of pigment production was measured per unit of cell growth (A_{466}/A_{620}). The value was adjusted to 0.05, 0.15 and 0.25 by adding required amount of 90% methanol gradually followed by spectrophoto metric measurements. Two ml of DPPH solution (0.1mM in methanol) was added to 1 ml of pigment extract. The mixture was incubated in the dark for 30 minutes and centrifuged at 2750xg rpm for 5 minutes. The absorbance of the supernatant was measured at 517 nm in a UV-Visible spectrophotometer (Systronics, India). The value was denoted as A_{517} sample. Reagent Blank (A_{517} blank) was prepared by adding 1 ml of distilled water to 2 ml of DPPH solution. Reagent Control (A_{517} blank) was prepared by adding 2 ml of ethanol to 1 ml of pigment extract. DPPH scavenging activity was measured by the following equation.

DPPH scavenging rate (%) = {1-(A_{517}sample - A_{517}control)/A_{517} Blank} × 100

Antimicrobial activity of the pigment was studied following Panigrahi *et al*., 2018**.** Six pathogens namely, *Escherichiacoli* MTCC 723, *Pseudomonasaureus* MTCC 741, *Vibrio cholerae* MTCC 3906, *Staphylococcus aureus* MTCC 902, *Klebsiella pneumoniae* MTCC109 and *Enterococcus cloacae* MTCC 7322 were used. Holes were made in the agar plates by using sterilized tips, and 50 µl methanolic extract (A_{466}=0.5) was put in those holes to check the antimicrobial activity. Methanol (50µl) was used as control.

Statistical data analysis

Statistical analysis of all data was done using Microsoft Excel 2010. All the values are presented as mean ± standard deviation of three replicates. Significance differences between the means were calcu-

lated by One-Way Anova Test.

Results and Discussion

Sample and Microbial Characterization

The Red mud or bauxite residues were collected in the month of November 2020. The moisture content was determined to be 74%. The pH of the soil was measured to be 11.8 ± 0.8.

Soil suspension (1% w/v) was serially diluted and spread on NA medium. Five different colonies could be distinguished according to the colony morphology. A bright yellow coloured bacterial colony was chosen for this study (Fig. 1). Morphologically the colony is round, small, convex, entire, smooth, yellow and opaque.

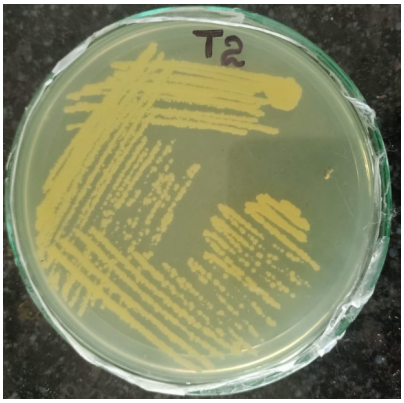

Fig. 1. Pure culture of *Glutamicibacter* sp. strain OURIIP2

Different phenotypical and biochemical properties of the isolate were studied (Table 1). The isolates showed a considerable growth on medium containing glucose, dextrose, maltose, and galactosebut not on other carbohydrates.

Molecular identification of the isolates was done through amplified 16S rDNA sequence analysis followed by phylogenetic analysis using MEGA X software. The16S rDNAsequence showed a maximum similarity of 99.7% with that of *Glutamicibacter halophytocola* strain KLBNP 580 and G. *mysorens* strain LMG16219 in Blast N analysis. Phylogenetically, the query sequence made a separate clade with G.*mysorens* LMG 16219 and G. *mysorens* DSM 12798 with a bootstrap value of 94 (Fig. 2). The strain was thus named *Glutamicibacter* sp. strain OURIIP2 and nucleotide sequence was submitted in NCBI under accession No. MW722790.Presence of *Glutamicibacter* in red mud has not been reported

Table 1. Phenotypic and physiological characteristics of *Glutamicibacter* sp. strain OURIIP 2

Characteristics	Result
Colony Characters	Round, small, convex. entire, smooth, opaque and yellow
Gram reaction	Gram +ve Coccus
Cell shape	
Motility	+
Carbon source	
Glucose, Dextrose, Maltose, Galactose, Lactose, Mannitol, Oxalate, Citrate, Oxalate,	+
Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Sorbitol	-
Nitrogen source	
Lysine, Ornithine, Urea, Nitrate	+
Other Biochemical tests	
Oxidase, Catalase, Methyl red, H_2S production	+

earlier. Krishna *et al*. (2014) have reported abundance of Gram +ve bacteria in red mud ponds of National Aluminium Company (NALCO), Damanjodi, Odisha, India.Many species of *Glutamicibacter* have been included under *Arthrobacter* (Busse, 2016).Lee *et al*., 2003 have reported isolation of A.*koreensis* from alkaline soil. Chen *et al*., 2009 have isolated A. *halodurans* sp. from sea water. Qin *et al*., 2018 have isolated 15 salt tolerantand plant growth promoting *Glutamicibacter* from halophyte *Limonium.* These reports suggest the alkali tolerant nature of *Glutamicibacter*.

Effect of stress on bacterial growth

The isolate was grown under various stress conditions such as pH, salt and heavy metals (Table 2). It could grow luxuriously on pH 5, 9 and 11 and the growth did not show significant differences at p=0.05 level as compared to control (pH 7). No significant differences at p=0.05 level could be found in growth of the isolate under 2%,5% and 8% salt concentrations as compared to the control. The LD_{50} values of As, Pb and Cr for the isolate were calculated to be 5.44mM, 6.68 mM and 1.57mM, respectively (Table 3). The red mud ponds are highly contaminated sites with higher levels of heavy metal concentrations (Santini *et al*., 2015). Presence of these metals force to develop resistance in the inhabiting

Table 2. Growth of *Glutamicibacter* sp. OURIIP2 under various pH and salt concentrations.

pH conditions	Cell Growth(A_{620})	NaCl Conc.	Cell Growth(A_{620})
pH7(Control)	1.83±0.04	0.5% (Control)	1.83±0.04
pH5	1.89±0.05	2%	1.72±0.6
pH9	1.80±0.06	5%	1.71±0.3
pH11	1.54±0.2	8%	0.88±0.12

Table 3. Percentage of growth and LD_{50} values of *Glutamicibacter* sp. OURIIP2 under various heavy metal concentrations.

Strains	Concentration (mM)	Percentage Growth	LD_{50} value (mM)
	0	100	
	1	91.80	
Arsenic	2	77.04	5.44
	5	49.18	
	10	12.56	
	0	100	
	1	83.60	
Lead	2	62.84	6.68
	5	49.72	
	10	38.79	
	0	100	
	0.5	83.88	
Chromium	1	54.75	1.57
	2	33.98	
	3	15.53	

microbes. Chromium and arsenic tolerance in *Arthrobacter* have been reported by many authors (Megharaj *et al*., 2003, Prasad *et al*., 2013, Sanjay *et al*., 2020).

Pigment production and characterization

The pigment analysis was done by extracting the bacterial pigments in methanol. The absorption maxima (A_{max}) for yellow pigment were found tobe at 436 nm and 466 nm. The range of absorbency lies within the range of yellow coloured carotenoid pigments (Sobin and Stahly, 1942; Sutthiwong *et al*., 2014). The FTIR spectrum of crude yellow pigment is presented in Fig3. The prominent peaks at 3308.49 (OH group of methanol) 2943.25 cm^{-1}(asymmetric stretch of CH_3), 2832.19 cm^{-1}(symmetric stretch of CH_2), 1449.31 $cm^{-1}$and 1416.12 cm^{-1}(â ionone rings), 1115.26 cm^{-1} (C-C stretching and C-H plane bending), 1020.24cm^{-1} (methyl rocking vibration) are in

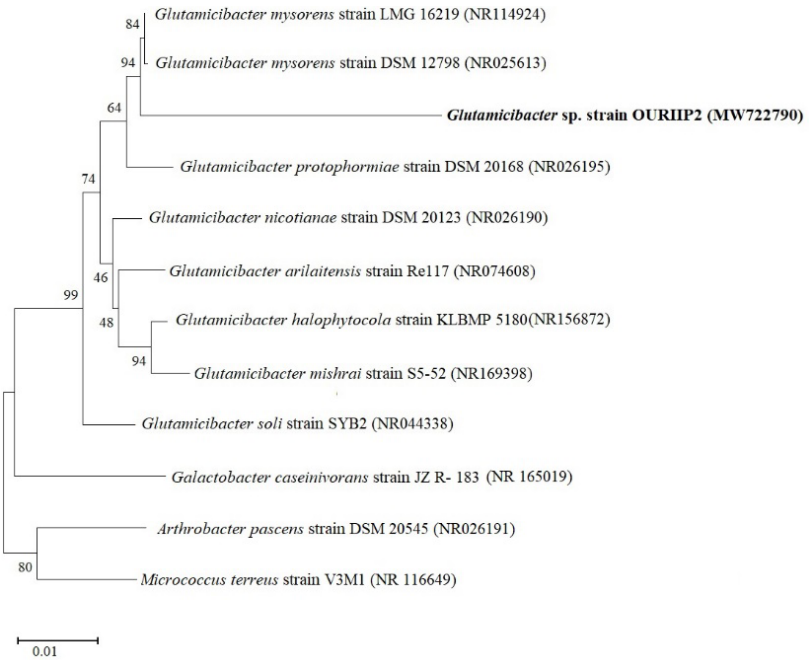

Fig. 2. Phylogenetic tree based on partial 16S rDNA sequence of *Glutamicibacter*sp. strain OURIIP2. Numbers in the parenthesis are GenBank accession numbers. Numbers at branch point indicate the bootstrap values. The scale bar indicates substitution per site.

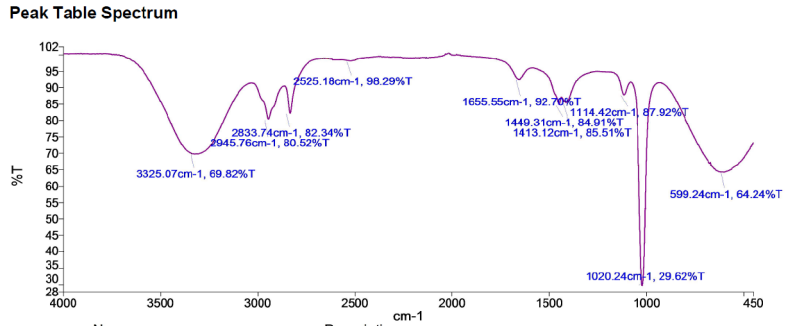

Fig. 3. FTIR analysis of extracted yellow pigment from *Glutamicibacter* sp. strain OURIIP2.

accordance with β carotene (Marshel *et al.*, 1998; Trivedi *et al.*, 2017).

Degree of pigment production

The degree of pigment production under various pH, salt and heavy metal conditions were studied. It increased significantly with increase in pH of the medium (Fig.4). Pigment production increased significantly and reached the highest at 8% NaCl concentration (F=63.18, P=0.0013) (Fig. 5). The degree of pigment production also showed significant increase as compared to control under metallic stress conditions. Pigment production was found to be maximum and significantly increased by 3.29 times (F=727.13, P=0.00001) when grown in NB medium supplemented with 1.0mM Chromium (Fig. 6). This suggests that the higher level of pigment production may be helping the strain to scavenge the free radicals.

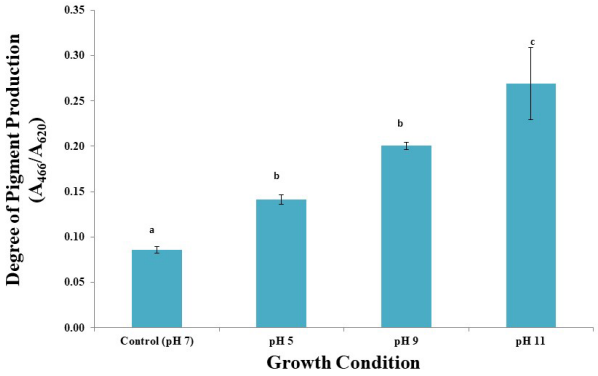

Fig. 4. Degree of pigment production by *Glutamicibacter* sp. strain OURIIP2 under various pH conditions. Different letters indicate significant difference (p≤0.05). Statistical analysis is based on three replicates of each.

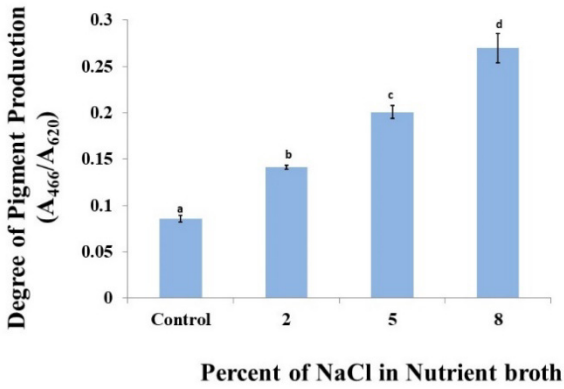

Fig. 5. Degree of pigment production by *Glutamicibacter* sp. Strain OURIIP2 under various salt (NaCl) stress conditions. Different letters indicate significant difference (p≤0.05). Statistical analysis is based on three replicates of each.

Free radical scavenging activity (DPPH Assay)

Free radical scavenging activity of the pigment was studied using DPPH assay (Fig. 7). The free radical scavenging activity increased significantly with increase in pigment concentration. Carotenoid pigments do show good free radical scavenging activity due to presence of the beta ionone rings, and the non- polar conjugated carbon-carbon double bonds (Galasso *et al.*, 2017). Our report is in accordance with Afra *et al.*, 2017, who have isolated carotenoid compound from *Arthrobacter* sp. G20 and reported the EC (50) value of DPPH scavenging activity to beof 4.5mg/ml.

Antimicrobial activity of the pigment

Antimicrobial activity of methanolic extract was studied against 6 pathogenic bacterial strains out of which the pigment showed effect only on *E.coli* strain MTCC723 only (Fig. 8). Other strainswere not

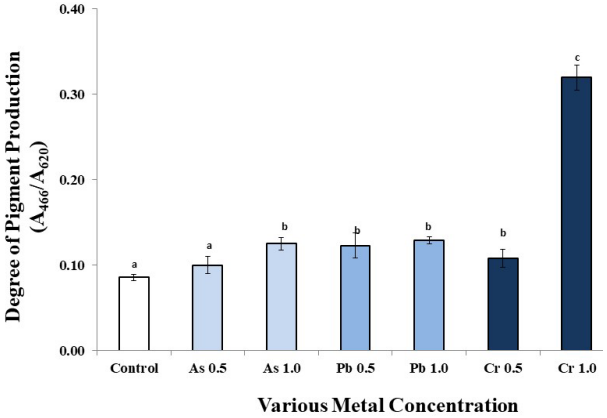

Fig. 6. Degree of pigment production by *Glutamicibacter* sp. Strain OURIIP2 under various metal stress conditions. Different letters indicate significant difference (p≤0.05). Statistical analysis is based on three replicates of each.

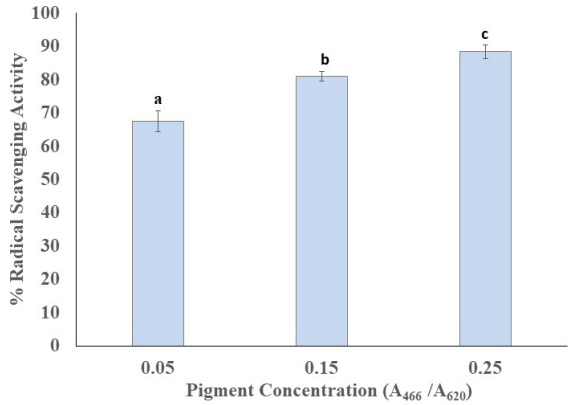

Fig. 7. DPPH scavenging activity of yellow pigment obtained from *Glutamicibacter* sp. strain OURIIP2. Different letters indicate statistically significant differences (p≤0.05). Statistical analysis is based on three replicates of each.

affected by the pigment. The results indicate a poor antimicrobial activity of the pigment against pathogens. Several authors have reported antimicrobial activity of beta carotene at higher concentrations (Ravikumar *et al.*, 2016; Keceli *et al.*, 2013; Boontosaeng *et al.*, 2016) in contrast to the present study. This may be due to absence of such pathogens in the red mud and the isomeric form of pigment may be playing a specific role of protection under stress.

Conclusion

Glutamicibacter sp. strain OURIIP2 is a potential strain isolated from red mud ponds from Utkal Alumina Rayagada, Odisha, India. The strain is alkalitolerant, salt tolerant, heavy metal tolerant and carotogenic. It produces a beta carotene pigment that is stable under a varied range of pH, and salt concentrations. It may find application in cosmetic and pharmaceutical industry. Tolerance of the strain to arsenic, chromium and lead may be useful in bioremediation studies.

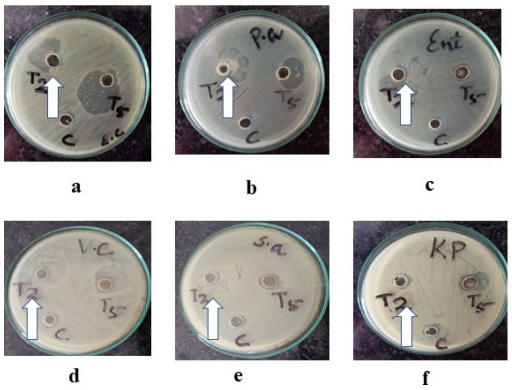

Fig. 8. Antimicrobial activity of yellow pigment obtained from *Glutamicibacter* sp. strain OURIIP2. Pathogens used were (a) *Escherichia coli* MTCC 723 (b) *Pseudomonas aeruginosa* MTCC 741 (c) *Enterobacter cloacae* MTCC 7322 (d) *Vibriocholerae* MTCC 3906 (e) *Staphylococcus aureus* MTCC 902 (f)*Klebsiella pneumoniae* MTCC109 (g) *Bacillus subtilis* (MTCC 121).

Conflict of Interest

The authors declare there are no conflicts of interest.

Acknowledgements

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Data availability

All datasets generated or analysed during this study are included in the Manuscript and /or Supplementary Files. The 16S rDNA sequence is available in the NCBI website.

Ethics Statement

This article does not contain any studies with hu-

man participants or animals performed by any author.

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