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BIOREMEDIATION OF CHROMIUM (VI) BY A MICROBIAL CONSORTIUM ISOLATED FROM TANNERY EFFLUENTS AND THEIR POTENTIAL INDUSTRIAL APPLICATION

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Highlights

- Four distinct isolates obtained from tannery effluents were biochemically characterized.
- All the isolates not only reduces chromium from its hexavalent to trivalent form but also accumulates it, with IS1 exhibiting the highest reduction capability.
- The isolates showed promising protein, leather, and lipid degradation ability, with IS4 demonstrating the highest efficiency.
- All the the isolates has been found to produce keratinase but maximal enzyme production was achieved by IS1 at pH 8 and 40 °C temperature.
- Thus, due to the promising multifaceted activities of IS1 and IS4, they were characterized by 16S rRNA sequencing followed by BLAST analysis where they corresponded to *Bacillus cereus* F4810/72, and *Brevibacillus brevis* US575 respectively, with high sequence similarity.
- Hence, IS1 and IS4 can be employed in the successful bioremediation of wastes from various sources like tanneries, agricultural industries, and poultries in a cost-effective and environment friendly manner.

Abstract. West Bengal has several leather industries and as such huge amount of leather are processed every year. The tannery effluents are discharged into the land and open water causing soil and water pollution respectively. Chromium is one of the most toxic inorganic contaminants which is well known for its carcinogenicity. Thus, our study focuses on investigating the bioremediation potential of common microflora isolated from tannery wastewater. In our study, Isolate 1 has the highest ability to reduce chromium (Cr^{6+}) as compared to others. Isolate 4 has the highest protease, lipase and leather degradation activities. Isolate 1 shows the maximum keratinase activity making it an effective strain for keratinase production. Also, it has been found that pH 8 and temperature 40 °C was most suitable for keratinase production. Owing to the multidimensional ability of these two isolates, they were identified by 16S rRNA sequencing and it reveals that Isolate 1 and Isolate 4 belong to *Bacillus cereus* F4810/72 and *Brevibacillus brevis* F4810/72 respectively. Thus, this study establishes the role and efficiencies of these microorganisms in combatting pollution, particularly in the water bodies in which harmful chemicals leak regularly owing to improper waste management by various industries.

Keywords: effluent, groundwater, chromium, tannery, bioremediation, keratinase.

Introduction

Air-water-land, the three essential components of the environment is being rapidly contaminated gradually owing to rapid urbanization, a massive increase in population density, and developing industrialization (Saranraj & Sujitha, 2013). This eventually leads to the accumulation of heavy metals (Benazir et al., 2010), especially chromium

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and lead that poses a significant risk to the health of the society (Igiri et al., 2018). When the toxic contaminants mobilize into the environment, they tend to persist, circulate, and ultimately accumulate in different levels of the food chain destroying the ecological balance (Garg et al., 2012). The Tannery industry is considered to be a major source of pollution and tannery wastewater has become a potential environmental concern nowadays as it contains significant concentrations of toxic inorganic contaminants like chromium, dissolved organic matter, and salts (Sharma & Malaviya, 2014; Bharagava et al., 2018).

Chromium is one of the most toxic hazardous waste found in tannery effluents (Bharagava et al., 2018). Different anthropogenic activities in tannery industries have led to excessive contamination of Chromium in the soil as well as water bodies and also have increased its bioavailability and bio-mobility as well (Raman et al., 2018). Chromium is a refractory metal that mainly exhibits two inorganic states namely trivalent Cr (III)) and hexavalent Cr (VI)). These are considered the most stable oxidation states found in the environment that differ greatly in physical, chemical and biological activities (Sanjay et al., 2020). The hexavalent state of chromium has largely been accepted to be toxic, carcinogenic, and mutagenic due to its high solubility in water, interaction with cellular proteins, and biological membrane permeability (Smith et al., 2002) while trivalent chromium is less water-soluble and less mobile. Plants exposed to environmental chromium cause oxidative metabolic stress (Srinath et al., 2001) that leads to the production of active free radicals. Such active radicals play a crucial role in modulating various signal transduction pathways that ultimately lead to the distortion of cellular membranes and organelles in plants (Oliveira, 2012).

In India, an appreciable concentration of chromium has been found in soil and groundwater samples, especially in the states of Tamil Nadu, Maharashtra, West Bengal places (Kumar & Riyazuddin, 2010; Gupta et al., 2011). Around 40% of the 69,000 tons of chromate products used in tanneries have been discharged to water bodies which can cause carcinogenicity upon coming in contact with humans (Iyer et al., 2006). Thus, effective treatment of chromium is an absolute need of the hour in terms of environmental protection management as well as sustainable development. Although treatment of tannery wastewater is carried out by different physicochemical methods like diffusion, reverse osmosis, sedimentation, etc. yet biological treatment of wastewater is much more favorable and cost-effective as compared to others (Mohanty & Patra, 2011). Bioremediation offers the possibility of using living organisms (bacteria, fungi, algae, or plants), but primarily microorganisms, to degrade or remove environmental contaminants and transform them into nontoxic or less-toxic forms. It involves different strategies that include biosorption, bioaccumulation, bioreduction, and immobilization of biomaterial(s). Simplistically speaking, microbes employ a variety of biological and chemical processes to reduce chromium at neutral pHs (Garg et al., 2012). Taking all these facts into consideration, our investigation deals with the characterization of the tannery effluents and the ability of microorganisms to biodegrade the components present in the effluent that is responsible for bioremediation. This method will not only be cost-effective but will also be highly efficient along with being environmentally friendly and will require little to low maintenance to keep it operational for a long duration of time.

1. Materials and methods

1.1. Water sample procurement

Water samples were collected aseptically from the Bantala Leather Complex in the district of South 24 Parganas, West Bengal from 5 cm below the surface to eradicate contamination from the ground surface and was then subjected to further analysis as detailed below.

1.2. Physicochemical characterization of the sample

The physical properties (colour, texture, and odour) of the collected water sample were duly noted. Besides, the pH, as well as the electrical conductivity of the sample, was determined using protocols which have been discussed previously (Jahan et al., 2015). Certain other critical parameters such as the inorganic phosphate (Pi) content, heavy metal (lead and chromium) content, and the total organic carbon content (TOC) of the sample were measured as described elsewhere (De La Luz-Pedro et al., 2019; Jahan et al., 2015; Ates et al., 1997).

1.3. Isolation and characterization of microbes present in the sample

The water sample obtained from the tannery was serially diluted to obtain four different dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. 0.1 ml of each of these diluted samples were inoculated separately on freshly-prepared nutrient agar plates, which were then incubated overnight at 37 °C. Four distinct colonies with distinct morphologies were identified in the plate inoculated with the 10^{-4} diluted sample. These four colonies were picked up and sub-cultured separately to obtain pure cultures which were named as Isolate 1 (IS1), Isolate 2 (IS2), Isolate 3 (IS3), and Isolate 4 (IS4) respectively. The Gram characteristics and the Motility test of these four isolates were individually determined using standardized protocols as described elsewhere (Trivedi et al., 2015; Adler & Dahl, 1967). Further, catalase and oxidase tests were also performed as outlined previously (Reiner, 2010; Shields & Cathcart, 2010). Finally, the isolates showing promising activity in terms of chromium reduction, leather and protein degradation, and keratinase production were identified using 16S rRNA sequencing, the details of which have been provided in section 1.9.

1.4. Chromium reduction assay

DPC or Diphenylcarbazide assay was used to determine the chromium reduction ability of the four isolates as mentioned previously (Leung et al., 2018). This assay

estimates the reduction of hexavalent Chromium (Cr^{6+}) to trivalent chromium (Cr^{3+}) which is indicated by a colour change in presence of the indicator DPC. For the assay, the chromate solution was prepared in nutrient broth at different concentrations of $K_2Cr_2O_7$ viz. 0.01 ppm, 0.1 ppm, 1 ppm, 10 ppm, and 100 ppm. The pHs of all these solutions was adjusted to 1 by the addition of 2M concentrated sulphuric acid followed by the addition of 2 drops of DPC. This immediately resulted in a colour change that showed different intensities of the colour at different concentrations of Chromium as confirmed by absorbance measurement at 590 nm (Elangovan et al., 2010). A standard curve was plotted accordingly taking the log of the chromium concentration along with their respective absorbance or O.D. (optical density) values at 590 nm.

To determine the actual chromium reduction by the isolates, they were first grown for 4 days in nutrient broth containing 50 ppm of chromium (VI). Later, they were centrifuged for 15 minutes at 5000 rpm and the supernatant was collected. The pellets, on the other hand, were resuspended in sterile water followed by another round of centrifugation at 5000 rpm for 15 minutes. The supernatants were collected and adjusted to a pH of 1 after which 2 drops of DPC were added to each of them. The colour change was observed and the absorbance at 590 nm was spectrophotometrically determined. Utilizing the standard curve, the absorbance values were converted to ppm values and thus, the actual amount of chromium reduction in ppm was calculated.

1.5. Leather degradation assay

The weights of the leather pieces (1 mm^3) were recorded after drying them in a hot air oven, after which they were sterilized by UV radiation (Pandi et al., 2019; Chatterjee et al., 2017). The four isolates were inoculated in nutrient broth media separately along with 1 piece of dried and sterilized leather. After one week of incubation at 37 °C, the leather pieces were removed from the media, dried, and weighed to determine the weight loss due to degradation by the isolates.

Since leather degradation is facilitated by certain enzymes such as lipase, keratinase, and protease, appropriate assays were done to determine the individual activity of these enzymes.

1.6. Lipid degradation assay

A lipid degradation assay was performed to determine the lipase activity of the isolates. Isolates incubated in nutrient broth medium were cultured at 37 °C for 2 days followed by centrifugation at 5000 rpm for 15 minutes. The supernatants were collected and used as crude enzymes to study the lipid degradation effect. Vanillin was used as the lipid and a 0.08 M solution of it was prepared which was used for all further experiments (Wang et al., 2014). Three setups were prepared. First, the control had 1 ml of 0.08 M vanillin, 1 ml of water, and 0.1 ml of concentrated H_2SO_4. Second, the blank had 2 ml of water along with 0.1 ml of concentrated H_2SO_4 and the experimental setups had 1 ml of the crude enzyme from one of the four isolates along with 1 ml of 0.08 M vanillin and 0.1 ml of concentrated H_2SO_4. To determine the amount of vanillin or lipid degraded, the optical density of each tube was measured at 490 nm after a few minutes using a spectrophotometer (Wang et al., 2016).

1.7. Protein degradation assay

Two-days old cultures of the isolates incubated at 37 °C were centrifuged at 5000 rpm for 15 minutes and the supernatant was used as the crude enzyme to test its protein degradation ability. Accordingly, bovine serum albumin (BSA) was used as the substrate. Four experimental sets were prepared – each of them had 1 ml of 1mg/ml BSA along with 1ml of the crude enzyme of any one of the four isolates. 4 ml of alkaline copper sulphate was added and the reaction was allowed to proceed for 10 minutes. Then, 0.5 ml of Folin-Ciocalteau reagent was added and finally, after 30 minutes, 0.5 ml of NaOH was added to stop the reaction, followed by absorbance measurement at 660 nm as per the standardized protocol as described elsewhere (Pescuma et al., 2012; Hess et al., 1978; Waterborg & Matthews, 1984). NaOH was added to prevent errors while absorbance measurement due to differential incubation times. Standard setups were also prepared with varying concentrations of BSA which were treated in the same fashion as the experimental sets except 1 ml of sterile water was used instead of crude enzyme. The absorbance values thus obtained were these setups used to plot the standard curve. The amount of BSA degraded by the isolates was derived from this standard curve using their respective absorbance readings. Finally, the amount of BSA degraded was divided by the total reaction time (40 minutes) to calculate the amount of BSA degraded per minute.

1.8. Keratinase assay and its optimization

Initially, the keratinase assay involves the preparation of the feather meal which was prepared by cutting chicken feathers into 3–4 cm pieces and washing them with sterile water. Chloroform-methanol solution in the ration of 1:1 was prepared in which the cut pieces of the feathers were soaked for 2 days followed by soaking in a 4:1:3 chloroform-acetone-methanol mixture for another 2 days to defat them. The solvents were recycled each day, and the feathers were finally washed several times with sterile water to remove solvent traces followed by drying at 60 °C and grinding to prepare the feather meal as described previously (Savita & Archana, 2014).

The media for submerged fermentation was prepared by combining 1% feather meal, 0.05% NaCl, 0.04% K_2HPO_4, 0.03% KH_2PO4, 0.01% $MgCl_2$, and 0.01% yeast extract and adjusted to a pH of 8. The isolates were inoculated into this media and incubated for 3 days at 37 °C under shaking conditions at 180 rpm (Savita & Archana, 2014).

This three-day-old culture was centrifuged at 10 000 rpm for 10 minutes and the supernatant was collected and used as the crude enzyme in further experiments. 50 mM Tris-HCl buffer of pH 8.0 was used to suspend 5 mg keratin (from the feather meal) which also acted as a substrate for the keratinase assay. 1 ml of this keratin suspension was added to 1 ml of the crude enzyme from one of the four isolates and then incubated at 50 °C for an hour. 2 ml of 1 M NaOH was finally added to stop the reactions and the substrate was then removed by filtration. The absorbance of the filtrate at 595 nm was determined (El-Refai et al., 2005; Jin et al., 2017). Finally, the amount of enzyme causing an increase in the absorbance at 595 nm by 0.01 of the sample as compared to the control after 1 hour of reaction at 50 °C was considered as one unit (U) of keratinase activity in accordance with a previous report (Savita & Archana, 2014).

Lastly, the optimal conditions with respect to pH and temperature for the keratinase production by the isolates were determined. To establish the optimal pH, the pH of the fermentation media was varied from 6 to 10 and the keratinase activity was measured after 3 days of incubation as described above. To identify the most effective temperature, the incubation of the isolates in the fermentation media was performed separately at 37 °C, 40 °C, or 45 °C.

1.9. Isolation of genomic DNA from bacteria

Owing to the multidimensional abilities in context to their high chromium reduction, keratinase, protease, and leather-degrading ability, the isolates 1 and 4 (designated as IS1 and IS4) were identified using 16S rRNA sequencing (Woo et al., 2008). For this, the genomic DNA of freshly cultured isolates 1 and 4 were isolated by CTAB/NaCl method as described elsewhere (Park, 2007; Ausubel, 1994). Briefly, 1.5 ml of bacterial culture was centrifuged for 5 minutes followed by resuspending the cell pellet in 600 µl of TE buffer. After that 30 µl and 5 µl of 10% SDS and 20 mg/ml Proteinase K were added respectively. After incubating the solution for approximately 1 h 30 mins, 100 µl of 5 M NaCl and 70 µl of CTAB/NaCl solution were added for the removal of cell wall debris, denatured proteins, polysaccharides, and other undesirable contaminants. The aqueous phase was extracted by adding an equal volume of Phenol, Chloroform, and Isoamyl alcohol in the ratio of 25:24:1. Finally, the chromosomal DNA was precipitated using isopropanol followed by centrifugation at maximum speed for 10 minutes. The DNA pellet was washed with 70% ethanol and finally was dissolved in 40 µl of TE buffer (Mukherjee et al., 2011).

1.10. Molecular identification of the isolates

For the molecular identification of our selected isolates (Isolate 1 and Isolate 4), the 16S rRNA gene was amplified via Polymerase Chain reaction (PCR) and sequenced using the primer sets F-73, 5'-AGAGTTTGATCCTGGCTCAG-3', and reverse primer, R-74, 5'-AAGGAGGTGATCCAAGCC-3'. The PCR fragments were further resolved on a 1.5% agarose gel followed by electrophoresis in 1X TAE buffer at 100 V for 40 minutes and were then subsequently visualized after staining with ethidium bromide. Finally, these amplified 16S rRNA fragments were purified and sequenced using Sanger sequencing (Chen et al., 2014) and were exported into BLAST (Basic Local Alignment Search Tool) provided by the NCBI (National Centre for Biotechnology Information) for identification of matches using characterized references sequences provided in the database. This provided useful information about the phylogenetic origin of these isolates and provided alignment scores which were then used to determine the identity of these isolates. The obtained DNA sequence was subjected to DNA and Noise editing using the Bioinformatics software Bio Edit version 7.5 (Sanjay et al., 2020). The 16S rRNA partial sequences were multiple aligned using the MUSCLE tool and subsequently, a phylogenetic tree was constructed through Neighbour Joining (NJ) method in Mega 7.0 (Kumari et al., 2016) keeping parameters default and the bootstrap value was kept 1000 to maximize the nodal supports.

2. Results

2.1. Physicochemical parameters of the sample

The various physicochemical parameters of the wastewater sample collected from the tannery have been listed in Table 1. The colour, texture, and odour of the sample along with some important chemical properties such as pH, electrical conductivity, inorganic phosphate content, heavy metal content (lead and chromium) were measured accordingly.

Table 1. Physicochemical parameters of the sample

Parameter	Observation
Colour	Yellowish-brown
Texture	Coarse with deteriorated leather particles
Odour	Extremely foul
pH	9±1.2
Electrical conductivity	17.66 Siemens
Inorganic phosphate (Pi)	15.7 mg/L
Lead	0.19 mg/L
Chromium	9.7 mg/L
Total organic carbon	1852.2 mg/L

2.2. Morphological and biochemical characterization of the isolates

4 distinct colonies with distinct morphological features were selected from the Nutrient agar plates and from the pure cultures. After that, their Gram characterization and biochemical characterization were observed and were summarized (Appendix Table 1A). The selected isolates were designated as IS1 – IS4.

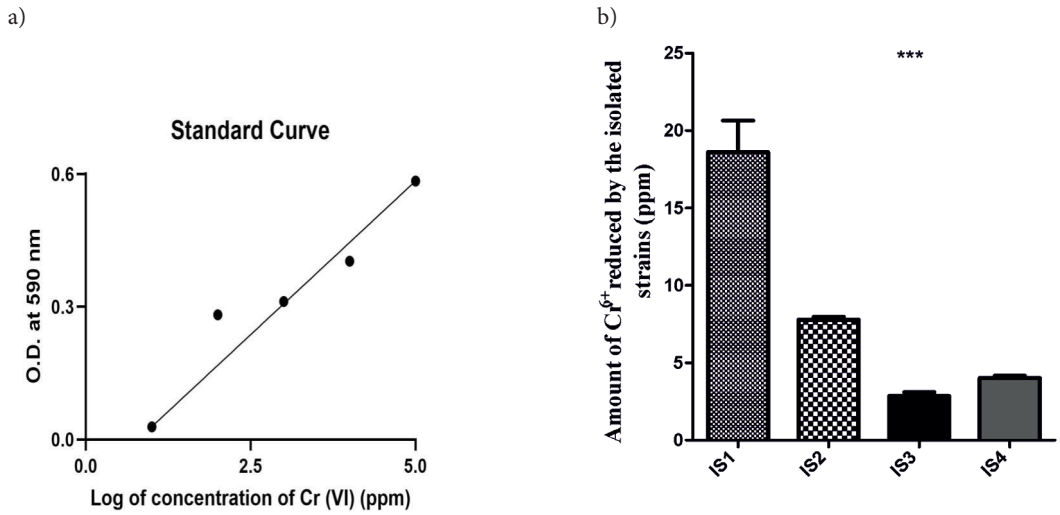

Figure 1. Observation of the amount of Chromium reduced by the isolates: a) Standard curve for determining the amount of Chromium reduced by the isolates by plotting the logarithmic value of Chromium concentrations at X-axis against O.D of the test culture conditions at Y-axis; b) Graphical representation showing the amount of Chromium reduced by the isolates. Y-axis indicates the amount of Cr^{6+} reduced to Cr^{3+} in ppm and the X-axis denotes the test isolates. Data (n = 3) illustrated in the bar diagram as mean ±SD. ***$p < 0.0001$ as determined by t-test (IS1 to IS4 represents the isolates)

2.3. Estimation of the amount of Chromium reduced by the tannery effluent isolated bacteria

The "Chromium Reductase Assay" is considered as one of the most important bioremediation strategy as it indicates the amount of Chromium reduction (Cr^{6+} to Cr^{3+}) by the microorganisms. 50 ppm Chromium that was present initially in the medium was reduced by the isolated strains as illustrated by graphical representations. Our results show that Isolate 1 (IS1) has the highest Cr reducing ability as compared to the other strains.

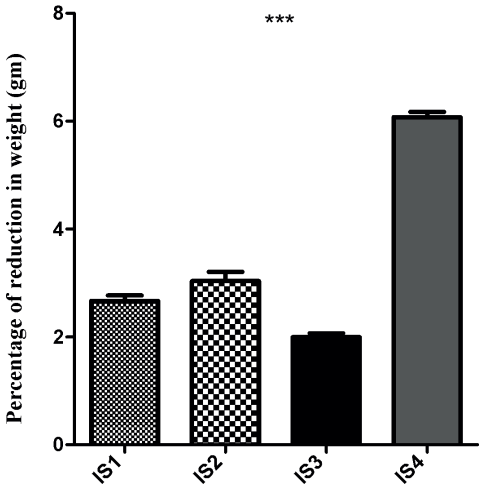

Figure 2. Observation of the amount of leather degraded by the isolates. Graphical representation showing the leather degrading activity of the isolates. Y-axis indicates the percentage of reduction in weight of the leather in gram (gm) and the X-axis denotes the test isolates. Data (n = 3) illustrated in the bar diagram as mean ±SD. ***$p < 0.0001$ as determined by One-way ANOVA (IS1 to IS4 represents the isolates)

2.4. The Isolates are potent leather degraders

Our results indicated that Isolate 4 (IS4) has the maximum ability to degrade leather making it a promising as well as an efficient strain for leather degradation.

2.5. The Isolates have the ability to degrade lipid and protein molecules

The "Lipase Assay" was performed to evaluate and estimate quantitatively the rate of lipid degradation through the production of industrially important lipase enzyme. Our study indicates that Isolate 4 has the highest lipase activity as estimated by the amount of the degraded substrate per ml of crude enzyme.

On the other hand, the Protease assay indicates the amount of protein degraded by the microorganisms. Isolate 4 shows the maximum protein degrading ability through the production of commercially important protease enzyme as determined by the amount of protease degraded per minute.

2.6. Keratinase activity and Optimization of culture conditions for keratinase production

Keratin plays a pivotal role in providing tensile strength and resistance to the cells. All the strains isolated from tannery effluents were found to produce Keratinase as estimated quantitatively. The strains were grown on a medium supplemented with feathers that act as an important source of carbon and nitrogen. The maximum keratinase activity as shown by Isolate 4 (IS4) was 36U/ml at a pH of 8. Maximum keratinase production of 52 U/ml was observed at a temperature of 40 °C which was considered as an optimum temperature for keratinase production after an incubation period of 4 days.

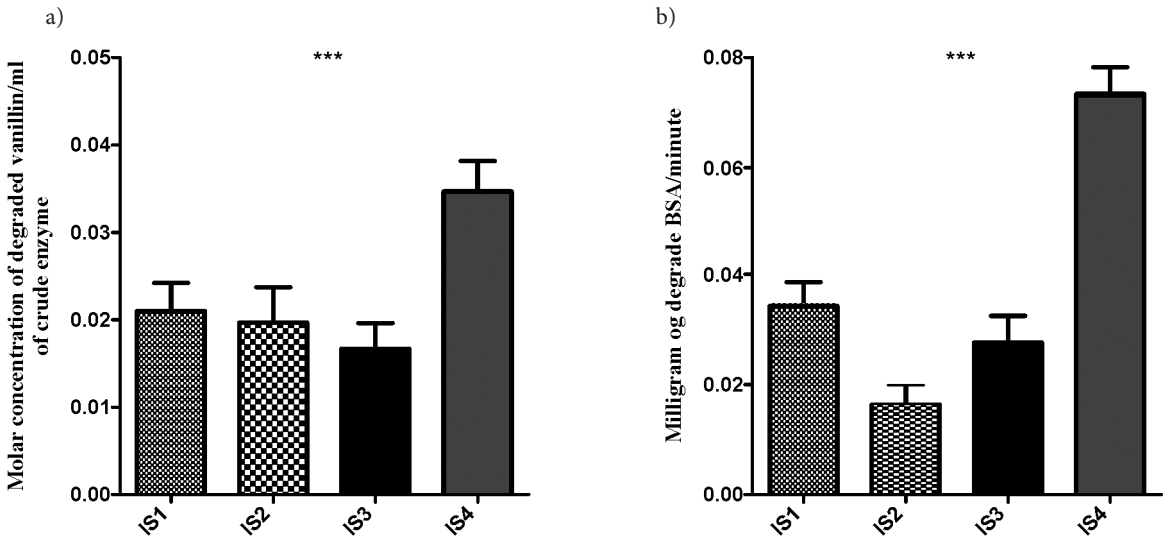

Figure 3. Observation of lipolytic and proteolytic activities of the isolates: a) Graphical representation showing the amount of lipid degraded activity by the microorganisms which was determined by the concentration of the vanillin degraded per ml of the crude enzyme. Y-axis indicates the molar concentration of degraded substrate per ml of enzyme and the X-axis denotes the test isolates; b) Graphical representation showing the protease activity by the microorganisms which were determined by milligram of degraded BSA per minute. Y-axis indicates the amount of degraded substrate per minute and the X-axis denotes the test isolates. All the data (n = 3) are illustrated in the bar diagram as mean ±SD. ***p < 0.0001 as determined by One-way ANOVA (IS1 to IS4 represents the isolates; BSA: Bovine Serum Albumin)

2.7. Identification of Isolate 1 (IS1) and Isolate 4 (IS4) through 16s rRNA

Analysis followed by sequencing of the PCR amplicon of 16s rRNA and its alignment in BLAST programme showed that Isolate 1 was ***Bacillus cereus* F4810/72** and Isolate 4 was found to be ***Brevibacillus brevis* US575** and IS1 and IS4 showed 96.5% and 98% similarity in sequence alignment respectively.

3. Discussion

Remediation of industrial effluents predominantly those heavily contaminated with toxic metals is essential in terms of environmental protection as well as human health (Al-Musharafi, 2016). Apart from humans, animals and plants are also adversely affected by chromium and other industrial wastes that causes degradation of their cell membranes (Mohanty & Patra, 2011). Recent

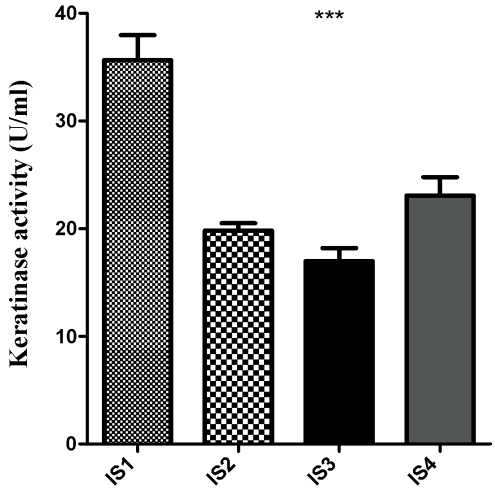

Figure 4. Observation of Keratinase activity of the isolates. Graphical representation showing the amount of keratinase activity by the microorganisms. Y-axis indicates the enzymatic activity expressed in U/ml and X-axis denotes the test isolates. Data (n = 3) illustrated in the bar diagram as mean +SD. ***p < 0.0001 as determined by One-way ANOVA (IS1 to IS4 represents the isolates)

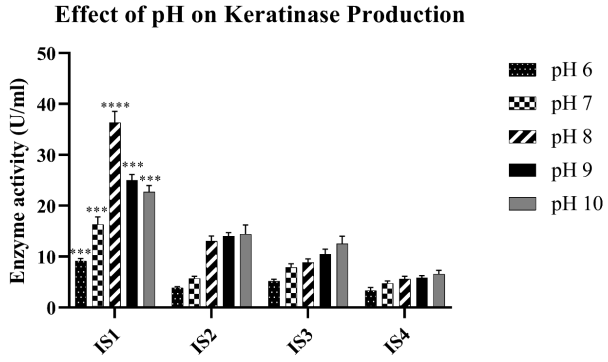

Figure 5. Effect of pH on the Keratinase activity of the isolates. Graphical representation showing the amount of keratinase activity by the microorganisms at different pHs. Y-axis indicates the enzymatic activity expressed in U/ml and X-axis denotes the test isolates. Data (n = 3) illustrated in the bar diagram as mean ±SD. ****p < 0.0001 as determined by Two-way ANOVA (IS1 to IS4 represents the isolates). Though the enzymatic activities of IS1 at different pHs showed similar statistical significance, the activity at pH 8 was the most significant. Also, the enzymatic activity at pH 8 was the highest. Hence, pH 8 was considered as the optimum pH for keratinase activity

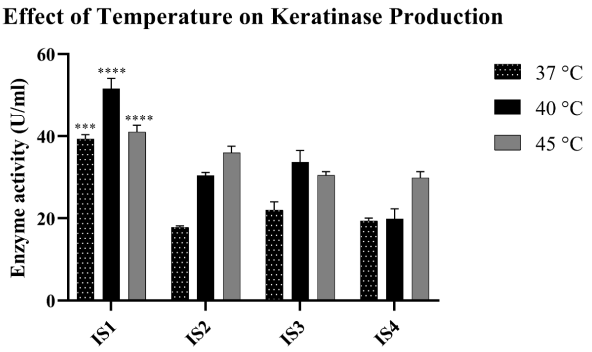

Figure 6. Effect of temperature on the Keratinase activity of the isolates. Graphical representation showing the amount of keratinase activity by the microorganisms at different temperatures. Y-axis indicates the enzymatic activity expressed in U/ml and X-axis denotes the test isolates. Data (n = 3) illustrated in the bar diagram as mean ±SD. ****$p < 0.0001$ as determined by Two-way ANOVA (IS1 to IS4 represents the isolates). Though the enzymatic activities of IS1 at 40 °C, and 45 have the same statistical significance, the activity at 40 °C was much higher than that at 45 °C.
Hence, 40 °C was considered as the optimal temperature for keratinase activity

literature has demonstrated the possibility of treatment of such wastes by bioremediation by using various microorganisms like *Stenotrophomonas maltophilia* (Raman et al., 2018), *Bacillus* spp., *Staphylococcus* spp. (Mythili & Karthikeyan, 2011; Yang et al., 2014), and fungal consortium (Sharma & Malaviya, 2016). Hence, it becomes clear that the efficient removal of toxic pollutants from the tannery effluents before their discharge to the natural waterways can be accomplished utilizing a sustainable, cost-effective, eco-friendly, and easy-to-apply green technology such as bioremediation (Fernández et al., 2018; Vijayaraj et al., 2018). Interestingly, much work has not been done regarding isolation or identification of novel bioremediators from Kolkata, West Bengal except for a few reports (Basu et al., 2014). Also owing to the different biogeochemical scenarios prevailing in different parts of India, the microbes used for bioremediation in areas like Chennai may not be suitable for use in Kolkata due to climatic diversification of these two places. Thus, there is an urgent need for isolation or identification of microbes that can be suitable for bioremediation in an area-specific manner. Accordingly, in this study, an effort was made to isolate

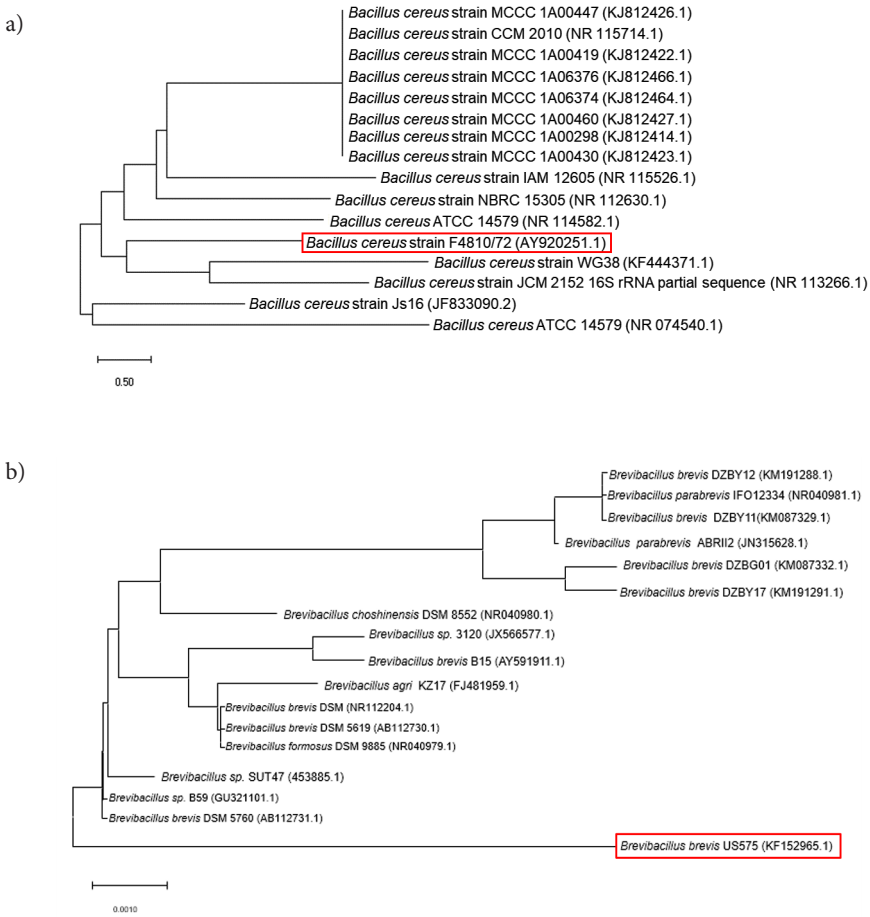

Figure 7. Phylogenetic tree obtained after sequence alignment of (a) Isolate 1, and (b) Isolate 4 by BLAST. A Phylogenetic tree was constructed through Neighbour Joining (NJ) method after aligning the partial sequence of 16S ribosomal RNA gene in Mega 7.0 keeping parameters default and Bootstrap value 1000. The Isolates 1 and 4 were identified as *Bacillus cereus* F4810/72 and *Brevibacillus brevis* US575 respectively and they were indicated and highlighted in a red box

microbes capable of bioremediation of wastes (particularly chromium from tannery effluents) particularly in Kolkata, West Bengal which can then also be employed for waste treatment in other areas with similar climatic conditions.

Thus, in the present study, the effluent collected from the tannery industrial sites was yellowish-brown in colour and had an extremely foul odour. Additionally, deteriorated particles of leather were present. The sample had a high content of inorganic phosphate and heavy metals such as lead and chromium along with a significant concentration of dissolved carbon (Table 1). These indicate a high level of pollutants in the effluent which is released untreated in the environment. Previous characterizations of tannery effluents from other areas have also reported similar observations (Tripathi et al., 2011; Kanimozhi et al., 2013; Jahan et al., 2015). Among the four isolates, IS1, IS2, and IS4 were Gram-positive while IS3 was Gram-negative. All of the isolates showed positive catalase activity while all except IS2 showed negative oxidase activity (Appendix Table 1A).

Numerous reports exist documenting the ability of microbes to reduce chromium (Mythili & Karthikeyan et al., 2011; Thakur & Srivastava, 2011). Additionally, microbes isolated from chromium polluted soil has also been shown to reduce chromium (Viti et al., 2003), whereas, heterotrophs and coliforms isolated from tannery effluents were tolerant of heavy metals like mercury and cadmium in addition to reducing chromium (Marzan et al., 2017; Verma et al., 2001). In our study as well, Isolate 1 (IS1) was found to reduce up to 16 ppm of hexavalent chromium which was the highest among the four isolates (Figure 1b). Also, it was found that Isolate 4 (IS4) caused a 5.22% reduction in the weight of leather after a week (Figure 2), which was highly significant as compared to other isolates. Thus, the isolates have some promising leather degradation ability which is probably due to their natural habitat and this could be one of the reasons of the presence of deteriorated leather particles in the effluent. Indeed, the presence of leather particles has also been reported in tannery effluents from various parts of the world (Hassen & Woldeamanuale, 2017; Chowdhury et al., 2015; Arias-Barreiro et al., 2010). Isolate 4 (IS4), also had the highest lipase and proteinase activates as demonstrated by the degradation of vanillin and BSA respectively (Figure 3a and 3b). Earlier reports have also indicated the lipase activity of certain bacteria (Kanimozhi et al., 2013). Thus, our findings establish the promising lipid and protein degrading capability of the Isolate 4 (IS4). Isolate 1 (IS1) had the highest keratin degrading ability (Figure 4). Since the isolates, especially IS1 showed promising keratinase activity, it is evident that physical conditions like temperature and pH may further enhance this activity. Accordingly, we performed the keratinase experiment under different pHs and found the maximum activity at pH 8 (Figure 5). This was highly significant in comparison to the keratinase activity of the other isolates. So, pH 8 was concluded as the optimum pH for keratinase production which also corresponds to previous reports (Jaouadi et al., 2013).

Similarly, different temperature conditions were also tested to elucidate their suitability for keratinase production, and 40 °C was found as the optimum temperature with the highest keratinase activity being exhibited by Isolate 1 (IS1) (Figure 6). Owing to their promising and multidimensional activities, they were identified using 16S rRNA and were respectively identified as *Bacillus cereus* F4810/72 and *Brevibacillus brevis* US575 with high sequence similarity (Figure 7). The high lipase activity shown by *B. cereus* (IS1) and *B. brevis* (IS4) coincides with the previous report of a lipase enzyme obtained from *Bacillus* sp. isolated from tannery wastes (Jaouadi et al., 2013). Thus, in this study two promising candidates-*Bacillus cereus* F4810/72 and *Brevibacillus brevis* US575 were not only found to reduce chromium but also degrade leather, keratin, proteins and lipids. Earlier reports have shown such properties exist in these bacteria (Zhao et al., 2012) and thus further validate our study. Apart from these, *Bacillus subtilis* has also been shown to exhibit certain keratinolytic properties (Pillai et al., 2011) with chromium reducing efficiency (Basu et al., 2014). The keratinolytic activity shown by IS4 (*B. brevis)* was also previously reported particularly concerning chicken feathers which also correlates nicely with our present findings (Jaouadi et al., 2017). Interestingly, previous reports have also mentioned the presence of *Bacillus cereus* in tannery effluents and have also demonstrated its chromium removal ability which correlates to our findings as well (Kumari et al., 2016). *B. cereus* could reduce hexavalent chromate by around 70% which is also in line with our results (Singh et al., 2013). Though fungi, algae, plants, and biomasses have been shown to possess the bioremediation ability of chromium (Mallick & Rai, 1994; Kapoor & Viraraghavan, 1995; Romero-Gonzalez et al., 2006; Sharma & Adholeya, 2011; Vajpayee et al., 2001), it appears that the bacterial world is more proactive in reducing heavy metals like chromium (McLean & Beveridge, 2001) with greater flexibility and under a variety of environmental conditions. Particularly, the *Bacillus* genus is well equipped with bioremediation capabilities, especially the reduction of chromium and tolerance to heavy metals like cadmium, and has been found in multiple tannery wastewater samples (Zahoor & Rehman, 2009; Tripathi et al., 2011). Our investigation reveals that the isolates not only reduce chromium but also accumulate it, thus changing it from cyclical pool to the reservoir pool. It also helps to precipitate chromium at neutral pH for further removal. The organisms are potent leather degraders. Thus, they can be used in various leather industries for the processing of leather. Also, the keratinolytic property may be useful in recycling keratin-rich agro-industrial and poultry waste byproducts.

Though the treatment of chromium is possible using low-cost adsorbers like activated carbon (Fahim et al., 2006), this approach has two potential downsides. Firstly, the tetravalent chromate can only be neutralized and not the hexavalent form which is the major pollutant in the effluents. Second, the activated carbon needs to be replenished to keep the system functioning. Though they can

treat wastes with good efficiency, other methods such as activated sludge, sawdust, or electrocoagulation (Sumathi et al., 2005; Verma et al., 1995; Espinoza-Quiñones et al., 2009), are not cost-effective as they often require additional inputs in terms of energy or regular maintenance. Thus, the most suitable process for treating such wastes appears to be bioremediation, or neutralization of chromate using microorganisms, which can function by biosorption, bioaccumulation, or bioreduction (Garg et al., 2012). Bioremediation of chromium from tannery waste becomes a lucrative option as it has negligible upkeep costs along with high efficacy and non-existent environmental damage. *B. cereus* is particularly promising because it has been found in tannery effluents from many areas (Kapoor & Viraraghavan, 1995) suggesting that it can operate successfully in a variety of environmental conditions and climate and treat wastes from tanneries by not only reducing chromium but also by degrading the leather bits in the wastewater along with degradation of keratins and lipids which may be resent in the waste discharge.

However, further optimizations might be required and further studies will need to be carried out to elucidate the effects of these bacteria on actual tannery effluents-whether they are efficiently able to reduce the chromium to acceptable levels and thereby lower the health hazards related to the discharge of the tannery effluents. Also, as the bacteria were isolated from tannery effluents, it may be said that are well-adapted to survive in the harsh niche provided by the tannery wastewater (containing various heavy metals and numerous pollutants) and hence, will most likely be able to reduce the chromium present in it. The study thus proposes a novel bioremediation strategy along with paving the way for further research with regards to optimization and efficiency studies on treating tannery wastes. We are hopeful that our findings will be utilized in the bioremediation of tannery effluents which will ultimately reduce health hazards such as cancer and help to reduce risk to society.

Conclusions

Overall, our study concludes that Bioremediation can be a cost-effective procedure if one organism can be used for tackling different pollution problems, in this case, *Bacillus cereus* F4810/72, *Brevibacillus brevis* US575 appears to be multidimensional organisms as it not only reduces but also bioaccumulates Cr^{6+} and at the same times producing enzymes like Protease, Lipase & Keratinase. Therefore, future research in this regard should expand the knowledge currently obtained through highlighting the activities of chromium tolerant strains in tannery effluents that may pave the way for a pollution-free clean environment in the nearby days.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Authors contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Arghyadeep Bhattacharjee, Rajarshi Chaudhuri, and Arup Kumar Mitra. The first draft of the manuscript was written by Arghyadeep Bhattacharjee and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Conceptualization: Arghyadeep Bhattacharjee, Arup Kumar Mitra; Methodology: Arghyadeep Bhattacharjee, Priyanshu Pandey; Formal analysis and investigation: Rajarshi Chaudhuri, Arup Kumar Mitra; Writing – original draft preparation: Arghyadeep Bhattacharjee; Writing – review and editing: Arghyadeep Bhattacharjee, Rajarshi Chaudhuri, Priyanshu Pandey; Resources: Arghyadeep Bhattacharjee, Rajarshi Chaudhuri, Arup Kumar Mitra; Supervision: Arup Kumar Mitra.

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APPENDIX

Table 1A. Morphological and biochemical characterization of the isolates

Designation of the Isolates	Gram Characterisation & Morphological features	Motility	Catalase Test	Oxidase Test
Isolate 1 (IS1)	Gram positive short rods with dull grey coloured and pale yellow coloured colonies with rough surface	Motile	Positive	Negative
Isolate 2 (IS2)	Gram positive short rods with yellowish, smooth round colonies	Non-motile	Positive	Positive
Isolate 3 (IS3)	Gram negative short rods with whitish, smooth and irregularly shaped colonies	Motile	Positive	Negative
Isolate 4 (IS4)	Gram positive shirt rods with whitish and grey round colonies.	Motile	Positive	Negative